Hepatitis B Virus (HBV) Infection in Peripheral Blood Mononuclear Cells of HBV Mono-infected and HBV/Human Immunodeficiency Virus Type-1 Co-infected Patients

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Hepatitis B Virus (HBV) Infection in Peripheral Blood Mononuclear Cells of HBV Mono-infected and HBV/Human Immunodeficiency Virus Type-1 Co-infected Patients

by

Zengina Lee

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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Abstract

It is unknown whether HIV-1 co-infection impacts HBV lymphotropism. We hypothesize that concomitant HIV-1 infection will affect HBV detection in PBMC subsets. We compared HBV genome detection in whole PBMC and CD4+ T, CD8+ T, CD14+ monocyte, CD19+ B and CD56+ NK cell subsets isolated from 14 HBV mono-infected (4/14 with a second sample collected after starting antivirals) and 6 HBV/HIV-1 co-infected patients on antivirals using nested PCR/nucleic hybridization and/or quantitative PCR assays. HBV DNA was detected in most target PBMC subsets regardless of HIV-1 co-infection and antiviral treatment; with the exception of the CD4+ T cell subset from HBV/HIV-1+ patients. All whole PBMC analyzed (13/13 HBV treatment naïve mono-infected, 4/4 follow-up cases on antivirals, and 3/3 HBV/HIV-1 co-infected) were HBV genome positive. The data suggests that HBV infection in CD4+ T cells is affected by concomitant HIV-1 infection.
Acknowledgements

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This thesis is fondly dedicated to my family: Julian, Judy, Storm, Charming and Evy Lee
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<th>Definition</th>
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<td>aa</td>
<td>Amino acid</td>
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<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine amino transaminase</td>
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<tr>
<td>Anti-HBc</td>
<td>Antibody to hepatitis B core antigen</td>
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<tr>
<td>Anti-HBe</td>
<td>Antibody to hepatitis B e antigen</td>
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<tr>
<td>ART</td>
<td>Antiretroviral therapy</td>
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<tr>
<td>C</td>
<td>HBV Core</td>
</tr>
<tr>
<td>cccDNA</td>
<td>HBV covalently closed circular deoxyribonucleic acid</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster differential</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<tr>
<td>CHB</td>
<td>Chronic hepatitis B</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin-antidigoxigenin</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DR</td>
<td>Direct repeat</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>DW</td>
<td>Water control from direct round of polymerase chain reaction</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ETV</td>
<td>Entecavir</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FT</td>
<td>Flow through after peripheral blood mononuclear cell sorting</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly Active Antiretroviral Therapy</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>HBcAg</td>
<td>Hepatitis B core antigen</td>
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<tr>
<td>HBeAg</td>
<td>Hepatitis B e antigen</td>
</tr>
<tr>
<td>HBsAg</td>
<td>Hepatitis B surface antigen</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks Balanced Salt Solution</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human immunodeficiency virus type-1</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IU</td>
<td>International Units</td>
</tr>
<tr>
<td>LAM</td>
<td>Lamivudine</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic activated cell sorting</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NA</td>
<td>Nucleoside/nucleotide analog</td>
</tr>
<tr>
<td>NAH</td>
<td>Nucleic acid hybridization</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>nPCR</td>
<td>Nested PCR</td>
</tr>
<tr>
<td>NW</td>
<td>Water control from nested round polymerase chain reaction</td>
</tr>
<tr>
<td>P</td>
<td>HBV Polymerase</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>pgRNA</td>
<td>Pregenomic ribonucleic acid</td>
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<tr>
<td>Poly-A</td>
<td>Polyadenylation</td>
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<td>Quantitative polymerase chain reaction</td>
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<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase-H</td>
<td>Ribonuclease-H</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>S</td>
<td>HBV Surface</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris base, Acidic acid, Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>TDF</td>
<td>Tenofovir</td>
</tr>
<tr>
<td>WHV</td>
<td>Woodchuck hepatitis virus</td>
</tr>
<tr>
<td>β-gal</td>
<td>Beta-galactosidase</td>
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</table>
“I call hepatitis B and liver cancer the Silent Killer …this is a disease that kills …1 person every 30 seconds. But how many times do you hear people talk about hep B? ... It is a problem for those in high-risk environments, such as those who undergo kidney dialysis, or doctor and dentists. A lot of people thought the vaccine would stop the problem, but unfortunately, there are millions of people who did not have access to the vaccine, or who were infected before it was available.”

- Dr. So (Expert Speakers Forum, 2006, Hepatitis B Foundation)
Chapter 1: Introduction

Hepatitis B virus (HBV) and human immunodeficiency virus type 1 (HIV-1) co-infection is common due to shared modes of transmission (1, 2). HIV-1 primarily replicates within cluster differentiation (CD)-4 positive (+) T lymphocytes but can also infect myeloid cells, including macrophages and dendritic cells (DC)s (3, 4). Although HBV is predominantly a hepatotropic virus infecting hepatocytes, data supporting HBV lymphotropism has been derived from in vitro studies and in the woodchuck hepatitis B virus model (i.e., woodchucks infected with woodchuck hepatitis virus, WHV) (5, 6). Additionally, HBV infection of lymphoid cells has been described in clinical studies where HBV persistence has been observed in liver transplant recipients (7-9), in patients despite long-term suppressive anti-HBV nucleotide/nucleoside analog (NA) therapy (10, 11), in acute HBV outbreaks on hemodialysis units, and in mother-to-child transmission (12-17) of the HBV. HBV DNA and HBV messenger RNA (mRNA) and the covalently closed circular DNA (cccDNA) transcriptional templates have been detected in extrahepatic tissues such as peripheral blood mononuclear cells (PBMC)s, bone marrow cells, spleen and lymphoblastoid cell lines (16, 18, 19). Similar to HIV-1 infection, HBV persistence within lymphoid cells may allow escape from immune recognition, and prevent eradication of the HBV despite long-term anti-HBV therapy (11, 16).

In HBV mono-infected and in HBV/HIV-1 co-infected patients, studies showed variable detection of HBV within whole PBMCs and subsets (16, 20-25). Previous studies however, were inconclusive regarding the effect of concomitant HIV-1 infection on HBV targeting and replication within PBMCs and subsets (16). The persistent state of immune activation in HIV-1 infected patients influences peripheral blood lymphocytes function (i.e., cytokine production) (26). We hypothesize that concomitant infection with HIV-1 will affect HBV detection in
CD4+/CD8+ T cells, CD14+ monocytes, CD19+ B and/or CD56+ NK cells as compared to HBV mono-infected patients (26). Our objective was to compare the detection of HBV DNA and its replicative intermediates in PBMC subsets as well as in total PBMCs i.e., CD4+ T cells, CD8+ T cells, CD14+ monocytes, CD19+ B cells, and CD56+ natural killer (NK) cells from HBV mono-infected versus HBV/HIV-1 co-infected patients.

1.1 The Epidemiology of HBV and HIV-1 Co-infection

The public health impact of infection with HBV and HIV-1 is significant and global. Separately, the World Health Organization estimates that the HBV infects 2 billion people and another 33 million people are infected with HIV-1 worldwide (27, 28). Chronic HBV (CHB) infection is common in HIV-1 infected individuals because of shared blood and body fluid routes of transmission, and approximately 3.3 million people worldwide have concomitant HBV/HIV-1 infection (29-31). Although the advent of highly active antiretroviral therapy (HAART) has decreased the progression of HIV-1 to the acquired immunodeficiency syndrome (AIDS), complications of HBV related liver disease has become a significant cause of morbidity and mortality in HBV/HIV-1 co-infected patients when compared to HBV mono-infected patients (30, 32).

HBV and HIV-1 are endemic in the same world regions with the majority of those infected living in sub-Saharan Africa and the Far East (33). The factors that influence co-infection rates range geographically and include age, efficiency of exposure and susceptibility among the patient population (33). For example in sub-Saharan Africa, adolescents and adults who are groups at risk for contracting sexually-acquired HIV-1, are also a population with a high prevalence of CHB due to perinatal and early childhood transmission of the HBV (33). In
comparison, there is a relatively lower prevalence of CHB (<1%) in Western Europe and North America with the majority of HIV-1 and HBV infections resulting from sexual contact and intravenous drug use (33). In low HBV endemic regions, there is a reported HBV/HIV-1 co-infection prevalence of 5-10% (10) and first and second generation immigrants from HBV endemic areas account for a disproportionately high number of CHB carriers (33). In Alberta, the prevalence of HBV/HIV-1 co-infection is lower than that reported in other developed nations (~3%) (10). It is most common in Caucasian males (62.4%) (34) who have acquired their infection from intravenous drug use, however, there is a recent increased incidence of HBV/HIV-1 co-infection in African immigrants (10).

