

**Associations between Hepatitis B Virus Mutations and Infection Kinetics, Host
Responses and Outcomes**

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Abstract

The hepatitis B virus (HBV) has infected more than 2 billion people worldwide and is associated with a significant global burden of disease due to its main associated complications of cirrhosis and hepatocellular carcinoma (HCC). The risk of developing cirrhosis or HCC is highly variable, with some people who have quiescent disease throughout their lifetime, whilst others develop aggressive disease associated with high mortality and morbidity rates.

HBV is prone to mutations during viral replication and, as a result, it acquires multiple mutations due to pressures either from exogenous vaccines or therapeutic agents or from the host's immune system. This propensity to mutate has resulted in the formation of genotypes and viral variants. Genotypes of HBV have a sequence variation of 8% or more and they have a specific geographical distribution. The most common viral variants of HBV are the basal core promoter (BCP) and precore (PC) mutations. BCP diminishes the production of the e antigen (HBeAg) whilst PC abolishes it completely. The role of the HBeAg is not completely understood. It is not required for infectivity or any major steps of the viral life cycle. It is thought to be an immune-modulator although the mechanisms of immune modulation are yet to be elucidated. There is a robust body of epidemiological evidence that links viral variants and genotypes to distinct clinical phenotypes. However, the actual host-virus mechanistic interactions are unknown. The HBV, in itself, is not generally cytopathic. It is the immune response to the virus that is thought to be responsible for the inflammatory response and subsequent scarring and liver fibrosis.

Currently, there is no definitive cure for chronic hepatitis B (CHB) infection. Antiviral therapy suppresses viral replication but does not eliminate the virus. Due to its close interaction with the immune response, an understanding of the host-virus immunopathogenesis is needed in order to explore novel immune-based therapies.

The first aim of this thesis was to understand the characteristics of CHB in Australia. Using a population sample from a large tertiary Victorian hospital, a detailed study of the virological characteristics including genotypes, and BCP and PC mutations was performed. We focused on the association between virological factors including genotypes and mutations and clinical phenotypes. Genotypes A, B, C and D were the most prevalent, with a clear preservation of the relationship between genotype and country of origin of the patient. Genotypes B and C were the most common, a reflection of the large migrant population from South East Asia. HBeAg-negative CHB was common and was strongly associated with the presence of the PC variant. A large

proportion of patients with HBeAg-negative CHB had an active hepatitis and they were in the immune escape phase.

BCP and PC mutations were highly prevalent and were independently associated with the presence of advanced fibrosis, regardless of age, sex or genotype. These findings suggest that there may be a role for sequencing for these mutations in clinical practice and use them as biomarkers. Based on this clinical observation, we also hypothesized that HBeAg was modulating the immune response to enable chronicity in early stages of disease but then becomes a liability in later phases. This formed the basis of studies of the immune response with a specific focus on HBeAg.

The second aim of this thesis was to explore some of the key immune pathways that modulate HBV infection. The hypothesis was that HBeAg modifies innate host immunity to establish chronic infection in the early phases of disease. Much of the literature in HBV has focused on the adaptive immune response but we were interested in the early phases of the natural history, where HBeAg is almost universally present and its absence is associated with reduced infectivity. The role of HBeAg in the early stages of HBV infection was studied using an immunocompetent mouse model transfected with HBeAg- negative strains of HBV. Pertinent findings included a sudden, early drop in viraemia in HBeAg- negative mutants compared to wild type (WT) HBV. A corresponding transaminitis strongly suggested that in absence of HBeAg, there was a more robust innate immune and inflammatory response to the virus. Complementation studies confirmed that the effect was due to HBeAg and no other viral components. Using gene-modified animals and selective antibody depletion, a series of studies were performed to examine individual arms of the innate immune response. We showed that the Type 1 interferon (IFN-I) response was much stronger in absence of HBeAg. Correspondingly, transcriptomic analysis revealed the downregulation of multiple IFN-I pathways and proteome/ phosphor-proteome studies showed changes in the expression of interferon regulating genes associated with HBeAg. Collectively our data showed that HBeAg allows the virus to evade the innate immune response by antagonising IFN-I in the initial stages of infection.

The emergence of HBeAg- negative mutants in the later stages of the natural history of CHB suggests that HBeAg presents a liability to the virus. In our mouse model, following a significant drop in viraemia in the initial phases of infection, there was a recrudescence in viral load in the later stages of disease in HBeAg deficient mutants. We hypothesized that in the later, adaptive immune response, HBeAg is an immune target and there is a strong selective pressure that favours HBeAg- deficient viral variants. A series of experiments using gene-targeted animals was performed to examine the roles of various mediators of the adaptive immune system. These studies

showed that the absence of HBeAg was associated with reduced function of CD4⁺ T cells and a reduction in TNF and IFN- γ activity. HBeAg deficiency was also associated with an escape from the antibody response.

In conclusion, HBeAg is an important antigen in the modulation of the immune response in HBV. Sequencing for HBeAg negative variants may have a role in clinical practice and further longitudinal, prospective studies are warranted. Further dissection of the proteomic and transcriptomic signatures associated with HBeAg negative mutants and their correlation with clinical samples would be the next steps to elucidate the intricate role of HBeAg in modulating disease behaviour and outcomes.

Declaration

I declare that this thesis is of my own composition and that it is a record of original work conducted between February 2015 and December 2019. The work described has not been submitted for any degree or diploma at this or any other university.

This thesis is not greater than 100, 000 words in length.

Zina Valaydon

January 2020

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“If I have seen little further, it is by standing on the shoulders of giants”.

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Preface

All the work in this thesis was performed and analysed by myself with the following exceptions:

Chapter 4: The proteome studies on mouse liver were performed by Dr Marcel Doerflinger (Pellegrini Lab, Infection and Immunity, The Walter and Eliza Hall Institute, Parkville, Melbourne), in collaboration with Dr Jarrod Sandow (Department of Medical Biology, University of Melbourne, Parkville, Melbourne). The transcriptomic analysis was performed in collaboration with Dr Waruni Abeysekara and Prof Gordon Smyth (The Walter and Eliza Hall Institute, Parkville, Melbourne and School of Mathematics and Statistics, The University of Melbourne, Parkville).

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1. "HBeAg defective variants of hepatitis B are associated with advanced fibrosis in chronic hepatitis B". Submitted for publication to the Journal of Gastroenterology & Hepatology on the 17th December 2019
2. "Hepatitis B virus e antigen modulates liver transcriptome, proteome and type I interferon responses". Submitted for publication to the Journal of Virology in December 2019

Conference Presentations

- 2017 European Association for the Study of the Liver, Amsterdam "The e antigen evades type 1 interferon in hepatitis B" - Young Investigator Travel Award
- 2016 Australasian Gastroenterology Week, Adelaide, Australia "The precore antigen is an interferon resistance protein in chronic hepatitis B"- Young Investigator Award finalist
- 2016 Asia Pacific Association for the Study of the Liver (APASL) Conference, Tokyo, Japan "Chronic Hepatitis B in Australia: High Prevalence of Variants Associated with Disease Progression- Young Investigator Award
- 2016 European Association for the Study of the Liver (EASL), Barcelona, Spain "Variants and mutations of hepatitis B: demographics and fibrosis rates"
- 2016 American Association for the Study of the Liver (AASLD), San Francisco "Precore and Basal core promoter mutations in HBV are independent risk factors for liver fibrosis"
- 2016 ACMD St Vincent's Research Week, Melbourne "Viral kinetics are significantly different in HBeAg negative mutants of hepatitis B"

- 2015 World Gastroenterology Organisation International Congress, Brisbane
“Virological characterisation of Chronic Hepatitis B in Australia: high prevalence of variants associated with disease progression”

Prizes and Awards

- 2017 Young Investigator Travel Award, European Association for the Study of the Liver (EASL)
2016 Young Investigator Award, Asia Pacific Association for the Study of the Liver (APASL)
2016 Young Investigator Award Finalist, Gastroenterological Society of Australia (GESA)
2016 Full Travel Scholarship, Australasian Conference for Viral hepatitis
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1 Literature review

1.1 Introduction

More than 2 billion people have been infected with hepatitis B virus (HBV) globally (1). The range of clinical outcomes in HBV varies widely. Some people can control the infection whilst others develop chronic active hepatitis that can progress to cirrhosis and hepatocellular carcinoma (HCC). Once persistent infection has been established, HBV is incurable and carries a significant lifelong risk of developing HCC, even in the absence of overt active hepatitis and cirrhosis.

HBV is the leading cause of liver cancer, the third leading cause of cancer-related deaths globally. HBV has a significant burden of disease globally and in Australia, where the incidence is rising as a result of migration from endemic countries.

Due to lack of proof-reading during viral replication, the HBV genome is susceptible to acquiring multiple mutations. This results in the formation of variants including different genotypes, sub-genotypes and mutants of HBV, which differ significantly in their genome. HBV mutants tend to become prevalent in the host in the later stages of disease, as a response to specific selection pressures. These selection pressures include both the endogenous host immune response and exogenous elements such as vaccines and the use of antiviral therapy, which select out escape mutants.

However, the underlying pathogenic mechanisms are unclear. There is currently no insight into what genetic features in these different HBV mutants contribute to the differences in outcomes. The interaction between the HBV genome and the host hepatocytes and immune cells is also poorly understood. There is an urgent need to understand how the HBV genetic features impact on the host response to infection and in turn how this correlates with disease outcomes.

The literature on HBV immunology largely focuses on the adaptive immune response. However, it is likely that the innate immune response is responsible for determining chronicity in HBV. The e antigen of HBV (HBeAg) has long been thought to be an immunomodulator but the specific interaction with the immune response is unknown. A study of the interaction between HBeAg and the innate and adaptive immune responses could offer some important insights in immunopathogenesis and form the basis for the development of novel therapies.

1.2 HBV Virology

1.2.1 Classification

The hepatitis B virus (HBV) is a member of the *Hepadnaviridae* family, a family of small, enveloped viruses that primarily cause hepatotropic infections. *Hepadnaviridae*

have two species-specific genera, *orthohepadnaviruses* that infect mammals and *avihepadnaviruses* that infect birds. Human HBV is the prototype of the *orthohepadnavirus* genus (2).

Like all hepadnaviruses, HBV causes a hepatotropic infection and it has not been isolated in tissues other than the liver. HBV itself is not directly cytopathic: hepatitis and any resulting liver damage are usually mediated by the immunological response of the host to the virus.

HBV requires specific host factors for various aspects of its life cycle and therefore is relatively species-specific. Primarily, HBV infects humans although infection is permissive in great apes, chimpanzees, macaques and in the laboratory setting, the tree shrew (3-5). In experimental models, surrogate HBV-like viruses have been successfully used to address particular questions concerning pathogenesis and replication, including ducks, squirrels and woodchucks and these models have provided crucial insights in HBV virology and subsequent advances in antiviral therapy (6).

1.2.2 Virus structure

HBV was first identified by electron microscopy and HBV virions were originally named Dane particles after the lead pathologist of the research team(7). The virion is 48-52 nm in diameter, consisting of a nucleocapsid core enclosed in a glycolipid envelope (8, 9). The envelope is made up of lipids and three surface proteins. The core is an icosahedral nucleocapsid of 30-32 nm in diameter typically made up of either 180 or 240 core subunits with triangulation numbers (T) 3 or 4, respectively. Capsids with the T=4 configuration are the predominant ones detected in active infection (10, 11). Cryoelectron microscopy studies have revealed that core particles form spikes on the surface of the particle and these spike tips have strongly immunogenic epitopes. The core contains the genome from which all viral proteins are transcribed.

1.3 Genome organisation

The HBV genome is a partially double-stranded DNA strand of approximately 3200 base pairs (dependent on the genotype), in a relaxed circular conformation (rcDNA) (12). The strands overlap at the 5' end and the minus strand is complete but the plus strand has a gap of about 600 nucleotides and the position of the 3' end is variable (13). HBV has an endogenous polymerase to incorporate nucleotides in order to complete the genome forming a full double strand during viral replication (13-15). The fully circularised HBV genome is made up of four overlapping and frame-shifted reading frames (ORFs) which are Pol (P), Core (C), Surface (S) and X and they

encode for the seven HBV proteins. They also encode for four promoter regions that help initiate transcription and two enhancers Enh I and Enh II that promote gene transcription when bound by transcription activators. The genome also has *cis*-regulating elements (CRE), which are regions of non-coding DNA that regulate transcription of nearby genes usually by acting as binding sites for transcription factors. The overlapping nature of the ORF has significant implications for mutagenesis as a mutation in one reading frame can affect the overlapping frame and its associated protein.

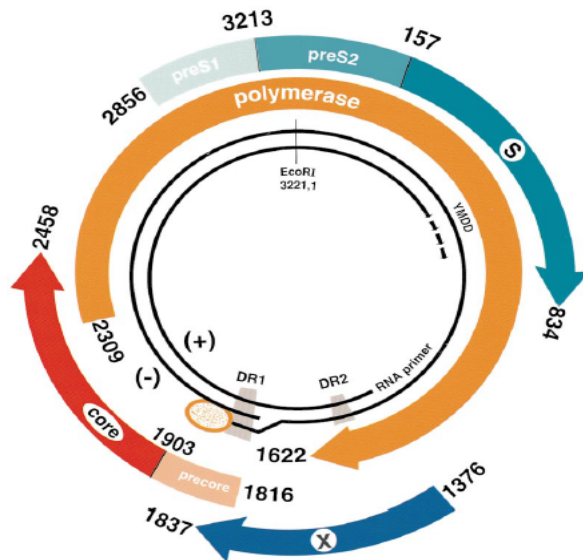


Figure 1.1 The HBV genome.

The inner circles represent the minus and plus DNA strands of the viral genome. The open reading frames encoded by the genome, designated as S, core, polymerase, and X, are indicated by the arrows. Nucleotide numbers designate the boundaries of each ORF. The nucleotide numbering of the genome is based on the unique EcoRI restriction enzyme site shown. Nucleotide numbers designate the boundaries of each ORF with position 1 mapped at the EcoRI site. The figure also shows positions for the viral direct repeats (DR1 and DR2) and the position of the YMDD locus in the HBV polymerase gene. Abbreviations: S surface antigen; Y tyrosine; M methionine; D aspartate; DR direct repeat segment

Pol ORF

Pol is the longest reading frame covering 70% of the viral genome and encodes the viral polymerase. HBV polymerase includes four functional sub-domains that are enzymes required for DNA synthesis: the reverse transcriptase required for synthesis of the plus strand, a carboxy terminal region that has ribonuclease H activity, a spacer region as well as a terminal domain which primes the pregenomic (pg) RNA for the synthesis of the minus strand of DNA.

Pre-S/S ORF

The S- ORF encodes for three HBV surface proteins: large (LHBs), middle (MHBs), and small (SHBs) protein but the proteins are translated from two different mRNAs: LHBs is translated from a long 2.4 kb transcript whereas MHBs and SHBs are translated from a slightly shorter 2.1 kb transcript. All three envelope proteins terminate

at the same 3' end but their starting 5' end is staggered such that L and M proteins have extra domains, the Pre-S1 and pre-S2 respectively.

SHBs

SHBs is the smallest of the three surface proteins, made up to 226 amino acids and is synthesised in the endoplasmic reticulum (ER). An unusual feature of HBV envelope proteins is that they have multiple transmembrane domains that span the ER with loops of amino acids internal and external to the cytosol. SHBs spans the ER membrane via four transmembrane domains (TM 1-4) which are linked by internal and external loops (16). The loop of amino acids linking TM2 and 3 is external to the ER and made of amino acids 99–161. This loop is known as the 'a' determinant and it is of particular virological and clinical significance as it is a major antigenic determinant of HBV. The 'a' determinant contains the major B cell neutralising epitopes of HBsAg (17, 18).

MHBs

The exact role of the MHBs remains unclear as it is not required for either viral or subviral particle secretion nor for infectivity (19).

LHBs

In contrast, LHBs is an important protein in the viral life cycle, essential for viral entry. The PreS1 domain of the L protein has a 75 amino acid domain at the N terminal that requires myristylation that is essential for viral infectivity (20, 21). LHBs also has an important role in encapsidation of the core during viral replication as the mature virion is formed and secreted.

Precore/ core ORF

The core gene encodes for two proteins: the core protein, HBcAg, and the secreted precore protein HBeAg. HBcAg is the major component of the viral nucleocapsid. It is 183 residues long, of which the N-terminal 149 residues are the assembly domain (22). The core contains the genome from which all viral proteins are transcribed (23). HBcAg is strongly immunogenic. Cryoelectron microscopy studies have revealed that core particles form spikes on the surface of the particle and these spike tips have strongly immunogenic epitopes.

The precore/core ORF also encodes for HBeAg. Full translation of the core ORF results in the formation of HBeAg, which is secreted in great excess of virions (24). HBeAg is the only protein to be modified post-translationally and prior to being

secreted, it is proteolytically processed at both C and N termini. The antigen is initially 29 residues long, but 19 residues are cleaved off upon entry into the endoplasmic reticulum (ER) to form a 22-kDa protein. Additional amino acids are cleaved off from the C-terminus upon transport into the Golgi compartment to form the 15-18 kDa HBeAg (25).

HBeAg is an accessory protein that is not required for structural stability, viral replication, secretion or infectivity (26). HBeAg is a serological marker for active HBV infection and replication and is regarded as necessary for the establishment of persistence. Its exact role is unknown but it is thought to enable immune escape by acting as an immunomodulator and tolerogen (27). The mechanisms are unclear, whether it is by suppressing the immune response to the virus (28, 29) or by acting as a decoy particle for the immune system (30).

X ORF

The X ORF encodes for HBx, the X protein. At the time of its discovery, the role of HBx was relatively unclear, hence its name(31). However, it is now known that is required for cccDNA transcription and is a key regulator of HBV replication. HBx is a small 16.5 kDa protein made up of only 465 base pairs but it contains important sequences of the genome including Enh II, the core promoter and the microRNA-binding region.

HBx activates HBV enhancers and promoters (32) to increase viral replication and tumorigenesis. It also inhibits the degradation of HBV by inhibiting proteasomes (33, 34) and enzymes that promote autophagy (35, 36) and by preventing deacetylation of cccDNA-bound histones (37).

HBx also influences the host innate immune response to promote chronicity. It downregulates the mitochondrial antiviral signalling (MAVS) protein which is essential in virus-activated immune signalling (38), blocks the induction of Type 1 interferon (39) and activates nuclear factor kappa B (NF- κ B) which allows resistance to pro-apoptotic ligands including Fas and tumour necrosis factor (TNF) (40, 41).

HBx is strongly associated with hepatocellular carcinoma. It alters gene expression and confers a carcinogenic potential to HBV (42, 43). It suppresses apoptosis by downregulating the tumour suppressor gene p53 (44-46), downregulates the anti-tumour retinoic acid receptor beta (47) and interferes with DNA repair (48, 49). HBx also interacts with the JAK-STAT pathway to promote proliferation of hepatocytes (50).

Subviral particles

Over and above the production of fully formed virions, subviral particles (SVP) consisting of surface proteins only are also formed during viral replication. SVP are either of spherical or filamentous conformation; spheres are 20-25 nm in diameter and filaments are 22 nm in diameter and are of variable length(19). SVP are made up of the three HBV envelope proteins that are collectively known as the surface antigen (HBsAg) (7, 51). SVP are secreted in large quantities in 1,000- 100, 000 fold excess of virions(52).

Since SVP do not contain any viral DNA, they are not infectious (53) but they have immunogenic properties. It is thought that they are secreted in such excess in order to neutralize anti-HBs antibodies hence promoting viral persistence (54). SVP have also been shown to enhance viral replication and gene expression and therefore, despite not being directly infectious themselves, they increase infectivity (55, 56). SVP are used in the current HBV vaccine as they contain the S protein but no capsid protein (56). As explained above, the current HBV vaccine elicits an antibody response that is predominantly directed at the 'a' determinant in the S protein and cases of vaccine resistance have been reported, due to mutations in the 'a' determinant. As SVP contain all the structural proteins but no genome, they could, in principle, help overcome the limitations of the current vaccine (57).

1.3.1 Viral replication cycle

The life cycle of HBV is well characterised and can be classified in 3 stages: early, middle, and late (57). The main events are depicted in Figure 1.2.

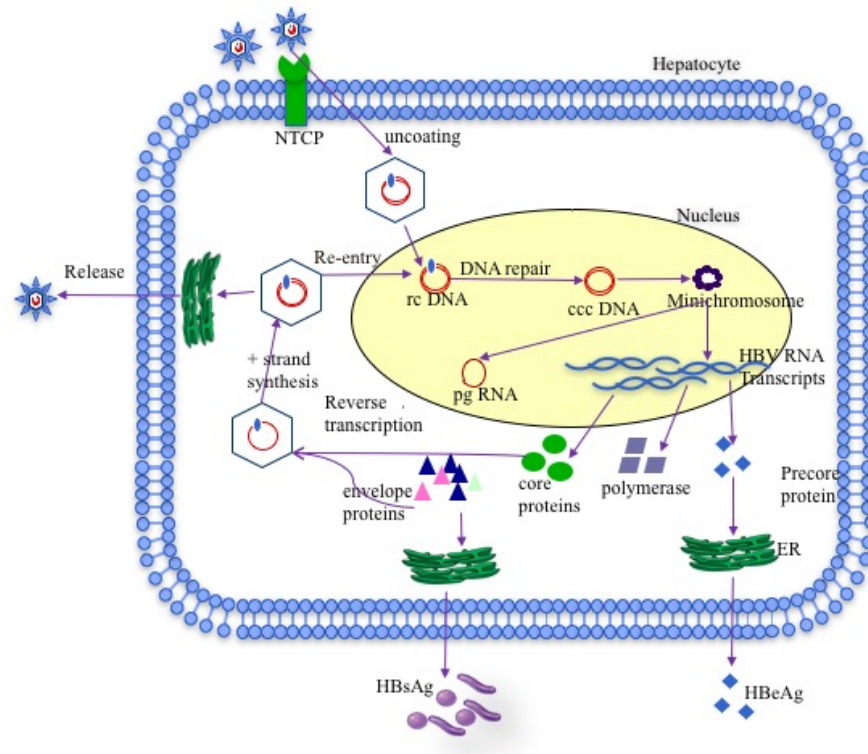


Figure 1.2 The HBV life cycle. HBV enters the hepatocyte via the sodium taurocholate co-transporting polypeptide (NTCP) receptor. The nucleocapsid is uncoated and migrates to the nucleus where it delivers the genome (rc-DNA). Rc-DNA is repaired to form double-stranded cccDNA and a mini chromosome from which the following HBV viral proteins are transcribed: core and precore antigen, surface antigens S, M and L, X protein and polymerase. After processing by the endoplasmic reticulum (ER), HBeAg and HBsAg are secreted. New virions are released, and genomic RNA is recycled back into the nucleus for ongoing viral replication.

Early events: attachment, entry and uncoating

HBV cellular entry has only recently been elucidated. HBV initially binds, in the space of Disse in the liver, to heparan sulphate proteoglycans (58) expressed on the surface of hepatocytes. It then binds with high specificity to the sodium taurocholate co-transporting polypeptide (NTCP) receptor via the preS1 attachment site and subsequently becomes internalised in endosomal vesicles (59, 60).

After entry into the hepatocyte, surface proteins are removed and the nucleocapsid is actively transported to the nucleus via microtubules, where it is uncoated and the genome is delivered to the nucleus (61). The small diameter of the capsid allows it to

pass through the nuclear pores. At this point in the viral life cycle, the genome is still in its usual relaxed circular conformation with an incomplete plus strand.

Reverse transcription and generation of the cccDNA

HBV is a pararetrovirus by virtue of its replication strategy: It is a DNA virus but it replicates via reverse transcription using an RNA intermediate (6, 62, 63). However, it differs from retroviruses in distinct ways. Firstly, in HBV replication, each protein is translated from its own mRNA except for Pol and Core, which are both, translated from the same mRNA, the pgRNA. Secondly, proteins are not processed post-translationally except for the precore protein, which undergoes proteolysis. Finally, unlike retroviruses, HBV DNA is first converted to a mini chromosome where the viral DNA can be found as a covalently closed circular DNA (cccDNA) molecule, the functional equivalent of an intracellular provirus.

To form cccDNA, rc-DNA is first converted to a fully double stranded genome. The rc-DNA has a complete minus strand but a gap in the plus strand, the 5' end of the minus strand is linked to the P protein whilst the 5' end of the plus strand contains a primer for plus strand synthesis. cccDNA binds to histones and other chromatinising proteins to form a mini chromosome that serves as the template for transcription of HBV mRNA from which HBV viral proteins are transcribed (64, 65). Transcription of the HBV mini chromosome is regulated by epigenetic changes of the histones that bind cccDNA, specifically the acetylation of H3/H4 histones (66).

Transcription of viral proteins

From the cccDNA mini chromosome template, the host cell RNA polymerase II transcribes five RNA species of different sizes. The cccDNA also encodes for four promoters and two enhancers. The enhancers have binding sites for transcription factors, which are liver specific, the hepatocyte nuclear factors 3 and 4 (HNF3 and HNF4), yet another mechanism by which the virus maintains hepatotropism (67). There are two genomic transcripts of 3.5 kb each and three sub-genomic transcripts of 2.4 kb, 2.1 kb and 0.7 kb. The sub-genomic 2.4 kb transcript serves as mRNA for the translation of the pre-S1 protein whilst the 2.1 kb is the mRNA for the translation of both Pre-S2, and S and the 0.7 kb transcript functions as mRNA for the translation of the X protein (28). The 5' end of the 2.1 kb mRNA is heterogeneous and the Pre-S2 start codon is only present in a proportion of transcripts such that the S protein is far more abundant than pre-S2.

The 3.5 kb genomic transcripts include the precore mRNA and pgRNA. The precore mRNA is similar to the core protein but has an extension at the N terminal end and it encodes for HBeAg, and as such is the first HBV protein synthesised.

PgRNA encodes for HBcAg and Pol. PgRNA is also the template for reverse transcription to generate HBV DNA. pgRNA is converted to double stranded DNA (ds-DNA) by reverse transcription. DNA synthesis starts when the viral polymerase binds to an encapsidation signal of the pgRNA. This initiates encapsidation to form new nucleocapsids (68-71). Reverse transcription of the RNA occurs inside the new nucleocapsid to form rc-DNA. A short DNA sequence is generated using a region of the epsilon loop as template (72-74). The template then switches to the 3' end and the minus strand synthesis is initiated. The plus strand is then synthesised, and the genome is circularised.

The newly synthesised DNA then either get recycled to the nucleus to maintain the cccDNA pool or it is packaged with surface proteins and exported as new virions. cccDNA and replication intermediates including pgRNA persist in the hepatocyte indefinitely (75). Splicing of the pgRNA can also occur with major deletions in the mRNA, producing variants. Splice variants are considered defective, but they have important clinical implications. Splice variants are not routinely tested in the clinical setting but they are common, with some case series reporting an incidence of up to 96% in serum samples of patients with chronic HBV (76).

Spliced pgRNA can form a novel protein called the HBV splice-generated protein (HBSP) (77). HBSP is associated with increased viral replication as manifested by an increased expression of core protein as well as secretion of HBeAg (76). Splice variants have also been associated with an increased progression to chronicity following acute infection with HBV (76), higher rates of liver cirrhosis and hepatocellular carcinoma (77, 78).

Viral assembly and release

In the cytosol, mature rc-DNA is assembled into new nucleocapsids and they are enveloped prior to being exported from the hepatocyte. Virions will only be enveloped once the genome is fully formed and mature with a minus DNA strand and a partial plus strand (63).

For the formation of new virions, capsids move towards intracellular membranes containing the three envelope proteins. These intracellular membranes act as budding sites for the formation of new virions. Surface proteins are essential for the final stages of viral assembly and the budding of new HBV capsids. The S protein consists of a luminal N-terminal sequence, two transmembrane regions with a cytosolic loop in

between and a luminal domain that includes the 'a' determinant also including the glycosylation site (79) at residue 146. PreS1 has a dual topology and in the virion half of PreS1 ends up on the internal surface of the virion, which is required for the envelopment of core particles and the other half of PreS1 is expressed externally as it is required for subsequent viral attachment (79). Following envelopment, the virions are deposited in the lumen of the ER and released.

Virions rely on the multivesicular body (MVB) pathway that generates intraluminal vesicles (80) in order to be released from the cell. The MVB pathway depends on the endosomal sorting complex required for transport (ESCRT) system that comprises the ESCRT-0, -I, -II, and -III complexes and associated proteins. The activation of ESCRT-III in particular brings about budding and membrane fission allowing the virion to be released out of the hepatocyte.

SVPs are formed independent of capsids as the envelope proteins for oligomers that bud off as spheres or filaments (81). They do not depend on the ESCRT complex for release from the cell (82), but are processed via the ER-Golgi pathway.

Integration in the host genome and HBV persistence

Unlike other hepatotropic viruses, HBV DNA can integrate into the host genome. A common site of viral integration is the human telomerase reverse transcriptase (hTERT). This is not necessary for the production of virions, but it ensures persistence in the hepatocyte. Integrated HBV DNA can act as a template for the expression of HBsAg, promoting immune tolerance and viral persistence(83). Integration also confers oncogenic potential to the virus as it can interfere with cellular signalling, proliferation and apoptosis.

In a small proportion of nucleocapsids, direct extension of the RNA primer leads to the formation of double-stranded viral DNA (dsl DNA) , a by-product of viral replication. Dsl-DNA does not lead to the formation of pgRNA but, unless it is degraded, it can be circularised by the non-homologous end-joining DNA repair pathway and form cccDNA-like molecule (84, 85). This process is susceptible to errors and most of the resulting molecules are often defective. dsl DNA can also integrate into the host chromosome, a phenomenon which is associated with carcinogenesis (86, 87).

cccDNA

A unique feature of HBV is its ability to form a mini chromosome during viral replication, which acts as a template for its RNA transcripts. cccDNA is an important feature of HBV infection that determines multiple significant clinical characteristics including chronicity, lifelong persistence and relative inefficacy of antiviral treatment.

cccDNA is a stable form of viral DNA that allows HBV to persist in the hepatocyte, hence ensuring the ongoing production of virus. cccDNA has yet to be identified in non-authentic host cells, such as transgenic animals, suggesting that specific host factors are required for its formation, which contributes to the species specificity of HBV. cccDNA is produced early, within 24 hours (88), and in large quantities. In a natural HBV infection there are more than 50 copies of cccDNA in a hepatocyte due to intracellular amplification (89-91) from pgRNA reverse transcription or from multiple rounds of virion reinfection. It can persist in the infected hepatocyte for up to 60 days and is not lost with cell division.

cccDNA is not susceptible to eradication with current antiviral therapy (52, 89, 92). Nucleos(t)ide analogues, lamivudine and adefovir, are designed to inhibit polymerase and reverse transcriptase activity and can therefore block the synthesis of new cccDNA (93, 94). However, animal studies have shown that pre-existing stable cccDNA persists (95) and rates of HBeAg seroconversion remain low, in the order of 12-15% (96, 97) and viral suppression is not sustained following cessation of therapy (98). Current antiviral therapy can efficiently suppress viral replication, but permanent eradication of HBV is virtually impossible to achieve due to the cccDNA reservoir. Once infected, patients will harbour cccDNA and replication intermediates such as pregenomic RNA (pgRNA) in the hepatocyte indefinitely until the hepatocyte is eliminated (75). Even after seroclearance of HBV, with the formation of antibodies to the surface antigen (anti-HBs), a risk of reactivation is still present in the context of immunosuppression (75, 99, 100).

1.4 Genotypes and mutations in HBV

One of the salient features of the HBV viral transcriptase is that it lacks proof reading, which renders HBV highly susceptible to acquiring mutations during each cycle of replication especially G-A hyper-mutation. This gives rise to various genotypes and mutations that have significant clinical implications to the natural history and the disease outcomes of HBV. Genotypes and mutations affect various aspects of viral pathogenesis, host immune recognition and response, viral replication and subsequently impact strongly on clinical outcomes in particular the development of fibrosis, cirrhosis and HCC (101).

1.4.1 HBV genotypes

To date 10 different HBV genotypes (A-J) have been identified along with multiple sub-genotypes within each genotype (102). Genotypes differ from one another in >8% of the nucleotide sequences and sub-genotypes vary by 4-8% (103). Genotypes have a

specific geographic distribution. Genotype A is common in Northern Europe, America and Africa whereas genotypes B and C are common in Asia, genotype D is prevalent the Middle East and southern Europe.

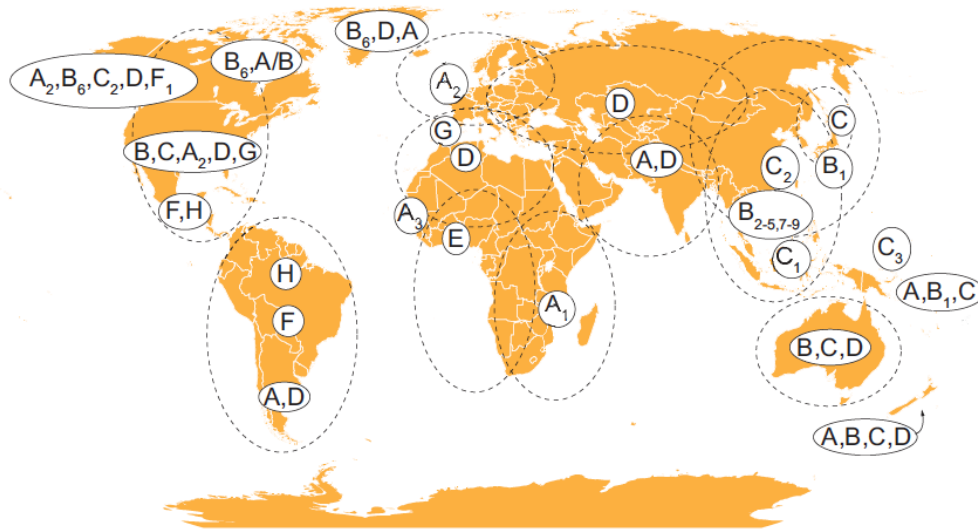


Figure 1.3 Geographic distribution of genotypes.

Genotypes have a specific geographic distribution. Genotypes A2 and D are common in Europe whereas genotypes B and C are highly prevalent in Asia. Reproduced with permission from Prof S Locarnini.

Genotypes are thought to influence multiple aspects of the natural disease progression including chronicity, HBeAg seroconversion, rate of HCC development and response to antivirals. For instance, Genotype C is associated with more aggressive hepatitis, less HBeAg seroconversion and a more rapid progression to cirrhosis compared to genotype B (104-106). Most of the clinical studies on genotypes have been performed in Asia so genotypes B and C have been studied the most. Very little data is available on genotypes E-J. As most studies are carried out in areas where either genotypes B and C are prevalent or genotypes A and D are prevalent, the research data tends to be a comparison between the two predominant genotypes i.e. B versus C and A versus D respectively. The major clinical differences amongst genotypes B versus C and A versus D are summarised in Table 1.1.