1.2 The Molecular Virology of HBV

1.2.1 The Hepadnavirus Family

Human HBV is the prototype member of the Hepadnaviridae family. The family Hepadnaviridae can be divided into two genera: that of the avianhepadnaviruses that infect birds such as Pekin ducks, herons, Ross geese, storks and parrots, and the orthohepadnaviruses that infect mammals such as woodchucks, artic and ground squirrels, bats and primates like orangutans and humans (35). HBV is mainly hepatotropic, but HBV DNA has also been found in the kidney, pancreas, and mononuclear cells (36). Hepadnaviruses share the capacity to cause acute and persistent infections within a narrow host range (37). Other defining features of the family are their use of reverse transcription to replicate a partially double-stranded relaxed circular (RC) (i.e., not supercoiled) genome of at least 3.0 kb in size and an envelope which surrounds the nucleocapsid containing the RC DNA genome (35, 38).
1.2.2 The Morphology of HBV

The HBV produces 3 different types of virus-related particles: the spherical double shelled 42-47 nm infectious Dane particle and the 20 nm non-infectious spherical and filamentous particles of variable lengths (39). The spherical and filamentous particles are produced up to 1000 fold in excess of the Dane particles but only the Dane particles contain replication competent HBV genome (39). The non-infectious spherical and filamentous particles are predominantly composed of HBsAg but devoid of the viral nucleic acid and polymerase activity (39). The Dane particle contains a 25-27 nm enveloped icosahedron nucleocapsid core composed of 240 copies of the core (C)-protein subunit which contains viral nucleic acid, viral reverse transcriptase/polymerase and associated viral proteins (40). The envelope is made up of host lipids and three membrane-associated S antigens (large, medium, and small) (36). The nucleic acid is a partially double-stranded 3.2 kb RC DNA with an incomplete plus strand and a complete minus strand (41). At the 5’ ends of the negative and positive strands are 11 nucleotide short direct repeat (DR) sequences crucial to priming viral replication; DR1 is responsible for the synthesis of pregenomic RNA (pgRNA) and the negative DNA strand and DR2 plays a role in the formation of the long cohesive end region of the positive strand used in the circularization of HBV DNA (42). Additionally, a terminal protein, which forms part of the viral polymerase (P) is attached to the minus strand and a capped RNA oligomer is attached to the incomplete plus strand (39).
1.2.3 The Genome Organization of HBV

HBV’s highly compact genome is made up of four partially overlapping open reading frames (ORF) which code for S, P, X and C proteins (43) (Figure 1). Using frame shift mechanisms via multiple in-frame initiation codons, more than one protein can be encoded using the same ORF (44). The preS/S ORF encodes for three viral surface antigens all with a common carboxyl-terminus end and variable amino-terminals (36, 39). The first initiation codon produces the preS1 protein or large HBsAg, the second initiation codon encodes the preS2 protein or medium HBsAg, and the typical HBsAg (i.e. small HBsAg) which contains only the S domain is a result of the initiation of the third start codon (45). The C coding region encodes for (1) the nucleocapsid structural protein, hepatitis B core antigen (HBcAg) (a first in-frame start codon product), and (2) hepatitis B e antigen (HBeAg) (using the upstream start codon). The HBeAg is secreted from infected cells (36, 38). The P reading frame spans 80% of the HBV genome and encodes the viral P which has multiple functions including (i) signalling for viral pregenome encapsidation, (ii) RNA dependent-DNA replication, (iii) RNAse-H activity, and (iv) terminal protein roles (46, 47). Finally, viral X protein, encoded by the X region, is believed to modulate host and viral gene expression and the in vivo replication and spread of HBV (36).
Figure 1: A schematic representation of the HBV genome

The size of the partially double stranded virion DNA (inner black circles) is noted at 400 base pair increments starting at the EcoR1 cleavage site. The complete minus DNA strand (solid black circle) has a 5’ attached protein primer. The positive DNA strand (inner most black circle with dashed line) has a variable 3’ region (dashed line) and a 5’ covalently attached RNA oligonucleotide primer (wavy line). The other broad arrows surrounding the DNA strands represent the viral core (C) (preC and C regions), surface (S) (preS1, preS2, and S regions), polymerase (P) and X gene open reading frames with their respective corresponding amino acid (aa) lengths of the translated protein products indicated beside the labels.
1.2.4 The Life Cycle of HBV

The HBV life cycle is complex and has two key processes: (1) the repair of RC DNA to form the HBV cccDNA which is used to produce viral subgenomic and pgRNA and (2) the reverse transcription of pgRNA within the viral nucleocapsid to form RC DNA (49) (Figure 2). Hepatocytes are the primary site of HBV infection but HBV infection of lymphatic cells has also been documented (12, 18, 46). Little was known about the receptors and/or co-receptors responsible for HBV binding to the surface of host cells until recently (38, 50). In 2012, the sodium taurocholate cotransporting polypeptide, which is mainly expressed in the liver, was identified as the functional receptor for HBV through its interaction with the large HBV S protein (50). Nuclear transport of the C particles after HBV entry into the host cell remains poorly understood but it is believed to be directed by a nuclear localization signal in the carboxyl-terminus of the C particle and via microtubules (51). Once uptake into the nucleus is accomplished, a supercoiled cccDNA is generated from RC DNA using host cellular repair mechanisms (36, 52). The positive cccDNA strand is completed and the 5’ terminal capped RNA oligomer and terminal protein are removed from the positive and negative strand respectively (38). The negative cccDNA strand is then used as a template by host RNA P II to transcribe (i) viral genomic and (ii) subgenomic RNAs which are transported to the cytoplasm to be translated (36). (i) The resulting 3.5kb genomic RNA (53) – otherwise known as pregenomic RNA which has an epsilon stem structure – translates into the preC, C and P proteins (54). (ii) The resulting 2.4 kb, 2.1 kb and 0.7 kb subgenomic RNA transcripts are translated into envelope preS1, preS2 and HBsAg proteins, and the X protein respectively (38). It has been observed that there is preferential reading and translation of the C ORF as compared to the P ORF (~100-150 C proteins produced to 1 P protein) (55). The mechanism behind this preferential reading of the
C ORF is still unknown, but it prevents viral nucleic acids from accumulating to cytotoxic levels, and ensures the correct ratio of C to P proteins is synthesized. Viral packaging in the cytosol is triggered by the binding of P to the epsilon stem loop structure at the 5’ end of pgRNA (55). Nucleocapsids are assembled with a P protein bound to the 3’ end of a single RNA molecule (36, 55). It is then encapsidated with C proteins (56) and the negative DNA strand is synthesized by initiating reverse transcription at the epsilon RNA stem loop by adding nucleotides to the amino-terminal hydroxyl group of a tyrosine residue on the P protein. The pgRNA template is degraded by P RNAse-H activity, as the negative DNA strand is synthesized and subsequently becomes the template for the positive strand synthesis (36, 55). The 5’ capped end of the pgRNA which contains the DR1 sequence remains undegraded and acts as an RNA primer for plus strand synthesis by directing base pairs to the complementary 5’ DR2 region of the minus strand. The plus strand DNA becomes annealed to the redundant 3’ end of the minus strand resulting in circularization of the genomic DNA (57). The premature termination of elongation on the positive strand is due to limited deoxyribonucleotide triphosphate (dNTP) availability inside the C particle; leading to a partially double stranded (ds)DNA. On rare occasions, the 5’ capped end of the pgRNA fails to translocate to the 5’ DR2 region on the minus DNA strand and results in a linear dsDNA. Linear dsDNA is capable of cccDNA formation, infectivity and viral DNA synthesis and is also the preferred form for host genome integration (58).

The HBV cccDNA is the basis for persistence and maintenance of infection in susceptible host cells and it is estimated from in vivo animal studies that 5-30 copies of cccDNA are present per nucleus (59). The nucleocapsids with RC DNA are recirculated back to the nucleus where they can be repaired to maintain a pool of cccDNA transcriptional templates (36, 60). Increased amplification of cccDNA occurs during early infection and it is surmised (based
on studies in the duck hepatitis B virus model) that it is regulated by a S protein concentration feedback loop (61). Once sufficient viral envelope proteins are accumulated, mature nucleocapsids bud into the ER membrane through the secretory pathway, instead of being recirculated back to the nucleus, where they acquire envelopes and are excised from the cell. Infectious particles are transported to the Golgi complex and released to infect neighboring cells or into the bloodstream to infect more remote sites. Surface proteins also bud through the ER with help from their transmembrane spanning domains to become non-infectious subviral particles (62).
Figure 2: A schematic diagram showing the replicative life cycle of the HBV

1. The large HBV S protein binds to the host cell via the sodium taurocholate cotransporting polypeptide and enters the cell. (2) The C particle is transported to the nucleus where (3) the relaxed circular DNA is repaired into a supercoiled covalently close circular (ccc)DNA.

4. The negative cccDNA strand is then used as a template by host RNA polymerase (P) II to transcribe the pregenomic (pg) RNA and subgenomic RNA transcripts. (5) The pgRNA translates into pre-C, C and P proteins. (6) The subgenomic RNA transcripts are translated into preS1, preS2, HBsAg and the X protein. (7) Viral packaging in the cytosol is triggered by the binding of P to the epsilon stem loop structure of the pgRNA.

8. The negative DNA strand, synthesized by reverse transcription, becomes the template for the positive strand synthesis. The nucleocapsids are either (9) recirculated back to the nucleus where they can be repaired to maintain the cccDNA pool or (10) bud into the endoplasmic reticulum (ER) where they acquire envelopes and are (11) excised from the cell.
1.2.5 The Genetic Variability and Genotypes of HBV

HBV viral variation and genotypes have been shown to be important determinants in predicting clinical outcomes (35). Stable variants in particular human populations are distributed geographically and can be grouped into 10 genotypes (A-J) based on more than 8% of nucleotide diversity of the full genome (35). HBV genome sizes range from 3182 nucleotides for genotype D to 3248 nucleotides for genotype G (35). Genotypes can be further differentiated into sub-genotypes which differ by at least 4% of their full genome sequence (35). The rate and emergence of viral variants is influenced by various exogenous selective pressures such as the state of the host immune system, antiviral drugs and vaccine immunoprophylaxis (38, 49). Additionally, the evolution of viral variants is greatly enhanced by the lack of proofreading function of the HBV polymerase and use of the inherently error prone pgRNA intermediate during replication (38). Nucleotide substitution rates for HBV are estimated to be 10 times higher than in other DNA viruses and on par with certain RNA viruses at around $1.4-3.2 \times 10^{-5}$ nucleotide substitutions per site per year (38, 49). HBeAg seroconversion in CHB generally corresponds to lower HBV DNA in serum and normalization of serum alanine transaminase (ALT) levels (63) (see Section 1.3). Some HBeAg negative patients, however, continue to have persistent viremia and active liver disease due to a point mutation in the preC or C promoter (63). Mutations in ORFs however, must be simultaneously advantageous in both reading frames if they are to be selected for in subsequent replication cycles (38). Persons infected with genotype C have been observed to remain HBeAg positive for years longer than those infected with other genotypes (64).
1.3 The Natural History of HBV

HBV is transmitted sexually, perinatally and through blood (65) and its natural history ranges from acute to chronic HBsAg positive infection to HBsAg clearance (66). In immunocompetent adults, acute HBV infection, diagnosed when the HBsAg is detectable in blood between 4 to 10 weeks after initial exposure (Figure 3), is usually a moderate to severe self-limiting illness with hepatocellular injury and inflammation (36). In acute infection, viremia is well established and shortly thereafter class immunoglobulin M hepatitis B core antigen (anti-HBc) is detectable; frequently along with circulating hepatitis B e antigen (HBeAg) (36, 67). Only 1-5% of healthy adults fail to resolve their acute infection and go on to become CHB carriers with persistent lifelong infection, as diagnosed by the persistence of HBsAg in serum for > 6 months (36) with class immunoglobulin G anti-HBc (Figure 3). In contrast, > 95% of HBV infection in neonates and 50% in children <5 years of age results in CHB (32) (Figure 4). Preventing early childhood transmission of HBV is thus important and more than 110 countries have widespread immunization of infants with the hepatitis B recombinant vaccine (67). A vaccine for HBV made from purified HBsAg and produced in recombinant yeast (68) has been available for the last 30 years (69). To date it remains the most effective means of preventing HBV infection and mass immunization campaigns in many countries are in place (67). Studies of CHB have shown that infected patients have a 100 fold higher risk than non HBV infected people to develop hepatocellular carcinoma, and over an average of 30 years, 25% of patients will develop liver cirrhosis (32, 36). Although a relatively lower viremia level often accompanies the transition from acute HBV to CHB, the continued presence of HBeAg often indicates HBV titers between $10^7$ and $10^9$ viral genomes per mL of blood (32). In carriers infected since birth, CHB can progress through different phases: immune tolerance, immune
active, inactive phase, reactivation (64) and clearance of HBsAg (Figure 4). **Phase 1:** The HBV immune tolerance phase is characterized by HBeAg positivity, normal ALT levels (i.e., minimal liver inflammation or fibrosis) and elevated levels of HBV DNA (>20,000 International Units (IU)/mL, >1 million copies) (64). The immune tolerant phase can last for years but there is minimal liver inflammation or fibrosis (64). **Phase 2:** During the immune active phase, damage to hepatocytes can occur as the host immune system recognizes the HBV infection and initiates an immune response (64). Persons in the immune active phase can either be HBeAg positive or negative and have elevated ALT, HBV DNA levels of at least 2000 IU/mL (10,000 virus copies) and active liver inflammation with possible liver fibrosis (64). HBeAg titres tend to decrease at a rate of 5 to 10% per year and are often accompanied by a rise in ALT or flares resulting from immune-mediated destruction of infected hepatocytes. **Phase 3:** Following seroconversion to antibodies to hepatitis e antigen (anti-HBe) and despite reduction of viremia (sometimes as great as fivefold), at least 70 to 85% of people with anti-HBe antibodies still have $10^3$ to $10^5$ HBV genomes per mL of blood (36). This may be due to the presence of a HBV pre-core mutant which is classified as the HBeAg negative CHB phase (see Section 1.2.5). **Phase 4:** HBeAg negative patients can either remain in the immune active phase or transition into the inactive HBV phase characterized by normal ALT levels, HBV DNA <2000 IU/mL, and improvement of liver fibrosis and inflammation (64). However, patients may remain at risk for reactivation (64) and vice versa. **Phase 5:** HBsAg clearance and anti-HBs seroconversion or recovery phase rarely occurs. **Phase 6:** Finally, some patients develop occult HBV infection defined by the presence of HBV DNA in the liver, serum and PBMC despite HBsAg clearance (70). Additionally, 30 to 50% of occult HBV positive patients are negative for all HBV serum markers with the exception of HBV DNA (70). Although serum HBV DNA levels can be near or below the detection
threshold of current assays in occult HBV infected patients, replicative HBV DNA remains detectable in liver tissue (70).

The immune response to HBV antigens can cause both viral clearance during acute HBV infection, and disease pathogenesis and hepatocellular injury (71). The variation and severity of hepatic injury is dependent upon the host immune response to virally infected cells (36). The HBV is not cytopathic in immune competent persons (64). The early phase of viral infection activates production of cytokines – i.e., type 1 IFN α and β secreted from infected cells and plasmacytoid DCs (71). Self-limiting acute HBV is attributed to a robust immune response characterized by major-histocompatibility-complex class II, CD4+ helper T cells and CD8+ cytotoxic T lymphocytic response with antibodies against HBV antigens, clearance of circulating virus particles, and elimination of infected cells respectively. The HBV can also evade immune recognition through viral suppression of toll like receptors which are responsible for activation of IFN regulatory factors. Thus, viral persistence may be linked to the ability of HBV to manipulate the innate immune system of its hosts. An inverse linear correlation between HBV disease progression and HBV specific T lymphocytes has also been noted which may have implications for sustained immunosuppression in the host, and may favor HBV replication (71).
Figure 3: The typical serologic progression to chronic HBV infection

Hepatitis B surface antigen (HBsAg) is the earliest serological marker of infection and is detectable around 4-6 weeks post exposure; persistence for > 6 months is diagnostic of chronic infection. The specific antibody to hepatitis B core antigen (anti-HBc) can be class immunoglobulin (Ig)M and IgG. The presence of the IgM anti-HBc with HBsAg is indicative of early acute infection, whereas HBsAg and IgG with undetectable IgM anti-HBc is characteristic of chronic infection. The presence of detectable hepatitis B e antigen (HBeAg) during weeks 3 to 6 is characteristic of acute active infection. HBeAg persistence beyond 10 weeks is a marker of chronic disease although some replicative mutant strains of HBV do not produce HBeAg (see Section 1.2.5). The continuous presence of the specific antibody to hepatitis B e antigen (anti-HBe) along with detectable HBsAg and anti-HBc occurs in chronic infection.
Figure 4: The natural progression of perinatal HBV infection

(1) The immune tolerant phase is characterized by normal ALT levels, serum HBV DNA >1 million copies/mL, and minimal liver inflammation.  (2) The immune active phase is characterized by elevated ALT levels, HBV DNA >10,000 copies/mL and liver inflammation. Patients can either (3) lose HBeAg- and still have ongoing active hepatitis due to a pre-core/core mutation (i.e., HBeAg negative CHB) or progress to the (4) inactive phase characterized by anti-hepatitis e antigen (HBe) positivity, normal ALT levels, <10,000 copies of HBV DNA and minimal or absent hepatic inflammation (and vice versa).  (5) In rare cases, some people can eventually clear hepatitis B surface antigen (HBsAg) and lead to recovery.  (6) Occult HBV infection can occur following HBsAg clearance and is defined by HBV DNA in the liver and PBMC while markedly absent serum from HBsAg.
1.3.1 Antiviral Therapy for Chronic HBV Infection

Currently nucleoside and nucleotide analogs (NA) – lamivudine (LAM), entecavir (ETV), adefovir, tenofovir (TDF) and telbivudine – and two interferon therapies (standard interferon α (IFN) and pegylated-interferon α 2a are approved for treating CHB (74). IFN belongs to a family of proteins with natural antiviral and immunomodulatory actions such as enhancing T cell helper activity, inhibition of T cell suppressors, maturation of B lymphocytes and human leukocyte antigen type 1 expression (75). NA conversely, inhibit P viral replication through premature chain termination of HBV DNA synthesis (65). Therapeutic intervention of CHB with antiviral drugs is usually recommended once clinical signs of liver disease, elevated ALT, high viral loads, or evidence of liver fibrosis are present (74). The goal of antiviral therapy is to prevent disease progression through the permanent suppression of HBV replication (74).

Anti-HBV therapy is recommended for other patients such as those with concomitant HIV-1 infection or undergoing immunosuppressive therapy or cancer chemotherapy (64). Clinical success in CHB treatment is defined by reaching undetectable HBV viral loads by a PCR assay, normalization of ALT, improvement of histology, HBsAg seroconversion, and ultimately, HBsAg loss (74). Despite current drug therapies, HBsAg loss and complete eradication of HBV is not achieved due to the persistence of HBV cccDNA (65).

The loss of NA efficacy can occur when HBV resistant mutations are selected for during treatment. The emergence rate of anti-HBV resistant mutations is variable and depends on the number of mutations needed to compromise the antiviral activity of the drug (i.e., genetic barrier to resistance). While some drugs require only a single mutation (i.e. LAM), others such as ETV, require an accumulation of multiple mutations in HBV P to compromise its efficacy (2). Long-term studies of ETV and TDF efficacy during treatment of antiretroviral therapy (ART)-naïve
patients, show a 0.4% rate of developing resistance to ETV after 4 years and no reported resistance to TDF to date (74). Conversely, LAM resistant strains have been known to develop at a rate of 20% per year on LAM monotherapy. Use of sequential therapy with less-potent NA with lower genetic barrier to resistance has contributed to a multidrug-resistant population. In these cases, the use of TDF either alone or in combination with ETV therapy has successfully been used as a rescue strategy (74).