Genotype	B	C	A	D
Transmission mode(107) (108)	Vertical	Vertical	Horizontal	Horizontal
Chronicity (107-111)	Lower	Higher	Higher	Lower
HBeAg seroconversion (112)	Early	Late	Early	Late
HBsAg seroconversion(101, 110)	More	Less	More	Less
Precore mutation (113)	High	Low	Low	High
Basal core mutation (113)	Low	High	High	Low
Response to interferon	Higher	Lower	Higher	Lower

Table 1.1 HBV genotypes and their associated phenotypes

It is common for more than one genotype to be dominant in a region. For example, genotypes B and C are prevalent in the Asia Pacific whereas genotypes A and D are prevalent in Western countries. Mixed infection with different genotypes is not unusual. Recombination of different genotypes can also occur and intergenotypic recombination is well documented (114). The most commonly reported recombinants are B/C or A/D, which is in keeping with the fact that these genotype pairs tend to co-exist in the same geographic region, namely Asia Pacific and Europe respectively (115). Genotype I is an intergenotypic recombination among genotypes A, C, and G, found in Vietnam and Laos (116). Sub-genotype B2 is a recombinant of B and C and the C/D recombinant is common in Tibet (117). Genotype J is closely related to gibbon strains and human genotype C (118).

1.4.2 Clinical implications of HBV genotypes

Due to the specific geographical distribution of genotypes, most studies are comparisons of the two predominant genotypes of that region, generally B versus C and A versus D.

Chronic infection

The risk of chronicity is higher in patients with genotype A infection compared to genotypes B and C (119). Genotype A was also found to have a greater tendency to chronicity compared to genotype D in Europe (109). However, the mode of transmission, and not the genotype, as such, may strongly influence the natural history of the infection, as detailed later in the text (see **Error! Reference source not found.****Error! Reference source not found.**).

Seroconversion of HBeAg and HBsAg

Early HBeAg seroconversion, characterised by the disappearance of the HBeAg and the formation of anti-HBe antibodies, usually is associated with a better clinical outcome. Compared to genotype B, genotype C is associated with lower rates of spontaneous HBeAg seroconversion and HBeAg seroconversion occurs on average 10 years later (120). This may be one of the factors contributing to the more aggressive phenotype of Genotype C compared to genotype B.

In Genotype A vs D patients, although the rate of HBeAg seroconversion is similar, the rate of sustained remission, whereby the negative HBeAg status is maintained, is higher in genotype A (110). Genotypes A and B also have a higher rates of HBsAg seroconversion compared to genotypes D and C respectively (110, 121).

Occurrence of mutations

Naturally occurring HBV mutants can modulate host immune response and dictate clinical outcomes. Specific genotypes are associated with mutants that could confer added virulence.

The two most common HBV mutants are the precore nucleotide 1896 stop codon mutation and basal core promoter A1762T/G1764A variants which are associated with HBeAg negative disease and poor outcomes. The role of these mutations will be detailed further. Genotypes tend to be associated with specific mutations. For instance, genotype C is associated with a high rate of basal core promoter (BCP) and envelope and HBx mutations whereas genotype B is associated with precore (PC) mutations (122). In contrast, PC mutations tend not to be present in genotype A as it can compromise the structural stability of the Epsilon loop and can compromise viral replication(123)(see 1.3.1 Viral replication cycle, sub-section Transcription of viral proteins).

It is unclear if the differences in clinical phenotype are attributable to the genotype *per se* or are due to the associated mutations (124). Genotype C is also primarily

associated with mutations in the X protein (HBx) although the actual mechanism is unclear.

1.4.3 HBV mutations

Envelope gene mutations

The 'a' determinant located at amino acids 124-147 is cluster of B cell epitopes and the main target for the antibodies produced following vaccination and those used in conventional diagnostic HBV assays. Antibodies to the 'a' determinant protect against all genotypes and serotypes of HBV and form the basis for the HBV vaccine. Mutations in the 'a' determinant region allow the virus to escape the vaccine and previously vaccinated individuals can become infected. Co-existing wild type HBV is neutralised by existing anti-HBs and is therefore undetectable by conventional assays, resulting in occult HBV infection (125, 126).

S mutations are likely due to selection pressure from immunoprophylaxis with vaccine or HBV immunoglobulin or antiviral therapy. The Pol reading frame completely overlaps the S frame in the genome. Therefore, Pol mutations that arise as a result of drug pressure can also cause mutations in HBsAg expression (127). For instance, the rtM204V mutation associated with lamivudine and entecavir resistance is associated with substitution mutations at codon 196 in the S gene including the formation of a stop codon (128). The most common mutations is the G145R, which is the substitution of a glycine for an arginine base at amino acid 145 (129). HBsAg with this mutation retains all its infective properties but is unrecognisable by the anti-HBs generated by vaccination or the antibody used in the diagnostic assay.

The emergence of HBsAg mutation is a significant public health concern. Universal vaccination is a highly effective public health measure that can greatly reduce the burden of disease(130). Mutations in the S gene, which codes for HBsAg has been reported in vaccine-resistant children (131). S gene mutation can also abrogate the production of HBsAg and cause occult HBV where despite an absence of HBsAg on conventional serologic assays, HBV DNA is present in the serum. This has important implications for blood donation and transfusion, organ transplantation and surveillance of HCC. Many blood banks have now expanded their screening tests to include HBV DNA and not HBsAg in isolation(132, 133).

Polymerase gene mutations

The polymerase gene mutation is classically associated with resistance to nucleos(t)ide analogues. Nucleos(t)ide analogues (NAs) inhibit the reverse

transcriptase and resistance to antivirals is usually due to mutations in the Pol gene. Clinically, development of resistance manifests as viral breakthrough, an increase in the serum viral load by more than 1 log₁₀ IU/ml from nadir HBV DNA or the detection of HBV DNA in the serum in an individual with previously undetectable serum viral load(134). Resistance may also lead to biochemical flares, characterised by a rise in aminotransferases. Both virological and biochemical breakthroughs can lead to decompensation of liver disease (135).

The mechanism of resistance to NAs depends on the structure of the drug (136). The most commonly encountered mutation in clinical practice is the tyrosine-methionine-aspartate-aspartate (YMDD) locus.

Lamivudine is associated with YMDD site and, not unexpectedly, the rate of resistance and therefore failure rate is high with over 70% individuals developing resistance in the first 5 years (137).

Drug resistance mutations frequently emerge with lamivudine therapy. The characteristic resistance mutation is an amino acid substitution over tyrosine~methionine aspartate~aspartate (YMDD) motif of the RNA-dependent DNA polymerase(138). Multiple mutations have been identified but the rtM204I and rtL180M/M204V are the most common (139, 140). The incidence of YMDD mutations increases with the duration of lamivudine therapy and reaches about 90% at 4 years of therapy. Multiple factors are associated with the selection of YMDD mutations including serum transaminases, HBV viral load and the presence of the BCP mutation (141-143). YMDD mutations reduce the efficacy of viral replication (144) additional P mutations may arise to restore replication fitness(139).

Newer agents, entecavir and tenofovir are therefore now recommended as first line treatment(145). Resistance to entecavir is less common than Lamivudine in <1% over 5 years (146) and resistance to tenofovir has only been reported in co-infection with HIV(147). Concurrent and compensatory mutations that occur in along with YMDD mutations can give a replicative advantage to the virus (148) or alter the expression of HBsAg as detailed above (149).

X gene mutations

The most common naturally occurring HBx mutations include the C1653T substitution in the Enh II and two mutations of the basal core promoter: T1753V and the classic BCP mutation A1762T/G1764A. The C1653T affects binding of nuclear factors and is associated with fulminant liver failure and HCC (150). T1753V modulates the binding of HBx to BCL2 and affects apoptosis, which leads to more severe liver disease and the development of HCC (122, 151, 152). Both the T1753V and C1653T are independent

poor prognostic factors for HCC survival (153) and both are strongly associated with the presence of the A1762T/G1764A BCP mutation (122). The A1762T/G1764A BCP is the most commonly encountered mutation associated with disease progression and development of HCC (122, 154, 155).

Mutations affecting HBeAg and HBcAg

The two most common mutations of the HBeAg are the basal core promoter (BCP) and precore (PC) mutations that reduce or abolish the production of HBeAg respectively. These mutations tend to arise relatively late in the natural history of the disease, during the phase of immune clearance and are associated with HBeAg seroconversion (155). The classic PC mutation is a G1896A substitution, which forms a stop codon (TAG) in the precore reading frame at nucleotide 1896 (codon 28) and causes a complete abrogation of the HBeAg production (156). Individuals with this mutation may have active liver disease and DNA replication but remain HBeAg negative. The G1896A PC mutation is genotype-specific. It is very common in genotypes B and C but rarely occurs in genotype A. In order for the G1896A to be stable, the opposing nucleotide in the DNA strand (nucleotide 1858) must be a T rather than a C as is the case for genotypes B and C.

The most common basal core promoter mutation is a dual mutation A1762T, G1764A (157). The BCP mutation is a mutation in the C reading frame. The most common mutation is a dual mutation A1762T, G1764A. The BCP spans nucleotides 1742–1849 in the HBV genome and it controls the transcription of both precore and core regions. The classic BCP mutation is an adenine (A) to thymine (T) substitution at nucleotide 1762 together with a guanine (G) to adenine (A) transition at nucleotide 1764 (A1762T/G1764A). This dual mutation diminishes transcription of the precore mRNA, which encodes for HBeAg therefore reducing levels of HBeAg.

The PC mutation determines HBeAg status(158). Unless it is directly transmitted during acute infection, PC tends to emerge and co-exist with WT virus in the immune tolerant phase. After the appearance of anti-HBe at seroconversion, PC variants are selected whilst the wild type (WT) is cleared(159). This can take years and a mixture of variants and WT are often observed (160). The presence of anti-HBe exerts selection pressure to select PC variants(161). PC becomes predominant during HBeAg seroconversion but the proportion of BCP tends to remain unchanged(162). BCP and PC mutations do not affect the production of pregenomic or core viral RNA as their transcription is initiated from a separate start codon downstream from the precore initiation site (157, 163).

BCP and PC mutations arise late in the natural history of HBV infection. These mutations are common and are the predominant virus species in patients with HBeAg negative CHB. Treatment of CHB with Lamivudine often selects drug-resistant strains, the most common of which are point mutations in the YMDD motif as detailed above. These drug-resistance mutations tend to be associated with reduced viral replication. However this could be significantly increased by the presence of BCP and PC mutations(164). BCP and PC mutations are associated with increased viral replication in lamivudine-resistant mutants as well as adefovir-resistant mutants. In fact, the presence of BCP mutations were independently associated with virological breakthrough in patients treated with Lamivudine and adefovir. The presence of BCP and PC mutations compensates for the reduction in replication capacity in lamivudine and adefovir-resistant mutants(157). This phenomenon does not seem to occur in entecavir-resistant mutants(165).

1.4.4 BCP and PC mutations and clinical outcomes

Both BCP and PC mutations have been associated with poor disease outcomes including acute fulminant hepatitis, cirrhosis and the development of HCC. HBeAg seroclearance is generally associated with a reduction in viral replication and relatively quiescent disease. However, BCP and PC mutations abrogate the production of HBeAg whilst allowing viral replication. In patients harbouring BCP and PC mutants, HBV DNA levels are high despite HBeAg negativity and HBeAg status is not representative of reduced viral replication (163).

BCP and PC mutations are independently associated with advanced fibrosis and cirrhosis (167) and an increased risk of HCC (168-174). They have also been associated with more liver inflammation as evidenced by higher rates of transaminitis (175), a higher number of disease flares and necro-inflammation(176, 177), fulminant liver failure (178) and cirrhosis- related complications such as decompensation(112). Response to treatment in particular pegylated- interferon is poor in patients with BCP/PC mutations (179).

1.4.5 Epidemiology of PC and BCP mutations

The prevalence of HBeAg negative disease is increasing, a phenomenon which is possibly a reflection of the ageing population. As expected, the geographical distribution of HBeAg negative disease varies. The median prevalence of HBeAg negative disease among patients with CHB is approximately 33% in the Mediterranean, 15% in the Asia Pacific and 14% in Northern Europe and the USA(180). There are marked geographical variations in BCP and PC variants, as they are genotype-dependent, as outlined above. Genotype C favours the BCP mutation (175) therefore

HBeAg negative disease is common in Asia with an estimated prevalence of about 77% amongst HBeAg-negative patients. PC mutants are almost universal in HBeAg negative patients in the Mediterranean basin, has a 50% prevalence in Asia and about 25% in USA and Northern Europe (180).

1.5 Laboratory diagnosis of HBV infection

HBV infection is diagnosed using serological markers and serum viral load. The natural history of HBV is largely dictated by the production of viral proteins and their interaction with the immune system to form corresponding antibodies. Serological markers used in clinical practice include HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc IgM and IgG. These markers allow both the identification of infection as well as the clinical phase of infection phase of infection, which form the backbone of a patient's assessment. Serological markers are used to make decisions regarding treatment initiation, and they are also used to monitor response to antiviral therapy.

1.5.1 HBsAg and anti-HBs

HBsAg is the serological hallmark of HBV infection and is the first antigen to become detectable in the serum of an infected individual. It appears early, within 6-12 weeks following infection. Persistence of HBsAg for more than 6 months indicates chronic HBV. The serum HBsAg level is a marker of viral replication and infectivity (181-183). The titre of HBsAg is usually higher in HBeAg positive disease. HBsAg correlates with HBV DNA serum titre and intrahepatic cccDNA in HBeAg positive patients but that correlation is not strong in HBeAg negative patients. This could be due to the fact that in HBeAg negative disease, there is a significant amount of translation from integrated sequences, which results in the formation of fewer fully formed capsids but large amounts of HBsAg (181, 184, 185).

Anti-HBs is a neutralising antibody and confers immunity against subsequent infections. In vaccinated people, anti-HBs without the presence of anti-HBc is detected in serum. There is an intriguing serological paradox in HBV whereby anti-HBs can co-exist with HBsAg and is not associated with functional cure or viral clearance, whereby HBV DNA is undetectable. It was initially thought that due to the high rates of mutations, anti-HBs and HBsAg that co-exist are genomically heterologous but this has been disproved. It is possible that all HBV carriers do in fact have anti-HBs but the large concentration of HBsAg renders those antibodies undetectable. Anti-HBs may be present but the response too weak or directed against other epitopes and viral clearance is not achieved (186, 187).

1.5.2 HBeAg

HBeAg is a marker of viral replication but is not used for diagnostic purposes as HBV DNA is a far more reliable marker of active viral replication. HBeAg seroconversion is, however, an important intermediate outcome in individuals with CHB. Seroconversion, characterised by the disappearance of HBeAg and the formation of anti-HBe antibodies, is a pivotal point in the natural history of CHB. Early seroconversion, which occurs before the age of 40 regardless of whether it is treatment-induced or spontaneous, is associated with clinical remission and a phase of inactive liver disease (188, 189), characterised by normal biochemistry and low HBV viral load. HBeAg seroconversion is also associated with lower rates of hepatic complications including liver fibrosis, cirrhosis and a reduced incidence of HCC (190-198). Conversely, delayed HBeAg seroconversion is associated with increased rates of cirrhosis (196, 199).

Although HBeAg seroconversion is not in itself a durable marker of clinical remission, it is associated with increased rates of HBsAg loss and seroconversion as well as viral clearance and increased survival (200).

Spontaneous HBeAg seroconversion is only in the order of 15% (201) but the rate of seroconversion is higher with treatment (202). Pegylated interferon treatment is associated with a 32% rate of HBeAg seroconversion at 6 months (203) and about 20% with NA therapy (204). As detailed above, the response rate can be variable across different HBV genotypes.

1.5.3 HBcAg and anti-HBc

HBcAg is not detectable in serum as it usually remains sequestered in the liver (205). Anti-HBc is the hallmark of HBV infection. With initial infection IgM levels peak then decline after about 6 months as IgG levels rise. IgG to HBcAg will usually persist about 20 years following infection making it a sensitive marker for infection and HBV reactivation is associated with a recrudescence in IgM (205). Anti-HBc levels peak in the immune active and immune reactivation phases of disease and are lower in the tolerant / inactive phases. Anti-HBc levels correlate with ALT levels, which suggests an important role of anti-HBc in the immune response against HBV (206). Anti-HBc is only induced by the presence of virus, not vaccination, and it is not protective against re-infection.

Isolated anti-HBc

A positive anti-HBc IgG serology in absence of HBsAg and anti-HBs can sometimes be observed. The first instance is in acute infection, there is a window during which

HBsAg has not appeared in the serum yet. The second instance is following the resolution of acute infection, when anti-HBs is present but the levels are too low to be detected. The third instance is seen in patients who have had longstanding CHB and the serum HBsAg has gradually dropped to an undetectable level. Finally, in patients with occult HBV, anti-HBc as well as HBV DNA are present but HBsAg is undetectable, due to the formation of HBsAg mutations that render it undetectable in commercial assays. This phenomenon is further discussed in the section on occult HBV (Error! Reference source not found. Error! Reference source not found.).

1.5.4 HBV Viral load

The detection of HBV DNA in the serum is diagnostic of HBV infection. The HBV DNA titre is used to determine the phase of disease from inactive carrier phase to immune escape phase, predict the risk of HCC development and prognosticate the outcome of disease. HBV DNA titre, along with the serum ALT levels and an assessment of the severity of liver disease, is also used to determine if antiviral treatment is warranted and to monitor response to antivirals including the development of drug resistant mutants (134).

A high HBV DNA level is the strongest predictor of increased cumulative, long-term risk of liver cirrhosis and HCC (207, 208), independent of HBeAg status and ALT levels. HBV DNA is also associated with infectivity (209). In patients receiving treatment, a lower viral load is associated with better response (210, 211). In patients treated with interferon, serum HBV DNA less than 5×10^7 IU/ml is associated with virological response and HBV DNA level $<10^9$ IU/ml predicts HBeAg seroconversion(211).

In patients undergoing treatment with NAs, serum DNA of more than 200 IU/ml after 6 months of lamivudine signals the development of drug-resistant mutants(212) and therefore on-treatment HBV DNA monitoring can help identify the emergence of drug resistance.

1.6 Epidemiology of HBV infection

HBV has infected more than 2 billion people globally and of those, an estimated 250 million people have become chronic carriers (213-216). The regions of highest prevalence are the Western Pacific region and the African continent (216).

1.6.1 Acute Hepatitis B

Symptomatic acute hepatitis B (AHB) predominantly affects adults, with the highest rates occurring amongst persons aged 30-39 years old (2.0/100,000), and the lowest

rates in persons less than 19 years old (0.04/100,000). The most common risk factors for the acquisition of AHB include sexual exposure and injecting drug use.

Vaccination has had a significant impact on the incidence of AHB. In the United States, the rate of acute infection has declined since 1990, falling from 8.5 to 0.9 / 100,000 population in 2011, the lowest rate ever recorded (217, 218).

In Europe, the overall rate of acute cases is 0.6 cases per 100 000, with a wide range of endemicity ranging from 0 cases in Luxembourg and Iceland to 3.4/ 100 000 cases in Latvia (1).

Again, vaccine uptake has risen from 30% in 2000 to 82% in 2014 and consequently there has been a steady decline in cases of AHB from 1.6 per 100,000 population in 2006 to 0.7 in 2014 (219). A similar trend has been observed in Egypt, where the frequency of acute HBV infection as a cause of symptomatic hepatitis decreased from 43.4% in 1983 to 28.5% in 2002, following the introduction of childhood immunisation in 1991 (220).

1.6.2 Chronic Hepatitis B

In contrast to AHB, the prevalence of chronic hepatitis B (CHB) is on the rise. This is likely due to the fact that in regions where CHB is endemic, the access to vaccination at birth is sub-optimal(221). Between 240 - 400 million people have been estimated to have chronic hepatitis B (215). HBV infection is the 10th leading cause of death worldwide (215).

Chronic hepatitis B is endemic, with an infection rate of more than 7% in South East Asia, China, sub-Saharan Africa, Micronesia and Polynesia. It is also endemic in some of the indigenous populations of Alaska, Northern Canada, Greenland, Australia, and New Zealand (222).

Most infections are acquired early in childhood and the risk of chronicity is inversely related to the age of infection. Perinatal infection leads to chronicity in > 90% cases. In contrast, infections acquired later in life tend to have a symptomatic acute phase but only a small proportion of immunocompetent patients develop chronic HBV (< 5%) (223). Approximately 45% of the global population lives in an area of high prevalence. Moderate prevalence rates of 2-7% are seen in the Southern regions of Eastern and Central Europe, the Amazon Basin, the Middle East, and the Indian subcontinent. Low prevalence regions include much of North America, the United Kingdom and Northern Europe, where the incidence of chronic HBV infection is less than 2%. In these countries, HBV is seen predominantly in immigrants from countries with high prevalence as well as in specific groups with percutaneous and sexual risk factors.

1.6.3 Transmission of HBV

The advent of the hepatitis B vaccine has greatly reduced HBV transmission worldwide. One of the most impressive outcomes has been shown in Taiwan where, following a national immunisation programme, the prevalence of HBV in children dropped from 9.8% to 0.5% with a corresponding drop in rates of HCC (224, 225). The mode of transmission is different in various geographical areas. In countries where HBV is endemic, perinatal transmission and horizontal transmission in early childhood are the main routes of transmission. In low-prevalence areas, unprotected sexual contact and intravenous drug use tend to be the main modes of transmission(226).

Perinatal transmission

HBV transmission from HBV positive mothers can occur *in utero*, but most will occur at the birth(227). The rate of transmission can be up to 90% but the risk is significantly reduced when the newborn is immunised and receives HBV immunoglobulin at birth(228). The risk of transmission is highest with a high maternal viral load and maternal HBeAg positivity at the time of delivery. Current standard of care includes treating pregnant mothers with CHB if they have advanced fibrosis or cirrhosis, or if they have a high HBV viral load of greater than 200 000 IU/ml or a HBsAg titre > 4log₁₀ IU/ml. Despite immunoprophylaxis, transmission rates are about 9% in mothers who are HBeAg positive and if the maternal HBV VL exceeds 10⁸ IU/ml (229).

HBeAg in perinatal transmission

HBeAg positivity is a risk factor for HBV transmission (230, 231). This association is independent of maternal HBV viral load. Studies in the field suggest that HBV transmission rate is low, in the order of 0.4% in HBeAg negative mothers and that a high titre of maternal anti-HBe may be protective against maternal- foetal transmission of HBV, regardless of viral load (232). It has been suggested that HBeAg can be used as an indicator of transmission and anti-HBe as an absence of transmission from mothers to children (233). However, there is a paucity of evidence in the area, as well as significant variability in both host and virus characteristics.

Although the exact mechanisms are still under investigation, it is thought that HBeAg can cross the placenta and may induce immune tolerance in the foetus, therefore establishing chronic infection in infants(234). Mouse studies have shown that maternal HBeAg can predispose the offspring macrophages to reduce CD8+ T cell responses hence allowing viral persistence (235).

1.7 Clinical manifestations of HBV

Clinical manifestations of HBV infection lie along a spectrum, both in acute and chronic disease. People who acquire HBV as children tend to have a fairly asymptomatic course but almost invariably, they will develop chronic disease. In contrast, adults who are acutely infected with HBV tend to be symptomatic and can even have a fulminant hepatitis.

In the chronic phase, clinical symptoms can range from being completely absent to manifestations due to advanced disease and complications of HBV including cirrhosis and HCC. HBV infection is also associated with a number of extra-hepatic manifestations, in both the acute and chronic phases.

1.7.1 Clinical manifestations of acute hepatitis B

Symptomatic acute hepatitis manifested by jaundice occurs in about 30% patients. Co-infection with other hepatotropic viruses or the co-existence of another form of liver disease is generally associated with a more clinically severe hepatitis. Fulminant liver failure from HBV infection is rare in the order of 0.1-0.5% (236).

The incubation period of HBV lasts 1-4 months. In the prodromal period a serum sickness-like syndrome may be present. Serum sickness symptoms generally include non-specific symptoms of fever, joint pain, fatigue and a rash. It can then progress to clinical symptoms of hepatitis including jaundice, nausea, anorexia and abdominal pain in the right upper quadrant. On average, these symptoms last 1-3 months although fatigue can persist beyond that time frame.

Biochemical disturbances occur during the acute phase with elevations in serum transaminases including alanine and aspartate aminotransferase levels (ALT and AST). Transaminitis resolves within the first 4 months and persistence of abnormal liver function tests beyond the 6-month mark suggests the development of chronic hepatitis. Patients may have normal levels of bilirubin or be icteric. Following recovery from AHB, serum antibodies to the surface and core antigens remain detectable and HBV DNA can also persist in the hepatocyte for a long period of time (237).

1.7.2 Fulminant Hepatitis B

Fulminant hepatitis B is rare, occurring in less than 1% of acute cases. Risk factors for fulminant hepatitis B include alcohol and drug use including the use of paracetamol and weight loss (238). Genotype D also appears to be associated with fulminant liver failure (238, 239).

1.7.3 Clinical manifestations of chronic hepatitis B

The risk of progression to chronic hepatitis is strongly dependent on the age of acquisition. The majority of patients who progress to chronic disease have acquired the infection in early childhood or perinatally and tend to be from areas of high endemicity. People with CHB can be asymptomatic or have non-specific symptoms such as fatigue and evidence of liver disease only becomes apparent during flares or in advanced disease.

In cirrhosis, typical clinical symptoms would include jaundice, ascites and peripheral oedema, encephalopathy and features of portal hypertension such as splenomegaly. These features are not specific to HBV infection. The biochemical profile of patients with CHB can reveal a mild transaminitis but many patients with chronic HBV infection do not have an active hepatitis.

Phases of disease in chronic hepatitis B.

CHB is categorised into five stages based on the presence of HBeAg, HBV DNA levels, transaminitis and the presence of liver inflammation. A new nomenclature has recently been developed with an aim to distinguish between the presence of infection versus the presence of infection with concurrent active hepatitis (134). However, some patients may not fit precisely into a specific phase and clinical approach needs to be individualised. Characteristics of each phase are outlined in **Error! Reference source not found..**

	HBeAg positive chronic infection	HBeAg positive chronic hepatitis	HBeAg negative chronic infection	HBeAg negative chronic hepatitis	Occult hepatitis
HBsAg	4.5-5 log ₁₀ IU/ml	3-4.5 log ₁₀ IU/ml	< 3 log ₁₀ IU/ml	3-4.5 log ₁₀ IU/ml	Negative
HBeAg	Positive	Positive	Negative	Negative	Negative
HBV DNA	>10 ⁷ IU/ml	10 ⁴ -10 ⁷ IU/ml	<2000 IU/ml	>2000 IU/ml	Positive
ALT	Normal	Elevated	Normal	Elevated	-
Liver Fibrosis	None/Minimal	Moderate/severe	Moderate/ severe	Moderate/severe	-

Table 1.2 Phases of disease as defined by EASL guidelines (134)

Phase 1 is a phase of HBeAg-positive chronic HBV infection, and was previously referred to as the 'immune tolerant' phase. Patients in this phase of disease have very high levels of viraemia and are therefore highly contagious. However, they do not have an active hepatitis as evidenced by normal transaminase levels and the absence of fibrosis on liver histology. This long period of low immunoreactivity is clinically asymptomatic and usually observed in children who acquire HBV perinatally. It was long thought that newborns have a deficient adaptive immune response and are therefore naturally tolerant to HBV. However, this view has been challenged as neonates have been shown to possess adequate immune maturation with the development of a T helper cell type 1 (TH1) response (240).

Phase 2 is one of HBeAg-positive chronic hepatitis B, and usually occurs after years or decades spent in phase 1. In this phase, there is an active hepatitis, characterised by an elevation in serum transaminases as well as evidence of inflammation and possible fibrosis on histology. This phase was previously referred to as a phase of immune clearance, during which the immune system begins to target HBV-infected cells. Patients may be symptomatic with a clinical syndrome of hepatitis. Biochemically, it coincides with an elevation in transaminases, a reduction in viral load, and a drop in HBsAg titre. This phase can lead to HBeAg seroconversion.

Phase 2 is characterised by flares of viraemia followed by immune mediated control that falls short of complete HBV clearance. Recurrent flares can lead to cumulative deposition of scar tissue that eventually culminate in extensive liver fibrosis and cirrhosis.

Phase 3 is a state of HBeAg-negative chronic HBV infection characterised by minimal viral replication and low-grade inflammation. It is a phase of relative immune control whereby the immune system can suppress viral replication. This phase was previously termed the 'inactive /silent carrier' phase as there are no clinical or biochemical manifestations of disease and the viral load is low, but this term has now been relinquished as progression of disease can occur despite the lack of overt clinical or biochemical features. During this period of viral latency, HBV replication is controlled by the adaptive immune system in particular HBV-specific CD4⁺ helper T cells, CD8⁺ cytotoxic T cells, B cells and cytokines such as interferon gamma (IFN-γ) and tumour necrosis factor (TNF) (241).

Phase 4 is a phase of HBeAg-negative CHB, whereby the virus escapes immune control and starts replicating actively again. Viral replication triggers an immune response and an active hepatitis ensues. This phase is generally associated with the development of a transaminitis that can fluctuate, a recrudescence in HBV viraemia and histological evidence of inflammation and fibrosis. Immune escape is often due to

either the formation of mutants that escape immune surveillance or immunosuppression of the host (242). Flares of disease can lead to liver failure and death.

Phase 5 is the HBsAg-negative phase also known as 'occult HBV infection'. This phase is characterised by an absence of HBsAg without the appearance of anti-HBs(134).

1.7.4 Complications of chronic hepatitis B

The main complications of CHB are the development of cirrhosis and HCC. The host immune response to the virus determines the risk of progression to fibrosis, cirrhosis and HCC.

Cirrhosis

The cumulative risk of developing cirrhosis over five years in an untreated adult with CHB varies from 8% to 20%. In patients with CHB who already have cirrhosis, the five year cumulative risk of decompensation is about 20% and the risk of HCC is high, in the order of 2-5% (243-245).

The risk of progression to cirrhosis is influenced by host and environmental factors as well as viral factors. HBV viral load, levels of inflammation (as indicated by the ALT level), and HBeAg status are strong determinants of overall risk for progression to cirrhosis (207, 246). Alcohol consumption is associated with a more rapid progression to cirrhosis and HCC and worse survival (247, 248). HBeAg positivity at the time of cirrhosis is also associated with worse survival outcomes whereas HBeAg seroconversion with viral control are associated with improved outcomes (249).

Hepatocellular carcinoma

HBV infection is associated with up to a 25-fold increase in HCC risk and it is the underlying aetiology for more than half the HCC worldwide (250, 251). As the incidence of HBV varies geographically, so does the incidence of HBV-related HCC. For instance, in China, HBV accounts for about 65% of HCC compared to 18% in Western and Northern Europe.

The risk of HCC development in a cirrhotic patient is about 2–5% cumulatively over 5 years (252). The majority of HCC occur in the context of cirrhosis but in CHB, about a third of HCC develop in absence of cirrhosis (253). In fact, even after HBsAg clearance from the serum, the risk of HCC is still present due to the presence of integrated viral DNA in hepatocytes (246, 254, 255).

HBV- related carcinogenesis is multifactorial. It is thought to be a combination of insertional mutagenesis whereby HBV integrates in the host genome causing genomic

instability, direct effect of viral proteins such as HBx, HBsAg and miRNAs (256) and the dysregulation of cellular processes such as apoptosis and DNA repair (257). Chronic inflammation may hasten the process as it places increased oxidative stress and increases necrosis and cell turnover.

HBV treatment can reduce the incidence of HCC but does not eliminate it completely (258). Pegylated interferon treatment has been shown to reduce HCC incidence especially in cirrhotic patients (259, 260). HCC risk also decreases with treatment with nucleos(t)ide analogues although the risk is still higher than an uninfected population or untreated patients who are in the immune control phase (261). A failure to maintain virological remission and HBeAg negative status at baseline are associated with higher HCC risk (262, 263). The risk of development of HCC is significantly reduced with HBsAg seroclearance but it is not eliminated altogether (264).

Both host factors and virus factors determine the risk of developing HCC. Host factors include the presence of cirrhosis, older age, male sex, being of African descent and a family history of HCC. Other factors that increase the risk of HCC include co-infection with hepatitis C or delta or HIV, duration of disease and male gender, alcohol consumption (265) and the co-existence of other carcinogens in particular tobacco and aflatoxin (250).

Significant virus-related factors include high viral load of greater than 2000 IU/ml, high HBsAg titres, HBeAg positivity, cirrhosis and certain genotypes and mutations as outlined above (198, 207, 246, 258, 266). Genotype C is associated with a high risk of HCC compared to genotype B (267). As previously detailed, the T1762/A1764 BCP mutation is associated with an increased risk of HCC. Other mutations associated with an increase in HCC risk include pre-S deletion mutants (268), stop codons at positions 172 or 182 of the S gene (269) and, as the S ORF overlaps with Pol, mutations induced by antiviral therapy in the Pol reading frame can affect the S gene. The rtA181T/sW172* mutation, which arises as a result of lamivudine or adefovir treatment can generate a stop codon on the S gene which has oncogenic potential and is therefore associated with HCC (270).

Multiple scores have been developed in an attempt to stratify HCC risk, including the GAG-HCC, the CU-HCC and REACH-B scores. The GAG (Guide with age, gender, HBV DNA, core promoter mutations and cirrhosis)-HCC score was initially derived to include the presence of BCP mutations. However, as BCP mutation is not routinely tested in clinical practice, the score was modified to exclude BCP (271). The score is heavily weighted to cirrhosis, male sex, age and HBV DNA. The negative predictive value for excluding HCC at 10 years is close to 100% but the positive predictive value is 26%. The sample size in developing the GAG-HCC score was quite small and the

cohort did not have a validation set.

The CU (Chinese University)-HCC score (CU-HCC) was developed in a prospective HCC study over a median follow-up of 10 years. It takes into account cirrhosis, HBV DNA and age as well as albumin and bilirubin levels(272) . The negative predictive value to exclude HCC at 10 years was 97% and the positive predictive value was 27%. The REACH-B score was developed using nomograms based on data from 3653 patients(273). REACH-B was independently validated in over 1500 patients (274). The REACH-B score factors are male sex, age, alanine aminotransferase (ALT), positive HBeAg and HBV DNA. REACH B does not include cirrhotic patients and offers a graded risk prediction ranging from 0-17.

The predicting factors for risk scores are similar but the weight attributed to each is variable. These scores have high negative predictive value to exclude HCC and can be used to stratify risk.

1.7.5 Extrahepatic manifestations

HBV primarily affects the liver but can also manifest in other organs. These manifestations are not due to the virus itself but rather due to the deposition of immune complexes, which reinforces the concept that HBV-related disease is primarily immune in origin. Immune complexes can affect the skin, musculoskeletal system and kidneys. 10—20% people with acute HBV infection will develop a transient serum sickness-like syndrome (275). Symptoms of low-grade fevers, skin rash and joint pains tend to precede the onset of jaundice and resolve after recovery. This is due to the presence of circulating immune complexes composed of HBsAg and complement factors. Renal involvement is relatively common in HBV with some studies citing an incidence of about 35% (276). The most common glomerulonephritis is membranous glomerulonephritis caused by the accumulation of immune complexes in the glomerular basement membrane, which then triggers an immune response. These patients tend to present with the nephrotic syndrome, a triad of proteinuria, chronic renal failure, and hypertension. The seroconversion of HBeAg usually heralds the resolution of membranous glomerulonephritis. Other extrahepatic manifestations include polyarteritis nodosa, mixed cryoglobulinaemia and neurological manifestations.

1.8 Management of HBV infection

1.8.1 Acute hepatitis B

In most immunocompetent adults, acute infection with HBV resolves spontaneously and treatment is therefore mainly supportive. Precautionary measures are taken to prevent the spread of infection to exposed contacts. Antiviral therapy is not

recommended for acute infection and may impede on the formation of neutralising antibodies if administered early in the course of disease. However, about 1% of patients with acute HBV can progress to liver failure and the use of antiviral agents may be instituted if there is any evidence that the patient is developing liver failure(134). Generally, only patients who show features of a severe or fulminant hepatitis require treatment and hospitalisation. Fulminant liver failure resulting from acute HBV infection is rare in the order of less than 1 %. It is characterised by massive necrosis of the liver parenchyma and manifests as jaundice, encephalopathy and the development of a coagulopathy Risk factors for acute liver failure due to hepatitis B include alcohol and drug use including the use of paracetamol and weight loss and co-infection with hepatitis D. There is also an association with genotype D infection (238, 239). Although the viral factors associated with the development of fulminant hepatitis B are not fully understood, mutations in the basal core promoter and the precore mutation have been associated with fulminant hepatitis. Cell culture studies showed increased viral replication and downregulation of HBeAg expression(277-279). However, this has not been a consistent finding in other studies, in which genomic variability did not seem to be associated with the incidence of fulminant or acute hepatitis(280). It is likely that the severity of infection is dependent on the interplay of genomic variation in the virus and host factors, by mechanisms that are yet to be elucidated.

1.8.2 Chronic hepatitis B

Principles of management

The primary goals of antiviral therapy in chronic HBV infection are to reduce mortality and morbidity by limiting disease progression and preventing the development of HCC. In patients with fibrosis, a regression in fibrosis can occur with adequate antiviral treatment. In patients with cirrhosis, the mortality benefit is not clear but the rates of decompensation of liver disease and HCC development are still significantly reduced (281). Additional benefits of antiviral therapy include the prevention of HBV- related extrahepatic manifestations and the reduction in disease transmission.

The stage of disease, timing of therapy and patient's age determine response to therapy and prognosis. Important milestones in CHB therapy include HBeAg loss and seroconversion, suppression of HBV viral load and HBsAg loss and seroconversion. HBeAg loss is associated with a state of immune control and is generally associated with a reduction in viral replication. However, in isolation, it is not a reliable endpoint to therapy as discontinuation of antiviral agents following HBeAg seroconversion can lead to seroreversion (282).

The main marker of disease progression and prognosis in CHB is viral replication (134). Suppressing viral replication reduces hepatic inflammation, fibrosis and can reduce the risk of HCC. Therefore, an important endpoint of therapy in CHB is the inhibition of HBV DNA (245, 283-285). An undetectable HBV viral load is usually accompanied by normalisation of liver enzymes. Persistently elevated transaminases despite HBV DNA being low or undetectable is usually due to the presence of a concurrent pathologic process such as co-infection with HDV, alcoholic liver disease or fatty liver disease (286).

HBsAg loss is the ultimate immunological therapeutic goal, an endpoint termed the 'functional cure'. HBsAg loss enables the cessation of treatment (264, 287) as the risk of reactivation is only a concern in severely immunocompromised patients (288). HBsAg clearance is not a definitive cure for HBV as cccDNA still persists in the hepatocytes which carries a small risk of reactivation and a small risk of HCC of 0.5% per year is still present even after HBsAg loss (289). Currently with our current antiviral therapy a complete cure, with full eradication of virus and cccDNA is not achievable.

Indications for therapy

The indications for treatment are based on viral load, levels of inflammation (as indicated by the serum transaminase levels) and the severity of liver disease.

Generally, patients who have active disease with elevated viral loads (> 2000 IU/ml in HBeAg negative patients and > 20 000 IU/ml in HBeAg positive patients), elevated ALT more than twice the upper limit of normal and/or at least moderate fibrosis ought to receive therapy. A lower threshold applies if the patient is cirrhotic. As a general rule, all patients with cirrhosis and a detectable DNA level should be treated (290). The cut-off of HBV DNA of 2000 IU/ml is chosen as complications tend to arise beyond above that level (207, 246). The cut-off for the upper limit of normal of the ALT can vary depending on society guidelines (134, 281). A lower threshold is also observed for patients deemed at higher risk such as those with a family history of cirrhosis and/or HCC, older patients and patients with extrahepatic manifestations.

In patients undergoing liver transplantation for HBV-related pathology, prophylactic treatment is routinely administered. In pregnancy, treatment is indicated in the third trimester in patients with high viral load to reduce the risk of vertical transmission. HBV carriers about to undergo immunosuppressive therapy including chemotherapy also receive prophylactic treatment to prevent reactivation (290).

1.9 Antiviral therapy for CHB

Treatment options for CHB infection includes interferon (IFN) therapy and oral nucleos(t)ide analogues (NA). NAs are more commonly utilised in clinical practice as they have a better safety and tolerability profile compared to IFN especially in cirrhotic patients. IFN is the only treatment option for hepatitis D infection although success rates are limited (291).

1.9.1 Nucleos(t)ide analogs

The most commonly used NAs in clinical practice are entecavir and tenofovir. Although lamivudine is still available, its use is limited by its broad resistance profile. NAs are very effective at reducing HBV DNA, but they do not affect transcriptional activity. Despite that, a reduction in cccDNA and intra hepatic HBV DNA is observed (292) but that effect is modest and does not lead to complete cccDNA eradication (293) such that long-term, if not lifelong, treatment is usually warranted (294-297). Furthermore, NA therapy leads to only minimal reductions in HBsAg (298) and does not eliminate the long term risk of HCC development in CHB (299). However, flares associated with NA cessation are associated with a decrease in HBsAg levels, which could be an interesting therapeutic strategy although further studies on the safety and feasibility are required.

To improve the efficacy of NAs in suppressing HBsAg, combination treatments have been studied. The combination of NAs and pegylated interferon alpha (pegIFN) seems appealing due to their vastly different modes of action but results from clinical trials so far have been disappointing. The combination of tenofovir and pegIFN is associated with an increase in HBsAg loss but the absolute rates of HBsAg clearance are still disappointingly low (300). The combination of entecavir and pegIFN can lead to a greater reduction in HBV DNA but the effect is not sustained (301). The concurrent use of ETV and pegIFN also appears to accelerate HBeAg seroconversion but in the long-term there is no significant difference in the rates of HBeAg seroconversion (302). Sequential therapy with tenofovir and pegIFN may increase the rate of HBeAg clearance and increases the likelihood of HBsAg clearance in HBeAg positive patients (303, 304). However, given the side effects of pegIFN, the small gain in HBsAg clearance is not justifiable clinically. The combination of two NAs has not demonstrated any clear benefit and dual NA therapy is only utilised for rescue therapy currently (134).

1.9.2 Pegylated interferon alpha

PegIFN modulates the innate immune response. PegIFN drives the proliferation and activation of NK cells (305) but the effect of pegIFN on restoring CD8⁺ T cell activity is minimal, which may explain its limited efficacy (306).

Treating with pegIFN for one year will achieve immune control, with undetectable HBV DNA in about a third of patients and achieve HBeAg seroconversion in about 70% patients. Over time, HBsAg clearance averages about 30% (190, 307, 308). The main appeal of pegIFN treatment is its finite duration, but side effects can be prohibitive. About a third of patients treated with pegIFN experience significant side effects including flu-like symptoms, headaches, myalgia, debilitating fatigue, depression, neutropenia and thrombocytopenia. PegIFN is also associated with a risk of flares whilst on treatment which can lead to decompensation in cirrhotic patients. For that reason, pegIFN is generally not suitable for patients with cirrhosis, patients with portal hypertension, pregnant patients and those with underlying haematological conditions, psychiatric and autoimmune disorders.

In HBeAg positive patients, predictors of response to pegIFN include a low HBV VL of 2000 IU/ml or less, a high ALT and a high activity score on histology. As serum ALT levels and HBV DNA tend to fluctuate over time, more robust predictors are required. A drop in HBsAg levels during treatment may be a better predictor of response (309, 310) and host genetic testing could provide a more reliable predictor of response but at present, they are not part of clinical practice (134).

Patients infected with genotypes A and B tend to have higher response rates compared to D and C respectively. Young and female patients tend to have more favourable response rates as well (311). In HBeAg negative patients with genotype D infection the addition of pegIFN is associated with a significant decrease in HBsAg levels (312).

1.9.3 Novel antiviral agents

As neither NAs nor pegIFN offer a permanent cure, novel therapeutic strategies are currently in various stages of preclinical and clinical testing. These include entry inhibitors, small interfering RNAs, and a plethora of immunomodulators.

Entry inhibitors

Entry inhibitors primarily target the NTCP receptor. The well-tested agent is Myrcludex B, a myristylated PreS1 peptide that binds competitively to the NTCP receptor (313). Myrcludex-B can reduce the cccDNA pool and prevent the spread of HBV to uninfected hepatocytes in vivo (314). It does not directly target cccDNA and as both cccDNA

decay and hepatocyte turnover are both slow processes, it is unlikely that Mycludex B will have a significant impact on the cccDNA pool, if used in isolation(315).

There is also a concern about the intrahepatic accumulation of bile acids associated with the use of Mycludex B that could be prohibitive in the clinical setting. However, clinical trials so far have demonstrated its potential, especially if used as part of a combination therapeutic strategy (316).

RNA Interference

Small interfering RNA (siRNAs) can target specific HBV gene expression and so far, they have been developed to target HBsAg. However due to the overlapping nature of the reading frames in the HBV genome, the transcription of other proteins is likely to be affected collaterally. There are also some concerns about the possible deleterious impact on other somatic genes (317). There are currently 3 agents that are being tested in clinical trials that have shown promise: ARC-520, which effectively reduces HBeAg and HBV core-related antigen, ALN-HBV, which can precipitate therapeutic flares characterised by an elevation in viral load and serum transaminases and TKM-HBV which reduces HBeAg, HBsAg, HBV DNA and cccDNA (318).

Toll-Like receptor agonists

Toll-like receptors (TLR) play a key role in recognising HBV and triggering the cascade of immune response. They recognise viral nucleic acids and induce innate immune response in particular IFN-I. A TLR agonist can enable the exploitation of the innate immune system to bring about viral eradication. GS-9620 targets TLR7 and stimulates production of IFN- α and downstream mediators of the IFN-I pathway including NK cells and T cells (319) with an accompanying rise in the levels of interferon-inducible gene 15 (320). Clinically, the drug has been tested in chimpanzees and has been shown to induce the clearance of HBV-infected cells with a reduction in serum viral load, HBsAg and HBeAg (321).

Retinoic acid- inducible gene ligands

Retinoic acid- inducible gene- 1 (RIG-1) is a potent antiviral that triggers a Type 3 IFN response upon recognition of HBV pgRNA and also interferes with the action of the HBV polymerase on pgRNA. SB 9200 is an immunomodulator that activates both the RIG-I and NOD2 pathways and has It has an antiviral effect on a large number of RNA viruses especially HCV (322). It has been shown to increase the production of interferon inducible genes (ISG) (323) and has demonstrated antiviral activity against HBV in a woodchuck preclinical model (324). Clinical trials have yet to be published.

Checkpoint inhibitors

T cell exhaustion is thought to be a significant contributor to the pathogenesis and persistence of HBV in chronic infection (325). T cell exhaustion is associated with the presence of inhibitory molecules, so-called checkpoints and the inhibition of those checkpoints could potentially restore T cell function. Nivolumab and pembrolizumab, are two checkpoint inhibitors currently in clinical trial. They are monoclonal antibodies against the Programmed Cell Death protein 1 (PD-1) and have been very effective in the treatment of cancer. However, the incidence of severe adverse events may make its use less attractive in CHB. Nivolumab has been tested in the treatment of HCC (326) and it is likely that it will be tested for use in CHB in future.

Cellular inhibitors of Apoptosis

The cellular inhibitors of apoptosis (cIAP) proteins are critical decision makers in determining whether the pleiotropic TNF will promote cellular activation and survival or cell death. In the absence of cIAPs, TNF signalling culminates in cell death. One study examined if endogenous TNF could be harnessed to promote the death of infected hepatocytes rather than promoting cellular activation. HBV infection was induced in cIAP deficient mice and these animals rapidly cleared infection with minimal collateral damage (327). In another study, a cIAP antagonist was shown to promote clearance of HBV in a preclinical model (328). The clinical potential of cIAP antagonists is still being explored.

1.10 Immunopathogenesis of CHB

HBV in itself is not a cytopathic virus and liver injury in HBV is primarily mediated by the immune response. Transgenic mice, which are genetically engineered to have HBV integrated within their genome, are tolerant to the virus and they do not develop liver injury despite high levels of viral replication (329). Similarly, in chimpanzees, at the peak of viral replication, T cell activity is minimal with no evidence of cytotoxicity (330). In humans the immune response determines the natural history of CHB as detailed above (331).

The immunology of HBV is complex, involving the interplay of various mediators. The mechanism of clearance is likely a combination of CD8⁺ T cells that kill HBV-infected hepatocytes, CD4⁺ T cells and innate immune cells that secrete cytokines which inhibit viral replication, kill infected cells and prevent re-infection. The virus is not passive during this immunological process as it has several characteristics that allow it to evade / escape the host response (332).

1.10.1 Innate immune system

The innate immune system is engaged in the early phases of infection and its primary purpose is to limit the spread of the infection and initiate recruitment of the adaptive immune response.

The innate immune system is activated upon recognition of pathogens or pathogen-derived structures by pattern-recognition receptors (PRRs). In viral infections, the main PRRs are Toll-like receptors, RNA helicases, such as RIG-I, melanoma differentiation associated gene 5 (MDA5), and double stranded RNA-dependent protein kinase (PKR) (333). When PRRs recognise virus-associated patterns, signalling pathways are triggered to produce cytokines to help eliminate the pathogen. The main events that occur in early phases of the innate immune response include the activation of natural killer cells and the activation of the type 1 IFN pathway.

1.10.2 Toll Like receptors

Toll-like receptors are crucial in the activation of the innate immune response following the recognition of pathogenic elements known as pathogen-associated molecular patterns (PAMPs) (334). TLRs consist of an ectodomain that binds and recognises PAMPs and a cytoplasmic region that mediates downstream signalling (335). TLR ligands include lipids, lipoproteins, proteins and nucleic acids and binding of those ligands induces conformational changes that activate transcription factors. Transcription factors include Interferon Regulatory Factors (IRF) 3/5/7, NF- κ B, and Activator Protein-1 (AP-1) and their activation leads to the induction of IFN-I and other cytokines that mediate the antiviral response (336, 337). Adaptor proteins including myeloid differentiation primary-response protein 88 (MyD88), TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor protein inducing interferon- β (TRIF), and TRIF-related adaptor molecule (TRAM) are also recruited (338). The MyD88 pathway results in the production of pro-inflammatory cytokines in particular IL-6, IL-10, IL-12, and TNF whereas the TRIF pathway activates IFN regulatory factors and leads to the production of type I IFNs (336, 338).

TLR in HBV

Injection of TLR3, TLR4, TLR5, TLR7, and TLR9 ligands can suppress HBV replication in an IFN-I -dependent manner (339). *In vitro*, murine cells that are stimulated by TLR3 and TLR4 significantly inhibit HBV replication. TLR3 activation leads to the production of IFN-I in particular IFN- β by hepatic non-parenchymal cells (340). IFN- β can inhibit HBV replication by either destabilising capsids or preventing capsid assembly (341, 342).

TLR4 is activated by lipopolysaccharides. A significant reduction in HBV replication has been demonstrated in the woodchuck model with administration of TLR4 ligands. However, TLR4 activation only leads to very low-grade production of IFN- β by Kupffer cells (340). It is likely that the TLR4 effect is largely independent of IFN-I (343). TLR2 ligands have also been shown to reduce HBV replication in an interferon-independent manner (344). Both TLR4 and TLR2 mediate the same antiviral pathways downstream of MyD88 activating pathways such as NF- κ B, MAPK, PI3k/ Akt pathways (343).

As is commonly observed in various aspects of HBV immunity, HBV has evolved to evade TLRs. It has been shown that HBeAg and HBsAg inhibit activation of non-parenchymal cells by TLR 3 ligands (340). HBsAg, HBeAg and HBV virions abrogate TLR –associated antiviral activity including inhibition of IFN and an increase in interleukin-10 (IL-10) activity (345). In patients with CHB, TLR3 expression on peripheral blood mononuclear cells (PBMCs) is significantly reduced but treatment with pegylated interferon alpha can help restore their expression partially (346).

TLR2 expression is also reduced in CHB, as demonstrated both in studies utilising PBMCs from infected patients as well as in preclinical models (347, 348). HBsAg has been shown to inhibit TLR2 activity in a dose-dependent manner (349). HBeAg also helps the virus evade TLR2. There is a significant downregulation of TLR2 in Kupffer cells and hepatocytes in HBeAg positive patients compared to HBeAg negative patients (29).

1.10.3 Natural killer cells

NK cells are activated early, within 48 hours, of HBV infection and their appearance is associated with a drop in HBV replication (350). They can either directly recognise and stimulate the elimination of virally-infected cells as well as inhibit HBV replication through the secretion of cytokines, in particular IFN- γ , TNF, granulocyte-macrophage stimulating factor, IL-10 and transforming growth factor- β (TGF- β) (351). NK cells target cells that express little or no major histocompatibility complex (MHC) class I on their surface in the context of upregulated stress ligands and the presence of certain cytokines in particular IFN, IL-12 and IL-18 (352). Hepatocytes generally express low levels of MHC class I and NK cells are abundant in liver, making up 30-40% intrahepatic lymphocytes. Therefore, NK cell activation can occur readily with HBV, as even a modest upregulation in stress ligands leads to a rapid engagement of NK cells (353). The exact mechanisms of how NK cells lead to viral clearance remain obscure, primarily because the studies performed so far have been in woodchucks and chimpanzees. Human studies are difficult as HBV acute infections do not become

clinically apparent until weeks after infection and by then, the activation and role of NK cells may have already become redundant (354). In the chronic phase of infection, NK cells have demonstrated impaired cytolytic activity (355), which may provide some explanation of viral persistence.

1.10.4 Kupffer cells

Kupffer cells (KC) are intrahepatic “scavenging” macrophages. In acute HBV infection, KCs produce a host of inflammatory mediators including IFN-I, TNF and IL-6 to mediate antiviral effects. HBsAg and KC interact via toll-like receptors (TLR). In chronic HBV infection, the role of KC is different: They secrete anti-inflammatory cytokines such as IL-10, which eventually leads to T cell exhaustion (356) and also inhibit the humoral immune system (357).

1.10.5 Dendritic cells

Dendritic cells (DC) are efficient antigen-presenting cells. The DC involved in HBV are either plasmacytoid DC or myeloid DC. DC present antigens to the immune system and also produce large amounts of IFN- α to inhibit viral replication as described in the mechanisms detailed below. It has been reported that the number of plasmacytoid DC is reduced and their function is lessened in patients with chronic HBV infection. This could be a possible mechanism of viral resistance but it is difficult to confidently ascertain that phenomenon given that DC in the peripheral circulation only accounts for 1% of the total DC population (358, 359).

1.10.6 Type 1 Interferon

Following infection with HBV, hepatocytes release IFN-I (IFN- α and IFN- β) that inhibit viral replication (341, 360, 361). IFN-I is produced in response to secreted proteins (HBsAg and HBeAg) as well as intracellular signalling via the signal transducers and activators of transcription (STAT)-1 pathway. The IFN-I family consists of 12 IFN- α subtypes, IFN- β , IFN-epsilon, IFN-kappa and IFN-omega (362). The two most well-characterised subtypes are IFN- α and IFN- β and will be the focus in this thesis.

IFN-I is produced by most nucleated cells upon detection of intracellular DNA and RNA in infected cells or in macrophages. Virus-derived RNA and DNA are recognised by pattern recognition receptors in the cytosol. RNA is recognised RIG-I-like receptors (363, 364) and melanoma differentiation associated protein 5 which is associated with the mitochondrial antiviral signalling protein (MAVS) (365). The receptors that recognise viral DNA include DEAD-box protein (DDX) 41, cyclic GMP-AMP synthase (cGAS) and IFN inducible protein 16 (366-369). These lead to the activation of the stimulator of IFN genes (STING). Following MAVS and STING activation, TANK-

binding kinase 1 is phosphorylated and interferon regulatory factors are activated, which leads to the production of IFN-I (370).

Both IFN- α and IFN- β bind to the same receptor the IFN- α / β receptor (IFNAR), which is expressed on most cell types. IFNAR consists of IFNAR1 and IFNAR2 subunits, which are associated with tyrosine kinase 2 (TYK 2) and Janus Kinase 1 (JAK 1) respectively. Binding to IFNAR triggers the activation of TYK 2 and JAK 1 and create docking sites for STAT. STATs are phosphorylated and migrate to the nucleus. STAT 1 initiates the transcription of interferon inducible genes (ISGs), which can interfere with different stages of viral replication (371) (372). STAT-1 and STAT-2 can form a heterodimer that recruits interferon regulatory factor 9 (IRF9) to form IFN-stimulated gene factor 3 (ISGF3). The ISGF3 complex then initiates the transcription of ISGs (373, 374). Independent of its role in inducing STATs, JAK also affects multiple signalling pathways including PI3K, NF- κ B and MAPK, which can augment Type 1 IFN signalling.

1.10.7 Type I Interferon in HBV

IFN-I has potent antiviral properties. Cell culture studies have demonstrated that the levels of the replication intermediate pgRNA are up to 10 fold less in cells treated with IFN-I (341, 342). IFN α / β also mediates the activity of antigen presenting cells including Kupffer cells and dendritic cells which then activate natural killer T cells (NKT) cells through the release of interleukin 18 (IL18) and Chemokine Ligand 3 (CCL3) (375, 376). IFN-I also directly promote apoptosis of virally infected cells by directly activating NK cells and upregulating pro-apoptotic proteins including Fas ligand (FasL), PDL-1 and TNF- related apoptosis-inducing ligand (TRAIL) (377).

IFN-I promotes the degradation of viral RNA and DNA via a number of mechanisms (377). It is associated with reduced acetylation of histones, which in turn, increases cccDNA decay (378, 379). It also induces PKR to inhibit RNA translation by blocking the recycling of guanine diphosphate. Furthermore, IFN α induce epigenetic modification of cccDNA and inhibits transcription (380). IFN-I in HBV is also IFN-I may also reduce the activity of Enh I and Enh II (381, 382) although the mechanisms so far remain unclear (382-384).

IFN α / β increases the transcription of ISGs which inhibit viral replication (385). The most well characterised ISGs in HBV include apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC), ISG20, Myxovirus resistance protein 1 (MxA) and Tripartite motif protein (TRIM).

The APOBEC gene encodes cytidine deaminases that disrupt DNA and RNA.

APOBEC3A/B is induced by IFN and can lead to cccDNA degradation via a process of hypermutation (386). ISG20 binds to the epsilon loop structure of pgRNA and inhibits

viral replication (387). MxA interacts with the HBV core protein, and disrupts capsid assembly(388). TRIM decreases the release of viral particles. TRIM 25 is elevated in HBV and can inhibit core promoter activity and viral replication (372). TRIM 14 and TRIM 31 have also been convincingly implicated in the control of HBV replication via the IFN pathway (389-391). IFN-I also modulates the adaptive immune response to enable elimination of virally infected cells.

1.10.8 Viral evasion of the innate immune response

Toll-Like receptors

HBV has evolved to evade various aspects of the immune response. HBV can evade immune recognition. It has been shown that HBeAg and HBsAg inhibit activation of non-parenchymal cells by TLR 3 ligands (340). HBsAg, HBeAg and HBV virions abrogate TLR –associated antiviral activity including inhibition of IFN and an increase in interleukin-10 (IL-10) activity (345). In patients with CHB, TLR3 expression on peripheral blood mononuclear cells (PBMCs) is significantly reduced but treatment with pegylated interferon can help restore their expression partially (346).

TLR2 expression is also reduced in CHB, as demonstrated both in studies utilising PBMCs from infected patients as well as in preclinical models (347, 348). HBsAg has been shown to inhibit TLR2 activity in a dose-dependent manner (349). HBeAg also helps the virus evade TLR2. There is a significant downregulation of TLR2 in Kupffer cells and hepatocytes in HBeAg positive patients compared to HBeAg negative patients (29).

NK cells

Cytolytic activity is also impaired in HBV, In the chronic phase of infection, NK cells have demonstrated impaired activity (355), which may provide some explanation of viral persistence.

Antigen-presenting cells

Antigen-presenting cells are also reduced in HBV . It has been reported that the number of plasmacytoid DCs is reduced and their function is lessened in patients with CHB infection. This could be a possible mechanism of viral resistance but it is difficult to confidently ascertain that phenomenon given that DCs in the peripheral circulation only accounts for 1% of the total DC population (358, 359).

Viral evasion of the Type 1 interferon response

Over time, HBV has evolved a number of strategies to evade the immune response and establish chronicity. There are no DNA sensing pathways in hepatocytes so the virus can establish itself without much interference (392). The IFN-I response in HBV is

significantly blunted as substantiated by *in vitro* studies that clearly demonstrate that IFN transcripts are significantly reduced in presence of HBV (393).

HBV induces a number of proteins that inhibit Type 1 IFN. NF- κ B essential modulator (NEMO) increases IFN- β production by activating IFN regulatory factor 3 (IRF3) (394). HBV induces rubicon, a protein that binds to NEMO to inhibit IFN-I production (395). HBV also induces parkin which modulates the ubiquitin chain on the MAVS complex, hence inhibiting IFN- β synthesis (396). HBV can also affect the IFNAR receptor and inhibit IFN signalling. The virus activates the collagen triple helix repeat containing 1 (CTHRC1), which reduces IFNAR expression thereby reducing IFN-I activity (397). HBV also induces matrix metalloproteinase 9, which promotes the degradation of the IFNAR receptor (398).

HBV viral proteins are also associated with the inhibition of IFN production. Both HBsAg and HBeAg suppress the activity of the Major Vault Protein that upregulates IFN-I production (399, 400) and HBsAg inhibits STAT3 (401). HBV Pol reduces the ubiquitination of STING and therefore decreases IFN production (402). HBx also interferes with IFN-I activity by suppressing TRIM 22 expression (403, 404).

1.10.9 Adaptive immune response

Whilst the innate immune system provides initial viral suppression, viral clearance depends largely on the adaptive immune response. The adaptive immune response comprises a combination of processes including cytolytic killing of infected cells as well as an antiviral cytokine response that promotes death of infected cells, suppresses viraemia and inhibits endogenous re-infection (405, 406). The humoral arm enables clearance of circulating virus in the serum and limits viral spread while the cellular arm eliminates infected hepatocytes.

1.10.10 T Cells

T cells are considered the main effectors of HBV clearance (407). Experiments in chimpanzees utilising both wild-type animals as well as CD4⁺ T cell and CD8⁺ T cell depleted animals have demonstrated an accumulation of CD8⁺ T cells in the liver during clearance (330, 407). Mouse studies, on the other hand, provide robust evidence that CD4⁺ T cells are crucial in the immune response to HBV infection, as mice in which CD4⁺ T cells were antagonised could not control viraemia (327). It is likely that the discrepancy in those animal studies stems from the fact that different phases of infection are being studied, there are gross differences in host genetic factors, including MHC alleles and viral factors including genotypes/ mutants are variable (408). However, it is likely that CD4⁺ T cells are essential to the adaptive

system by promoting CD8⁺ T cell activity and facilitating B cell antibody responses (409).

The activation of CD8⁺ and CD4⁺ T cells starts with antigen presentation by APCs. Cytokines, in particular IL-12 and TNF are released and they induce the production of IFN- γ and proliferation of CD8⁺ T cells. IL-12 also induces CD4⁺ T-cell differentiation into TH1 cells. TH1 CD4⁺ T-cells are directed against peptides c50–69, found in both HBcAg and HBeAg (410, 411). IFN- γ is predominantly produced by CD8⁺ T cells but can also be produced by NKT cells and CD4⁺ T cells (376).

Cytotoxic T cells (CTL) mediate apoptosis of infected hepatocytes both directly and indirectly. Transgenic mouse studies using labelled CD8⁺ T cells have shown a direct engagement of infected cells by CTLs (56) but it is unlikely that that mechanism alone can clear HBV (412, 413). It is probable that non cytotoxic mechanisms are also at play to supplement the activity of CD8⁺ T cells.

Transgenic mouse studies have demonstrated the importance of antibody-mediated viral clearance as well as IFN- γ and TNF mediated viral control (414) using mechanisms that destabilise the nucleocapsid, degrade viral proteins and post-transcriptional RNA (415, 416). It is likely that specific immunological mediators are more prominent during various stages of disease (417, 418).

1.10.11 Interferon gamma

IFN- γ is primarily secreted by NK cells, NK T cells and T cells but the IFN- γ receptor is expressed on almost every cell type. Binding of IFN- γ to its receptor initiates downstream signalling, which leads to the activation of JAK1 and JAK 2(419).

Activated JAKs phosphorylate the IFN- γ receptor which enables the activation of STAT1 hence triggering the transcription of target genes(420). Although the JAK-STAT pathway is the main signalling pathway associated with IFN- γ , other pathways independent of STAT1 are also implicated including MAP kinase, PI3K and NF- κ B signalling pathways (421). IFN- γ can also directly translocate to the nucleus, together with its receptor and STAT1 to induce gene expression of IFN inducible genes by binding to IFN- γ activated site (GAS) (422).

IFN- γ induces the expression of antiviral proteins including PKR, oligo adenylate synthetase and Mx GTPase, the functions of which are detailed above. IFN- γ also blocks viral replication by inducing the expression of dsRNA-specific adenosine deaminase (ADAR), which edits and impairs translation of viral proteins. IFN- γ also induces FasL and promotes the apoptosis of virally infected cells.

IFN- γ induces pro-inflammatory cytokines to attract T cells, macrophages and neutrophils to the site of infection and helps to augment the expression of IFN-I to

further promote antiviral activity. IFN- γ is also important in engaging the adaptive immune system after the innate system has been activated (423). For instance, IFN- γ increases IL-12 expression by APCs, which further helps develop CD4⁺ TH1 T cells and activates NK cells.

The exogenous administration of IFN- γ as a therapeutic agent is controversial. The results in HIV have been disappointing, but in HBV there is a suggestion that it can improve fibrosis score (424). However, this needs to be validated and it is not part of current clinical practice.

1.10.12 Tumour Necrosis Factor

First characterised in the mid 1980's, TNF earned its name as it was seen to cause disintegration of tumours (425). The group of cytokines making up TNF includes TNF itself, also known as cachectin, as well as lymphotoxin, CD40 ligand, Fas L, GITR ligand, OX40 ligand, RANK and TNF-related apoptosis inducing ligand (TRAIL) amongst others. For the purposes of this thesis, we will exclusively focus on TNF.

TNF is released by activated monocytes, T cells, NK cells, mast cells, B cells and Kupffer cells in the liver. Its production is augmented by IFN- γ , which is released by NK cells and T cells (426, 427).

TNF signalling pathways are so intricate that they have been compared to a 'spider's web' (428). TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) are present on most cell types but differ in their expression, their binding affinity, cytoplasmic tail structure and downstream signalling mechanisms. Secreted TNF binds preferentially to TNFR1. TNFR1 appears to be more important in the function of TNF as TNFR1 knockout mice better recapitulate the phenotype of TNF deficient mice compared to TNFR2 knockout mice (429). TNFR1 is constitutively expressed on virtually all cell types except erythrocytes, whereas TNFR2 is generally inducible and is preferentially expressed on endothelial and hematopoietic cells.

One of the remarkable characteristics of TNF is that it can either induce gene expression by activating transcription factors and therefore increase the production of inflammatory mediators and survival proteins or it can induce cell death depending on the metabolic state of the cell (428). TNF binds to TNFR1 on the surface of a cell, numerous adapter proteins are recruited to form a signal complex. The constituents of this signalling complex critically determine the outcome of ligand / receptor interaction. If cellular inhibitor of apoptosis (cIAPs) proteins are recruited to the complex, then the transcription factor NF κ B is activated resulting in production of cytokines and

upregulation of cell survival proteins. However, in the absence of cIAPs the default outcome is programmed cell death caused by apoptosis, mediated by Caspase-8. TNF is a pleiotropic cytokine, being both an important mediator of apoptosis as well as a major sentinel cytokine whose main biological role is to defend against local injury. TNF plays a central role in inflammation and immune regulation as well as cellular differentiation and proliferation. TNF stimulates the recruitment of neutrophils and monocytes at sites of infection and it activates those cells to enable pathogen clearance. In conjunction with IL-1 and IL-6, TNF induces the production of acute phase reactant proteins such as serum amyloid protein and fibrinogen from the liver. At low concentrations, these pro-inflammatory effects will generally achieve successful eradication of pathogens with minimal collateral damage. However, when the production of TNF is dysregulated or excessive, outcomes can be deleterious. One of the most well recognised destructive effects of excessive TNF is septic shock, which is observed in severe infections. Disturbances in TNF are also implicated in the pathogenesis of various autoimmune conditions including multiple sclerosis, rheumatoid arthritis, and inflammatory bowel diseases, where it causes inappropriate inflammatory reactions against host tissues. One of the revolutions in the treatment of those autoimmune conditions has been the use of TNF inhibitors (427, 430). But paradoxically the use of these agents illustrated TNF's critical role in preventing infectious diseases related morbidity and mortality. Antagonizing TNF can cause reactivation of latent or quiescent infections including HBV (431).

1.10.13 TNF in HBV

TNF is a potent antiviral in HBV. Low levels of TNF are associated with a failure to clear HBV (432). TNF levels are increased in the serum in patients with HBV (433) and in patients with acute, self-limiting hepatitis, levels of TNF are significantly higher than in patients who harbour chronic HBV (415, 434). In the chimpanzee model, viral clearance is immediately preceded by a rise in TNF and IFN- γ (330). Similarly, in a mouse model of HBV infection, peaks in TNF as well as IFN- γ , IL-6, IL-12, and IL-1 β were seen in early phases of infection (327) and conversely TNFR1 knockout mice are unable to clear HBV (409). Blocking TNF signalling causes impairment in virological control (327, 435), a reduction in Toll-like receptor 9 (TLR9) activation and T cell exhaustion (436).