1.3.2 The Lymphotropism of HBV

1.3.2.1 The Woodchuck Hepatitis Virus Model of HBV

The WHV model is a valuable animal model for the study of human HBV infection as both are orthohepadnaviruses (see Section 1.2.1). It has been suggested based on studies in the WHV model that lymphoid cells may serve as an extra-hepatic site for viral replication (13, 76). WHV is similar to human HBV in that both viruses are closely related molecular structures and noncytopathic hepadnaviruses which result in similar patterns of liver disease (76, 77). WHV cccDNA and pgRNA have been detected in peripheral and organ lymphoid cells (76, 77). In vitro mitogen-stimulation of mononuclear cells has produced infectious WHV, suggesting that virus replication is possible in these cells (78) (see Section 1.3.3.2). A study by Coffin et al. observed that woodchuck mothers with occult WHV infection were able to transmit pathogenic virus to their offspring, inducing a persistent infection within the lymphatic system that was not always present in the liver (77).
1.3.2.2 Clinical and Laboratory Observations of HBV Lymphotropism

In HBV infected patients, a diversity of HBV molecular forms have been found in extrahepatic lymphoid cells, including circulating PBMCs, hematopoietic cells of bone marrow and spleen of HBV patients (12, 18, 46). A specific lymphoid cell derived viral variant and HBV replicative intermediates have been implicated in HBV reinfection of liver grafts in liver transplant recipients (9). *De novo* HBV infection occurs in up to 80% of liver grafts from HBsAg negative but anti-HBc positive donors (13). There is also evidence of HBV graft reinfection after liver transplantation in patients with detectable HBV DNA in their PBMCs after receiving a HBsAg negative liver graft (13, 79). Cabrerozo et al. detected HBV DNA in the PBMCs from 54% of HBsAg-negative chronic hemodialysis patients sampled (14). In general, the frequency of PBMC infection has been observed to correlate with the level of ongoing HBV replication in patients (13). Similar to HIV-1 (see Section 1.4.5), chronic stimulation of B cells during CHB infection has been linked to an increased risk of developing non-Hodgkins lymphoma than in the general population (80). In studies of mother to child (i.e., vertical) transmission, HBV infected maternal PBMCs can cross the placental barrier and cause intrauterine infection of the fetus (13, 15). Up to 30% of PBMCs from newborns of HBsAg positive mothers have been found to be infected with a level of viremia correlating with that of the HBV DNA status in PBMCs of the mother (81). A small study by Shen et al. showed elevated serum ALT levels and HBV DNA present in cord blood leukocyte samples from two infants despite absence of viral DNA in their sera (82). Similarly, in three maternal samples analyzed by Shimizu et al., HBV DNA was detected in PBMCs but absent from plasma; while two of the four infants born to those mothers developed acute or fulminant hepatitis within three months after birth (82). The results of these studies suggest that HBV may be transmitted
vertically (from mother-to-child) through PBMCs and result in \textit{in utero} infection. Clinical observations have been further supported by \textit{in vitro} studies. Both HBV RNA and HBsAg expression in PBMCs can be upregulated by \textit{ex-vivo} mitogen stimulation, indicating that PBMCs may represent a site of full expression of the HBV genome (17). In studies on unstimulated PBMCs HBV DNA and specific RNA sequences were also detected (16) as well as the observed expression of HBsAg and HBeAg in PBMCs via immunoelectron microscopy (83).

In the setting of HBV/HIV-1 co-infection, data from a few studies suggest that HBV DNA is detectable in PBMCs (25). Yoffe et al. (20) and Bartolome et al. (84) used PCR and less sensitive hybridization assays respectively to detect HBV in PBMC subsets of HBV/HIV-1 co-infected individuals as compared to HBV mono-infection patients (84). HBV sequences were detected in PBMCs of HBsAg positive CHB and HBsAg negative/occult HBV/HIV-1 patients with AIDS (20). HBV sequences were also found in HIV-1 positive asymptomatic carriers (84). The detection of HBV within PBMC did not correlate with the patient’s clinical status such as CD4+T cell count or CD4+/CD8+ T cell ratio (84).

1.3.2.3 The HBV Genome Detection in Peripheral Blood Mononuclear Cell Subsets

Infection of specific PBMC subsets by HBV may affect the host immune response and serve as sites for replicating virus, thus contributing to the persistence of HBV. A few published studies to date have investigated HBV infection in subpopulations of PBMCs (20, 21, 85). In a study by Yoffe et al., PBMC were collected from 14 HBsAg positive patients untreated for steroids or antiviral drugs (20). Five of fourteen HBsAg carriers were positive for HBeAg and 8/14 carriers tested positive for anti-HBe. PBMCs were isolated from whole blood and
monocytes and macrophages were fractionated using a panning method. The nonadherent cells were separated into T and B cell fractions by passing the cells through plastic straw columns. Following incubation, T cells were eluted and adherent B cells recovered by repeated compression of the column. The T cell population was further fractionated into CD4+ and CD8+ T cell subsets by complement-dependent cytotoxic depletion with CD4+ and CD8+ monoclonal antibodies respectively. The viability of recovered cells was >95%. Phenotypic analysis of PBMC subsets using commercial monoclonal antibodies and flow cytometry was performed to verify the effectiveness of the depletion procedure, although subset purity was not reported.

HBV genomes were detected with an estimated sensitivity of 0.05 HBV genome equivalents per cell in digests of total cellular DNA, via PCR. Fast-migrating, low molecular weight species of HBV DNA (similar to the pattern seen in replicative intermediate sequences) were detected in the PBMCs of all 5 HBeAg-positive patient samples and 1/9 HBeAg-negative patient samples. The most intense signal of hybridization was seen in monocytes followed by low level signal in the B cell enriched fraction, and none was detected in either of the T cell fractions (including NK cells eluted with the T cell fractions). In the PBMCs of the other 2 HBeAg negative patient samples, high molecular weight viral genomes were detected and thought to represent non-integrated relaxed circular (i.e., non-replicative) HBV DNA sequences (20).

Pasquinelli et al. detected HBV DNA in CD4+ T cell, CD8+ T cell and B lymphocytes enriched by a similar panning method as Yoffe et al. (20) from 7 HBV mono-infected patients (HBeAg status was not stated) (85). The authors used conventional PCR methods and a hybridized $^{32}$P labelled PCR probe and detected HBV lengths (specificity $3-4 \times 10^8$ virus copies/$\mu$g) suggestive of both integrated and non-integrated HBV viral sequences (similar to those found in the liver) (85). No HBV DNA replicative forms however, were observed in
PBMC subsets (85). Trippler et al. quantified HBV DNA viral load within the CD4+, CD8+, CD14+, CD16+, CD19+, CD16+, and CD57+ PBMC cell subsets (21). They used whole blood from two acute and three chronically infected HBsAg positive patients. PBMC subpopulations were separated either by a fluorescence activated cell sorter (FACS) or Miltenyi® magnetic bead separation using specific monoclonal antibodies. The purity of enriched PBMCs was >95% with exception to the B-cell subpopulation where a high proportion of contaminating monocytes were found. A sensitive HBV-specific PCR was carried out to evaluate for HBV genomes. The frequencies of HBV DNA positive cells were ~ 50 to 500 fold higher in samples from chronic compared to acute HBV patient samples. HBV genomes were detected with the highest frequency in monocytes and B-cells followed by CD8+ T cells, NK cells, and CD4+ T cells respectively. HBV viral loads were found in 63% of specific PBMC subsets with an estimated one HBV genome per infected cell.

1.4 An Overview of HIV-1 Molecular Virology

1.4.1 HIV-1 as a Member of the Retroviridae Family

The HIV-1 belongs to the Retroviridae family and genus lentivirus characterized by a diploid viral genomic RNA that is reverse transcribed into DNA by a viral enzyme and subsequently integrated into the host cell genome (i.e., proviral DNA) (86). The integrated proviral DNA can encode viral genes which can be inherited as cellular genes (86). Similar to other lentivirus, HIV-1 infection is characterized by long incubation periods, in vitro cytopathogenicity, lack of oncogenicity, and chronicity of infections (86, 87).
1.4.2 The Infection of Peripheral Blood Mononuclear Cells by HIV-1

The primary targets of HIV-1 are DCs, CD4+ T cells and cells of a monocyte-macrophage lineage (88). HIV-1 disease pathogenesis is attributed to active viral replication with nearly $10^{10}$ HIV-1 virions produced per day (89). The CD4+ T lymphocyte levels decline significantly as HIV-1 kills memory CD4+ T cells over others with different specificities (90, 91). The first line of host defense against further mucosal penetration by HIV-1 are DCs which take up HIV-1 and migrate to the draining lymph nodes where CD4+ T cells become primed for a HIV-1 specific immune response (91). DC contact with CD4+ T lymphocytes, however, likely contributes to productive infection of CD4+ T cells (91).

1.4.3 An Overview of the Natural History of HBV/HIV-1 Co-infection

HIV-1 and HBV share the same perinatal, sexual and blood borne routes of transmission (33, 92). In the setting of HIV-1 co-infection, the risk of developing CHB increases fivefold in co-infected patients with <200 CD4+ cells/µL – likely due to immune mediated destruction of HIV-1 infected CD4+ T cells (32, 92). This supports clinical observations where a strong HBV-specific T-cell response is important for clearance of acute HBV infection (Section 1.3 above) (36). In addition, concomitant HIV-1 infection can lower rates of serum HBeAg loss (1). In turn, it has been suggested that HBV X protein may trans-activate HIV-1 replication and lead to an accelerated decline of CD4+ T cells in HBV/HIV-1 co-infected individuals (92).
1.4.4 An Overview of Antiviral Therapy in HBV/HIV-1 Co-infection

Five major types of antiretrovirals exist to treat HIV/AIDS: entry inhibitors (which interfere with binding of HIV-1 to host cell receptors), fusion inhibitors (which interfere with fusion of HIV-1 to the host cellular membrane), RT inhibitors or NAs (which act as a faulty DNA building block or bind and interfere with RT), integrase inhibitors (which block HIV-1 integration into host DNA), and protease inhibitors (which inhibit protease’s ability to cut long chains of HIV-1 proteins into smaller individual proteins for new viral particle assembly) (93). A combination of at least two antiviral types make up HAART (93). HAART can effectively suppress HIV-1 replication but cannot eradicate it (90, 94). In the case of HBV/HIV-1 co-infection, combination therapy with two HBV-active agents is recommended (95) (see Section 1.3.2). NA or RT inhibitors such as a coformulation of TDF and Emtricitabine (FTC) can be used (95). TDF remains the preferred choice in HBV/HIV-1 co-infection treatment as it has dual antiviral activity, minimal side-effects and a high genetic barrier to resistance (95). ETV also has high efficacy against HBV, however it is less effective in patients with LMV resistance. It also has weak ART activity against HIV-1 and is only recommended for HBV/HIV-1 co-infected individuals with well controlled HIV-1 viral loads (95). The availability of dual viral suppression by current therapeutic agents which target both HIV-1 and HBV replication can prevent significant HBV-related liver disease development in those with concomitant HBV and HIV-1 co-infection.
1.4.5 An Overview of the Host Immune System Dysregulation by HIV-1

The alteration of cytokine production by HIV-1 allows the virus to increase its production by disrupting the host immune response (26). The persistent state of immune activation in HIV-1 infected patients influences peripheral blood lymphocytes (such as circulating CD4+ and CD8+ T lymphocytes) to upregulate cell cycle dependent proteins such as cyclin B (96). Cyclin B is instrumental in cell binding and subsequent activation of p34 cdc2 kinase; which phosphorylates various proteins important for the completion of the cell life cycle (96). Inappropriate activation of the cyclin B/p34 cdc2 kinase complex lowers the threshold for activation-induced apoptosis of lymphocytes and even uninfected lymphocytes may become more susceptible to apoptosis when challenged with a viral antigen (96). Additionally, cell cycle dysregulation causes abnormal nucleolar structure which can further act as a proapoptotic factor (96). The increased T cell turnover during chronic immune activation increases cell targets for viral replication and subsequently depletes naïve T lymphocytes (91).