TNF exerts antiviral activity via multiple mechanisms. It can decrease viral entry into the hepatocyte (437) and reduces viral replication. TNF also contributes to cccDNA decay by through activation of APOBEC3 deaminases (415, 434). TNF signalling can

modulate the levels of cellular FADD-like IL-1 β -converting enzyme-inhibitory protein (c-FLIP), which inhibits HBV transcription and replication (438).

The use of TNF antagonists in the treatment of autoimmune conditions has been associated with recrudescence of HBV in HBsAg positive patients. The risk of reactivation with TNF inhibitors ranges between 1% to 10% (439). HBsAg positive patients are at significantly higher risk, and one retrospective study quotes a reactivation rate as high as 39% as well as a risk of fulminant liver failure in 5 patients, four of whom died. However, that data is difficult to interpret due to the concurrent use of other immunosuppressive agents (440). The risk of viral reactivation is significantly reduced with the administration of prophylactic antiviral agents (440). Screening for HBV is therefore recommended prior to the commencement of immunosuppressive therapy.

1.10.14 B cells

Although the bulk of HBV research has focused on T cells, the role of B cells in the adaptive immune response in HBV is increasingly being recognised. Patients with CHB appear to retain intact antibody production and there is solid evidence of adequate B cell activation in CHB (441, 442). Clinically, the definition of the disease phases and subsequent therapeutic decisions rely heavily on the appearance of specific serum antibodies in the serum (134). Furthermore, the role of B cells appears to be central in the immune control of HBV as the use of B cell-depleting therapy is associated with a high risk of HBV reactivation.

As detailed above, antibodies are produced by the host against all the HBV proteins. Acutely, anti-HBs and anti-HBc are produced and of the two only anti-HBs is protective against future infections, whilst anti-HBc is merely a marker of infection. A clinical report has described that acute liver failure in two patients were associated with massive infiltration with anti-HBc suggesting that humoral immunity may well play a central role in the pathogenesis of acute liver failure associated with HBV (443). Anti-HBs is of great clinical importance, by virtue of the fact that it is a protective neutralising antibody. Antibodies against the antigenic 'a' loop and the PreS1 domain can inhibit viral entry into the hepatocyte and can therefore neutralise the virus. This is exploited clinically with the use of immunoglobulins and in vaccination (444). In contrast, antibodies directed at other regions of the S antigen do not have neutralising properties (445-447). Vaccine failure is often attributed to the fact that the antigenic determinant has undergone a mutation (448, 449). Since antibodies prevent entry into the hepatocyte, they might help prevent the spread of virus in people who are already

infected. There are some promising pre-clinical results in therapeutic vaccination with anti-HBs. However, clinical trial data is yet to be published (450-453).

B cell response appears to be different in acute and chronic infection. For instance, in acute infection, anti-HBs-producing B cells are more abundant in serum compared to chronic infection (454-456). This could be attributable partially to the fact that B cell production is T cell dependent and tolerance is induced by regulatory T cells (457).

1.11 Models to study HBV

1.11.1 Cell culture

Cell culture studies have provided the basis of a seminal discoveries in HBV. *In vitro* cell culture systems include primary human hepatocytes, Tupaia Belangeri (tree shrew) hepatocytes and human hepatoma cell lines, all of which are susceptible to human HBV infection (472-474). Primary human hepatocytes are the gold standard for cell culture as they possess almost all the attributes of the human liver and are 100% infectable with HBV under the correct conditions (475). However, primary human hepatocytes and Tupaia hepatocytes are difficult to source and the experimental results are not always reproducible due to high variability in the characteristics of the cells. Furthermore, they only remain susceptible to HBV for a few days.

Immortalised hepatoma cells (HepaRG) are used after two weeks of differentiation and replicate many of the attributes of PHH (462, 463). HepaRG cells can also express the NTCP receptor but NTCP is only expressed in about 10% of the cells and therefore the susceptibility to infection is not as good as PHH. HepaRG cells are easily available and are widely used in drug testing.

The HepG2 hepatoma cell line has also been used in but the susceptibility to HBV is poor. However, with the discovery on the NTCP receptor has greatly improved the scope of cell culture utilisation in HBV research(60). HepaRG-NTCP cells and HepG2-NTCP cells can now be engineered.

1.12 Animal models

In vivo systems include the duck model, the woodchuck model, the chimpanzee model and the tree shrew model. The duck and woodchuck utilise surrogate viruses, duck hepatitis B (DHBV) and woodchuck hepatitis B (WHB) respectively whereas the chimpanzee and the tree shrew can be infected with human HBV.

1.12.1 The Duck model

Duck cells are readily available and have been extremely valuable in the testing of antiviral agents as well as viral replication of hepadnaviruses (464-466). However, the duck model is limited in various respects. First, the DHBV is genetically very different to human HBV, sharing only 40% homology. In fact, of all the animal models, it is the most distantly related to the human liver. Secondly the duck liver does not appear to develop active liver disease or hepatocellular carcinoma with DHBV infection. Therefore, there are gross host and virus differences that make the duck model somewhat limited in its use in HBV research.

1.12.2 The Woodchuck model

The woodchuck model shares some similarities with human infection. First, infection of newborn woodchucks causes a chronic infection in contrast to older animals that develop an acute hepatitis. The woodchuck HBV (WHBV) infection also shares some immunological similarities with human HBV. The transcriptome for instance demonstrates an attenuated IFN-I response and T cell exhaustion, both features of human HBV infection (467). The woodchuck model has been crucial in the evaluation of antiviral drugs that are used in the treatment of CHB infection (95, 468, 469) as well as the new agents including PDL-1 inhibitors and TLR 7 agonists (470, 471). The main limitations of the woodchuck model include the fact that WHB integrates in the host DNA but forms an oncogene such that chronically infected woodchucks invariably develop HCC (472). Furthermore, woodchucks are difficult to breed in captivity and the model poses multiple logistic problems.

1.12.3 The Chimpanzee model

The chimpanzee is an immune competent model that supports human HBV infection and the kinetics of HBV infection are similar to those of human infection (473). Similar to human HBV infection, there is both cccDNA formation and integration in the host DNA (474, 475) therefore recapitulating many aspects of human HBV infection. The chimpanzee model has been used extensively in HBV research in pharmaceutical safety studies, vaccine testing, the development of therapeutic antibodies and therapeutic vaccines, as well as the testing of novel antiviral agents including TLR7 agonist and small interfering RNA ARC-520 (476-479). It has also been used in immune studies, showing a strong CD8⁺ T cell response in HBV clearance (407). However, there are significant ethical issues regarding the use of chimpanzees, and they are no longer used in HBV research.

1.12.4 The Tupaia model

Tree shrews are also susceptible to human HBV infection. Infection of neonate animals leads to chronic infection with the development of fibrosis and HCC (480)(34).

However, the efficiency of *in vivo* infection is limited and Tupaia cells have been used *in vitro*. The *in vitro* use of Tupaia cells has been pivotal in the identification of the NTCP receptor (481).

1.13 Mouse models in HBV

1.13.1 The transgenic mouse model

Transgenic animals have been successfully used to study the virology of HBV and determine oncogenic pathways. They have also been used to study antiviral therapy including some of the new agents detailed above namely siRNAs and cytokines (329, 482). Transgenic mice are produced with an integrated HBV DNA viral construct and viral replication occurs in the mouse hepatocytes at high levels. The mouse is tolerant to the virus and does not mount any cytotoxic or immune response to HBV, which constitutes the main limitation of the model in the study of pathogenesis or viral clearance.

Transgenic mice are immune tolerant, and they do not develop a hepatitis or any form of liver injury. This further confirms the fact that in absence of an immune response, HBV is not cytopathic. From a research point of view, the main limitations of the model are that active hepatitis or liver injury cannot be studied. Furthermore, certain aspects of the viral life cycle pertaining to cccDNA formation cannot be studied either as they are not found in murine hepatocytes. Mice also do not have the NTCP receptor and the processes of cellular entry and re-infection cannot be studied in those mouse models.

1.13.2 The chimeric mouse model

Humanised mouse models have also been used in the study of HBV infection.

Transgenic mice in which the urokinase gene is driven by the human albumin promoter/enhancer have been developed and they have accelerated hepatocyte death and consequent chronic stimulation of hepatocyte growth (483). These uPA transgenic mice are then mated with immunodeficient SCID mice to create uPA/ SCID mice.

Human hepatocytes are transplanted into uPA/SCID mice and human hepatocytes replace the apoptotic mouse livers. The chimeric mice can then be infected with HBV by injection of virus or virally infected serum (484, 485). Active viral replication occurs as evidenced by the presence of HBV VL in serum as well as HBcAg on immunohistochemistry. The uPA/ SCID mouse model has been valuable in the study of drug metabolism (486, 487). However, the main drawback of this model is that, in mice

with a high level of viral replication, HBV becomes directly pathogenic due to a lack of adaptive immune response(488, 489).

1.13.3 The hydrodynamic mouse model

Hydrodynamic injection of HBV to transfect mouse hepatocytes is a relatively recent innovation but a successful way to establish a small animal research model for *in vivo* HBV studies. In this model the mice utilised are immune competent and they are therefore suitable for the study of the pathogenesis and immune response (490, 491). The hydrodynamic injection (HDI) is a rapid and pressurised injection of a large volume of HBV-DNA solution in the tail vein of the mouse, usually 8-10% the body weight of the mouse. This generates a large hydrodynamic force, which overcomes the cardiac capacity. The resulting cardiac congestion leads to an increased pressure in the inferior vena cava and drives the injected solution back into the liver via the hepatic vein (492-494).

The high pressure generated by the injection widens the fenestrations of the Disse space and opens up the hepatocyte membrane, which allows the DNA to enter the hepatocyte (494-496). The cardiac congestion is transient and the technique is safe and ethical. The rates of animal loss through the injection are minimal and liver damage sustained from the injection itself is transient, only lasting up to 72 hours post injection (497).

Following the injection, hepatocytes with express HBcAg and start replicating virus. Virions and HBV antigens are detected in the peripheral circulation and products of viral replication including pgRNA and mRNAs are detected in the hepatocyte (498). Using this method, HBsAg is detectable for up to 6 month and mimics chronic infection in humans. Moreover the mouse is immunocompetent and allows for the study of immune responses as well as immune manipulation by novel therapies (327, 328).

The AAV vector favours transgene expression in the hepatocyte (507). The plasmid backbone likely influences the long-term maintenance or expression of the transgene in the livers(508). The genetic background of the mouse determines the immune response and therefore the outcome if the transfection. Despite its growing applications in medical research, there are some aspects of intrahepatic gene delivery following hydrodynamic injection that remain unclear. It is unclear, for instance if genes are exclusively delivered to the liver or if other organs are transfected as well. There is some evidence that even capillaries that are not fenestrated can allow gene delivery(509) (510). Furthermore, even though the C57BL6 mice, the immune

response fails to clear the input DNA as well as HBV antigens there is no evidence of cccDNA being formed in this mouse model (500).

1.14 Conclusions and gaps in knowledge

Chronic Hepatitis B remains a significant public health problem, in Australia and worldwide. To date, despite an expanding body of research, there is no known definitive cure for CHB, and mortality and morbidity rates remain high. The host immune response to the virus is critical in HBV infection: disease outcomes are largely mediated by the immune response and evasion of the immune response to maintain viral persistence is a critical barrier to cure.

The HBeAg is thought to play an important role in modulating the immune system but the exact mechanisms by which HBeAg enabled immune modulation of the host are unknown. There are significant gaps in our knowledge of the immunopathogenesis of HBV. The literature, to date, has demonstrated that it is a complex interplay of various immune mediators, however, the exact role of these immune mediators and their interaction with the virus is unclear. The relative importance of each of those immune mediators in human disease is also unknown.

There is also an unmet need to further understand the role of HBeAg, specifically with regards to its effect on the immune response. Whilst there is abundant data on associations between HBeAg status and various aspects of clinical phenotypes, the pathways by which HBeAg can modulate host responses is unknown. Additionally, the presence of mutations that influence HBeAg appear to be associated with significant differences in disease outcomes, but their exact role at a mechanistic level is unclear. An understanding of the significance of the pathways by which HBeAg modulates the immune response and how mutations in HBeAg influence clinical outcome can provide significant insights that can be exploited in developing novel therapies.

1.15 Aims of this thesis

The aims of this thesis are:

- 1) To perform a detailed clinical and virological characterisation of patients with CHB in Australia, with a particular focus on HBeAg status, genotypes and BCP and PC mutations
- 2) To investigate the role of HBeAg in the immune-pathogenesis of CHB, using an immune-competent mouse model, with a specific focus on the relationship between HBeAg and various mediators of the innate and adaptive immune system.

2 Materials and Methods

2.1 Methods for the population-based study of HBV characterisation

2.1.1 Patient recruitment

A retrospective database audit was performed to identify patients from the St Vincent's Hospital (Melbourne). HBV database with available data for HBV genotype and BCP and PC sequencing. The demographic data collected in the database included age, sex, country of birth and the presence of other forms of liver disease including co-infection with hepatitis C (HCV), alcohol, fatty liver and haemochromatosis. Patients with other forms of liver disease were excluded. No patients were co-infected with the Human immunodeficiency virus (HIV). Only patients who were treatment-naïve at the time of sequencing were included in the study.

Liver function tests, HBV serology including surface antigen (HBsAg), HBV surface antibody (anti-HBs), HBeAg, HBV e antibody (anti-HBe) and HBV viral load (HBV DNA) were performed at the time of genotyping and sequencing. A high ALT was defined as 35 IU/ml for women and 40 IU/ml for men as it was the cut off for our pathology laboratory. A liver biopsy was performed in a subset of 194 patients. Liver biopsies were graded using the METAVIR scoring system and advanced fibrosis was defined as F3 and F4. Disease phases were defined as per the guidelines of the European Association for Study of the Liver (EASL) as summarised in **Error! Reference source not found..**

2.1.2 Hepatitis B virus testing

Hepatitis B serology including HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc was performed using commercial immunoassay kits (Abbott Laboratories, North Chicago, IL, USA). Hepatitis B viral load was measured at the time of sequencing at the Victorian Infectious Diseases Reference Laboratory (VIDRL) using the Digene Hybrid Capture Assay (Digene Diagnostics, Beltsville, United States), Digene Hybrid Capture II Assay (Digene Diagnostics, Beltsville, United States) or Bayer VERSANT HBV 3.0 (Bayer Diagnostics, New York, United States). HBV DNA results were standardised to IU/ml. Genotype analysis and population-based PCR sequencing for the PC G1896A and BCP A1762T/G1764A mutations was performed at VIDRL. The methods have previously been described (499).

2.1.3 Statistical analysis

Data were analysed using SAS® statistical software. Version 9.4 (North Carolina, United States of America). Comparisons between HBeAg positive and negative cohorts

were analysed using Chi square test. Univariate and multivariate analyses were used to establish an independent correlation between demographic variables, mutations and clinical outcomes.

2.2 Methods for the Immunopathogenesis of HBV

2.2.1 Plasmid preparation

HBV WT plasmid pAAV-HBV1.2

The WT HBV encoding pAAV-HBV1.2 vector (genotype A2, serotype *adw*) was kindly provided by Pei-Jer Chen, National Taiwan University, Taipei (491). The vector contains a 1.2 over-length HBV genome spanning nucleotides 1,400–3,182/1–1,987 and it is flanked by inverted terminal repeats (ITR) of an adeno-associated virus (AAV)(327). The AAV vector is important in ensuring viral persistence. The AAV vector favours transgene expression in the hepatocyte (507). The plasmid backbone likely influences the long-term maintenance or expression of the transgene in the livers(508).

Site Directed Mutagenesis (SDM)

The pAAV-HBV1.2 vector constructs containing the G1896A Precore (PC) and A1762T/G1764A Basal Core promoter (BCP) mutations were created through *in-vitro* site-directed mutagenesis using the QuikChange II Site-Directed Mutagenesis kit (Stratagene Technologies, San Diego, United States) as outlined below. The method is based on amplification of the HBV DNA using complementary primers (Table 2.1) that encode for the required mutation.

For the HBV PC mutation construct, the G1896A, a C to T mutation is required at nucleotide 1858 to stabilise the epsilon, as previously described in Chapter 1(123). This was achieved with 2 consecutive rounds of site-directed mutagenesis to construct PC-HBV. Primers (Table 2.1) were obtained from Geneworks, South Australia and were diluted in nuclease-free water to yield a final concentration of 100ng/μl.

Mutagenesis primer	5'-3' sequence
C1858T forward	GTA CAT GTC CTA CTG TTC AAG CCT CCA AGC
C1858T reverse	GTA GTT TCC GGA AGT GTT GA
G1896A forward	GCCTTGGGTGGCTTTAGGGCATGGACATCGACC
G1896A reverse	2.2.1.1 GGTCGATGTCCATGCCCTAAAGCCACCCAAGGC
A1762T/G1764A forward	GGGGGAGGAGATTAGGTTAATGATCTTTGTACTAGGAGGC
A1762T/G1764A reverse	2.2.1.2 GCCTCCTAGTACAAAGATCATTAACCTAATCTCCTCCCCC

Table 2.1 Primers used for mutagenesis

In general, 20ng of pAAV HBV 1.2 vector template DNA was added to a Master Mix solution made up of 38.5µl nuclease free water, 5µl 10x Buffer, 1µl deoxyribonucleotide triphosphate solution (dNTP), 1.25µl forward Primer, 1.25µl reverse primer and 1µl Pfu Turbo DNA polymerase.

Amplification of mutant constructs were performed by endpoint PCR of 18 cycles with the following parameters:

- 1) Initial denaturation: 95°C, 5min
- 2) Denaturation: 95°C, 30 sec
- 3) Annealing: 55°C, 60 sec
- 4) Extension: 68°C, 18 sec

Steps 2-4 were repeated for 18 cycles

- 5) Final extension 68°C, 7min
- 6) Sample kept at 4°C on "Hold" until further usage

Following PCR, samples were digested with restriction enzyme DpnI, specific for methylated and hemi-methylated DNA, to digest the parental DNA template and to select for mutation-containing synthesised plasmid DNA only. Samples were incubated for 1 hour at 37°C. Following digestion with DpnI, the DNA was analysed on a 1% agarose gel to ensure it was similar to the original SDM product.

Plasmid DNA amplification

To amplify the vector DNA, *E.coli* bacterial cells were transformed as described below (0

Transformation of chemically competent *E.coli*)

Briefly, mutant plasmids were transformed into DH5 α competent cells and selected clones were screened using Big Dye sequencing (Roche Diagnostics). 'Mini preparation' was used to amplify plasmids (0 Plasmid production), which were then sequenced at MicroMon, Monash University to ensure that no other mutations were inserted. Sequencing primers are outlined in **Error! Reference source not found.** and **Error! Reference source not found.** Sequence verified, correct plasmid DNA clones were further amplified by 'Maxi preparation', see below (0 Plasmid production).

Primer name	Position *	Forward (F) or Reverse (R)	Region PCR and/or Sequencing (Seq)	Sequence (5' to 3')
Seq2	300-318	F	Pol PCR and Seq 1.2 mer clone Seq	TTG GCC AAA ATT CGC AGT C
P1	1821-1841	F	Full genome PCR	CCGGAAAGCTTGAGCTCTTCTTTT CACCTCTGCCTAATCA
P2	1806-1825	R	Full genome PCR	CCGGAAAGCTTGAGCTCTTCAAAAA GTTGCATGGTGCTGG
SeqP1	1821-1841	F	Full genome Seq	TTT TTC ACC TCT GCC TAA TCA
SeqP2	1806-1825	R	Full genome Seq	AAA AAG TTG CAT GGT GCT GG
865	2301-2320	F	Full genome Seq 1.2 mer clone Seq	TTC GGA GTG TGG ATT CGC AC
2996	1175-1193	R	Pol PCR and Seq 1.2 mer clone Seq	GCG TCA GCA AAC ACT TGG C

JM	3114-3134	F	Full genome Seq 1.2 mer clone PCR and Seq	TTG GGG TGG AGC CCT CAG GCT
1798	3237-3257	F	Full genome Seq 1.2 mer clone Seq	CCA CTG CAT GGC CTG AGG AT G
903	2337-2362	R	Full genome Seq	GTT GAT AAG ATA GGG GCA TTT GGT GG
OSX1	807-827	F	Full genome Seq 1.2 mer clone Seq	TTT TCT TTT GTC TTT GGG TAT
2254	412-403	R	Full genome Seq 1.2 mer clone Seq	GAA GAT GAG GCA TAG CAG CAG G
PC5	1604-1623	F	BCP/PC PCR and Seq 1.2 mer clone Seq	TCG CAT GGA GAC CAC CGT GA
PC2	1940-1959	R	BCP/PC PCR and Seq 1.2 mer clone Seq	GGC AAA AAC GAG AGT AAC TC

*Numbering from the *EcoR1* site

Table 2.2 Primers used for PCR and sequencing of HBV plasmid

Transformation of chemically competent *E.coli*

100ng of pAAV-HBV1.2 plasmid DNA was added to 50µl of chemically competent DH5α cells and incubated for 5 minutes on ice. The bacteria-DNA mix was heat shocked for 90 seconds at 42°C and placed on ice for 2 minutes. 500µl of super optimal broth (SOB) medium (1 L SOB: 20 g tryptone, 5 g yeast extract, 2 ml of 5 M NaCl, 2.5 ml of 1 M KCl, 10 ml of 1 M of MgCl₂, 10 ml of 1M MgSO₄ in 1 L of deionised H₂O) were added mix incubated at 37°C for 30 min - 1 hour on a shaker. 100 µl of the bacterial culture was plated on Lysogeny Broth (LB) agar plates (1 L LB broth/agar: 10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g of agar in 1 L of deionised H₂O) and incubated at 37°C overnight.

Plasmid production

Mini preparation: Bacterial colonies were picked and transferred in 5 ml of Lysogeny B (LB) medium (1 L Lysogeny broth: 10 g tryptone, 5 g yeast extract, 10 g NaCl) with 100mg/ml carbenicillin. Bacteria were amplified in selection LB medium at 37°C in a shaker overnight. To isolate amplified plasmid DNA, the endotoxin-free Wizard Plus SV Miniprep DNA Purification kit (Promega, Wisconsin, United States) was used as per manufacturer's instructions. Vector sequence was verified by restriction enzyme digestion and gel electrophoresis.

Maxi preparation: To further amplify plasmid DNA, 300ml of LB with carbenicillin were inoculated with 1ml of the overnight culture and again incubated at 37°C in a shaker overnight. Plasmid DNA was isolated using the EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany), as per manufacturer's instructions and resuspended in TE buffer at 1µg/µl and stored at -80 °C. HBV mouse model of infection

2.2.2 Mice

All inbred mice used in experiments had been backcrossed to C57BL/6. All experiments were approved by the Walter and Eliza Hall Institute animal ethics committee (AEC Project 2012.013, 2014.005). C57BL/6 mice of both genders, aged between 7 and 9 weeks were used for all experiments except for transcriptome studies, in which only female mice were utilised.

IFN γ ^{-/-} and IFNAR^{-/-} mice

IFN γ ^{-/-} and IFNAR^{-/-} animals were provided by Prof Benjamin Kile and have been previously described (500, 501). IFN γ ^{-/-} mice do not generate a functional IFN- γ protein

and therefore cannot signal through this pathway. IFNAR deficient animals lack the receptor required for signalling through type I IFNs, including IFN- α and IFN- β .

Prf1^{-/-} mice

Perforin deficient mice (Prf1^{-/-}) have a normal number of CD8⁺ T cells and NK cells but are not able to induce CD8⁺ T cell mediated cytotoxicity and lyse virus-infected cells. Prf1^{-/-} mice has been described elsewhere (502).

OT-II mice

OT-II mice transgenically express an α - and a β -chain of the T-cell receptor (TCR) an epitope in chicken ovalbumin (323-339 peptide). The transgenically expressed receptor pairs with the CD4 co-receptor and interferes with the expression of other TCRs, thus skewing the whole CD4⁺ T-cell compartment and impeding responses to antigens other than ovalbumin. Generation of OT-II mice has been described elsewhere (503).

Fc γ R^{-/-} mice

Fc γ R^{-/-} mice are unable to phagocytose antibody-coated cells, despite normal binding and no inflammatory response to immune complexes can be generated. T cell development is unaffected. Generation of Fc γ R^{-/-} mice has been described elsewhere (504). Fc γ R^{-/-} mice (B6.129P2-Fcgr3^{tm1Jsv}/J) were obtained from the Walter and Eliza Hall Institute animal facilities.

Antibody mediated depletion of TNF in C57BL/6 mice

TNF-neutralizing antibody (rat IgG1, XT22) and rat IgG isotype control antibody were injected intraperitoneally at a dose of 200 μ g twice weekly for 3 weeks, starting 3 hours before HBV DNA hydrodynamic injection of C57BL/6 mice.

2.2.3 HBV mouse model of infection

Induction of HBV infection by hydrodynamic tail vein injection

The hydrodynamic tail vein injection has been well described (491, 505). The principle is that large bolus of HBV plasmid in phosphate buffered saline (PBS) is injected rapidly in the tail vein of the mouse. The forceful, rapid and large volume injection precipitates cardiac congestion, which in turn causes a backflow of the solution from the right heart into the mouse liver. The high hydrodynamic pressure of the injection effectively forces the plasmid- containing solution into the hepatocytes resulting in about 20% of liver cells being infected (327).

Injections were carried out in a sterile fume hood. Mice were weighed and the volume to be injected was calculated as 8% of the weight. Plasmid solution was prepared so that every mouse received 10µg plasmid-DNA in the required volume of PBS.

The appropriate DNA/PBS solution was injected intravenously through the tail vein within 5 seconds and mice were monitored for 10–15 minutes to ensure recovery.

Serum isolation

For determination of viral titres in the serum of mice over time, 100µl of blood was collected in non-heparinised tubes via retro-orbital bleed or puncture of the sub-mandibular vein, at 3 days or 1 week after induction of infection to validate efficient infection, in addition to indicated time points. Blood was left at room temperature for 1 hour to coagulate, then centrifuged at 3500g for 10 min and serum was collected and stored at -20°C until needed.

2.2.4 Alanine Aminotransferase and Aspartate Aminotransferase quantification

To determine transaminases in the serum of mice, terminal intra-cardiac bleed was performed to collect around 500µl of blood. Serum was isolated as indicated before (2.2.5.1 serum isolation). Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using electrochemilluminescence on a Cobas 6000 analyser (Roche Diagnostics, Mannheim, Germany) per the manufacturer's instructions.

2.2.5 Quantitative measurement of serum HBV DNA

Viral DNA extraction

HBV viral DNA was extracted from the serum using the Invisorb Virus DNA HTS 96 Kit (Stratec Biomedical Systems, Birkenfeld, Germany) as per manufacturer's instructions. Briefly, 50µl of serum was added to 150µl PBS in each well of the collection plate. 200µl Lysis buffer was mixed in followed by 40µl proteinase K/Carrier RNA mix and the plate was incubated for 10 minutes at 56°C, then cooled to room temperature. Following lysis, binding of the viral DNA was achieved by the addition of 200µl binding buffer HL and 640µl of the lysate was transferred to a DNA binding plate. The plate was centrifuged at 1500g for 3 minutes to run the lysate through the filter. The binding plate was washed with 600µl wash buffer I and centrifuged at 1500g for 3 minutes at room temperature, then washed twice with 600µl wash buffer II and centrifuged at 1500g for 3 minutes at room temperature.

Following the wash, the filtrate was discarded and the binding plate centrifuged at 1500g for 10 minutes at room temperature to eliminate any traces of ethanol. Viral

DNA was then eluted with 50µl elution buffer, incubated at room temperature for 3 minutes and centrifuged at 1500g for 5 minutes at room temperature. Eluted viral DNA was either quantified immediately with real-time PCR or stored at -20 °C.

Viral DNA quantification

Quantification was performed using real-time RT-PCR and the DNA Amplification SYBR Green I (Roche Life Science, Penzberg, Germany) on a LightCycler® 480 II Machine (Roche Life Science, Penzberg, Germany) with Absolute Quantification Software (Roche Life Science, Penzberg, Germany). LightCycler® 480 SYBR Green I Master is a ready-to-use reaction mix designed specifically for hot start PCR that significantly improves the specificity and sensitivity of PCR by reducing the formation of non-specific products (506, 507). SYBR Green I intercalates into the DNA helix and PCR products can be quantified by measuring the SYBR Green I fluorescence signal.

Preparation of the PCR Mix:

Primers HBV1745fw (GTTGCCCGTTTGTCTCTAATTC) and HBV1844rev (TGAGGGAAACATAGAGTTGCCTTGA) were diluted to a final concentration of 20 µM, which was previously determined to result in the lowest crossing point (Cp) and adequate fluorescence on PCR (327, 328).

A standard reaction was first prepared with 3µl PCR grade water, 2µl primer and 10µl SYBR green Mastermix for each sample. 15µl of the reaction mix was loaded onto each well of the LightCycler® 480 Multiwell plate 96 and 5µl sample was added. Negative controls were prepared by replacing the template with nuclease free water. Four negative controls were run with the experiment. A plasmid containing one copy of the HBV genome and known copy number was used as a standard, which has also been standardised against the WHO HBV Reference Sample. A series of five ten-fold dilution were used to generate a standard curve.

The plate was prepared in a fume hood in a different laboratory to ensure no contamination. The plate was sealed with a sealing foil, centrifuged at 1500g for 2 minutes and loaded on the LightCycler® 480 instrument. The PCR program was started as per the operator's instructions (1 cycle pre-incubation, 45 cycles amplification, 1 cycle melting curve, 1 cycle cooling).

The PCR parameters were as follows:

- 1) Initial denaturation: 95°C, 5min
- 2) Denaturation: 95°C, 25 sec
- 3) Annealing: 60°C, 15 sec
- 4) Extension: 72°C, 30 sec

PCR results

HBV DNA analysis:

The LightCycler® 480 has an optical filter that is set to match the wavelengths of fluorescence of SYBR Green. DNA binding significantly increases the fluorescence of SYBR Green. During PCR, the increase in SYBR Green I fluorescence is directly proportional to the amount of double-stranded DNA generated. Fluorescence is measured at the end of each PCR cycle to monitor the amount of DNA being amplified. Our limit of detection of serum HBV DNA was 500 IU/ml.

Melting curve analysis:

Melting curve analysis was performed with each PCR run to confirm that the desired PCR product had been amplified. To perform a melting curve analysis the reaction mixture is heated to 97°C, which denatures the double-stranded DNA, which is indicated by a drop in the SYBR Green signal. The decrease in fluorescence is displayed as a melting peak. Each melting peak has a characteristic melting temperature (T_m) for a specific DNA product. PCR product has a specific melting point that has been previously determined (327, 328).

2.2.6 Quantitative measurement of HBeAg

Serum HBeAg was quantified by an electrochemiluminescence immunoassay on the Cobas Elecsys (Roche Diagnostics, Mannheim, Germany). Although this kit is not specifically designed as a quantitative assay, HBeAg levels can be calculated by comparison with a standard curve of fluorescence rate vs. HBeAg concentration. The fluorescence signal is linear within a restricted range (508).

The HBeAg reference preparation was obtained from the Paul Ehrlich (PE) Institute (Paul-Ehrlich Institute, Langen, Germany) and had a defined HBeAg activity of 100 PE IU/mL. A working HBeAg standard was prepared at the VIDRL from serum samples with known high HBeAg concentration and calibrated against the PE reference preparation. Serial dilutions were prepared using the Diluent HepB (Roche Diagnostics, Mannheim, Germany) to plot a standard curve. Mouse serum samples were tested undiluted and then diluted 1:10 or 1: 100 if their value fell outside the range of interpolation.

2.2.7 Quantitative measurement of HBsAg titre

Serum HBsAg was quantified using the Elecsys e411 platform (Roche Diagnostics, Mannheim, Germany) and the Roche Elecsys HBsAg II Quant Immunoassay. Mouse serum was diluted using Elecsys HBsAg Manual Diluent (Roche Diagnostics, Mannheim, Germany) and the sample tested at a dilution of 1:1000. Quantitative

HBsAg levels are reported in IU/ml. Assays were performed at the VIDRL by Rachel Hammond, Danni College and Hugh Mason.

2.2.8 Quantitative measurement of anti-HBe and anti-HBs antibodies

Serum anti-HBeAg and anti-HBsAg were measured performed using electrochemiluminescence on the Cobas e411 analyser (Roche diagnostics, Mannheim, Germany) as per manufacturer's instructions.

2.2.9 Next Generation Sequencing

To determine the presence of BCP and PC mutations, amplification and sequencing of a 356 bp fragment of the BCP/PC region was performed using primers PC5 and PC2 (Table 2.2).

PCR products generated by whole-genome sequencing were pooled in a 1:1 molar ratio, processed using the Nextera XT kit (Illumina, San Diego, United States), sequenced on the MiSeq platform (Illumina, San Diego, United States) and further processed using a four-stage verification process. The average total number of nucleotide reads generated per sample was $312\,187 \pm 12\,327$, with an average coverage per site of $14\,790 \pm 648$. The threshold for mutation classification was set at 1%. Nucleotide substitutions present at <1% of the total reads were deemed as no change and were considered wild type.

2.2.10 Immunohistochemistry and histology

Tissue preparation

Mouse livers were collected and flushed with ice cold PBS, fixed in 10% buffered formalin and embedded in paraffin blocks. Tissue sections were cut into 4 μ m sections and mounted on adhesive slides. Prior to staining, tissue sections were incubated in xylene solution for 10 minutes to remove the paraffin. They were then rehydrated by incubation in graded dilutions of ethanol (100%, 90% and 70%)

Tissue staining

Rehydrated tissue was washed in PBS for 5 minutes. Slides were incubated in 0.3% hydrogen peroxide-PBS for 30 minutes and then washed in distilled water and PBS. The tissue was then blocked with 3% bovine serum albumin (BSA) dilutes in Tris-buffered saline for 20 minutes before antibodies were added.

Tissue sections were stained with HBV core protein-specific antibody (rabbit anti-HBcAg; 1:200; Dako), Haematoxylin and Eosin stain, or antibodies that recognise CD3 or CD8.

Histology

Slides were washed with PBS and horseradish peroxidase (HRP)-streptavidin diluted in 10% FCS was added and incubated for 1 hour. Histologic slides were developed by adding chromogen provided in the Liquid DAB+ kit (Dako Agilent) and incubating for 5 minutes. Slides were washed in distilled water and incubated in Lillie-Mayer Haematoxylin for 10 seconds, then washed in PBS. Slides were then dried and mounted for viewing.

Slides were scanned using a Panoramic SCAN II scanner (3D Hitech, Budapest, Hungary). Images were analysed using FIJI with a custom written analysis pipeline that made use of the colour deconvolution function to separate the stains into separate channels. A median filter was applied and each channel binarised using user defined thresholds. Object counts were recorded and binary masks output for user verification. Due to the size of the files involved, each image was split into tiles for memory management and speed of processing. The object counts were summed across all tiles.

2.3 Transcriptome analysis

Liver RNA extraction was performed using the RNeasy® Mini Kit (Qiagen, Hilden, Germany). An input of 100ng of total RNA were prepared and indexed separately for sequencing using the TruSeq RNA sample Prep Kit (Illumina, San Diego, United States) as per manufacturer's instruction. Each library was quantified using the Agilent Tape-station and the Qubit™ DNA BR assay kit for Qubit 3.0® Fluorometer (Life Technologies, California, United States). The indexed libraries were pooled and diluted to 1.5pM for paired end sequencing (2x 81 cycles) on a NextSeq 500 instrument using the v2 150 cycle High Output kit (Illumina, San Diego, United States) as per manufacturer's instructions. The base calling and quality scoring were determined using Real-Time Analysis on board software v2.4.6, while the FASTQ file generation and de-multiplexing utilised bcl2fastq conversion software v2.15.0.4.

Between 24 and 63 million read pairs were generated for each sample and reads were aligned to the hybrid genome built by concatenating *Mus musculus* genome (mm10) and the HBV genome using the Subread aligner (509). The number of read pairs overlapping each mouse Entrez gene or the HBV genome was summarised using featureCounts (510) and Subread's built-in NCBI gene annotation. Genes were filtered if they failed to achieve at least 0.7 counts per million (CPM) in at least three libraries. Genes without current annotation, ribosomal genes and reads mapping to the HBV genome were also filtered. Differential expression (DE) analyses were undertaken using the edgeR (511) and limma (512) software packages. Library sizes were

normalised using the trimmed mean of M-values (TMM) method (513). Log2 fold-changes were computed using voom(514). Differential expression was assessed using a robust empirical Bayes procedure(515). The false discovery rate (FDR) was controlled below 0.05 using the method of Benjamini and Hochberg (516). Over-representation of Gene Ontology (GO) terms and KEGG JAK STAT pathways for the differentially expressed genes were identified using the functions goana and kegg in limma package. Barcode plots illustrating the enrichment of interested pathway genes were drawn using barcode plot function in limma package. Gene set enrichment tests used the ROAST method with two-sided (UpDown) p-values(517).

2.4 Proteome studies

2.4.1 Mass Spectrometry - proteome analysis

Livers were perfused with PBS, harvested and homogenised in lysis buffer containing 4% SDS, 100mM TrisHCl pH 7.4, 5mM TCEP and 10mM chloracetamide using Tissue Lyser II (Qiagen, Hilden, Germany). Lysates were cleared by centrifugation (10.000g, 10min, 4°C). For each sample, 400µg of cleared lysate was resuspended in 6M Urea, 100 mM DTT and 100 mM Tris-HCl pH7.0 and subjected to protein digestion using FASP (filter aided sample preparation) before lyophilisation to dryness using a SpeedVac AES 1010 (Savant, Thermo Fisher, Waltham, MA, USA) (518). The tryptic digests were subjected to stepwise high-pH fractionation. Peptides were resuspended in 10mM Ammonium Formate pH10 and loaded onto a stage-tip (home-packed, 4 C18-matrix discs; activated using Methanol, washed in 5mM Ammonium Formate/50% acetonitrile (ACN) and equilibrated 2 times with 10mM Ammonium Formate pH10). The samples were fractionated by stepwise elution in 10mM Ammonium Formate with increasing concentrations of ACN: 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20%, 60%. The fractionated peptide solutions were then concentrated by centrifugal lyophilisation. Peptides were resuspended in 2% acetonitrile, 1% formic acid and injected and separated by reversed-phase liquid chromatography on a M-class UPLC system (Waters Corporation, Milford, MA, USA) using a 250mm × 75µm column (1.6µm C18, packed emitter tip; IonOpticks, Parkville, Victoria, Australia) with a linear 60-min gradient at a flow rate of 400 nl/min from 98% solvent A (0.1% Formic acid in Milli-Q water) to 34% solvent B (0.1% Formic acid, 99.9% acetonitrile). The UPLC was coupled on-line to a freshly cleaned Q-Exactive HF-X mass spectrometer (ThermoFisher). The Q-Exactive HF-X was operated in a data-dependent mode, switching automatically between one full-scan and subsequent MS/MS scans of the 15 most abundant peaks. Full-scans (m/z 350–1,600) were acquired with a resolution of

60,000 at 200 m/z. The 15 most intense ions were sequentially isolated with a target value of 100000 ions and an isolation width of 1.4 m/z and fragmented using HCD with normalised collision energy of 27. Maximum ion accumulation times were set to 50ms for full MS scan and 40ms for MS/MS. Dynamic exclusion was enabled and set to 20 seconds.

The raw files were analyzed using the MaxQuant software (version 1.5.8.3) (519, 520) and the database search was performed using mouse and HBV protein sequences obtained from Uniprot including isoforms with strict trypsin specificity allowing up to 3 missed cleavages. The minimum required peptide length was set to 7 amino acids. Carbamidomethylation of cysteine was set as a fixed modification while N-acetylation of proteins N-termini and oxidation of methionine were set as variable modifications. During the MaxQuant main search, precursor ion mass error tolerance was set to 4.5 ppm and fragment ions were allowed a mass deviation of 20 ppm. PSM and protein identifications were filtered using a target-decoy approach at a false discovery rate (FDR) of 1%.

Further analysis was performed using a custom pipeline developed in R, which utilises the MaxQuant output files (allPeptides.txt, peptides.txt and evidence.txt). A feature was defined as the combination of peptide sequence, charge and modification. Features not found in at least half the number of replicates in each group were removed. Proteins identified from hits to the reverse database and proteins with only one unique peptide were also removed. To correct for injection volume variability, feature intensities were normalised by converting to base 2 logarithms and then multiplying each value by the ratio of maximum median intensity of all replicates over median replicate intensity. Features assigned to the same protein differ in the range of intensity due to their chemico-physical properties and charge state. To further correct for these differences, each intensity value was multiplied by the ratio of the maximum of the median intensities of all features for a protein over the median intensity of the feature. Missing values were imputed using a random normal distribution of values with the mean set at mean of the real distribution of values. A peptide feature was defined as the combination of peptide sequence, charge and modification. Each peptide was associated with a leading razor protein and Uniprot Ids were mapped to gene symbols using Uniprot.

DE analyses were undertaken using the limma (512) software package. Peptides were filtered if they were detected in fewer than 6 samples or could not be mapped to a gene. Missing peptide intensities were set to value of 500,000, which was near the minimum of observed peptide means. Intensities were log2-transformed and quantile normalised. Differential expression was conducted at the gene-level using limma's fry

method. Imputed intensities were assigned a precision weight of 1/50 and sample quality weights were estimated using the method of Ritchie et al (514). An average log₂-fold-change was computed for each gene as the weighted average of peptide log₂-fold-changes with squared standard errors as inverse weights.

2.4.2 Mass spectrometry - phosphoproteome analysis

Livers were perfused with PBS, harvested and homogenised in lysis buffer containing 4% SDS, 100mM TrisHCl pH 7.4, 5mM TCEP and 10mM chloracetamide using Tissue Lyser II (Qiagen, Hilden, Germany). Lysates cleared by centrifugation (10,000g, 10min, 4°C). 500µg of protein was precipitated using 80% acetone at -20°C overnight. Precipitated protein was collected by centrifugation for 10min at 4,000g 4°C. Pellets were washed twice in -20°C 80% acetone before air drying for 10min. Protein was resuspended in 100mM ammonium bicarbonate/10% 2,2,2-trifluoroethanol and sonicated in water bath for 5min. Trypsin and LysC were added at an enzyme:protein ratio 1:50 in 100mM ammonium bicarbonate/10% 2,2,2-trifluoroethanol. Samples were incubated overnight 37°C, thermomixer (Eppendorf, Hamburg, Germany) at 1200rpm. Digest solution was acidified with 10% trifluoroacetic acid (TFA) (final 1% TFA). Solution was spun at 21,000g for 10min to remove precipitate. Neat ACN containing 0.12% TFA was added such that the solution became 80% ACN/ 0.1% TFA. 50µl of FeIII-NTA loaded magnetic beads (25% slurry; CUBE biotech; washed 2 x 500ul 80% ACN/ 0.1% TFA prior to use) was added and mixed for 30min at room temperature. Beads were washed 3 x 250µL Wash buffer (80% ACN, 0.1% TFA). Beads were transferred with 40 µL of Wash buffer into the top of a pre-wetted (using ACN) C8 stage-tip (2 discs). 30µL of 10% TFA was placed in the bottom of each collection tube. Phosphopeptides were eluted by adding 20µL 50% ACN, 2.5% Ammonium Hydroxide to the top of the C8 tip containing the beads and centrifuged at 500 x g, 1-3 mins, into the collection tube. This step was repeated once. Samples were lyophilised to near dryness, avoiding complete drying. Samples were resuspended in 5% FA and loaded onto stage-tips (home-packed, 2 C18-matrix discs; activated using isopropanol, washed in 60% ACN, 5% FA and equilibrated 2 times with 5% FA). Samples were washed twice using 5% FA before elution using 60% ACN, 5% FA. Samples were lyophilised until dry and stored at -80°C. Before analysis, peptides were resuspended in 6µl 2% ACN, 1%FA with 5µl loaded onto the instrument. Peptides were injected and separated by reversed-phase liquid chromatography on a M-class UPLC system (Waters Corporation, Milford, MA, USA) using a 250mm × 75µm column (1.6µm C18, packed emitter tip; IonOpticks, Parkville, Victoria, Australia) with a linear 90-min gradient at a flow rate of 400 nl/min from 98% solvent A (0.1% Formic acid in Milli-Q

water) to 34% solvent B (0.1% Formic acid, 99.9% acetonitrile). The UPLC was coupled on-line to a Q-Exactive mass spectrometer (Thermo Fisher, Waltham, MA, USA). The Q-Exactive was operated in a data-dependent mode, switching automatically between one full-scan and subsequent MS/MS scans of the 10 most abundant peaks. Full-scans (m/z 350–1,850) were acquired with a resolution of 70,000 at 200 m/z . The 10 most intense ions were sequentially isolated with a target value of 100,000 ions and an isolation width of 1.4 m/z and fragmented using HCD with normalised collision energy of 27. Maximum ion accumulation times were set to 50ms for full MS scan and 110ms for MS/MS. Dynamic exclusion was enabled and set to 20 seconds.

The raw files were analysed using the MaxQuant software (version 1.5.8.3) (519, 520) and the database search was performed using mouse and HBV protein sequences obtained from Uniprot including isoforms with strict trypsin specificity allowing up to 3 missed cleavages. The minimum required peptide length was set to 6 amino acids. Carbamidomethylation of cysteine was set as a fixed modification while N-acetylation of proteins N-termini, oxidation of methionine and phosphorylation of serine, threonine and tyrosine were set as variable modifications. During the MaxQuant main search, precursor ion mass error tolerance was set to 4.5 ppm and fragment ions were allowed a mass deviation of 20 ppm. PSM identifications were filtered using a target-decoy approach at a false discovery rate (FDR) of 1%.

Further analysis was performed using a custom pipeline developed in R, which utilises the MaxQuant output files allPeptides.txt, peptides.txt and evidence.txt. A feature was defined as the combination of peptide sequence, charge and modification. Features not found in at least half the number of replicates in each group were removed. To correct for injection volume variability, feature intensities were normalised by converting to base 2 logarithms and then multiplying each value by the ratio of maximum median intensity of all replicates over median replicate intensity. Missing values were imputed using a random normal distribution of values with the mean set at mean of the real distribution of values minus 1.8 s.d., and a s.d. of 0.3 times the s.d. of the distribution of the measured intensities. The probability of differential peptide expression between groups was calculated using ANOVA. Probability values were corrected for multiple testing using Benjamini–Hochberg method.

2.4.3 Statistical analysis

For statistical analysis of HBV DNA, data were subjected to an unpaired, 2-way ANOVA. For time for seroconversion analysis for HBeAg and HBsAg, data were subjected to Log-rank (mantel-Cox) test. For ALT and AST comparisons at a single

time point, data were analysed using 1-way ANOVA. All statistical analysis was carried out using Prism 8 software (Graph Pad Software, La Jolla, CA).

3 HBeAg defective variants of Hepatitis B are associated with advanced fibrosis in chronic hepatitis B

3.1 Introduction

Chronic hepatitis B is a significant public health issue in Australia. Despite a widespread national immunisation programme, the prevalence of CHB is rising due to migration from endemic countries, in particular from the Asia Pacific region.

HBV genomic variation has been associated with differences in clinical outcome. There are 10 recognised HBV genotypes, which by convention differ from one other by a sequence variation of 8% or more across the entire genome (103) or 4% or more in the S reading frame(521).

BCP and PC mutations are the most common mutations acquired by HBV during viral replication. They reduce and abolish the production of HBeAg respectively. There are multiple epidemiological studies that have shown an association between the presence of the BCP and PC mutations and aggressive clinical phenotypes, in particular, the development of cirrhosis and an increased risk of HCC (168-172), disease flares and liver failure (23, 24)(112).

In Australia, due to migration from various endemic regions including, the Asia Pacific, Africa, and the Mediterranean, there is a broad spread of genotypes. However, there is little published data about the genotypes and mutations in HBV in the Australian population(522). Currently genotyping and sequencing are not reimbursed and not part of routine clinical practice. The aims of this study were to determine the demographics, virological profile including genotype profile and frequency of BCP and PC mutations in the Australian population and to study the association between HBV genotype, BCP/PC variants and disease severity.

3.2 Manuscript

The work presented in this chapter has been submitted for publication in the peer-reviewed Journal of Gastroenterology & Hepatology.

HBeAg defective variants of Hepatitis B are associated with advanced fibrosis in chronic hepatitis B

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Abbreviations: HBV: Hepatitis B virus, BCP: basal core promoter, PC: precore, CHB: chronic hepatitis B, HBeAg: Hepatitis B e antigen, ALT: alanine transferase, WHO: world health organisation

Conflict of interest: None

3.3 Abstract

Background: Hepatitis B virus (HBV) genotype and variants including the basal core promoter (BCP) and precore (PC) variants may influence the natural history of patients with chronic hepatitis B (CHB).

Aim: To determine the association between HBV genotype, BCP/PC variants and clinical features and virological profile in a large cohort of Australian patients.

Methods: A retrospective single-centre cross-sectional analysis of treatment-naïve Australian patients infected with genotype A-D was performed. HBV genotypes, BCP/PC variant were correlated with age, gender, ethnicity, hepatitis B e antigen (HBeAg) status, serum ALT and HBV DNA level and liver fibrosis stage on biopsy.

Results: 279 patients were identified. They were predominantly male (n= 184, 66%), Asian (n=217, 78%) and HBeAg-negative (n=108, 60%). HBV genotypes were A (n=18, 7%), B (n=119, 43%), C (n= 92, 33%) and D (n= 46, 17%). Genotype was strongly correlated with ethnicity, with genotype B/C HBV prevalent in people of Asian ethnicity. Of the 162 patients who had BCP/ PC sequencing performed, 103 (73%) had a detectable mutation. On univariate analysis, age > 40yrs, male sex and the presence of BCP/PC mutations were significantly associated with the presence of advanced fibrosis, but HBV genotype was not. Using multivariable analysis, the presence of BCP or PC mutation was independently associated with advanced fibrosis (OR 5.18, CI= 1.07-25.18, p= 0.04).

Conclusions: HBeAg negative disease was common in Australian patients with CHB and associated with the selection of BCP/ PC mutants. BCP/ PC mutations were independently associated with advanced liver fibrosis.

Keywords: Hepatitis B, Mutations, Genotype, Fibrosis

Word count: 249

3.4 Key Points

- HBV genotypes and variants can affect clinical outcomes
- Basal core promoter and precore mutants abrogate the production of HBeAg and are increasingly common.
- Basal core promoter and precore mutations are associated with a higher rate of advanced fibrosis, independent of genotype or HBeAg status.
- BCP and PC mutations may be independent markers of poor prognosis, which would justify the use of sequencing in the clinical setting.

3.5 Introduction

HBV has infected more than 2 billion people globally and an estimated 257 million people are currently chronic carriers (523). Up to 40% of patients with chronic hepatitis B (CHB) develop serious liver disease. CHB is the leading cause of hepatocellular carcinoma(524), the 10th leading cause of death worldwide (215) causing 1.2 million deaths per year (523). In Australia, an estimated 218 000 people are infected and CHB accounts for approximately 600 deaths annually (525). Despite a successful national immunisation program, the prevalence of CHB is rising due to migration from endemic countries, from the Asia Pacific region.

The natural history and outcomes of CHB are variable. HBV genomic variation has been associated with differences in clinical outcome. There are 10 recognised HBV genotypes, which by convention differ from one other by a sequence variation of 8% or more across the entire genome (103) or 4% or more in the S reading frame(521). The geographical distribution of HBV genotypes is well characterised with a high prevalence of genotypes B and C in Asia whilst A2 and D are common in Europe and A1 and E are predominantly found in Africa. Genotype C has been associated with more severe disease outcomes compared to genotype B, whilst genotype D has been associated with worse prognosis than genotype A (526-528) (102, 110, 267, 529-532). Few studies have included people infected with all 4 HBV genotypes (A-D) as most endemic populations tend to be enriched in one or two genotypes. Furthermore, the exact role of HBV genotype in determining the natural history of CHB remains poorly defined.

BCP and PC mutations are the most common mutations acquired by HBV during viral replication. The BCP spans nucleotides 1742–1849 in the HBV genome and it controls the transcription of both precore and core regions. The A1762T/G1764A dual mutation reduces transcription of the precore mRNA, leading to consequent reduction in HBeAg translation and HBeAg levels. The core reading frame overlaps the reading frame for the X protein (HBx) and as a result the A1762T/G1764A mutation is often associated with codon changes in X at nucleotides 130 and 131(533). The X protein is a key regulatory protein that is essential for viral persistence and it is strongly implicated in carcinogenesis (533) (87). The PC mutation is a substitution of guanine (G) to adenine (A) at nucleotide (G1896A), which forms a stop codon (TAG) in the 76recure reading frame at nucleotide 1896 (codon 28) and completely abolishes HbeAg production(157). BCP and PC mutations have been associated with advanced fibrosis and cirrhosis and an increased risk of HCC (168-172). They have also been associated with a higher number of disease flares and necro-inflammation(176, 177), fulminant liver failure and

cirrhosis-related complications such as decompensation (112). Finally, the frequency of BCP / PC mutations has been observed to vary by HBV genotype. For example, genotype C HBV has been associated with a high rate of BCP mutations and genotype B HBV with PC mutations. In contrast, PC mutations are rare in genotype A HBV as the mutation would compromise the structural stability of the Epsilon loop and therefore the efficiency of viral replication (123). It is unclear if differences in clinical phenotype are attributable to the HBV genotype *per se* or are due to the associated mutations.

The Australian CHB epidemic is unique for the broad spread of HBV genotypes. However, despite the increasing recognition of the clinical importance of HBV genotypes and mutations in HBV clinical outcomes, there is little published data about the Australian population (522). Currently genotyping and sequencing are not reimbursed and not part of routine clinical practice. The aims of this study were to determine the demographics, virological profile including genotype profile and frequency of BCP and PC mutations in the Australian population and to study the association between HBV genotype, BCP/ PC variants and disease severity.

3.6 Patients and Methods

3.6.1 Patients

The methods for patient recruitment has been described in the Materials and Methods chapter (2.1.1 Patient recruitment).

3.6.2 Hepatitis B virus testing

The assays for Hepatitis B serology, Hepatitis B viral load, genotype analysis and PCR sequencing have been described in the Materials and Methods chapter (2.1.2 Hepatitis B virus testing)

3.6.3 Statistical analysis

Data were analysed using SAS® statistical software Version 9.4 as described in the Materials and Methods chapter (2.1.3 Statistical analysis).

3.7 Results

3.7.1 Patient characteristics

279 treatment-naïve patients with CHB were identified (Table 3.1). The cohort was predominantly male (n= 184, 66%) and HBeAg negative (n=171, 60%) with an average age of 41 years. The majority of the cohort (n=242, 87%) was born overseas with only 37 (13%) being Australian-born. Most patients were of Asian ethnicity (n=217, 78%) and the rest of the population originated from the Mediterranean basin (n= 29, 11%) or Africa (n=10, 4%). There was a significant age difference between the HBeAg positive and the HBeAg negative groups (34 years vs 46 years, p-value <0.01). 201 patients (77%) had an elevated ALT at baseline with a median baseline ALT of 60 IU/ml (IQR 35- 120). The majority of patients in our cohort had active hepatitis, with a large number of patients in the HBeAg positive chronic hepatitis phase of disease (n=92, 33%) or in the HBeAg negative chronic hepatitis phase (n=145, 53%). Among patients with chronic hepatitis, patients who were HBeAg-positive were younger (34 vs 46, p <0.01), and had higher levels of ALT (80 vs 55 IU/ml, p<0.001) and HBV DNA (7.3 vs 4.9 log₁₀ IU/ml, p <0.001). Liver biopsy was performed in 194 patients as part of the assessment for treatment. 20% (n=37) of patients had advanced fibrosis (F3-4).

	Overall	HBeAg +ve	HBeAg -ve	P value
Number	279	108 (40%)	171 (60%)	
Age, yrs (median, IQR)	41 (31-50)	34 (27-42)	46 (35-54)	<0.01
Male (n, %)	184 (66%)	63 (58%)	121 (71%)	
Ethnicity				
Asian	217 (78%)	86 (79%)	131 (77%)	NS
Caucasian	18 (6%)	11 (10%)	7 (4%)	
Mediterranean	29 (11%)	8(7%)	21 (12%)	
African	10 (4%)	2 (2%)	8 (5%)	
Other	5 (2%)	1 (1%)	4 (2%)	
ALT, IU/mL (median, IQR)	60 (35-120)	80 (36-134)	55 (33-101)	<0.001
HBVDNA, log ₁₀ IU/mL (median, IQR)	6.3 (4.5-7.3)	7.3 (7.0- 7.8)	4.9 (3.6-6.5)	<0.001
Disease Phase				
HBeAg positive chronic infection	22 (8%)	22 (20%)	-	
HBeAg positive chronic hepatitis	86 (32%)	86 (80%)	-	
HBeAg negative chronic infection	26 (7%)	-	26 (15%)	
HBeAg negative chronic hepatitis	145 (53%)	-	145 (85%)	
HBV genotype (n, %)				
A	18 (7%)	8 (7%)	10 (6%)	NS
B	119 (43%)	43 (40%)	76 (44%)	NS
C	92 (33%)	43 (40%)	49 (29%)	NS
D	46 (17%)	14 (13%)	34 (20%)	NS

BCP/PC, n= 162 (n, %)				
BCP alone	43 (27%)	27/ 66 (42%)	16/ 96 (17%)	NS
PC alone	40 (25%)	4/ 66 (6%)	36/ 96 (37%)	<0.001
BCP + PC	40 (25%)	4/ 66 (6%)	36/ 96 (37%)	NS
Liver Fibrosis, n= 194 (n, %)				
METAVIR F0-2	157 (80%)	69 (85%)	88 (78%)	NS
METAVIR F3-4	37 (20%)	12 (15%)	25 (22%)	NS

Table 3.1 Patient demographics, biochemistry, virology and histology

3.7.2 HBV Genotypes

Four main genotypes of HBV were identified: A, B, C and D, with B and C being the predominant ones (n=119, 43% and n=92, 33% respectively). There was a strong correlation between HBV genotype and ethnicity. Asian patients were almost exclusively genotypes B or C, African patients were genotype A and the majority of Mediterranean patients were genotype D (Table 3.2). The Australian-born Caucasian population (n=18, 6%) included a mix of all 4 genotypes with a greater proportion of genotype D.

Genotype	A (n=18)	B (n=119)	C (n=92)	D (n=46)
Ethnicity (n, %)				
Asian	4 (2%)	109 (50%)	102 (47%)	2 (1%)
African	10 (27%)	3 (9%)	5 (14%)	19 (50%)
Caucasian	1 (5%)	0	0	18 (95%)
Mediterranean	7 (70%)	0	0	3 (30%)

Table 3.2 Relative distribution of genotypes amongst ethnic groups

3.7.3 Basal core promoter and precore mutations

Population sequencing data for the PC (G1896A) and BCP (A1762T/G1764A) mutations was available in 162 patients. The mutations were common: 25% (n=40) had the PC mutation only, 27% (n=43) had the BCP mutation only and a further 25% (n=40) had both PC and BCP mutations. Only 24% (n= 39) patients did not have a BCP or PC mutation detected. The presence of the PC mutation was strongly

associated with HBeAg negative disease. Only 12% (n=8) of HBeAg positive patients had the PC mutant detected vs. 74% (n=71) of HBeAg negative patients ($p < 0.01$).

BCP was common in both HBeAg positive and negative disease (n= 31 [48%] vs. n= 52, 54%] respectively, $p = 0.23$). Only a minority of HBeAg negative patients had no BCP/ PC variants (n=9, [9%]).

BCP and PC mutants were associated with specific HBV genotypes (Table 3.1). The PC mutation was predominant in genotype B HBV (p -value < 0.001) where 35% (n= 24) had the PC mutation only and an additional 18% (n= 12) had both PC and BCP mutations. The BCP mutation was strongly prevalent in genotype C HBV (p -value < 0.02) where 47% (n= 24) had the BCP mutation alone and an additional 26% (n=13) had both BCP and PC mutations. The PC mutant was most common in Genotype D HBV (67%, n= 19); the combination of both BCP / PC mutants was also common. BCP / PC variants were uncommon in patients Genotype A HBV.

Genotype	A (n=7)	B (n=71)	C (n=55)	D (n=28)
HBV BCP / PC variants (n, %)				
BCP	2 (3%)	5 (7%)	26 (47%)	4 (14%)
PC	1 (1%)	25 (35%)	4 (8%)	8 (30%)
BCP+PC	0	13 (18%)	14 (26%)	10 (37%)

Table 3.1 Frequency of BCP / PC mutants according to HBV genotype

3.7.4 Predictors of advanced liver disease

As noted, 20% (20/194) of the cohort had advanced liver fibrosis on histology (METAVIR F3-F4). Age > 40 years, male gender, and the presence of a BCP / PC variant was associated with advanced liver fibrosis (Table 3.2). HBeAg antigen status and HBV genotype were not significantly associated with advanced fibrosis (p -value 0.02 and p -value 0.04 respectively). The presence of either a BCP or PC mutation was also associated with advanced fibrosis. Logistic regression for the effect of age > 40 years old, male sex, HBeAg status, genotype and presence of a BCP or PC mutation revealed that the presence of BCP or PC mutation was independently associated with advanced fibrosis (odds ratio 5.18, CI= 1.07-25.18, $p = 0.04$).

Univariable analysis		Multivariable analysis		
Variable	P-value	Variable	OR (95% CI)	P-value
Age > 40 years	0.02	Age > 40 years	1.226 (0.378- 3.98)	0.74
Male gender	0.04	Male gender	3.518 (0.734- 16.9)	0.12
HBeAg status	0.31	*		
HBV genotype AD vs BC	0.09	*		
B	0.011	B	1.13 (0.47- 2.7)	0.78
C	0.07			
BCP / PC variant vs WT	0.017	BCP / PC variant	5.18 (1.07-25.18)	0.04
HBV DNA				
>107	0.933	*		
>104	0.933			
>2000	0.37			
ALT	0.61	*		
Ethnicity				
Asian	0.021	Asian	1.08 (0.42-20.0)	0.279
African	0.574	*		
Caucasian	0.377	*		
Mediterranean	0.024	Mediterranean	0.58 (0.16- 3.11)	0.521

Table 3.2 Regression modelling for clinical and virological predictors of advanced liver fibrosis (METAVIR F3-F4)

* There was no statistically significant difference on univariate analysis

3.8 Discussion

This study is the first detailed analysis of HBV in Australia that focuses on the association between virological factors and clinical outcomes. There were four prevalent HBV genotypes in our cohort, A-D, with a clear ethno-geographic distribution consistent with Australian migration patterns. The Australian demographics of CHB is made up of a large proportion of patients from Asia and a genotype profile enriched in genotypes B and C, which reflects the steady increase in migration from endemic regions, in particular South East Asia (525).

More than half of our cohort had HBeAg negative chronic hepatitis. HBeAg negative disease was associated with older age, consistent with the global trend seen over the past 2 decades for HBeAg-negative disease to be more common as the HBV population ages. HBeAg negative disease was also associated with selection of the PC variants that abrogates HBeAg production.

There was a high prevalence of BCP and PC mutations in our cohort, consistent with a high proportion of patients with HBeAg negative active hepatitis in the immune escape phase. Mutations were genotype specific, being very low in the genotype A group although it should be noted that the number of patients was small. This is in keeping with the fact that genotype A does not readily support the precore G1896A substitution due to the absence of a C at the 1858 nucleotide. Only 1 patient with genotype A had the precore G1896A mutation and in that patient, the compensatory C1858T mutation was also present.

Furthermore, the duration of viraemia also likely has an effect on the development of mutants. In our cohort, a large proportion of patients originated from an endemic area, where vertical transmission and early childhood horizontal transmission are the most likely modes of HBV acquisition. Age, therefore, may be considered a surrogate marker of duration of viraemia. Our Genotype D patients were significantly older and genotype D was notable for the presence of both the BCP and PC mutations.

The presence of the PC mutation was strongly associated with HBeAg negative disease but the BCP mutation was not. This could be explained by the fact that BCP diminishes but does not completely abrogate the production of HBeAg. The associations of a more severe disease phenotype in BCP mutants compared to wild type may therefore not all be attributable to the loss of HBeAg. It is possible that the overlap between the BCP and the X ORF is responsible for that effect. Further mechanisms to explain the immunopathogenesis would be required.

The presence of BCP or PC mutations was independently associated with advanced fibrosis. This finding has been reported in other studies (534, 535). These studies

define fibrosis score as F2 or above and they were limited to the study of only one genotype.

The association between BCP/PC variants and advanced liver fibrosis was independent of HBV genotype, suggesting a direct pathogenic role for these variants. In contrast to previous descriptions, we did not observe an association between HBV genotype and advanced liver fibrosis. In many epidemiological studies, Genotype C has been associated with more aggressive disease compared to genotype B HBV. However, in our cohort, there was no independent association between genotype C and advanced fibrosis. Genotype C is strongly associated with the BCP mutation, and we hypothesise that in our cohort, it may be responsible for the clinical phenotype. There were some limitations to our study. It is a retrospective study and there is potentially a selection bias in our patient cohort. Our cohorts consisted of who had presented to the hospital for assessment for treatment. Our observation that a large proportion of the cohort was in the immune active or immune escape phases of disease could be influenced by selection bias, and not a true reflection of CHB in the community. Our study was not powered to be able to dissect the individual role of BCP and PC mutants separately as the number of patients with one mutation alone was small. Furthermore, in that patient population, the co-existence of both BCP and PC is common and the individual contribution of either mutation is difficult to delineate and the clinical relevance of such a distinction may be academic.

Although it is likely that genotypes do play a role in pathogenesis, this study emphasises the importance of specific HBeAg negative mutants. We postulate that this clinical observation corresponds to the immune-modulatory effect of HBeAg. In the early phases of disease, HBeAg allows the virus to evade the immune system and establish chronicity. However, in the later phases of disease, HBeAg negative mutants are preferentially selected, associated with the formation of anti-HBe antibodies. It is possible that the absence of HBeAg now confers a selective advantage to the BCP/PC mutant in the setting of an adaptive immune response and the presence of antibodies directed at the HBeAg. A HBeAg deficient mutant can therefore escape immune control by the adaptive immune system and replicate relatively unhindered. This leads to an active hepatitis, which in turn results in a more rapid progression to fibrosis. However, this hypothesis warrants further evaluation.

This study has significant implications for the ongoing management of CHB. The prevalence of BCP/ PC mutants is high in endemic populations and their association with advanced fibrosis would justify the use of sequencing in clinical practice. Sequencing for BCP / PC variants is not currently a standard clinical test and prospective studies are warranted to evaluate the clinical utility of BCP / PC variants as

clinical biomarkers for HCC risk in the era of NA therapy. Indeed, the data suggest this may be more important than HBV genotype testing.

3.9 Conclusion

The Australian CHB population has a broad spread of HBV genotypes. HBeAg negative disease is common and associated with the selection of BCP and PC mutants. BCP / PC mutants are strongly associated with advanced fibrosis, which is in keeping with international data. This suggests a role for BCP / PC sequence as a biomarker for more aggressive natural history, warranting vigilant surveillance and monitoring.

4 Studies on the role of HBeAg on the Innate Immune Response

4.1 Introduction

The interaction between the virus and the immune response is central to the pathogenesis of chronic hepatitis B.

A key viral protein in immune-modulation is HBV e antigen (HBeAg). Multiple clinical and animal studies have established the association between HBeAg and clinical outcomes. For instance, in the clinical context, transmission of HBeAg-negative HBV is associated with an acute but self-limiting hepatitis, whereas the presence of HBeAg appears to be associated with immune tolerance and subsequent lifelong viral persistence (Li 26060603), . This is in keeping with the concept that HBeAg somehow allows the virus to escape the innate immune response and allows persistence (see 1.10.8 Viral evasion of the innate immune response)

Most of the research in the immunology of CHB has focussed on the adaptive immune response. The role of the innate immune response is relatively unclear. IFN-I is of particular interest, as it is known to be a key mediator of the innate response in various viral infections. Its role in HBV, however, remains unknown. Animal and patient data suggest that IFN-I signalling is weak in HBV infection, but the mechanisms by which the IFN-I response is suppressed are unknown.

One hypothesis is that HBeAg allows the virus to establish persistence by evading the innate immune response and is a key element in the early stages of infection. The aims of the study was to:

1. Examine the role of HBeAg in the early stages of HBV infection by comparing HBeAg deficient variant with wild type virus using an immunocompetent mouse model.
2. Analyse specific mediators of the innate immune response, with a specific focus on the interaction with HBeAg.

4.2 Manuscript

The work described in this chapter has been submitted for publication in the peer-reviewed journal Gastroenterology.

Hepatitis B virus e antigen modulates liver transcriptome, proteome and type I interferon responses.

Short title: Hepatitis B virus e Antigen abrogates immunity

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Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCP, basal core promoter; CHB, chronic hepatitis B; HBV, Hepatitis B virus; anti-HBe, anti-HBeAg antibodies; HBeAg, hepatitis B virus e antigen; anti-HBs, anti-HBsAg antibodies; HBsAg, hepatitis B surface antigen; IFN, interferon; IFN-I, type I interferon; IFNAR, interferon α / β receptor; IFN - γ , interferon gamma; IRG, interferon regulated gene; PC, precore; TNF, tumour necrosis factor

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Transcript and Proteomic Profiling: RNA-seq data is available as GEO series GSE123570; proteome and phospho-proteome data is available in the PRIDE repository, reference number PXD011325.

Authors' contribution: Z.V. designed and performed experiments, analysed and interpreted data and prepared the manuscript, A.I.W., C.A., G.E., G.K.S., H.M., J.J.S., L.M., M.P.C., M.D., P.V.D., R.E., S.B., S.P., V.S. and W.A., assisted with experimental techniques, interpretation and analysis of data, A.J.T., G.E., M.P. and P.R., designed experiments, interpreted data and assisted with the preparation of the manuscript.