HIV-1 infected patients are also at a higher risk of developing non-Hodgkin’s lymphoma than the general population (97). These lymphomas are characteristically related to B-cell hyperactivation and hyperproliferation as a result of deregulated chemokine and chemokine receptors (97). HIV-1 does not infect neoplastic B cells directly so the manipulation of active T cells, macrophages, and endothelial cells which make up the tumor microenvironment is important for its proliferation (88).
1.5 The Hypothesis of the Study

The HBV likely infects some of the same target PBMC subpopulations as HIV-1. It remains unclear how HBV/HIV-1 concomitant infection impacts HBV detection in PBMC subsets. Since the persistent state of immune activation in HIV-1 infected patients influences peripheral blood lymphocytes (i.e., cytokine production), we hypothesize that concomitant infection with HIV-1 will affect detection of the HBV in CD4+/CD8+ T cells, CD14+ monocytes, CD19+ B cells and/or CD56+ NK cells as compared to HBV mono-infected patients.

1.6 The Purpose of the Study

The aim of this study is to characterize the presence of HBV DNA and HBV replicative intermediates in isolated PBMC subsets (i.e., CD4+ T cells, CD8+ T cells, CD14+ monocytes, CD19+ B cells, and CD56+ NK cells) from HBV mono-infected as compared to HBV/HIV-1 co-infected patients.
Chapter Two: Patients, Methods and Materials

2.1 Patients, Clinical and Laboratory Tests

Clinical information was collected from both HBV mono-infected and HBV/HIV-1 co-infected patient cohorts as indicators of infection status as well as to assess differences between factors that may affect HBV natural history. In total, 14 HBV mono-infected patients (13 M/1F, median age 46.2 y, range 26-62y) and 6 HBV/HIV-1 co-infected patients (5M/1F, median age 45 y, range 22-60y) patients, all with HBsAg positivity > 6 months (indicator of CHB, see Section 1.3), were enrolled (Table 1). The study was approved by the University of Calgary conjoint health research ethics board according to the Declaration of Helsinki. The clinical and demographic data collected included age, sex, comorbid liver disease and ART to suppress HBV and HIV-1 viral loads. Laboratory information included CD4+ T cell count (an indicator of HIV immune restoration), HIV-1 and HBV viral load (measurement of circulating virus, treatment response, possible noncompliance, and the development of treatment-resistant mutations) (69) and liver enzymes (i.e., ALT), which in CHB are periodically or consistently elevated and may indicate a higher risk of long-term liver damage (98)). The HBV DNA levels in serum were tested by a clinical kinetic PCR assay (detection limit <20 – <55 IU/mL or ~100–300 copies/mL, Roche Molecular Systems, Inc., Branchburg, NJ, USA). The HIV-1 RNA was tested using the Abbott Real Time HIV-1 assay m2000 (Abbott, Mississauga, ON, Canada). Transient elastography (FibroScan®, Echosens, France) data, a non-invasive ultrasound test to measure liver stiffness as a means to assess for liver fibrosis, was performed (69). HBsAg and HBeAg serological markers were evaluated by commercial chemiluminescent microparticle immunoassays (Architect Anti-HBsAg Qualitative, anti-HBcAg II and anti-HBs; Abbott
Diagnostics, Mississauga, ON, Canada). HBsAg reflects HBV’s transcriptional activity, cccDNA genomes and immune response to the virus (see Section 1.3) (69). HBeAg is a general indicator of active viral replication and immune control. In some patients, HBeAg negativity may indicate a preC mutational form which is still virally active (see Section 1.2.5 and Figure 4) (98).
### Table 1: Summary of clinical information from 6 HBV/HIV-1 co-infected patients and 14 HBV mono-infected patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>HBV/HIV-1 Co-infected</th>
<th>HBV Mono-infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex N= M/F</td>
<td>6 = 5M/1F</td>
<td>14 = 13M/1F a</td>
</tr>
<tr>
<td>Median Age, years (range)</td>
<td>45 (22-60)</td>
<td>46.2 (26-62)</td>
</tr>
<tr>
<td>HBeAg Positive</td>
<td>3/6</td>
<td>7/14</td>
</tr>
<tr>
<td>Median HBV DNA (IU/mL) b (range)</td>
<td>313 (&lt;55 – 690)</td>
<td>Baseline: 5.4 x 10^3 (&lt;20 - 3.6 x 10^3)</td>
</tr>
<tr>
<td>Median alanine aminotransferase (U/L) (range)</td>
<td>43 (15 – 54)</td>
<td>47.5 (23 – 236)</td>
</tr>
<tr>
<td>Median CD4+ T cell (cells/mm^3) (range)</td>
<td>240 (114 – 800)</td>
<td>N/A c</td>
</tr>
<tr>
<td>Median HIV RNA (copies/mL) d (range)</td>
<td>&lt;40 (&lt;40 – 10^4)</td>
<td>N/A</td>
</tr>
<tr>
<td>FibroScan® e</td>
<td>Unknown</td>
<td>Stage 0 (N=7), 1 (N=1), 2 (N=3), 2-3 (N=1), 3-4 (N=2)</td>
</tr>
<tr>
<td>Antiviral Treatment</td>
<td>5/6 on HAART with anti-HBV activity f</td>
<td>14/14 baseline naïve 1/5 follow up naïve 4/5 follow up on ART g</td>
</tr>
</tbody>
</table>

a 5/14 additionally sampled in follow-up (4/5 on antiretroviral therapy). b HBV DNA was tested using kinetic PCR assay with detection limit of <20 or <55 IU/mL (<100-300 copies/mL; TaqMan Roche). c Normal CD4 count is 500 cells/mm^3 to 1,000 cells/mm^3 (99). d HIV RNA was tested using real time PCR assay with sensitivity <40 or 75 - 10^10 virus copies/mL (Abbott m2000). e FibroScan = liver stiffness according to METAVIR staging (100). f Highly active antiretroviral therapy. g Antiretroviral therapy
2.2 Isolation of Unsorted Peripheral Blood Mononuclear Cells

To obtain specific PBMC subsets, unsorted PBMCs were isolated from ~60-80 mL of ethylenediaminetetraacetic acid (EDTA) treated blood on a Ficoll-hypaque gradient. Briefly, whole blood was centrifuged for 10 minutes at 2000 revolutions per minute (rpm). The top layer of plasma was removed and the remaining packed blood was diluted by a 1:1 ratio with Hank’s Balanced Salt Solution (HBSS) (Gibco©, Life Technologies, Grand Island, NY, USA) which provides a pH and osmotically balanced solution for dilution (101). The diluted packed blood was then transferred onto Ficoll at a 2:1 ratio before centrifuging at 2000 rpm for 10 minutes. The buffy coat was collected with a transfer pipet and washed with a 1:7 ratio of buffy coat to HBSS. The buffy coat was centrifuged for 10 minutes at 2000 rpm to collect the blood cells; then 4 mL of ammonium-chloride-potassium lysing buffer was added (Gibco©, Life Technologies, Grand Island, NY, USA) to lyse remaining blood cells for 10 minutes at 37˚C. The cells were washed with 12 mL of HBSS, centrifuged at 2000 rpm for 10 minutes, resuspended in 2 mL of HBSS and the cell count determined using a Moxi™ Z Mini Automated Cell Counter (ORFLO Technologies, VWR, Mississauga, Ontario, Canada). Approximately 2.0 x 10^7 cells were saved for cryopreservation by resuspending in 4 mL of dimethyl sulfoxide (DMSO) with 10% fetal calf serum (FCS) in order to reduce ice formation and support the structural integrity of the cell membrane (102). The remaining cells were diluted with HBSS to a concentration of 1 x 10^7 cells/mL and split into 5 equal aliquots in anticipation for cell subset purification.

In parallel, whole PBMCs were isolated from 7 healthy HBV and HIV-1 uninfected individuals (2M/5F, median age 37 years (23-55 years) for subsequent cell subset separation to serve as additional controls during isolation of unsorted PBMCs from patients. Downstream
PCR and nucleic acid hybridization (NAH) assays (see Section 2.6 and 2.9) from healthy samples were consistently negative for HBV DNA.
2.3 Peripheral Blood Mononuclear Cell Subset Purification and Fluorescence Activated Cell Sorting

The optimal conditions for PBMC subset separation were determined in an experiment on cell survivorship involving four different cell preparation conditions. After collection of 64 mL of whole blood from a healthy person, samples were either 1) left overnight at 4°C, 2) diluted with HBSS overnight at 4°C, 3) placed on a Ficoll-hypaque gradient to isolate PBMCs which were then cultured overnight with RPMI media (Gibco®, Thermo Scientific, Burlington, ON) with 20% FCS 1 x penicillin/streptomycin at a concentration of $10^7$ cells/mL or 4) placed on a Ficoll-hypaque gradient to isolate PBMCs which were then immediately enriched through positive magnetic activated cell sorting (MACS) (Miltenyi Biotec®, Auburn, CA, USA). In summary, Miltenyi® magnetic bead separation on freshly isolated PBMCs was determined to be the optimal condition for cell viability in all PBMC cell subsets (median 62.4%, range 58.4 – 64%) and was subsequently used when separating PBMC subsets from patient blood.