4.4 Abstract

Background and aims:

A conundrum in the natural history of hepatitis B virus infection is that the virus appears to rely on the production of HBe antigen protein for transmission and establishment of infection, but HBV mutations conferring a loss of HBe antigen prevail in chronic stages of infection. It is unclear why this viral protein changes from being an early asset to an apparent late liability for the virus. We aimed to address this knowledge gap by dissecting the causal consequences of HBeAg production *in vivo*.

Methods:

We compared and contrasted the viral kinetics, liver transcriptome, liver proteome and outcomes in mice hydrodynamically injected with wild type (WT) HBV and mice injected with two naturally occurring viral mutants, basal core promoter (BCP) and precore (PC), that are deficient in HBeAg production.

Results:

HBV mutants that were deficient in HBeAg production were attenuated in their ability to establish early persistent infection compared to WT virus. However, mutants and WT virus were equivalent in initiating persistent infection in mice that were deficient in type I interferon (IFN-I) signalling. Additionally, when HBeAg was supplied *in trans* through co-infection with WT virus, mutant virus was able to establish robust infection. Liver transcriptome and proteome data showed that HBeAg abrogates the early innate immune response by antagonizing IFN-I responses.

Conclusions:

Our studies define an early role for HBeAg in establishing persistent HBV infection through the modulation of IFN-I responses. We speculate that this may become a liability for the virus during late stages of infection.

Keywords: HBV, HBeAg, HBeAg mutants, interferon, liver transcriptome, liver proteome, liver phospho-proteome.

4.5 Introduction

Chronic hepatitis B (CHB) is a major contributor to the burden of liver related morbidity and mortality. The initial host-virus interaction is key in determining both the natural history and clinical outcomes of hepatitis B virus (HBV) infection. Activation of host type I interferon (IFN-I) responses contribute to early innate control of several viral infections (334, 341, 351, 360, 361, 376, 385), but their role in the early stages of HBV infection remains controversial (342, 381, 382, 386, 536). Clinically, polymorphisms in the interferon receptor (IFNAR) promoter have been linked to the susceptibility of developing chronic HBV infection(537). However, a combination of *in vitro* (538), mouse(341), chimpanzee (539) and patient (540) data have indicated that IFN-I signalling is not robustly activated by HBV. It is plausible to suggest that host and or viral factors conspire to mitigate the magnitude and impact of IFN-I signalling on initial HBV infection.

One viral product that may play a role in modifying host immunity is HBV e antigen (HBeAg) (26-30, 541). Clinical and chimpanzee studies have provided insights regarding the role of HBeAg in the pathogenesis of HBV infection and disease. People acutely infected with HBV variants that are deficient in the production of HBeAg develop an acute self-limiting hepatitis(542), but in some cases the hepatitis can be fulminant (543-548). The apparent dichotomy in clinical outcomes can be reconciled with the knowledge that HBV is a non-cytopathic virus and host immunity / inflammation is responsible for both hepatocyte death and reduction in viral loads. Therefore, these clinical reports suggest that acute HBV infection, in the absence of HBeAg, is associated with strong immunological and inflammatory responses that result in viral control and / or excessive liver damage. This is consistent with the notion that HBeAg abrogates early host responses. Chimpanzees infected with HBeAg deficient virus also develop an acute severe hepatitis replicating the strong immunological host responses seen in humans (549).

In the natural history of vertically transmitted HBV infection, HBeAg is only present in the early phases of disease, with HBeAg negative variants emerging in later phases (reviewed in (234)). The two most common HBV mutations are those causing a loss of HBeAg, namely the basal core promoter (BCP) and precore (PC) variants (Supplementary Figure 2A). The classic PC mutation is the G1896A substitution, often accompanied by a G1899A mutation. The former results in a translational stop codon, and no HBeAg is translated (157, 176). The classic BCP variant is a double substitution at nucleotides 1762 and 1764 (A1762T/G1764A), which reduces promoter activity and decreases transcription of HBeAg (550, 551). The BCP mutation greatly

reduces the level of HBeAg but it does not result in the complete loss of this viral protein. Additionally, because of overlapping reading frames in the HBV genome, BCP mutations cause amino acid substitutions in the HBV X protein (K130M / V131I) (552). Both BCP and PC mutants are associated with increased risk of cirrhosis and hepatocellular carcinoma in the later phases of disease (106, 553, 554). In this study we examined the role of HBeAg in the early stages of HBV infection by taking advantage of the naturally occurring and clinically relevant BCP and PC variants. We used an immunocompetent mouse model that recapitulates aspects of persistent HBV infection to dissect the impact of HBeAg. We quantified *in vivo* viral kinetics and host endpoints comparing WT and HBeAg deficient variants. This allowed us to delineate the role of HBeAg in modulating early host immunity.

4.6 Materials and Methods

4.6.1 Generation and preparation of HBV WT, PC and BCP mutant plasmids

This has been described in the Methods and Materials chapter (See 2.2.1 Plasmid preparation).

4.6.2 Animal model

The Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee reviewed and approved all animal experiments (AEC Project 2012.013 and 2014.005). Mice, including gene-targeted animals, are described in the Materials and methods chapter (2.2.2 Mice).

4.6.3 Hydrodynamic tail vein injection

Approximately 10 µg of HBV plasmid was rapidly injected, within 5 seconds, intravenously through the tail in a volume of saline equivalent to 8% of the mouse body weight. The method has been described in the Materials and methods chapter (2.2.3 HBV mouse model of infection).

4.6.4 Serum HBV DNA Quantification.

HBV DNA was extracted from the serum of animals and quantified as previously described (2.2.5 Quantitative measurement of serum HBV DNA)

4.6.5 HBV Serology, Aspartate Aminotransferase and Alanine Aminotransferase Quantification.

Quantification of serum HBsAg, HBeAg, anti-HBeAg antibodies (anti-HBe) and anti-HBsAg antibodies (anti-HBs) were performed as described in sections 2.2.6 Quantitative measurement of HBeAg, 2.2.7 Quantitative measurement of HBsAg titre,

and 2.2.8 Quantitative measurement of anti-HBe and anti-HBs antibodies. Aminotransferases were quantified as described in section 2.2.4 Alanine Aminotransferase and Aspartate Aminotransferase quantification.

4.6.6 Histologic Analysis

Mouse livers were collected, fixed in formalin and the number of CD3 positive cells was quantified as described in the materials and methods chapter (**Error! Reference source not found. Error! Reference source not found.**).

4.6.7 Next Generation Sequencing

Next generation sequencing was performed as described in section 2.2.9 Next Generation Sequencing.

4.6.8 Transcriptomic analysis

RNA extraction and transcriptomic analysis has been described in section 2.3 Transcriptome analysis.

4.6.9 Mass Spectrometry – proteome and phospho-proteome analysis

The preparation of mouse livers and proteome and phosphor-proteome analysis has been described in section 2.4 Proteome studies.

4.6.9.1 Data accessibility

RNA-seq data was deposited into the GEO repository, reference number GSE123570. Proteome and phospho-proteome data were deposited into the PRIDE repository, reference number PXD011325.

4.6.10 Statistical Analysis

Statistics were performed using Prism 7 software (GraphPad). The various analyses are described in section 2.4.3 Statistical analysis.

4.7 Results

The initial magnitude and kinetic of serum viral DNA loads was highly abrogated in animals with BCP and PC mutants compared to those infected with WT HBV. We induced infection in animals using WT HBV and HBV that was deficient in its ability to produce HBeAg (BCP and PC mutants). Mice with WT HBV showed a gradual decline in serum viral DNA loads over a 7-week period. In contrast, when BCP and PC mutants were used to induce infection, there was a precipitous drop in serum viral DNA levels resulting in a 3-log difference between mutant and WT viral loads in infected mice (Figure 4.1A).

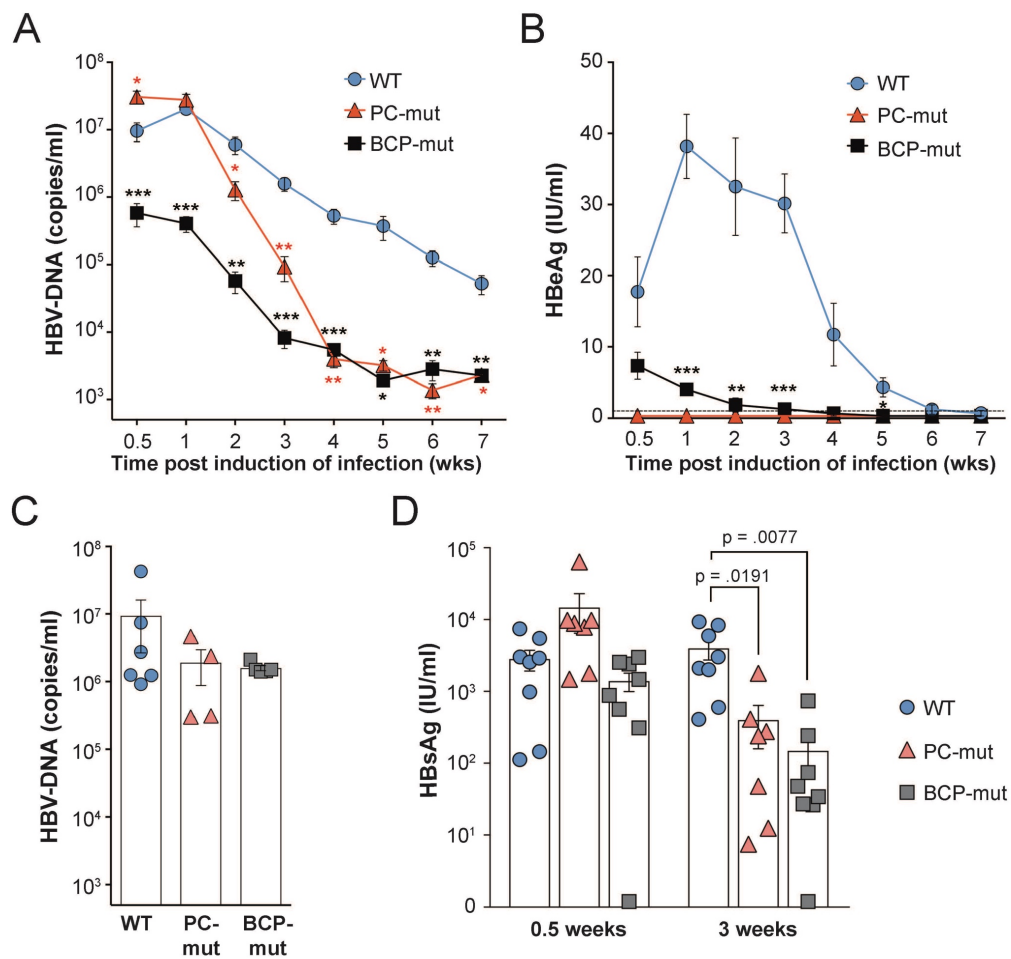


Figure 4.1 A – D Persistent PC and -BCP infection is constrained in a preclinical model of HBV infection. (A) Serial measurement of serum HBV DNA levels (n = 25 - 29 per group). (B) Serial measurement of serum HBeAg levels (n = 5 - 6 per group). (C) Serum HBV DNA levels in mice one day post induction of infection (n = 4 - 6 per group). (D) Serum HBsAg levels at the indicated time points (n = 7 - 8 per group). Mean and SEM are shown. Data are pooled from three independent experiments (A) or are representative of two independent experiments (B, C and D). Experiments were blinded. Each symbol represents an individual animal. Red asterisks indicate comparisons between HBV WT and PC-mutant and black asterisks indicate comparisons between HBV WT and BCP-mutant, *P < .05; **P < .01; ***P < .001. We confirmed that BCP and PC produced little or no HBeAg, respectively, compared to WT HBV (Figure 4.1B). HBeAg was quantifiable over a 5 - 6 week period in mice infected with HBV WT, but the levels of this viral protein declined thereafter, mirroring viral kinetics in these animals. Mice with PC virus had no detectable serum HBeAg and mice with BCP virus had very low levels of HBeAg at early time points (Figure 4.1B).

Mice with BCP mutant quickly appeared to control the amount of HBeAg within 4 weeks and all mice with BCP developed anti-HBeAg antibodies (anti-HBe) by 6 weeks (Figure 4.2B). In contrast mice with WT virus maintained high levels of serum HBeAg (Figure 4.1B) consistent with their high serum viral loads and there was a marked delay in the development of anti-HBe (seroconversion) (Figure 4.2B).

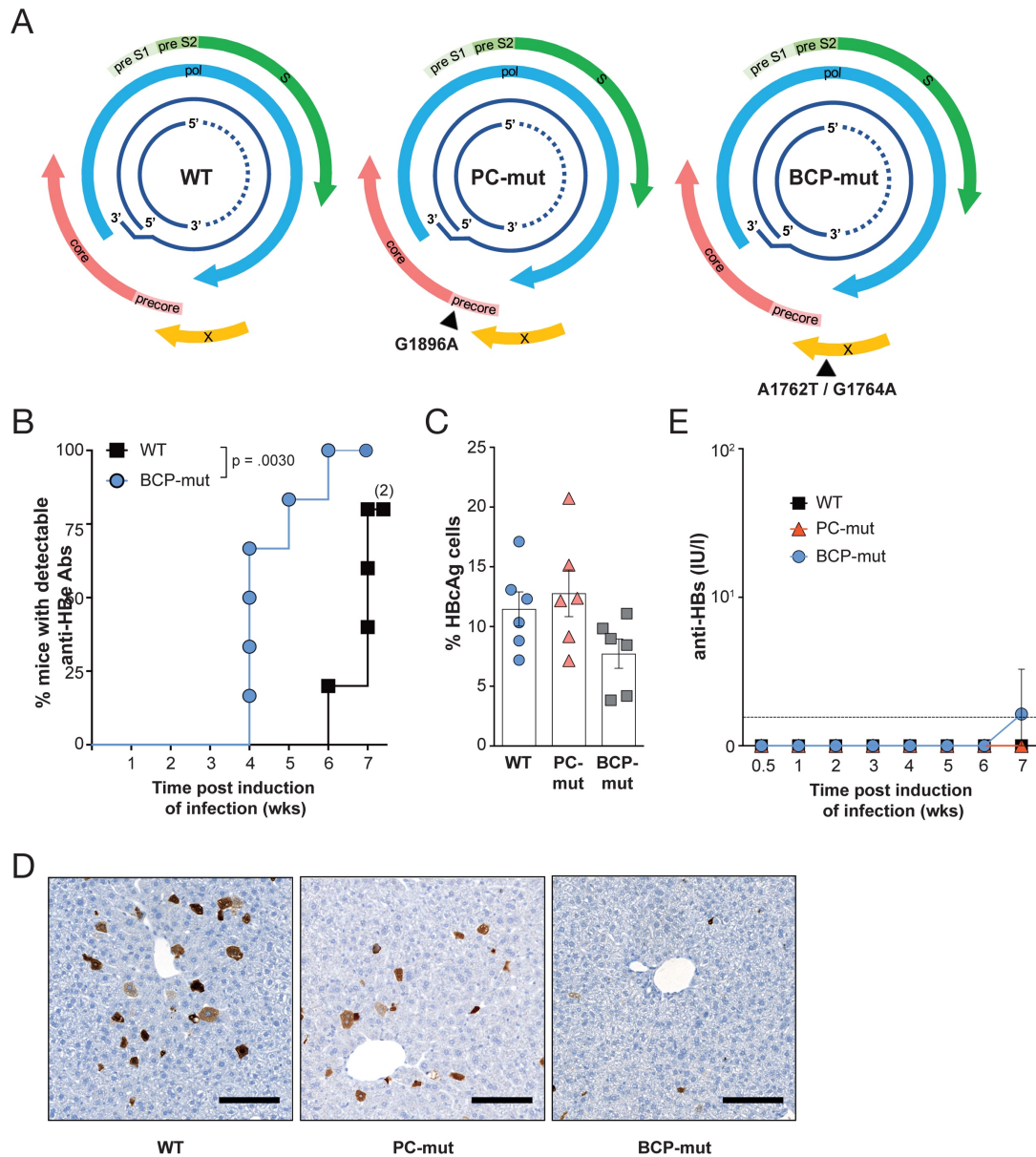


Figure 4.2 A – E (A) Schematic representation of the viral open reading frames of HBV WT (left), PC mutant (middle) and BCP mutant (right). (B) Quantitative analysis of HBcAg positive cells in liver sections of mice one week post induction of infection (n = 6 per group). (C) Representative immunohistochemistry staining of HBV core antigen positive cells in liver sections of C57BL/6 mice one week after induction of infection with the indicated HBV mutants (n = 6 per group, scale bars: 125 μm). mutants

Although both HBeAg mutant viruses were associated with dramatic reductions in viral loads at 4 weeks post induction of infection compared to WT virus, the reduction in viraemia occurred earlier in BCP mutant virus compared to PC mutant and WT virus (Figure 4.1A). To exclude the possibility that mutant viruses, and particularly BCP virus, possessed intrinsic replicative defects, we quantified viral loads 24 hours post induction of infection. At this time point we did not detect any differences in viral loads between the different viruses indicating that BCP and PC viruses did not have an intrinsic replication defect (Figure 4.1C). Additionally, all viruses resulted in the production of HBV core protein (HBcAg) in the liver (Figure 4.2 C and D). These data indicated that infection could be induced with WT and mutant viruses but in the absence of HBeAg mutant viruses failed to robustly establish persistent infection.

4.7.1 Serum levels of HBsAg declined rapidly in mice with mutant virus compared to mice with WT virus

WT, PC and BCP viral mutants all produced HBsAg when infection was induced in mice. Serum levels of HBsAg mirrored the corresponding HBV DNA levels across viral genotypes. Both mutant viruses were associated with a rapid decline in serum HBsAg compared to the kinetics of serum HBsAg clearance in mice with WT virus (Figure 4.1D). Despite the decline in HBsAg observed in mice with BCP and PC viruses compared to WT virus, very few animals developed detectable anti HBsAg antibodies (anti-HBs) during the follow-up period (Figure 4.2E).

4.7.2 Transaminitis was an early event in mice with BCP and PC mutants compared to mice with WT virus.

Collectively our data indicated that deficiency of HBeAg was associated with a more robust early innate immune / inflammatory response with a more rapid decline in serum HBV DNA titres and HBsAg and early acquisition of anti-HBe antibody compared to infections in which WT levels of HBeAg were present. We speculated that such a robust immune response in mice with BCP and PC mutant virus might manifest as transaminitis with elevations in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) similar to patients acutely infected with HBeAg deficient variants. We quantified the levels of serum ALT and AST in mice with PC, BCP and WT viruses one week post induction of infection and found that both serum ALT and AST were elevated in mice with mutant viruses compared to WT virus (Figure 4.3A). Therefore, the transaminitis was coincident with the rapid reduction observed in viral loads and HBsAg in mice infected with HBeAg deficient mutants.

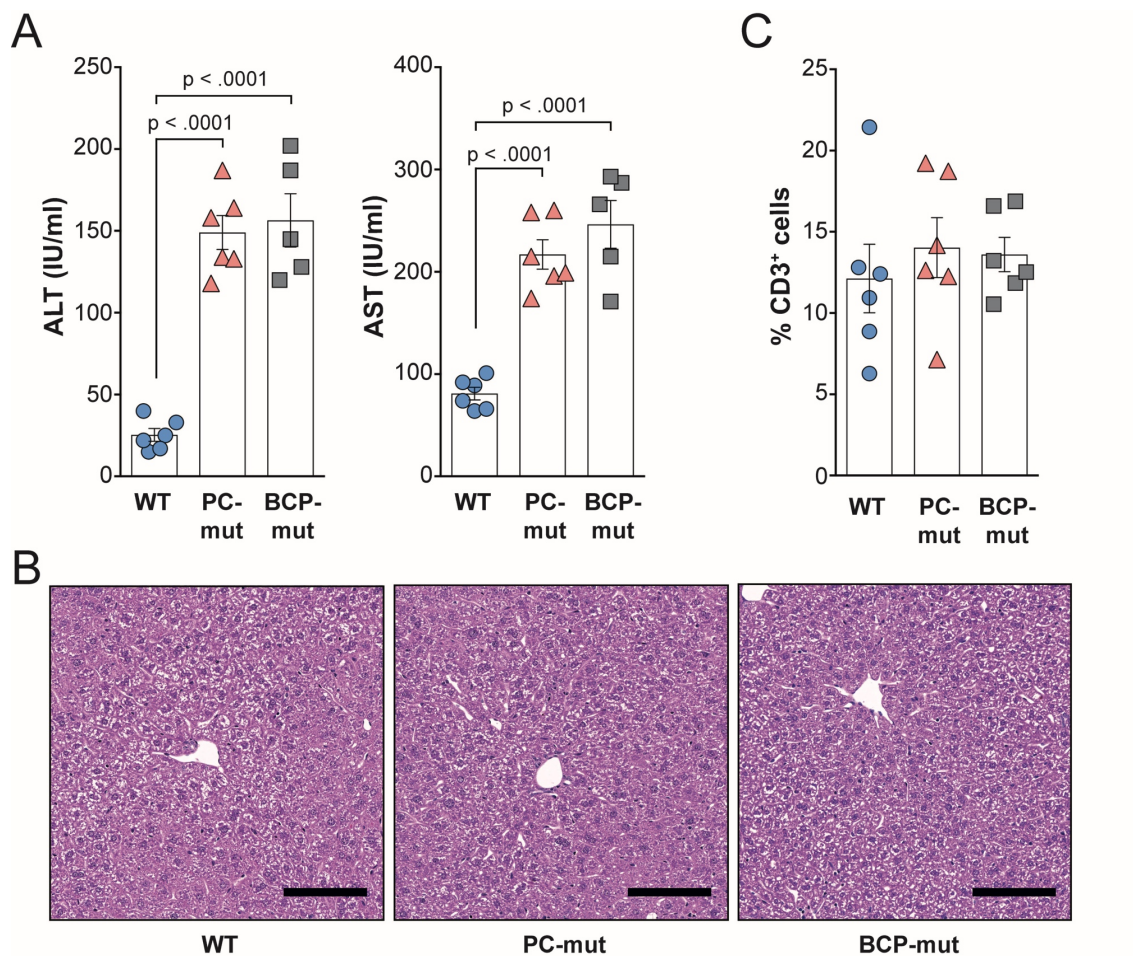


Figure 4.3 A - C Transaminitis associated with HBV PC and -BCP mutants. (A) Serum ALT (left) and AST (right) levels one-week post induction of infection (n = 5 - 6 in each group). (B) Representative hematoxylin and eosin stained liver sections one-week post induction of infection (scale bar: 125 μ m). (C) Proportion of CD3⁺ T cells in liver sections one-week post induction of infection (n = 6 per group). Mean and SEM are shown. Data in (A) and (C) is representative of two independent experiments. Experiments were blinded.

4.7.3 BCP, PC and WT viruses caused similar histopathological phenotypes in mice.

There was no major histopathological difference between livers from mice with BCP, PC and WT virus at one week (Figure 4.3B) or 3 weeks (Figure 4.4A) following induction of infection. Additionally, we did not observe any differences in the proportions of CD3⁺ T cells infiltrating the livers one-week (Figure 4.3C and Figure 4.4B) or 3 weeks (Figure 4.4 C and D) post induction of infection, regardless of viral

mutation. Therefore, we speculated that a very early innate immunological event was responsible for the reduction in HBsAg and transaminitis in mice with HBeAg deficient virus. To further dissect this possibility we induced infection, with WT and mutant viruses, in animals that were deficient in aspects of immune signalling.

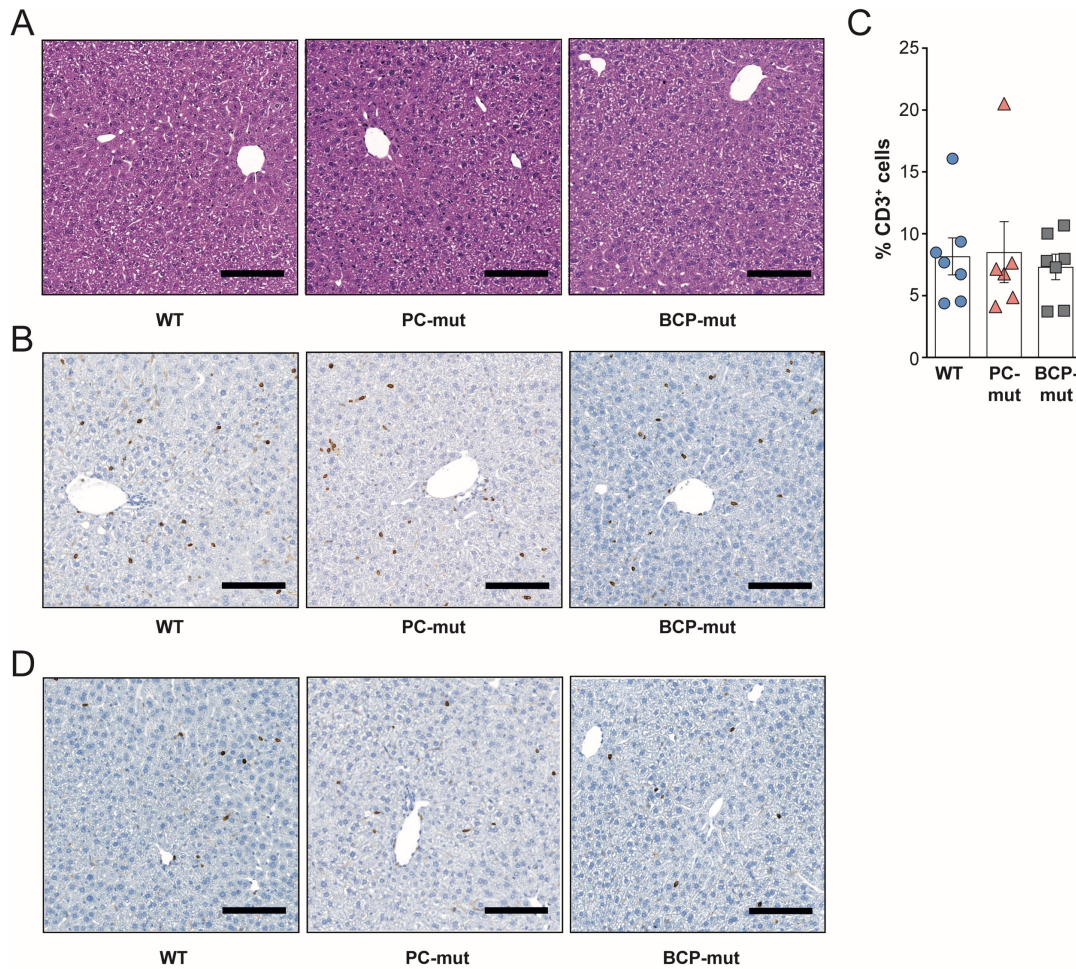


Figure 4.4 A – D Immunohistochemistry (A) Representative immunohistochemistry staining for CD3⁺ T cells in liver sections of C57BL/6 mice one week after induction of infection with the indicated HBV mutants (n = 6 per group). (B) Representative immunohistochemistry staining for CD3⁺ T cells positive cells in liver sections from C57BL/6 mice three weeks after induction of infection with the indicated HBV mutants (n = 6 per group). (C) Quantitative analysis of CD3⁺ T cells positive cells in liver sections from C57BL/6 mice three weeks after induction of infection with the indicated HBV mutants (n = 6 - 7 per group). (D) hematoxylin and eosin stained liver sections from C57BL/6 mice three weeks after induction of infection with the indicated HBV

mutants (n = 6 per group, scale bars: 125 μ m). Mean and SEM are shown. Experiments were blinded. Data is representative of two independent experiments.

4.7.4 Antagonizing TNF signalling did not alter the early kinetics of viral control in mice with mutant viruses

Tumour necrosis factor (TNF) is a cytokine produced by innate immune cells, such as macrophages and Kupffer cells and it is also produced by the adaptive immune system (426, 555). In addition to its early role in activating immune cells it also plays a role in killing target cells, including HBV infected cells (409, 432, 555). To examine if TNF was contributing to the early virologic control observed in mice with HBeAg deficient virus, we injected animals with TNF neutralising antibodies. The initial viral kinetics were not altered in mice treated with TNF neutralising antibodies compared to mice receiving an isotype control regardless of the HBV mutation (Figure 4.5A).

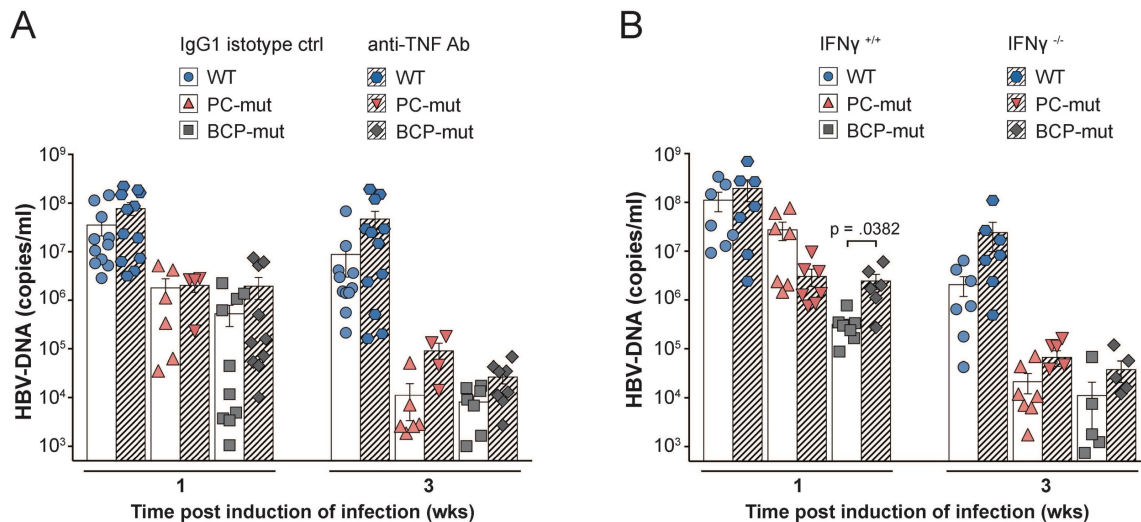


Figure 4.5 A – B TNF and IFN- γ do not contribute to the very early control of viraemia in mice infected with HBV mutants. (A) Serum HBV DNA levels. Mice were infected with the indicated HBV mutants and treated with TNF neutralizing antibodies (hatched) or isotype control (n = 4 - 12 in each group). (B) Serum HBV DNA levels in wildtype or IFN- γ deficient animals (hatched) at the indicated time points post induction of infection (n = 5 - 7 per group). Mean and SEM are shown. Data in (A) and (B) was pooled from two independent experiments. Experiments were blinded. Despite anti-TNF treatment, mice with HBeAg deficient viruses still displayed the same precipitous drop in serum viral DNA loads that we observed in our initial experiment and in mice that were not treated with TNF neutralising antibodies (Figure 4.5A and

Figure 4.1A). These results indicate that TNF is not the main immunological mediator responsible for the early improved viral control observed in mice with HBeAg deficient virus.

4.7.5 Interferon γ did not contribute to the early control of serum DNA viral loads in mice with HBeAg deficient HBV

Interferon gamma (IFN γ) is primarily produced by cells of the adaptive immune system. We have previously shown that this cytokine is very important in determining the host's long-term ability to control HBV infection (327). To assess if HBeAg was antagonizing IFN γ signalling early during establishment of infection, and therefore responsible for the rapid drop of serum viral DNA loads in mice with BCP and PC mutant virus, we infected IFN γ deficient mice. A slight increase in serum BCP mutant HBV DNA levels was observed in IFN γ deficient mice compared to WT mice one week after infection. This difference diminished 3-weeks post infection. PC mutant and WT virus displayed the same viral kinetics regardless of mouse genotype (Figure 4.5B). These data indicate that IFN γ is not a major contributor to the very early precipitous drop in serum viral DNA loads observed in mice with BCP and PC virus. Therefore, HBeAg is unlikely to be having a major antagonist effect on IFN γ signalling during the very early stages of infection. Alternatively, there may be very little IFN γ during this early stage of infection. Regardless, IFN γ is not the key driver of early viral control in BCP and PC infected mice.

4.7.6 In the absence of IFN-I signalling BCP and PC mutant viruses behaved like WT virus in infected animals

IFN-I is one of the earliest cytokines produced by the innate immune system in response to infection. We reasoned that this cytokine might be the driver of the dramatic drop in serum viral DNA loads in mice with BCP and PC mutant viruses. To examine this possibility, we induced infection in mice that are deficient in interferon- α/β receptor subunit 1 (*Ifnar1*^{-/-}). We established infection with WT HBV in *Ifnar1*^{-/-} mice and found that the kinetic of serum viral DNA loads was identical to the kinetics we observed when WT HBV infection was induced in WT mice (Figure 4.6A)

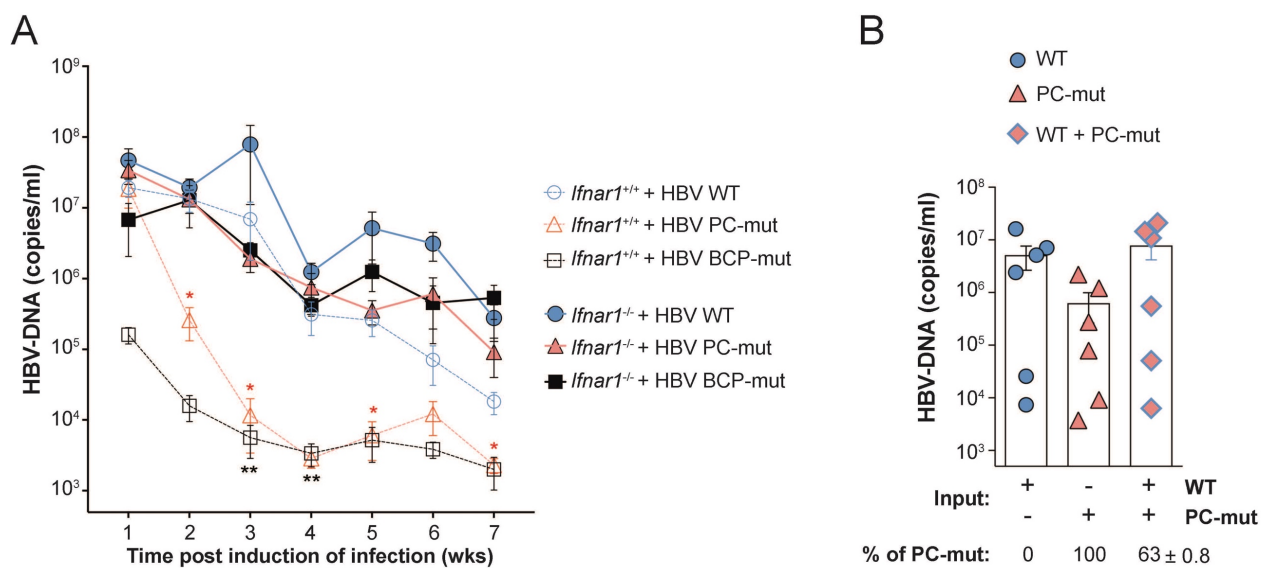


Figure 4.6 A – B IFN-I mediated responses promote early control of HBV viraemia in mice infected with HBV mutants but not in mice with WT HBV. (A) Serum HBV DNA levels in $Ifnar1^{+/+}$ and $Ifnar1^{-/-}$ mice (n = 4 - 13 per group). Mean and SEM are shown. **(B)** Serum HBV DNA levels three weeks post induction of infection in C57BL/6 mice infected with either HBV WT, PC-mutant, or an equal mix of WT / PC-mutant (n = 6 per group). Percentage of PC-mutant genotype contributing to the HBV-DNA levels is indicated below the bar graph. Data are representative of two independent experiments. In (A), red asterisks indicate comparisons between $Ifnar1^{+/+}$ and $Ifnar1^{-/-}$ mice infected with PC-mutant, black asterisks indicate comparisons between $Ifnar1^{+/+}$ and $Ifnar1^{-/-}$ mice infected with BCP-mutant, *P < .05; **P < .01; ***P < .001. Mean and SEM are shown. Data in (A) and (B) was pooled from two independent experiments. Experiments were blinded.