To obtain the CD4+ T cell, CD8+ T cell, CD14+ monocyte, CD19+ B cell and CD56+ NK cell target cell subsets, they were isolated using MACS from total PBMCs isolated from patient blood (see Section 2.2). Subset purity was verified using FACS when possible (3/6 HBV/HIV-1 co-infected, 7/14 HBV treatment naïve mono-infected, 4/5 HBV ART follow-up). In the case of FACS analysis, 1 million cells were taken out to serve as the isotype and unstained cell control. PBMC subsets, using the aliquots from Section 2.2, were separated by positive selection using MACS monoclonal antibody beads for CD4+ T cells, CD8+ T cells, CD14+ monocytes, CD19+ B cells and CD56+ NK cells. A previous study by Trippler et al. determined that though comparable purity yields were attained by FACS and MACS beads for separating PBMC subsets, the method of MACS beads was much faster (21). The CD14+ monocytes were
isolated prior to CD4+ T cell positive selection to address the monocyte contamination observed by Trippler et al (21). Two aliquots were pooled and incubated with 20 µl of CD14+ microbeads/10^7 cells for 15 minutes at -4°C. The other 3 respective aliquots were incubated with CD8+, CD19+ or CD56+ microbeads/10^7 cells for 15 minutes at -4°C. Each aliquot was washed with 10 mL MACS buffer (phosphate buffered saline, 2% FCS, 1 mM EDTA), centrifuged at 300 g for 10 minutes and each aliquot resuspended in 1 mL of MACS buffer. A 10 µl aliquot of the samples was taken from each sorted cell subset to serve as unsorted comparison if FACS analysis was to be conducted. Labeled cells were applied to MACS columns prepared with 3 mL of MACS. The resulting flow through (FT) was collected with 50 µl taken for FACS sampling. The column was washed with an additional 2 mL of MACS, the aliquots spun down and the resulting FT pellet resuspended in 2 mL of DMSO with 10% FCS for future studies. The column was removed from the magnet and the positive fraction was eluted from the column using 5 mL of MACS buffer. An aliquot of 1 mL from the CD14+ FT was removed to serve as the CD14+ FT sample and prepared as mentioned above for the other FT samples. The remaining CD14+ FT aliquot was incubated with CD4+ microbeads/10^7 cells for 15 minutes at -4°C and treated as mentioned above for other aliquots. If FACS analysis was to be conducted, 100 µl was removed from each positively enriched fraction to determine the subset purity. All FT and positive fractions were spun down at 300 g for 10 minutes and resuspended in FCS with 10% DMSO until further analysis. All samples for FACS analysis were centrifuged at 2000 rpm for 5 minutes, resuspended in 20 µl of MACS buffer and incubated for 10 minutes at -4°C with 2 µl of either FITC (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) or phycoerythrin (PE) antibodies specific for the enriched subset (Invitrogen, Human R-PE conjugate, Frederick, MD, USA). Cells were washed with 1 mL of MACS buffer, centrifuged at 2000 rpm for 5
minutes, and the precipitate resuspended in 200 µl of 2% paraformaldehyde in MACS buffer for 20 minutes at -4°C. Samples were centrifuged at 2000 rpm for 5 minutes to remove the paraformaldehyde, resuspended in 300 µl of MACS buffer and transferred to FACS tubes for FACS analysis. FACS analysis was conducted by dual color immunofluorescence (BD Biosciences LSR II, San Jose, California) using Kaluza software (Beckman Coulter, Mississauga, Ontario). PBMC subsets determined to have >80% purity were included in the data set (Figure 5).
Figure 5: Representative fluorescence activated cell sorting density plots and histogram analysis of peripheral blood mononuclear cell subset purity

Fluorescence activated cell sorting purity analysis of peripheral blood mononuclear cell (PBMC) subsets enriched via positive magnetic bead cell sorting (Miltenyi®) isolated from a hepatitis B virus (HBV) mono-infected treatment naïve patient (ID# 9). PBMC subsets determined to have >80% purity when compared to a background sample of unlabelled cells were considered suitable for further HBV genome detection assays. In this representative patient sample, the purity of each isolated subset was determined to be 96.8% for CD4+ T cells, 94.8% for CD8+ T cells, 82.8% for CD14+ monocytes, 89.0% for CD19+ B cells and 83.2% for CD56+ natural killer cells.
2.4 Treatment of Peripheral Blood Mononuclear Cell Subsets with Trypsin and DNase Digestion

To address possible HBV DNA contamination from adhering extracellular viral particles or freely circulating unenveloped HBV DNA molecules, total PBMCs and cell subsets were treated with trypsin/DNase digestion (103). The final wash was saved for control analysis and treated cells were used in subsequent nucleic acid isolation (see Section 2.5). PBMC subsets were washed 3 times with 9 mL of phosphate buffered saline (PBS) and the cell pellet resuspended in 900 µl PBS after spinning at 2000 rpm for 15 minutes. The samples were then incubated on ice for 30 minutes after adding 10 µl of 0.1 M CaCl\(_2\) and 10 ng/mL trypsin. Afterwards, each subset was incubated with 100 µl of 10x DNase I buffer and 10 µl of 1 milligram/mL DNase I for 30 minutes at 37˚C. The samples were incubated again at room temperature with 1 mL of 20% FCS for 5 minutes. The cells were washed in 5 mL of PBS, centrifuged for 15 minutes at 2000 rpm, and the cell pellet resuspended in 900 µl PBS.

2.5 Nucleic Acid Isolation from Peripheral Blood Mononuclear Cell

Whole PBMCs and PBMC subset samples, including those with > 80% purity for the target subset as verified by FACS analysis, were tested for HBV genomes (Figure 1). After isolation of nucleic acid from 6 HBV treatment naïve mono-infected and 5 HBV/HIV-1 co-infected patient samples using commercial nucleic acid extraction kits (Illustra TriplePrep kit, GE Healthcare, Chalfont St Giles, Buckinghamshire), the concentration and yield of HBV DNA was determined to be insufficient for detecting HBV with our sensitive two round nPCR using 3 primer pairs standardized at 1 ng of input DNA and cccDNA standardized at 5 ng of input DNA. The DNA input template often had to be adjusted for cccDNA nPCR to what remaining template
was available (~1-3 ng). Therefore for subsequent patient samples, phenol-chloroform extraction method (Section 2.5.1) was used in an effort to increase DNA yield in the remaining 8 HBV treatment naïve mono-infected, 5 HBV ART follow-up and 1 HBV/HIV-1 co-infected patient PBMC subset samples (yields increased from ~ 15-100 ng/µl DNA to ~ 400 ng/µl DNA).

Similarly, phenol-chloroform extraction method was also used for nucleic acid isolation from whole PBMC from 13/14 HBV treatment naïve mono-infected, 5/5 HBV follow-up mono-infected and 3/6 HBV/HIV-1 co-infected patients.

### 2.5.1 DNA Extraction Using Phenol-Chloroform/Ethanol Precipitation

Briefly, $1 \times 10^7$ cells were lysed with 350 µl of lysis buffer (Illustra TriplePrep kit, GE Healthcare, Chalfont St Giles, Buckinghamshire) and 3.5 µl 2-mercaptoethanol to protect DNA against oxidation and degradation. A “mock” sample was also set up using water instead of cells to serve as an extraction contamination control. Samples were incubated at 42°C for 1 hour. Buffer saturated phenol (300 µl) (UltraPure™, Invitrogen, Carlsbad, CA, USA) was added to each tube and the samples were rocked for 15 minutes. The samples were centrifuged at 10,000 rpm for 2 minutes and the upper aqueous layer collected. Another 300 µl of buffer saturated phenol was added and the samples were rocked and centrifuged as before. The upper layer was collected and 300 µl of chloroform isoamyl alcohol 24:1 (UltraPure™, Invitrogen, Carlsbad, CA, USA) was added and the samples were rocked as before. Samples were centrifuged at 10,000 rpm for 2 minutes, the upper aqueous layer was collected and 600 µl of cold 100% ethanol and 30 µl of sodium acetate were added to each tube to be left overnight at -20°C to allow DNA precipitation. The following day, the samples were spun down at 14,000 rpm for 30 minutes at
-20°C and the cell pellet washed with 1 mL of 70% cold ethanol. The samples were spun down again at 14,000 rpm for 30 minutes at -20°C. The cell pellets were resuspended and left in 20 µl of elution buffer overnight (Illustra TriplePrep kit, GE Healthcare, Chalfont St Giles, Buckinghamshire) before determining the DNA concentration ~ 400 ng/µl median (range ~ 93 – 900 ng/µl).

2.5.2 RNA Extraction Using TRIZol® Reagent

Total RNA was isolated from whole PBMC (13/14 HBV mono-infected and 3/5 HBV/HIV-1 co-infected) using TRIZol® reagent (Roche technologies, CA, USA). Briefly, 0.75 mL of TRIZol reagent was added to every 0.25 mL of cells and incubated the samples for 5 minutes at room temperature to allow for the complete dissociation of the nucleoprotein complex. After adding 0.2 mL of chloroform per 1 mL TRIZol® reagent used, the samples were vigorously shaken for 15 seconds before incubation at room temperature for 2 minutes. The sample was then centrifuged at 12,000 g for 15 minutes at 4°C. The colorless aqueous phase was removed and 0.5 mL of 100% isopropanol for every 1 mL of TRIZol® reagent was added before incubating at room temperature for 10 minutes. The samples were then centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was washed with 1 mL of 75% ethanol for every 1 mL of TRIZol® reagent initially used. The samples were vortexed briefly before centrifugation at 7500g for 5 minutes at 4°C. The final wash was discarded and the samples were allowed to air dry for 10 minutes before eluting in elution buffer overnight (Illustra TriplePrep kit, GE Healthcare, Chalfont St Giles, Buckinghamshire).
2.6 HBV Genome Detection by Nested Polymerase Chain Reaction Amplification

Multiple specific HBV gene primers were used in HBV DNA detection to increase specificity and sensitivity. PBMC cell subsets were analyzed for HBV genomes using HBV specific C, S, and P primers via nPCR followed by NAH to a digoxigenin-antidigoxigenin detection (DIG) fluorescent labelled probe (Roche Diagnostics GMBH, Manheim, Germany) (sensitivity ~10 virus copies/mL) (11). The gold standard for detection of occult HBV infection (see Section 1.3, Figure 4) is to amplify HBV DNA using two rounds of PCR (i.e., nPCR) with multiple primers specific to HBV DNA (104). The recommendations were thus adapted and C, S, P primer sets (Table 2) were used for the purpose of our assay as both the HBV mono-infected patient samples on ART and the HBV/HIV-1 co-infected samples were expected to have low/undetectable plasma HBV DNA. Additionally, preferential reading of some HBV ORFs, loss of HBsAg in occult infection and integrated forms can occur (refer to Section 1.2.4 and 1.3). Thus the use of multiple primer pairs to detect low-level HBV genomes is expected to increase the sensitivity of our assay.