This is consistent with our previously published data showing that endogenous IFN-I plays a redundant role in the control of WT HBV infection(327). In contrast, and intriguingly, we found that the early viral control we observed in WT mice with BCP and PC was lost when the host was deficient in IFN-I (Figure 4.6A). Indeed, we found that BCP, PC and WT HBV showed the same serum DNA viral kinetics in $Ifnar1^{-/-}$ mice. This data indicates that in the absence of HBeAg, IFN-I plays a major role in promoting the control of HBV infection. The corollary is that HBeAg appears to antagonise IFN-I signalling and it assists in the initial establishment of persistent infection by abrogating the early host innate immune response.

4.7.7 BCP and PC HBV viral serum DNA kinetics mirrored those of WT HBV when HBeAg is complemented in trans.

To confirm that HBeAg was the predominant factor responsible for modulating the effects of IFN-I signalling, we induced infection in WT mice with a 1:1 mix of PC mutant and WT HBV. We found that under these conditions, WT HBV appeared to compensate for the loss of HBeAg in PC mutant HBV at 3 weeks post induction of infection such that serum viral DNA loads were restored in co-infected mice to levels detected in animals that were only infected with HBV WT (Figure 4.6B). Analysis of HBV mutants present in the serum revealed WT and PC mutant viral DNA loads were equivalent in co-infected mice (Figure 4.6B). These data showed that *trans* complementation with a virus that produces HBeAg was sufficient to convert the PC HBV *in vivo* phenotype to that of WT HBV. Collectively, our data showed that HBeAg was acting to antagonise host Type I IFN signalling and promote establishment of persistent infection. To confirm this role of HBeAg, we performed mouse liver *ex-vivo* transcriptional and proteomic assays comparing HBeAg deficient viral infection to WT infection.

4.7.8 Expression of HBeAg protein is associated with transcriptional down regulation of IFN-I signalling

To examine the impact of HBeAg on the host transcriptional landscape in the liver, we induced HBV infection in WT mice using HBV WT or PC mutant virus incapable of producing HBeAg and compared the differentially expressed genes one week post induction of infection. We chose this early time point to minimise any impact on transcriptional outcomes attributable to differences in HBV DNA levels and the amounts of HBV structural proteins between WT or PC mutant infected animals (Figure 4.1A and B). In animals infected with PC HBV compared to animals infected with WT HBV, 74 of a total of 12832 mapped genes were significantly down-regulated and 49 were significantly upregulated at the transcriptional level (Figure 4.7A). Among the significantly deregulated genes were a number of genes that have previously been implicated in chronic inflammatory HBV disease and hepatocellular carcinoma such as *smad7*⁵¹ and *gas5*.^{52,53} Pathway analysis revealed a significant upregulation in IFN-I pathway genes in animals infected with PC mutant compared to animals with HBV WT (Figure 4.7B). There was no significant deregulation in transcription of *Ifnar1* and *Ifnar2* or *Ifngr1* and *Ifngr2* and type I IFNs, indicating that HBeAg was not directly impacting the transcription of these receptors and cytokine.

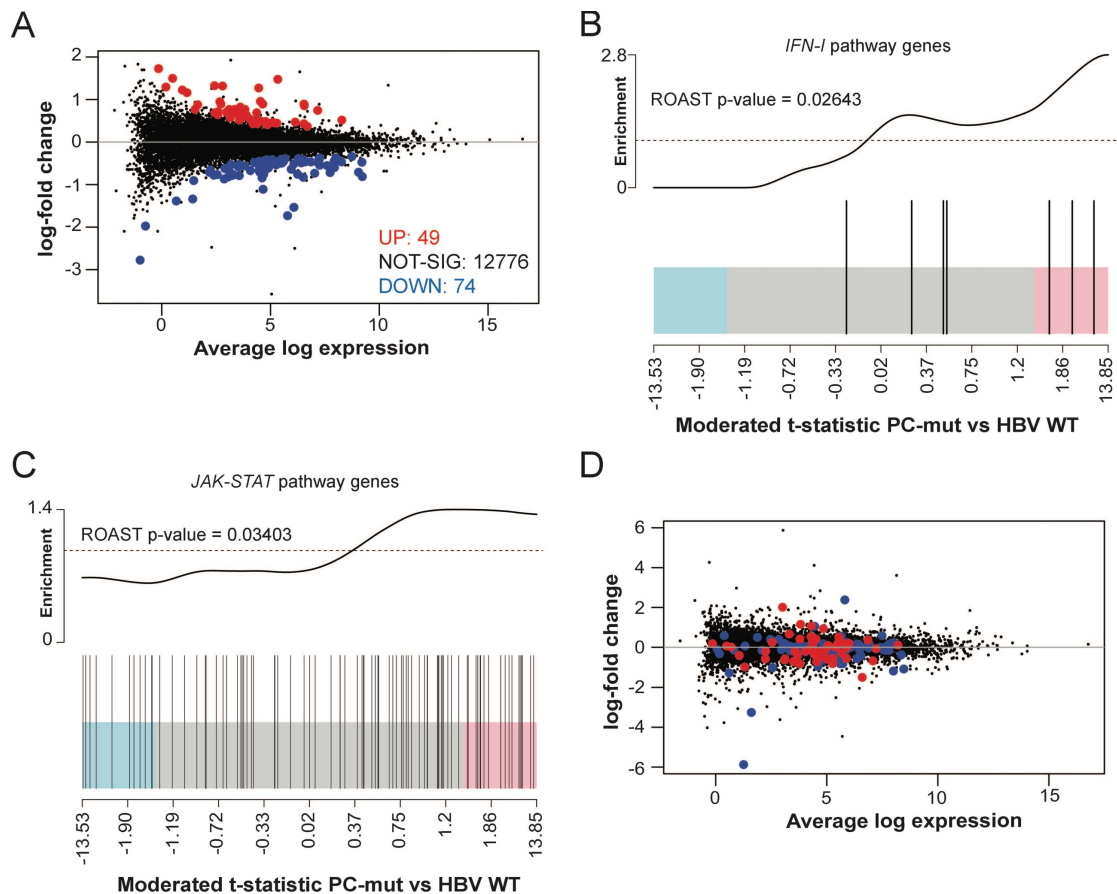


Figure 4.7 A - D. HBeAg is associated with transcriptional downregulation of IFN-I responses in the liver of infected mice. (A) Mean-difference (MD) plot showing log₂ expression fold-changes in WT mice infected with PC-mutant vs WT HBV. The plot shows fold-changes and average expression for 12832 genes in livers of C57BL/6 WT mice one week post induction of infection (mean of n=4 per group). Colour code indicates significantly differentially expressed genes: Red - 49 genes significantly up regulated in PC-mutant compared to WT HBV; blue - 77 genes significantly down regulated in PC-mutant compared to WT (FDR < 0.05). Barcode plots show enrichment of IFN-I pathway (B) and the JAK-STAT signaling pathway (C) amongst those genes that were up-regulated in PC-mutant infected mice relative to WT HBV infected mice. Each plot ranks the 12832 expressed genes right to left from most up- to most down-regulated in PC-mutant infected mice, with genes in the pathways marked by vertical bars. (D) MD plot for IFNAR deficient mice infected with PC-mutant vs WT HBV. The plot shows the same genes as in (A) in livers of IFNAR deficient mice one week post induction of infection (mean of n=3 per group). Red and blue coloured dots correspond to the same dots in (A).

In keeping with a transcriptional signature that indicated an upregulation of IFN-I signalling in mice infected with PC HBV, we also observed a significant upregulation of the JAK-STAT signalling pathway in those animals (Figure 4.7C). More specifically, gene ontology analysis showed that there was a trend towards increased expression of a number of genes in the Type I IFN pathway including STAT-1, STAT-3, IRF9, JAK-1 and JAK-2. Interestingly, there were no commensurate differences in the expression of genes in the IFN-gamma pathway (Figure 4.8A). This is consistent with our analysis of gene-targeted mice where we found that type I IFN signalling, but not IFN γ signalling, was linked to the early differences in viral kinetics between mice infected with PC mutant compared to HBV WT. Given that PC mutant virus behaved like WT HBV in mice with defective type I IFN responses, we next sought to determine if the transcriptional differences, described above in WT mice, diminished in IFNAR $^{-/-}$ mice.

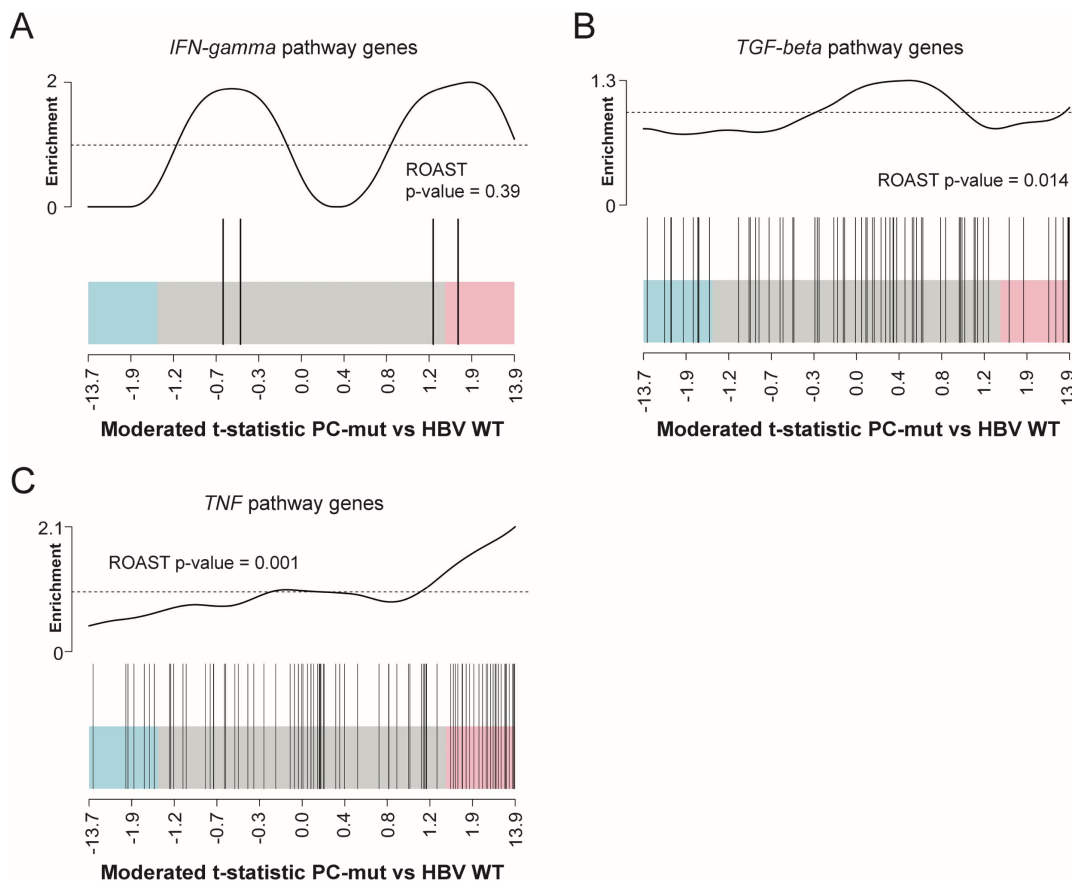


Figure 4.8 Barcode plot analysis of genes involved in (A) interferon-gamma signalling pathway (B) TGF-beta signalling pathway and (C) TNF signalling pathway in C57BL/6 mice infected with PC-HBV compared to wildtype WT HBV (gene enrichment tested using ROAST method). Indeed, the differences described above collapsed and diminished in type I IFN deficient mice such that the transcriptional profile of PC mutant infected livers was similar to livers infected with WT HBV (Figure 4.7D).

Collectively, these data indicate that HBeAg antagonises type I IFN signalling. Additionally, a broader analysis suggested that expression of transforming growth factor β (TGF- β) signalling pathways was upregulated in mice infected with PC mutant compared to HBV WT infected animals (Figure 4.8B). Similarly, expression of TNF signalling pathways was also upregulated in mice with HBV PC compared to WT (Figure 4.8C). It is interesting to speculate how these changes might confer a long-term advantage to mutant viruses and their implications for liver disease.

4.7.9 HBeAg protein induces global changes in liver proteome and phosphoproteome reflecting changes in expression of Interferon Regulated Genes (IRGs)

In order to interrogate the global changes in protein expression induced by HBeAg, we conducted a full proteome and phospho-proteome analysis of livers from C57BL/6 mice infected with WT HBV or PC mutant one week following infection. Amongst the total 7243 proteins identified, 403 proteins were significantly over-represented and 22 were under-represented in livers infected with WT virus compared to PC mutant virus (Figure 4.9A).

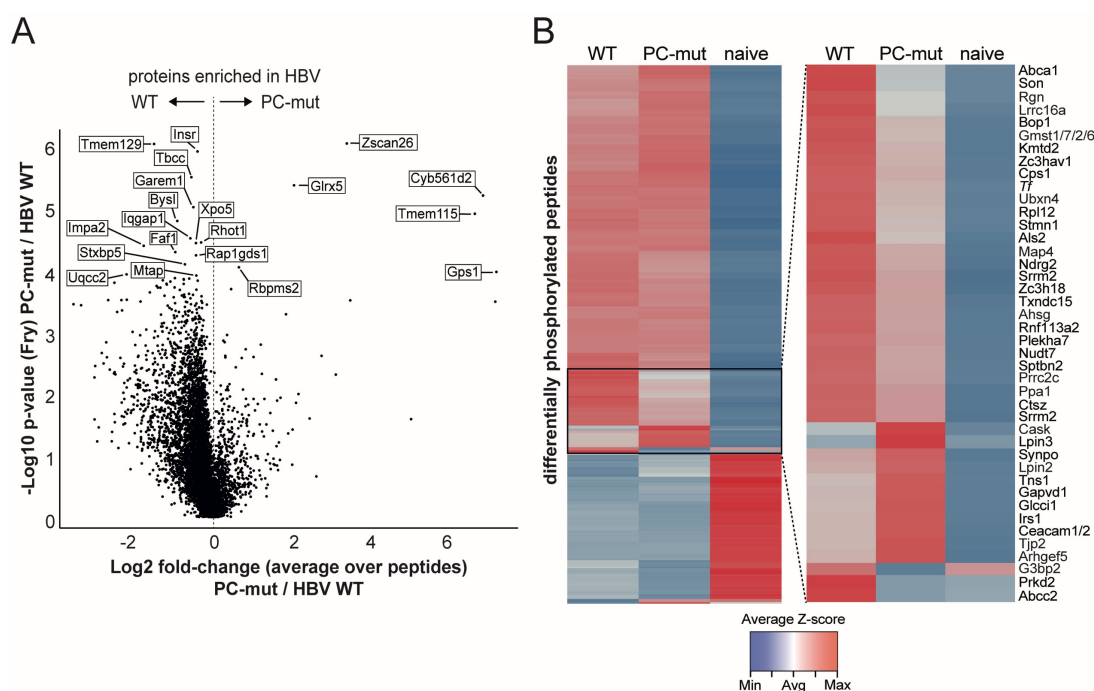


Figure 4.9 A – B Liver proteome and phospho-proteome changes associated with HBeAg

(A) Volcano plot of 7243 detected proteins in livers of C57BL/6 mice one week post induction of infection (mean of n=6 per group). Named proteins are amongst the top 20 differentially expressed proteins. (B) Heatmap of significantly differentially phosphorylated peptides (adj. P-Value < 0.05) in livers of C57BL/6 mice one week post induction of infection (mean of n=6) compared to uninfected control mice. Insert: peptides differentially phosphorylated between PC-mutant and WT. For clarity, only the gene name for each phospho-peptide is displayed. A full table of differentially phosphorylated peptides, specific modified peptide sequences and associated statistical analyses can be found in the PRIDE database submission.

Some of these differentially expressed proteins have been implicated in type I IFN mediated antiviral responses. In contrast to the transcriptomic analysis there was no definitive global downregulation of type I IFN related proteins, rather, some interferon related proteins were enriched in WT virus infection and some in PC mutant infection. Differential protein turnover and post-translational modifications likely impact on the role that each of the differentially expressed proteins plays in determining infection outcomes. Indeed, phosphor-proteome analysis indicated differential post-translational modifications in the presence and absence of HBeAg.

Gene name	Protein name	enriched in WT or PC-mut	Reference
Tmem129	E3 ubiquitin-protein ligase TM129	WT	(556)
Insr	Insulin receptor	WT	(557)
Glr5	Glutaredoxin-related protein 5	PC-mut	(558)
Iqgap1	Ras GTPase-activating-like protein IQGAP1	WT	(559)
Rhot1	Mitochondrial Rho GTPase 1	WT	(560)
Faf1	FAS-associated factor 1	WT	(561)
Rbpms2	RNA-binding protein with multiple splicing 2	PC-mut	(562)
Mtap	Microtubule-associated protein 1A	WT	(563)

Table 4.1 Differentially expressed proteins implicated in type I IFN mediated antiviral responses – proteome

Gene	Protein name	max. phosphorylat ed in WT or PC-mut	Reference
Abca1	ATP-binding cassette sub-family A member 1	WT	(564)
Gstm1/7/2/6	Glutathione S-transferase Mu 1,7,2,6	WT	(565)
Zc3hav1	Zinc finger CCCH-type antiviral protein 1	WT	(566)
Map4	Microtubule-associated protein 4;Microtubule-associated protein	WT	(563)
Ppa1	Inorganic pyrophosphatase	WT	(567)
Lpin2	Phosphatidate phosphatase LPIN2	PC-mut	(568)
Gapvd1	GTPase-activating protein and VPS9 domain-containing protein 1	PC-mut	(569)
Irs1	Insulin receptor substrate 1	PC-mut	(557)
Ceacam1/2	Carcinoembryonic antigen-related cell adhesion molecule 2	PC-mut	(570)
G3bp2	Ras GTPase-activating protein-binding protein 2	WT	(571)
Prkd2	Serine/threonine-protein kinase D2	WT	(572)
Abcc2	Canalicular multispecific organic anion transporter 1	WT	(573)

Table 4.2 Differentially phosphorylated proteins implicated in IFN-I mediated antiviral responses – phospho-proteome

WT and PC HBV infection induced broad changes in liver protein phosphorylation compared to uninfected control mice (Figure 4.9B). The liver protein phosphorylation pattern associated with WT virus infection was different to that induced by PC mutant virus (Figure 4.9 insert). Some of the differentially phosphorylated proteins have been implicated in IFN-I mediated antiviral responses (Table 4.1, Table 4.2). Collectively proteome and phospho-proteome analysis highlighted that WT and PC mutant viruses

differentially impacted on the expression and phosphorylation of numerous IFN-I related liver proteins.

4.8 Discussion

Using two naturally occurring mutants of HBV that are totally (PC HBV) or partially (BCP HBV) deficient in the production of HBeAg we were able to dissect the role of this viral protein in the early establishment of HBV infection. We found that the absence of HBeAg did not alter our ability to induce infection as serum HBV DNA levels in mice were equivalent on the first day post injection across all mutants. During the initial weeks, however, mice with BCP and PC HBV showed a rapid decline in serum HBV DNA levels compared to mice with WT HBV. Additionally, mice with BCP HBV, which make a small amount of HBeAg, showed a quicker HBeAg seroconversion response compared to mice with WT HBV.

Interestingly, the decline in serum HBV DNA levels in mice with BCP HBV appeared greater compared to mice with PC HBV. It is plausible, given that the BCP mutation also alters HBV X protein, that the differences in PC and BCP serum HBV DNA levels may be attributable to the additional HBV X mutation in BCP that is not present in PC. HBV X has been implicated in many facets of HBV viral life cycle and disease pathogenesis (574). Nonetheless BCP HBV, in most of our analyses, phenocopied PC HBV.

We showed that the abrogated viral kinetics observed in mice with PC HBV was attributable to the loss of HBeAg production using *in trans* complementation experiments. This led us to speculate that HBeAg was antagonizing a very early host immune response against HBV. This was supported by the observation that mice with PC and BCP HBV had a clear transaminitis that was not seen in mice with WT HBV. This transaminitis was likely due to an augmented host response because as noted BCP and PC viral DNA levels were lower compared to mice with WT HBV. In human infection, BCP and PC mutations are associated with a more severe hepatitis(277-279). However, this finding has been inconsistent, and no causality has been established (280).

We were able to dissect the host mediators that were responsible for attenuating viral kinetics in mice with PC and BCP HBV using a combination of gene-targeted mice and injection of cytokine neutralizing antibodies. We found that IFN-I signalling was responsible for causing the rapid reduction in viral DNA levels in mice with mutant viruses. Other studies in transgenic mice have also shown a similar relationship. HBV transgenic mice, when crossed with IFNAR^{-/-} mice showed a higher level of viral replication when transfected with HBV compared to control mice (quot McClary

10666256 .Interestingly, the absence of IFN-I signalling had no impact on the early viral kinetics of WT HBV in mice in our study.

To substantiate the causal link between HBeAg and abrogation of host IFN-I signalling beyond our mouse genetic and complementation experiments we performed liver transcriptomic and proteomic, including phospho-proteomic, analyses in WT mice and *Ifnar1*^{-/-} animals. We were not able to attribute the HBeAg induced inhibition IFN-I signalling to a single gene. Rather there was a strong collective signature showing abrogation and or modulation of IFN-I responses in the presence of HBeAg. The *in vivo* liver transcriptional and proteomic data sets will be a powerful resource to link HBeAg to molecular and biological consequences. Our data will help inform future studies to mechanistically examine how HBeAg directly or indirectly antagonises IFN-I signalling.

Future studies studying the actual cell types responsible for the interferon response would be warranted in future. Generally, IFN- α and IFN- β are produced by leukocytes and fibroblasts respectively. In the liver, the natural IFN- α -producing cells appear to be precursor dendritic cells and Kupffer cells ([114](#), [381](#)) and not hepatocytes and further studies could be done to address that.

We purposefully restricted our analysis to examine the early impact of HBeAg in a mouse model of HBV infection using two mutants that arise spontaneously in the majority of people over the course of chronic infection. We have previously shown the levels of HBeAg in mice with WT HBV decline with time, coincident with the decline in HBV DNA, and this is mediated by an adaptive immune response ([327](#), [575](#)). Partial immunological control of HBV in people who are chronically infected is also associated with the decline and loss of serum HBeAg, most often caused by the emergence of HBV mutations that abrogate HBeAg expression ([176](#), [543](#)). The natural history of HBeAg serum levels during infection suggests that that this protein plays an early important role but at later stages there is a strong selection for viral mutants that are deficient in HBeAg expression. In many infections, host IFN-I signalling plays an important early role in promoting immunity and controlling infection. However during the late stages of chronic infection, persistent production of IFN-I abrogates immunity and worsen outcomes ([576-578](#)). Therefore, it is plausible to speculate that although HBV favours expression of HBeAg to antagonise IFN-I signalling during early phases of infection, at later stages of chronic HBV infection, persistent IFN-I production may be advantageous to the virus. This may then coincide with the evolution of HBeAg deficient HBV mutants. Prolonged production of HBeAg, beyond establishment of

persistent infection, may be counterproductive for the virus in the later phases of disease, where it is an additional epitope for immune recognition. HBeAg represents an immune target and mutants that are deficient in HBeAg will be strongly selected for during the late stages of infection when HBeAg likely serves no further purpose in antagonising IFN-I.

In conclusion, collectively our data are consistent with the clinical scenario and explain the early requirement for HBeAg expression to subvert innate immunity by antagonising IFN-I signalling. As explained above, this function of HBeAg together with its antigenicity may represent a liability for the virus at later stages of infection explaining the strong selective pressure driving the emergence of HBeAg deficient mutants in chronic disease

5 The Adaptive Immune system in BCP/PC mutants vs. WT

5.1 Introduction and Aims

The findings in Chapter 4 show significant differences in viral kinetics between infection with HBeAg negative/ deficient mutants and WT HBV. The early stage of infection was explored in detail and studies of the innate immune system revealed that Type 1 interferon (IFN-I) was a key mediator of those differences. Based on the transcriptome analysis as well as complementation studies, it was concluded that HBeAg abrogates the IFN-I response in the early stages of HBV infection. Further investigation of viral kinetics of HBV was then carried out to study the later phase of disease and the role of the adaptive immune response.

5.2 Results

5.1.1 The serum viral DNA loads rebounded and became significantly higher in animals infected with BCP and PC mutants compared to WT HBV at late stages of infection

Infection was induced in immunocompetent mice using WT HBV and HBeAg negative mutant strains (BCP and PC). In mice infected with WT HBV, following the peak of viraemia at week 1, there was a steady decline in VL until virus was undetectable at weeks 14-15.

In contrast, in mice infected with BCP and PC mutants, despite a rapid drop in VL in the initial phases of infection, there was an increase in VL after week 7 (Figure 5.1) with up to a log 2 difference in VL at week 13 for both BCP and PC mutants (Table 5.1 and **Error! Reference source not found.** respectively).

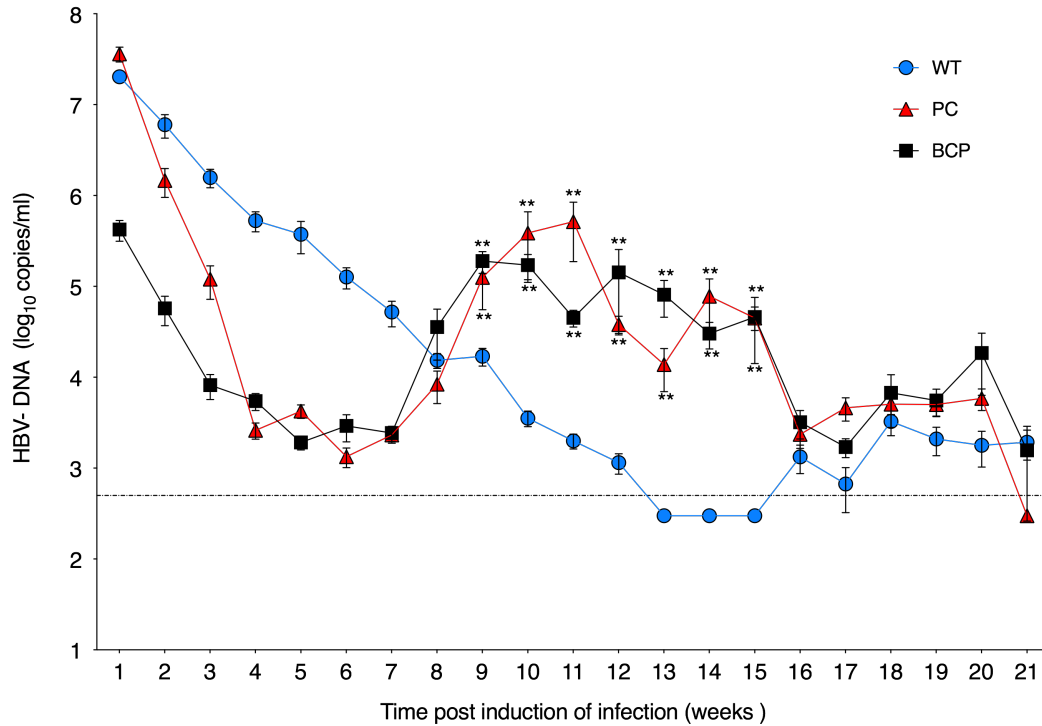


Figure 5.1 Abrogated HBe-antigen expression by BCP and PC HBV mutants, mitigated the immunological control in the later phase of disease and delayed clearance kinetics in mice. Serial measurements of serum HBV DNA (n= 15-17 per group). Mean and SEM are shown. Data are pooled from three independent experiments. All experiments were blinded. Asterisks represent comparisons between HBV WT and both mutant strains. An unpaired t-test with a Holm-Sidak correction was used. The dotted line indicates the reliable limit of detection. ** p <0.001

	HBV DNA (log10 IU/ml) Mean (95% CI)		P value
	WT (n=15)	BCP (n=17)	
Week 9	4.17 (3.63- 4.7)	4.77 (4.15- 5.4)	0.004
Week 10	3.27 (2.87-3.68)	4.52 (3.64- 5.4)	< 0.001
Week 11	2.94 (2.31-3.56)	4.25 (3.45-5.05)	< 0.001
Week 12	2.88 (2.38- 3.38)	4.16 (3.62- 4.71)	< 0.0001
Week 13	1.90 (1.17- 0.74)	4.21 (3.39- 5.01)	< 0.0001
Week 14	2.60 (2.14-3.06)	4.00 (2.92- 5.09)	< 0.001
Week 15	2.15 (1.62-2.69)	3.97 (2.89- 5.05)	< 0.001

Table 5.1 Mice infected with HBV BCP mutant were unable to control viraemia in the later stages of infection compared to WT mice.

	HBV DNA (log10 IU/ml) Mean (95% CI)		P value
	WT (n=15)	PC (n=15)	
Week 9	4.17 (3.63- 4.7)	4.69 (5.34- 4.77)	0.05
Week 10	3.27 (2.87-3.68)	3.85 (5.63- 4.51)	< 0.001
Week 11	2.94 (2.31-3.56)	4.24 (5.48- 4.25)	< 0.001
Week 12	2.88 (2.38- 3.38)	3.83 (5.01- 4.17)	< 0.0001
Week 13	1.90 (1.17- 0.74)	3.85 (3.33- 4.38)	< 0.0001
Week 14	2.60 (2.14-3.06)	3.95 (2.80- 5.10)	< 0.0001
Week 15	2.15 (1.62-2.69)	3.67 (2.37- 4.96)	< 0.0001

Table 5.2 Mice infected with HBV PC mutants were unable to control viraemia in the later stages of infection.

5.1.2 Minimal transaminitis during late stages of infection

The rebound in serum viral loads in mice infected with HBeAg deficient virus, compared WT virus could be attributable to an abrogated adaptive immune response caused by the lack of an important viral antigen and immune epitope. My data indicates that HBeAg antagonises innate immunity and IFN-I signalling during the early phases of infection but at later stages, when chronicity has been established, it appears that HBeAg contributes to virological control. The late recrudescence of viraemia in mice infected with HBeAg deficient virus coincides with the initiation and expansion of adaptive immunity. We speculated that in the later phase of disease, HBeAg no longer confers an advantage to HBV but may be detrimental because it becomes an important immunological target for the adaptive immune system.

To investigate this possibility, I examined the level of serum liver transaminases that I speculated would correlate with liver damage and potentially liver immune attack. I quantified the levels of serum ALT and AST in mice with PC, BCP and WT viruses 9 weeks following the induction of infection. Surprisingly, I found minimal transaminitis in mice nine weeks post infection regardless of HBV genotype. Serum AST and ALT levels were similar in mice infected with WT and mutant virus (Figure 5.2).

It is possible that serum transaminitis is an insensitive measure of adaptive immune attack at this late stage. Indeed my histological data did not show any major immunohistochemical changes in chronically infected animals. It is possible that the adaptive immune response is sufficiently subtle in infected animals such that a single cross sectional analysis may not be representative of magnitude of immune attack. To further explore if HBeAg deficiency negatively impacted on adaptive immunity in HBV, I performed a series of investigations using gene-targeted animals that possessed adaptive immune defects. Using this loss of function approach, I attempted to identify an adaptive immune element that promoted immune clearance of WT virus but had minimal effect on control of HBeAg deficient virus.

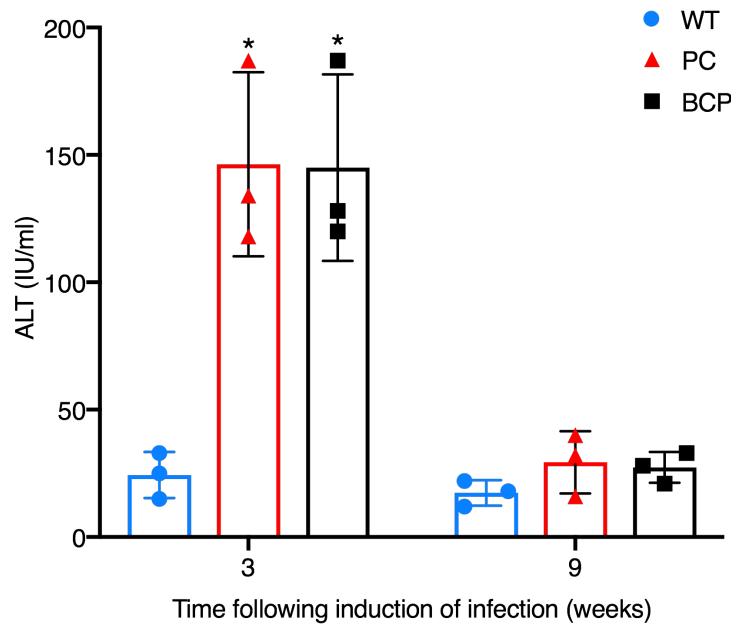


Figure 5.2 ALT levels were significantly lower in BCP/ PC mutants in the later phases of infection suggestive of an escape from the immune response. At later time points, there was no statistically significant difference between WT and mutant strains (n=3 per group). Mean and SEM are shown. Asterisks represent comparisons between HBV WT and mutant strains. Each data point represents one mouse. Data were analysed using a 1-way ANOVA and statistical significance was determined setting confidence intervals of 95%. *p < 0.05

5.1.3 CD8⁺ T cell dysfunction results in an inability to control viraemia

Although cytolytic T cells may play a role in promoting clearance of HBV in people with acute remitting infection, CD8⁺ T cells fail to eradicate the persistent viral reservoir in individuals who are chronically infected. To compare and contrast the contribution of cytolytic T cells in promoting clearance of WT versus mutant virus, I hydrodynamically infected perforin deficient mice (Prf1^{-/-}) and control animals. Prf1^{-/-} mice have a normal number of CD8⁺ T cells, compared to control animals, but these T cells lack cytolytic function and they are unable to lyse virally infected cells.