HBV cccDNA is the template for viral gene expression and transcription of the viral pregenome which is reverse transcribed within viral capsids (30) and is thus indicative of replicative competent virus. HBV cccDNA represents < 2.0% of total DNA in whole PBMC (22) consequently only HBV PBMC subsets which tested HBV DNA positive with at least one HBV gene specific primer were analyzed for HBV cccDNA (sensitivity <10^2 virus copies/mL, Table 2). HBV DNA was digested with S1 nuclease (Invitrogen Canada Inc, Burlington, Ont, Canada) to cleave dsDNA (i.e., HBV RC DNA). Briefly, for every 5 µg DNA, 6 µl of 5x reaction buffer (Invitrogen Canada Inc, Burlington, Ont, Canada), 1 µl of S1 nuclease and enough water to bring the mixture to a total volume of 30 µl was used. The mixture was
incubated at room temperature for 30 minutes and the reaction was stopped by adding 2 µl of 0.5M EDTA at 70°C for 10 minutes. The resulting DNA template was then amplified using primers spanning the gap region of the HBV genome by nPCR (see Figure 1).

Whole PBMC from 13/14 HBV treatment naïve mono-infected, 5/5 HBV follow-up mono-infected and 3/6 HBV/HIV-1 co-infected were also analysed for HBV DNA, mRNA (another indicator of replicative competent virus as described in Section 1.2.4), and cccDNA (as mentioned above) in order to verify results from PBMC subsets as we surmised that copies of cccDNA in individual subsets was below our assay sensitivity. For detection of HBV mRNA, reverse transcription of total RNA to complementary (c)DNA was completed by using random hexanucleotides (Oligo(dT)12-20 Invitrogen, Carlsbad, CA, USA) to prime RNA species and RT enzyme (SuperScript® II, Invitrogen, Carlsbad, CA, USA) to synthesize cDNA followed by nPCR using HBV specific C primers.

All experiments were performed under rigorous contamination control precautions. The nucleic acid extraction and PCR controls included isolation of total PBMC and subsets from healthy HIV-1 and HBV negative individuals (Section 2.2), done in parallel to patient samples, which were tested for cell subset purity and confirmed to be HBV DNA negative in downstream PCR/NAH assays, mock water extractions during nucleic acid isolation, and PCR waters in both first and second round amplification. The reverse transcription step was carried out in parallel both with and without RT enzyme, to ensure specificity for detection of cDNA transcribed from mRNA, and not carry-over HBV total DNA (105). The positive control was a HBV genotype A DNA plasmid for C, S, and P gene detection and a HBV genotype A EcoR1 enzyme digested DNA plasmid for cccDNA detection (provided by Dr. T.I. Michalak).
2.7 HBV Genome Amplification by Quantitative Polymerase Chain Reaction

To improve cccDNA detection in whole PBMC and subsets a quantitative (q) real-time PCR with greater assay sensitivity was optimized (3.7 x 10^1 copies/mL). The range of detection is increased when compared to nPCR due to a cleaved probe which provides a record of cccDNA amplification in real-time. A 10-fold serial plasmid dilution with either a homo β-galactosidase (beta-gal) – molecule used for quantitating expression in mammalian cells (1 beta-gal molecule/cell) – or a HBV cccDNA insert was used to generate standard curves in duplicate (Table 2, Figure 6 and 7). On 96 well plates, assays were set up triplicate assays using 10 µl of PerfeCTa® FastMix® II, ROX™ (Quanta Biosciences, Gaithersburg, MD, USA) as our qPCR reagent system, 2 µl of cccDNA or beta-gal primers, 1 µl of a 21 base pair (bp) cccDNA or 22 bp beta-gal Taqman labelled probe (Table 2) and 7 µl of HBV DNA template for each qPCR reaction. A negative control containing water instead of DNA template was used.
Table 2: Primer sequences and amplicon sizes for HBV DNA detection using nested and real-time polymerase chain reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Round and amplicon size (bp)</th>
<th>Primer sequences 5’-3’ (sense, antisense)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core</td>
<td>Direct – 509</td>
<td>GTTCATGTCCCCACTGTTCAGC ANATAGGGGCATTGGTG</td>
</tr>
<tr>
<td></td>
<td>Nested – 403</td>
<td>TTTGGGCGTGGGACATTTGACC AATAGCTGGAGGAGTCGAATCC</td>
</tr>
<tr>
<td>Surface</td>
<td>Direct – 876</td>
<td>TTGTGGGTCACTATATCTGGG CCGGCAACATACCTTGAGTCC</td>
</tr>
<tr>
<td></td>
<td>Nested – 408</td>
<td>ATCCTCAGGCGCATGAGTCC GAGACACATCCAGGCTATAACC</td>
</tr>
<tr>
<td>Polymerase</td>
<td>Direct – 1140</td>
<td>GTTGGCTAGCAGAATCTTTGGG CCTGCTGGTGCTGCCAGTCAG</td>
</tr>
<tr>
<td></td>
<td>Nested – 960</td>
<td>ACCCTGATCCGAAAATGGG CAACCTCCGAAATACATCGC</td>
</tr>
<tr>
<td>cccDNA</td>
<td>Direct – 150</td>
<td>ACTCCTGGACTCTACATTGGG GTATGGTAGGAGGTAGCATT</td>
</tr>
<tr>
<td></td>
<td>Nested – 144</td>
<td>AGGCTGTAGGGAATTTGGG GTCTTAGGGTCTTCTACA</td>
</tr>
<tr>
<td>cccDNA (qPCR)</td>
<td>One round – 151</td>
<td>GTGCCTTCATCTCGCCGG GCAAGAGAATCTAGGCAA</td>
</tr>
<tr>
<td>cccDNA (qPCR probe)</td>
<td>N/A – 21</td>
<td>AGGCCTGATGGGCATAAATTGGGT</td>
</tr>
<tr>
<td>Homo B-glo (qPCR)</td>
<td>One round – 151</td>
<td>TGCACGTGGGTCTCTGTGACAATTTTCA TGATACCTTTGGGGAATCTACTTA</td>
</tr>
<tr>
<td>Homo B-glo (qPCR probe)</td>
<td>N/A – 22</td>
<td>TGCTGGCCCATCATCTTTGGCA</td>
</tr>
</tbody>
</table>
Figure 6: A representative figure of a HBV covalently closed circular DNA quantitative real time polymerase chain reaction standard curve

A 10-fold serial dilution of a HBV covalently closed circular DNA (cccDNA) cloned plasmid was used to produce a standard curve in duplicate in real time quantitative PCR. The average required minimum number of amplification cycles from triplicate assays to produce a cccDNA labelled probe fluorescent signal which exceeds the control background level is indicated on the y axis. The corresponding number of HBV cccDNA copies (log<sub>10</sub>) is indicated on the x axis. The linear regression equation as determined from the standard curve is \( y = -3.4017x + 40.016 \). The \( R^2 \) coefficient of correlation obtained from the standard curve was 0.9986 (> 0.99 \( R^2 \) value is considered a good match between observed data and the values expected by theory).
Figure 7: A representative figure of a homo β-galactosidase quantitative real time polymerase chain reaction standard curve

A 10-fold serial dilution of a homo β-galactosidase (beta-gal) cloned plasmid was used to produce a standard curve in duplicate in real time quantitative PCR. The average required minimum number of amplification cycles from triplicate assays to produce a beta-gal labelled probe fluorescent signal which exceeds the control background level is indicated on the y axis. The corresponding number of beta-gal copies (log_{10}) is indicated on the x axis. The linear regression equation as determined from the standard curve is \( y = -3.3004x + 40.975 \). The \( R^2 \) coefficient of correlation obtained from the standard curve was 0.9981 (> 0.99 \( R^2 \) value is considered a good match between observed data and the values expected by theory).
2.8 Agarose Gel Electrophoresis

Ten µl of amplified HBV DNA nPCR product was mixed with 1 µl of 5X blue/orange loading dye and loaded it onto a 1.0% agarose gel with 0.1% SafeView™ (Applied Biological Materials, Richmond, BC, Canada). A 1000 bp or 100 bp molecular DNA ladder (Invitrogen, Frederick, MD, USA) was loaded in the first well to determine the size of the amplified PCR product. Electrophoresis was carried out in 1X tris base, acidic acid, and EDTA (TAE) buffer at 95 volts for 55 minutes. Resulting DNA bands were imaged on VersaDoc using Quantity One software (Life Science, Mississauga, Ont, Canada).