If the impaired virological control observed in BCP HBV infected mice was due to an inability of CD8⁺ T cells to recognise infected cells, due to the lack of HBeAg expression, then we would expect the phenotype of Prf1^{-/-} mice infected with WT HBV to mimic the phenotype of wildtype animals infected with mutant virus. However, we found that viral clearance kinetics were similar in perforin deficient and sufficient mice

infected with WT virus. Perforin deficient mice infected with BCP HBV showed the same viral clearance kinetic as wildtype animals infected with BCP HBV (Figure 5.3).

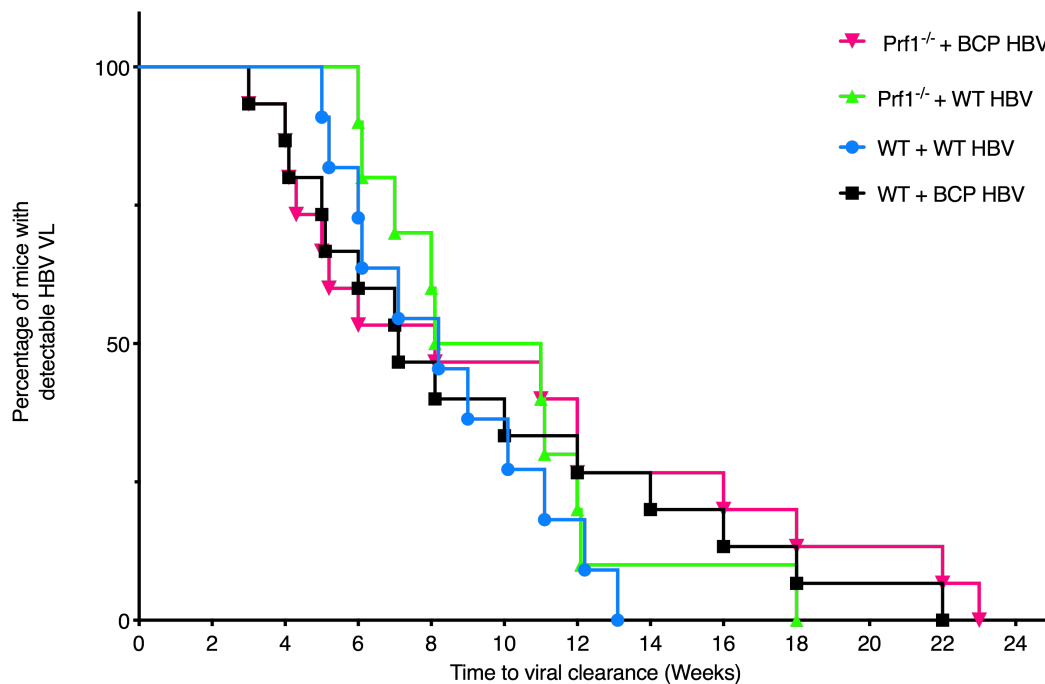


Figure 5.3 The cytotoxic activity of CD8⁺ T cells was not responsible for the differences in viral clearance kinetics between WT HBV and BCP mutant HBV.

The proportion of animals with detectable viraemia at each time point is shown (n= 9-12 per group). Data are pooled from two independent blinded experiments. Data were analysed using a Log-rank (mantel-Cox) test and no statistically significant differences were observed.

Collectively, these data indicate that CD8⁺ T cells are not responsible for the viral load discrepancies observed between mice infected with BCP and mice infected with WT HBV.

5.1.4 CD4⁺ T cell deficiency recapitulates BCP HBV phenotype in mice infected with WT HBV

CD4⁺ T cells are known to play a significant role in controlling HBV infection. It is possible that in the absence of HBeAg the functional capacity of CD4⁺ T cells is compromised leading to the late viral rebound seen in mice infected with BCP and PC mutant viruses. To investigate this we utilised transgenic OTII mice that have a numerically normal complement of CD4⁺ T cells but all of these T cells possess

receptors that only recognise an irrelevant ovalbumin antigen. Consequently, these CD4⁺ T cells have no capacity to recognise hepatitis B antigens, including HBeAg.

Transgenic OTII animals infected with HBeAg expressing HBV showed protracted viral persistence compared to control animals. Viral load in these transgenic animals was significantly higher for nearly all time points analysed compared to wild type mice (

Figure 5.4 A). Indeed the viral kinetics of WT HBV in OTII mice mimicked the rebound kinetics observed in control animals infected with BCP mutant virus. OTII mice infected with BCP mutant HBV showed similar kinetics to control animals infected with BCP HBV. A similar phenomenon was observed in OTII animals infected with PC HBV (

Figure 5.4 B and C).

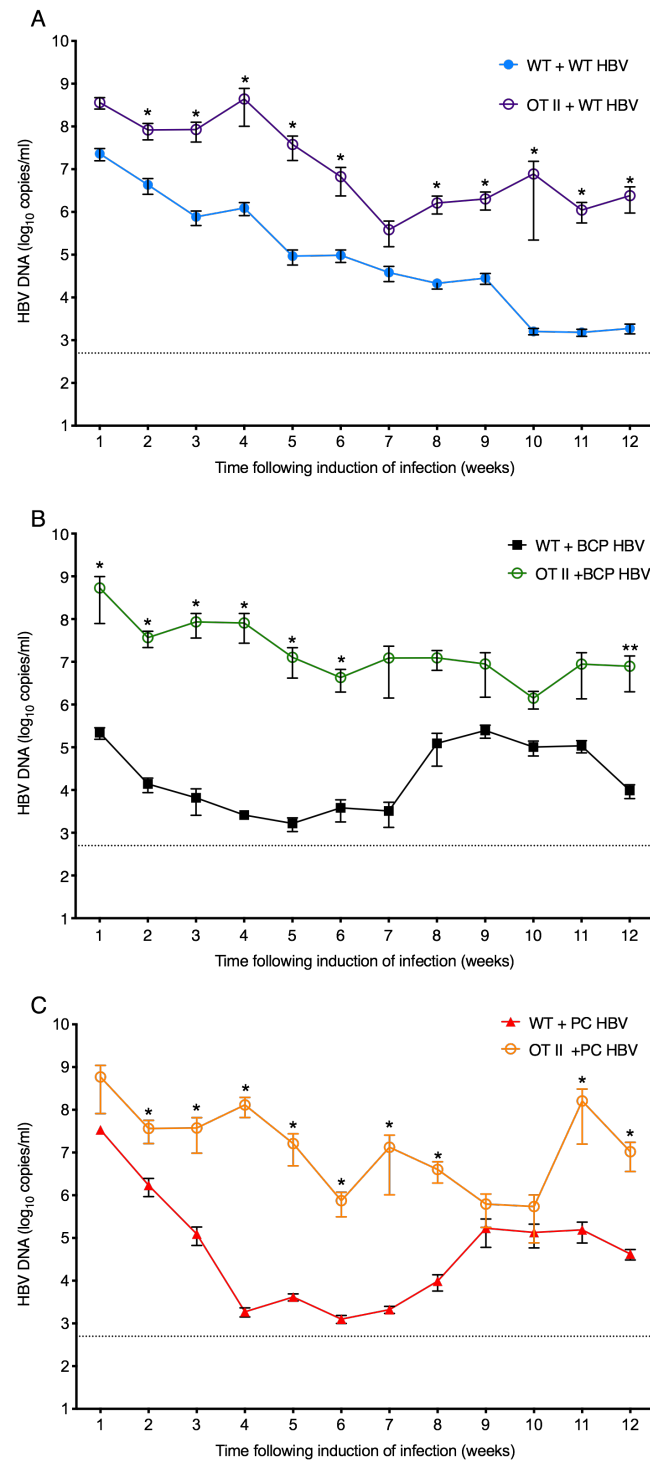


Figure 5.4 A - C HBV-specific CD4⁺ T cells are essential for the control of both WT and PC/BCP mutant HBV. Serial HBV DNA measurements are shown, comparing WT and OTII transgenic mice infected with (A) WT HBV (n=11-12 per group), (B) BCP mutant HBV (n=10-12 per group) and (C) PC mutant HBV (n= 12 per group). Mean and SEM are shown. Data were analysed using an unpaired, 2-way ANOVA and statistical

significance was determined setting confidence intervals of 95%. The dotted line indicates the reliable limit of detection. * $p < 0.05$ ** $p < 0.01$ **

Collectively this data shows that, in absence of CD4⁺ T cells, mice infected with WT virus phenocopy the viral kinetics observed in control animals infected with BCP and PC mutant virus. Therefore, the absence of HBeAg is compromising the function and ability of CD4⁺ T cells to control viral infection. It is plausible to conclude that HBeAg represents an important target antigen for CD4⁺ T cells and in its absence viral persistence is promoted.

5.1.5 Interferon gamma deficiency recapitulates BCP HBV phenotype in mice infected with WT HBV

HBV specific CD4⁺ T cells can contribute to the control of HBV through various mechanisms. Antigen activated CD4⁺ T cells secrete IFN- γ which has potent antiviral effects. We next sought to determine if, much like CD4⁺ T cell deficiency, WT HBV infected mice lacking IFN- γ mimicked control animals infected with BCP HBV. IFN- γ deficient mice were infected with WT and mutant strains of HBV and compared to WT animals. IFN- γ deficient animals infected with WT HBV showed a significant increase in HBV VL in the later weeks of infection (weeks 7-9 and week 11) compared to WT mice infected with WT HBV (Figure 5.5 A). This phenocopied the kinetics of control animals infected with BCP mutant virus. IFN- γ deficiency did not dramatically alter the viral kinetics in animals infected with BCP and PC and mutants compared to WT animals infected with the same viruses (Figure 5.5B and C respectively). These data suggest that the absence of HBeAg compromises CD4⁺ T cell and IFN- γ responses resulting in prolonged and more pronounced viraemia.

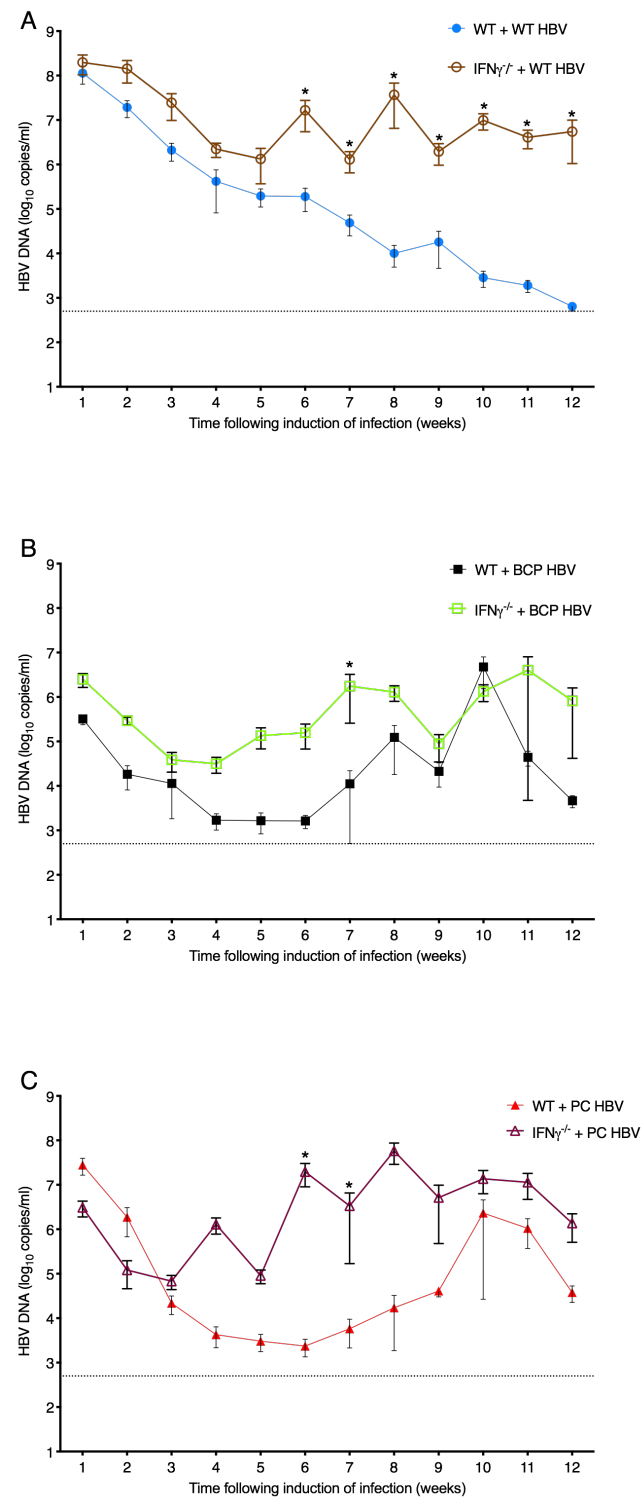


Figure 5.5 A – C IFN- γ contributes to the control of WT and PC/BCP mutant HBV.

Serial HBV DNA measurements are shown comparing WT and IFN- γ ^{-/-} animals. (A) WT HBV (n=6 per group), (B) BCP mutant HBV (n= 6 per group) and (C) PC mutant HBV (n= 6 per group). Mean and SEM are shown. Data were analysed using an unpaired, 2-way ANOVA and statistical significance was determined setting confidence intervals of 95%. The dotted line indicates the reliable limit of detection. * p < 0.05.

5.1.6 Antibody responses contribute to the control of HBV infection and these responses may be compromised in animals infected with HBeAg deficient virus.

CD4⁺ T cells and B cells cooperate across many aspects of immunity. Antigen activated CD4⁺ T cells are generally required to support the maturation of antibody producing B cells. Antibodies can contribute to the control of infection through numerous mechanisms. Although antibody neutralisation of virus would not abrogate viral persistence in our model, given the nature of hydrodynamic infection, antibodies can contribute to antibody dependent cell cytotoxicity (ADCC) and antibody dependent cellular activation (ADCA). The latter mechanisms are reliant on the expression of antibody receptors, FcγR, on effector cells. To investigate these possible mechanisms and their role in promoting control of HBV in the presence or absence of HBeAb we utilised FcγR deficient (FcγR^{-/-}) mice. In animals infected with WT HBV, there was a significant increase and persistence in HBV VL in FcγR^{-/-} mice compared to WT mice in the later phases of disease. HBV VL was significantly increased at weeks 7,8 and 10-12 but not in the earlier time points. This data is in keeping with the fact that the antibody response matures late in HBV infection (Figure 5.6).

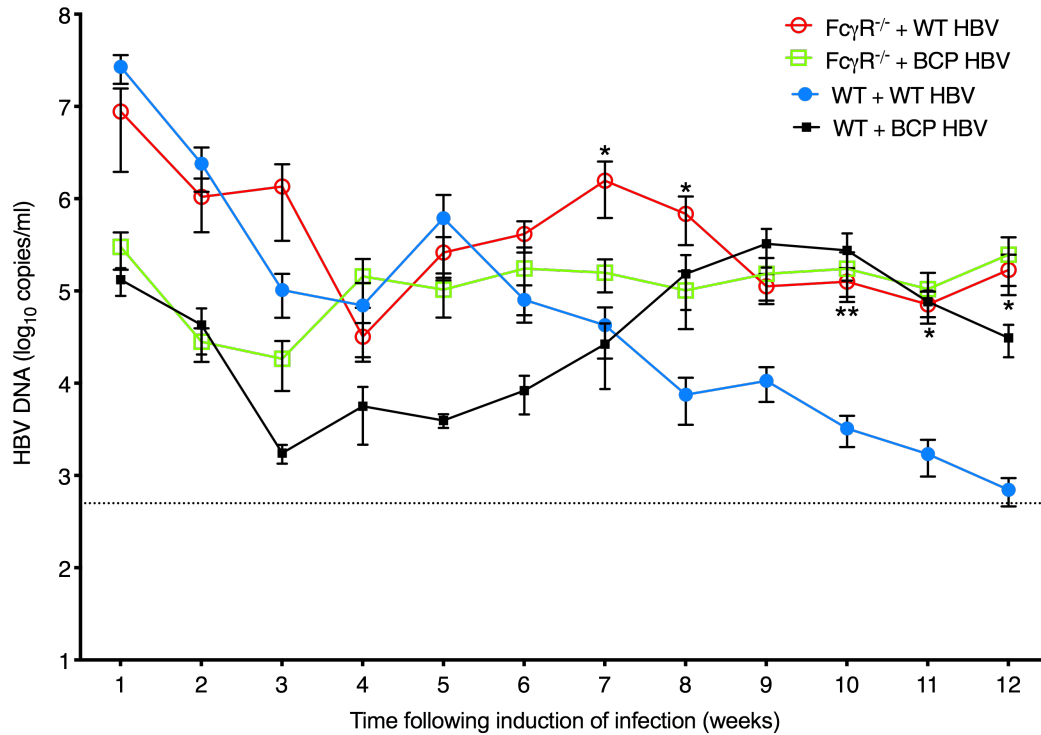


Figure 5.6 The antibody dependent cell cytotoxicity (ADCC) and antibody dependent cellular activation (ADCA) are attenuated in animals infected with HBeAg deficient virus. Serial HBV DNA measurements were taken for WT and FcγR^{-/-} mice infected with WT or BCP mutant HBV (n=6 in each group). Mean and SEM are shown. The black asterisks represent comparisons between WT and FcγR^{-/-} mice infected with WT HBV. Data were analysed by unpaired, 2-way ANOVA and statistical significance was determined setting confidence intervals of 95%. The dotted line indicates the reliable limit of detection. * p value <0.05, ** p value <0.01. In contrast, there was no significant increase in HBV VL in animals infected with BCP mutants, when comparing FcγR^{-/-} mice to WT mice. This suggests that HBeAg loss compromises both CD4⁺ T cell responses and aspects of ADCC and ADCA in animals infected with BCP mutant virus (Figure 5.6).

5.1.7 TNF suppression was associated with a failure to control viraemia in both WT and BCP/PC HBV

In addition to secreting IFN-γ, activated CD4⁺ T cells produce TNF, which has been correlated with virological control in both animal models of HBV infection and in patients infected with HBV. To examine the role of TNF in preventing late virologic recrudescence, we treated infected mice with TNF neutralizing antibodies. As a control, we used an irrelevant antibody of the same isotype as the anti-TNF antibody. With anti-TNF treatment, mice infected with WT HBV and mutant virus failed to control viraemia

(**Error! Reference source not found.**). In animals treated with anti-TNF antibody, there was viral persistence beyond 20 weeks for the majority of animals. These results are consistent with the fact that TNF is a key mediator in viral control in the later phase of disease. In keeping with the studies described above it is plausible that HBeAg helps to promote a TNF response directed against HBV-infected hepatocytes.

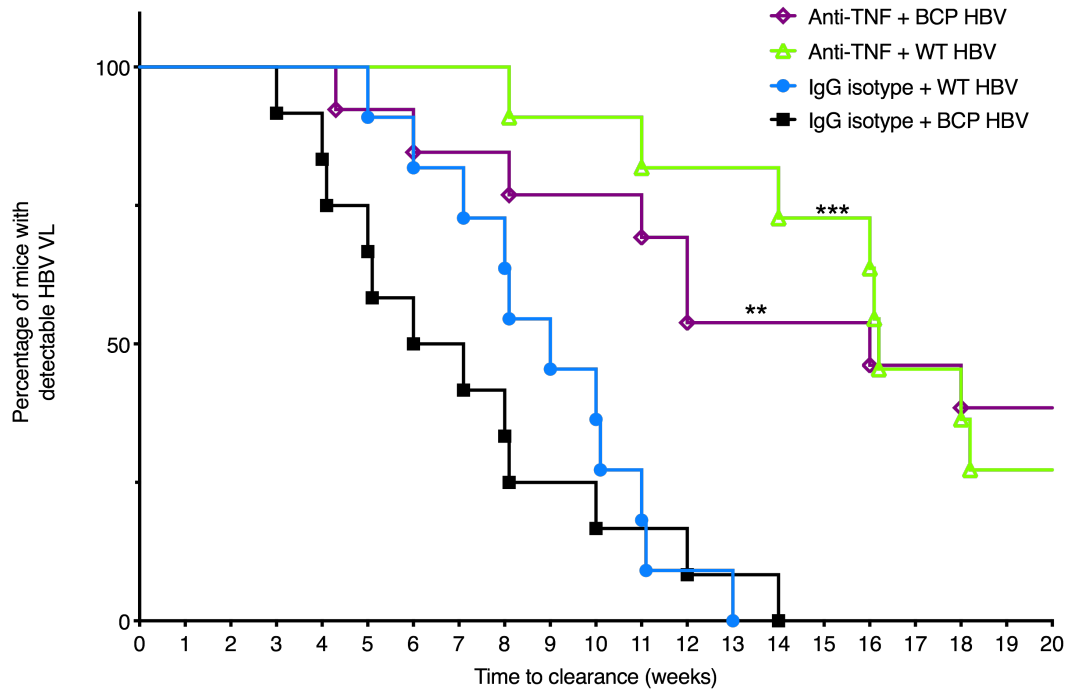


Figure 5.7 TNF suppression resulted in viral persistence in both BCP and WT HBV. Proportion of animals with detectable viraemia at each time point is shown (n= 8-10 per group) are shown. Data are pooled from two independent blinded experiments. Asterisks represent comparisons between animals treated with anti-TNF antibodies and control animals treated with IgG isotype for each construct. Data were analysed with a Log-rank (mantel-Cox) test. ** $p < 0.01$, *** $p < 0.0001$

5.2 Discussion

Our studies show that in mice infected with BCP and PC HBV there is an initial precipitous drop in serum viral loads followed by an equally dramatic recrudescence in viraemia at later stages of infection. This contrasts with the gradual and progressive decline in viraemia observed in mice infected with WT HBV. We reasoned that the loss of HBeAg in the later stages of HBV infection is advantageous to the virus. I speculated that HBeAg represents a target for the adaptive immune system and its expression facilitates immune mediated clearance of infected cells, which is consistent with the clinical scenario. To examine which facet of the adaptive immune system was targeting HBeAg and promoting virological control, the hydrodynamic HBV injection model was used to infect several gene-targeted animals. We reasoned that in the absence of a critical immune component, WT HBV infection would mirror the kinetics of BCP and PC HBV infection with elevated viral loads during late stages of infection and protracted clearance kinetics.

The titres and kinetics of WT hepatitis B viraemia in perforin deficient mice that lack cytolytic function, were similar to control animals. Similarly, perforin deficient mice infected with BCP, showed the same impaired virological control observed in control mice. Therefore, cytolytic function was not contributing to the clearance of HBeAg expressing virus. Both NK cells and CD8⁺ T cells rely on perforin for their function. However, our data showed no difference in viraemia in either the early or late phases of disease.

In contrast, transgenic OTII mice that cannot mount an anti-HBV CD4⁺ T cell response showed impaired virological control of WT HBV that mirrored the impairment observed in control animals infected with BCP HBV. This suggests that the late increase in VL noted in mice infected with HBeAg- negative mutants is in part due to compromised CD4⁺ T cell function. I speculated that the impaired CD4⁺ T cell function is linked to the loss of HBeAg, which likely represents an important target for these cells.

This possibility was further explored by examining the role of CD4⁺ T cell immune mediators. Both TNF and IFN- γ replicated the OTII phenotype. This underscores the notion that in the absence of HBeAg, the function of CD4⁺ T cells and their ability to produce and promote TNF and IFN- γ activity is compromised. Other studies in transgenic animals have shown similar results. When HBV transgenic mice are crossed with IFN- γ -/- mice, the levels of viral replication are higher, implicating a significant contribution to viral control (McClary quote).

Finally we examined if antibodies were contributing to control of WT HBC infection, through ADCC and ADCA, and whether this antibody mediated control was

compromised in mice infected with HBeAg deficient virus. Mice lacking FcγR are deficient in ADCC and ADCA. These mice were infected with WT HBV and they demonstrated impaired virological control mimicking control animals infected with HBeAg deficient virus. These results suggest that ADCC and ADCA responses directed against HBeAg expressing hepatocytes promote virological control.

5.3 Conclusion

Collectively our data show that CD4⁺ T cells and antibodies targeting HBeAg promote immune control of HBV infection. Therefore, expression of HBeAg during late stages of infection is detrimental for HBV and immunological pressures may select for HBV variants that are deficient in HBeAg. Interestingly this hypothesis supported by our experimental system is consistent with the natural history of HBV and the development of HBeAg deficient mutants in humans.

6 Synopsis and Future directions

The hepatitis B virus has infected more than 2 billion people worldwide. About a quarter of people chronically infected will die from HBV-related complications such as liver cirrhosis or hepatocellular carcinoma. The disease phenotype of chronic hepatitis B is variable, which some people maintaining a quiescent infection throughout their life and others rapidly developing cirrhosis or HCC. Such variability in disease outcomes may be due to genotypic differences in the virus.

HBV is susceptible to mutations during replication, which gives rise to the formation of a quasispecies made up of viral variants and genotypes. Genotypes and certain specific mutations have been associated with variations in clinical phenotype in epidemiological studies. However, in clinical practice, sequencing and genotyping the virus are not part of routine clinical care.

The immune response in HBV is crucial to its pathogenesis: The virus itself is not generally directly cytopathic but the immune response of the host leads to liver injury. The virus interacts actively with the immune system throughout its life cycle and has developed multiple strategies to evade the immune response to avoid clearance. We were particularly interested in the HBeAg, which has long been thought to be an immunomodulator although the specific interaction with the immune response is unknown.

The exact mechanisms of how HBV evades the innate immune response to first establish itself in the hepatocyte and then later further manipulates the immune response to evade immune recognition are unclear. We postulated that, although the virus most likely has multiple strategies for evading immune recognition and response, it is highly probable that HBeAg plays a central role.

In this context, the aims of this thesis were to:

1. Determine the clinical relevance of genotypes and variants in a 'real world' clinical cohort
2. Examine the viral kinetics of HBeAg-deficient mutants and correlate them to immune pathways
3. Investigate the role of HBeAg in modulating the liver transcriptome and proteome
4. Investigate the role of HBeAg in modulating the innate and adaptive immune response

6.1 Virological characteristics of HBV in a real world cohort

A large cohort of patients with CHB were studied with respect to their demographic and virological characteristics, including genotype and BCP / PC sequence. The cohort was predominantly male, and had HBeAg-negative disease. The majority of patients had migrated from South East Asia, the Mediterranean basin or Africa, where CHB is endemic and rates of vertical and early childhood horizontal transmission are high. Corresponding to the region of origin, the genotypes identified were A, B, C and D, with the majority of patients having either genotypes B or C as our cohort were largely of Asian origin.

HBeAg negative disease was prevalent in our study cohort. Most of the patients were in a phase of immune escape with active disease and HBeAg negative status was not synonymous with inactive carrier state. However, it is important to highlight the presence of selection bias in the study. The population was selected from a tertiary hospital, which is a referral centre for the treatment of CHB, so the study population may have been enriched for people who qualify for treatment due to the presence of active disease.

BCP and PC mutations were common with only a quarter of the population that did not harbour a mutation. This could be due to the fact that our population is older and age correlates with duration of viraemia in vertically acquired HBV. The study population was also enriched for genotypes B, C and D which support these mutations.

The PC mutation was almost universally associated with HBeAg negative disease, whereas the presence of BCP mutation was found in both HBeAg positive and HBeAg negative disease. This is likely due to the fact that BCP reduces the amount of HBeAg produced whilst the PC mutations completely abrogates the production of HBeAg. The PC mutation was predominantly found in genotype B HBV and BCP was highly prevalent in genotype C, which is in keeping with published data.

Advanced liver fibrosis was common in our cohort and one of the most pertinent findings of our study was that the presence of a BCP or PC mutation was associated with advanced fibrosis. Importantly, this was an association independent of other factors including age, sex and genotype. It is important to emphasise that in our study there was no independent association between genotype and fibrosis. In previous epidemiological studies genotype C in particular has been associated with poor clinical outcomes and we would postulate that, in our cohort, it is the mutation, not the genotype *per se* which is responsible for the aggressive clinical phenotype.

The main limitations of our study were that it was a retrospective study. There was also a selection bias as the cohort was a group of patients referred to a large tertiary

hospital for consideration of treatment. A prospective study with participants recruited from both the hospital and the community setting would be the next step to further advance this project. Sequencing for other mutations such as HBx would also be of great value.

This study has significant implications for the management of CHB. Currently, genotyping and sequencing are not part of routine clinical practice. BCP and PC mutants are the most common mutations of the HBV. We have demonstrated a clear association between fibrosis and the presence of these two common mutations. Prospective longitudinal studies would be required to establish the utility and cost effectiveness of BCP/ PC sequencing as a clinical tool.

This study has significant implications for clinical medicine. Increasingly, there is a tendency to treat patients with HBV earlier in order to prevent cirrhosis and reduce the risk of hepatocellular carcinoma. For instance, the recognition that age and ethnicity play a role in determining outcomes now shapes current guidelines. It is possible that the underlying genomic variability of the virus, which varies with both duration of viraemia and age as well as ethnicity, may be contributing to clinical phenotypes. The increasing recognition of the clinical significance of genotypes and mutations is important, and I predict that it will filter down to the clinical setting to incorporate genotyping and sequencing in future.

This study was also the basis for the formulation of further hypotheses regarding the role of the HBeAg in the pathogenesis of HBV. HBeAg is almost universally present in the early phases of disease suggesting an important role for the protein, but then natural selection of HBeAg-negative variants occurs later on which suggests that HBeAg could present a selective disadvantage to the virus. The next phase of our work was to gain further insights in the immune mechanisms behind the phenomenon.

6.2 The role of HBeAg on mediators of the innate immune response

The natural history of the virus suggests that HBeAg is important in the initial phases of disease, for the establishment of chronicity, but later on is not advantageous to the virus and mutations that abrogate the production of HBeAg tend to be naturally selected. It is generally thought that HBeAg is an immune-modulator but the actual host-virus immunological interactions are unclear. I aimed to dissect some of those mechanisms of the innate immune response in HBV with a specific focus on the role of HBeAg.

An immunocompetent mouse model was utilised and infection with BCP and PC mutants that are naturally deficient in HBeAg was induced. Wild type HBV virus was used as control. This study provided novel insights in the pathogenesis of HBV. The

absence of HBeAg did not affect the ability to induce infection, which is in keeping with the clinical observation that HBeAg is not required for infectivity.

Mice infected with HBeAg-deficient virus strains demonstrated a rapid drop in viraemia, with a significant corresponding transaminitis. This supported the concept that the immune response was more vigorous in animals infected with HBeAg- defective variants. Complementation experiments showed that that sudden drop in viraemia was attributable to HBeAg and I deduced that HBeAg was allowing the virus to escape an early innate immune response of the host.

I then proceeded to study individual host mediators that could be responsible for the differences in viral kinetics. I dissected different arms of the immune system with the use of gene-targeted animals and neutralising antibodies. A key finding was that, in absence of IFN-I, the viral drop was mitigated, which suggested a significant interplay between HBeAg and the IFN-I response. Transcriptomic and proteomic studies revealed a strong molecular signature that showed significant modulation of IFN-I - related immune pathways in the presence of HBeAg.

My data supports the concept that HBeAg is necessary to modulate the early innate immune response and establish chronicity. We have shown, convincingly that HBeAg allows the evasion of the IFN-I pathway, a key component of the innate immune response. Moreover, we have established the first liver transcriptome and proteome repository to be published and will form the basis of multiple associations between HBeAg and clinical outcomes.

The main limitations of this study is that it is a mouse model and despite the fact that it is an immune-competent animal, there are significant genomic differences between the model and a human host. Our model is suitable to test immune pathways but how this translates to human infection is yet to be tested. Furthermore, my studies were limited to genotype A2 and BCP and PC mutations. To gain a more thorough understanding of the interaction of the host immune response with the viral genome, it would be valuable to study different genotypes, in particular B and C , and other mutations including but not limited to mutation in HBx.

Despite many advances in the field, there is still no definitive cure for CHB. Current antiviral therapy is lifelong and does not result in complete viral eradication. As we now recognise that pathogenesis is largely dictated by the immune response of the host, manipulation of immune pathways may be used for therapeutic advantages. It is of crucial importance to understand the immune response in order to identify elements

that can be either enhanced or antagonised in order to bring about viral eradication by the host.

6.3 The role of HBeAg in the adaptive immune response

The first part of my studies demonstrated that HBeAg allowed the virus to evade type 1 interferon in the initial phases of HBV infection. However, from clinical data, including my own clinical study, HBeAg negative variants seem to have a selective advantage over wild type virus in the later phases of disease. This led to the exploration of the adaptive immune response in greater detail.

In our mouse model, there was an increase in viraemia in the later phases of disease in mice infected with HBeAg- defective variants. I speculated that the loss of HBeAg in the later phase of disease confers a selective advantage to the virus. Whilst HBeAg can help evade the host immune response during early phases of disease, in the later phase, it could potentially be a target for recognition by the adaptive immune system. To dissect the elements of the adaptive immune response that could be contributing to the phenomenon, I used gene-targeted animals and found that the CD4+ T cell response was impaired in absence of HBeAg and this was associated with an impaired production of TNF and IFN- γ . I also showed that antibody-mediated immunity through ADCC and ADCA responses were impaired in absence of HBeAg. These results show that HBeAg is a target for CD4+ T cells as well as the antibody response. The natural selection of HBV mutants that do not secrete HBeAg confers a survival advantage to the virus.

This is in keeping with the clinical observation that the majority of people with active disease in the later phases of disease harbour HBeAg-negative mutations. An innovative therapeutic strategy using SMAC mimetics to restore the TNF pathway has been explored. Preliminary data showed that the restoration of the TNF pathway could promote clearance of HBV-infected hepatocytes and clear HBV. Although the clinical application may not be direct and further developments are required, it substantiates the concept that manipulation of the immune response can lead to viral clearance.

6.4 Summary and future prospects

The natural history and clinical outcomes in chronic hepatitis B are highly variable. Increasingly, we are recognising that the clinical approach to HBV needs to be individualised due to genomic variations in the virus and variable immune responses in the host. The ability of the virus to mutate in response to immune pressures has contributed to its longevity and the host immune response is largely responsible for the dire clinical consequences in HBV.

In this thesis, I first performed a study of a clinical cohort of patients with CHB which showed that there was a high prevalence of BCP and PC mutants and importantly, the presence of these mutations is independently associated with advanced fibrosis. I then proceeded to investigate the underlying mechanisms for that phenomenon. I used a small animal model and studied viral kinetics of HBV and HBeAg-deficient variants of HBV. I showed that HBeAg deficiency was associated with an inability to escape the type 1 interferon response in early phases of disease. Transcriptomic and proteomic data further supported that finding. I also showed that in the late phases of disease, CD4⁺ T cell responses are diminished in HBeAg negative variants.

The future steps are now to understand how these new insights in immune pathways can be used in the development of the next generation of therapeutic agents in HBV. With ongoing research in the immune-pathogenesis of HBV, and the establishment of a transcriptome and proteome library, as well as the availability of small animal models, the goal of a definitive cure in HBV may be achievable.

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