2.9 Nucleic Acid Hybridization and Southern Blot Analysis

To confirm PCR amplicon authenticity, agarose gels with electrophoresed HBV genome sequences were transferred to a nylon membrane (GE Healthcare Limited, Amershan Hybond™ -N+, Little Chalfont, Buckinghamshire, England) using the classic method by Southern et al. and hybridized with a recombinant HBV DNA probe (see below). Briefly, using a vacuum blotting apparatus, the agarose gel was laid out on top of a plastic cut-out, slightly smaller than the size of the gel, which sits on extra thick filter paper (Life Science, Mississauga, Ont, Canada). The apparatus was filled with transfer buffer (58 g sodium chloride, 16 g sodium hydroxide, 1 liter water) until the agarose gel was submerged and the vacuum was turned on for 45 minutes. Afterwards, the nylon membrane was removed and washed in 5X saline-sodium citrate solution for 5 minutes and allowed to dry.

The dried membrane was sealed in a glass tube with 10 mL of DIG Easy Hyb (Roche Diagnostics, Manheim, Germany) at 42°C for 30 minutes. The DIG Easy Hyb was decanted and
the membrane was incubated overnight with 14 µl of PCR probe. The PCR probe was synthesized with a deoxyribonucleotide mix, random hexamers and labeling grade Klenow enzyme (DIG probe labelling kit, Roche Diagnostics, Manheim, Germany), specific PCR primers (see Section 2.6) and plasmid. Following addition of the PCR probe to 7 mL of DIG Easy Hyb, the mixture was denatured for 5 minutes at 102°C. The following day, the buffer was decanted and the membrane incubated twice at room temperature in 10 mL of low stringency buffer (Roche Diagnostics, Manheim, Germany). The membrane was rinsed in 1X washing buffer (Roche Diagnostics, Manheim, Germany) and then incubated in 20 mL blocking solution (Roche Diagnostics, Manheim, Germany) with 1µl anti-digoxigenin antibody (Roche Diagnostics, Manheim, Germany). The membrane was washed twice for 15 minutes in 1X washing buffer and incubated it for 3 minutes in 20 mL of 1X detection buffer (Roche Diagnostics, Manheim, Germany). The used detection buffer was decanted and the membrane was incubated for an additional 15 minutes in 990 µl of 1X detection buffer with 10 µl of the chemiluminescent CSPD substrate (DIG Luminescent Detection Kit for Nucleic Acids, Roche Diagnostics, Manheim, Germany) before exposure using the VersaDoc and Quantity One software.
2.10 Statistical Data Analysis

With the assistance of a biostatistician, a nonparametric analysis using Mood’s median test was used for the comparison of categorical data (HBV DNA from whole PBMC from 11 HBV treatment naïve vs 2 HBV/HIV-1 HAART treated patients and 4 HBV ART follow-up vs 2 HBV/HIV-1 HAART treated patients). The statistical analysis of the data was done using Minitab 17 software. A multivariate analysis of frequency distribution using the Fisher exact test was used for the comparison of categorical data (5 PBMC subsets from HBV treatment naïve vs HBV ART follow-up vs HBV/HIV-1 HAART treated patients). The statistical analysis of the data was done using SAS 9.2 software. Statistical significance between the HBV/HIV-1 HAART co-infected to HBV mono-infected (treatment naïve and on treatment) groups and HBV mono-infected treatment naïve to HBV mono-infected on ART follow-up groups were analyzed using a one-way ANOVA and paired t-test respectively using SAS 9.3 software. Two-tailed P values of <0.05 were considered statistically significant for all tests.
Chapter Three: Results

3.1 Summary of Clinical and Virological Data

Clinical information was collected to assess infection status as well as differences between factors that may affect HBV natural history for both HBV mono-infected and HBV/HIV-1 co-infected patient cohorts. Overall, both the HBV mono-infected and HBV/HIV-1 co-infected cohort had comparable clinical parameters with the notable difference of a significantly lower HBV DNA viral load observed in the HBV/HIV-1 HAART treated co-infected cohort as compared to the HBV treatment naïve mono-infected cohort ($P = 0.01$). The HBV DNA viral loads were not statistically significant between HBV/HIV-1 HAART treated co-infected patients and HBV ART treated follow-up monoinfected patients ($P = 0.49$). At the time of enrolment, 14/14 HBV mono-infected patients were treatment naïve, 6/14 were HBeAg-/anti-HBe+, with a median plasma HBV DNA of 5.4 X 10$^5$ IU/mL (<20 – 3.6 x 10$^7$ IU/mL or ~100 - 1.8 x 10$^6$ copies/mL), median ALT 47.5 U/liter (23 – 236 U/L), 3/14 had moderate to severe liver fibrosis. Follow-up samples were collected from 5/14 HBV mono-infected cases, 4/5 of which had suppressed plasma HBV DNA after starting anti-HBV therapy (e.g. TDF or ETV, median duration 22.6 months, range 16-32). In the HBV/HIV-1 co-infected cohort, 2/6 HBeAg-/anti-HBe+, 5/6 were on HAART, with median HBV DNA 313 IU/mL (<55 – 690 IU/mL or ~300 – 3.5 X 10$^3$ copies/mL COBAS TaqMan HBV test), median ALT 43 (range 15 – 54 IU/L), median CD4+ T cell count 240 cells/mm$^3$ (114 - 800 cells/mm$^3$) and median HIV-1 RNA <40 copies/mL (<40 – 10$^4$ copies/mL) (Table 1). In summary, all our patients have clinical and serological markers consistent with CHB infection. Despite significantly higher HBV plasma viral loads in HBV mono-infected treatment naïve patients at baseline when compared with
HBV/HIV-1 co-infected patients on HAART, HBV plasma viral loads were subsequently comparable HBV mono-infected patients after commencement of ART.
3.2 Summary of Florescence Activated Cell Sorting Purity Analysis of Peripheral Blood Mononuclear Cell Target Subsets Isolated by Magnetic Beads

Where available, PBMC subset purity was verified by FACS analysis to establish the likelihood of contamination by non-target subsets and cellular debris in subsequent downstream HBV genome detection assays. PBMC subsets isolated in parallel from healthy HBV and HIV-1 negative individuals also served as controls (see Section 2.2). The majority of PBMC subsets verified by FACS (3/6 HBV/HIV-1 co-infected, 7/14 HBV treatment naïve mono-infected, 4/5 HBV ART follow-up) had > 80% purity (Table 3 and 4). Of the 3/6 co-infected patients, 4 subsets did not have enough cells for FACS analysis (1/3 CD4+ T cells, 1/3 CD14+ monocytes, 1/3 CD19+ B cells and 1/3 CD56+ NK cells). In the remaining 11 subsets which were analyzed by FACS, 8 had > 80% purity and were included in subsequent analysis (1/2 CD4+ T cell, 3/3 CD8+ T cells, 2/2 CD14+ monocytes, 1/2 CD19+ B cells, 1/2 CD56+ NK cells) (Table 3). Of the HBV mono-infected cohort, PBMC subsets from 7/14 HBV treatment naïve patients and 4/5 HBV ART follow-up patients were verified for purity by FACS. Overall, in HBV mono-infected subsets, 9 individual subsets from 4 patients (2 treatment naïve and 2 ART follow-up) had insufficient cells to complete FACS purity analysis (1/11 CD4+ T cells, 3/11 CD14+ monocytes, 2/11 CD19+ B cells, 2/10 CD56+ NK cells). However, 32/46 (70%) individual subsets from 7 HBV treatment naïve and 4 HBV ART follow up had >80% purity and were included in subsequent analysis (8/10 CD4+ T cells, 8/11 CD8+ T cells, 8/8 CD14+ monocytes, 3/9 CD19+ B cells, 5/8 CD56+ NK cells) (Table 4). In summary, positive selection enrichment using magnetic beads yielded >80% purity of target PBMC subsets the majority of the time; supporting that downstream detection of HBV genomes was likely to correspond with the assayed target PBMC subset.
**Table 3: Summary of fluorescence activated cell sorting analysis in peripheral blood mononuclear cell subsets isolated from 3 HBV/HIV-1 co-infected patients to determine cell subset purity**

<table>
<thead>
<tr>
<th>HBV/HIV-1 Co-infected</th>
<th>PBMC Subset Purity (%) as Determined by FACS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ID#</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Total subsets (n) &gt; 80% pure</td>
<td>1/2</td>
</tr>
</tbody>
</table>

N/A – samples with insufficient cells for FACS
Table 4: Summary of florescence activated cell sorting analysis in peripheral blood mononuclear cell subsets isolated from 11 HBV mono-infected, either treatment naïve (A) or after starting antiviral therapy (B)

<table>
<thead>
<tr>
<th>HBV Mono-infected</th>
<th>PBMC Subset Purity (%) as Determined by FACS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID#</td>
<td>CD4+</td>
</tr>
<tr>
<td>1B</td>
<td>93.97</td>
</tr>
<tr>
<td>3B</td>
<td>60.8</td>
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<tr>
<td>6</td>
<td>N/A</td>
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<td>7</td>
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<td>10</td>
<td>93.51</td>
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<tr>
<td>11</td>
<td>73.9</td>
</tr>
<tr>
<td>12A</td>
<td>81.85</td>
</tr>
<tr>
<td>12B</td>
<td>96.52</td>
</tr>
<tr>
<td>13B</td>
<td>93.75</td>
</tr>
<tr>
<td><strong>Total subsets (N) &gt; 80% pure</strong></td>
<td>8/10</td>
</tr>
</tbody>
</table>

N/A – samples with insufficient cells for FACS analysis. FACS analysis was not completed for patient ID# 1A, 3A 8B and 14.
3.3 Detection of HBV DNA and Covalently Closed Circular DNA in Peripheral Blood Mononuclear Cell Subsets

A sensitive nPCR/NAH was used to detect HBV DNA and cccDNA in target PBMC subsets isolated for HBV treatment naïve and ART follow-up mono-infected and HBV/HIV-1 HAART treated co-infected patients. With exception to the CD4+ T cell subset from HBV/HIV-1 co-infected patients, HBV DNA was detected in all cell subset types from all cohorts, including low-level HBV cccDNA detection in the CD8+ T cell and CD56+ NK cell subsets of 3/6 HBV/HIV-1 co-infected patients. Using HBV C, S and P specific gene primers, HBV DNA was detected in at least one subset from 13/14 treatment naïve HBV mono-infected patients at baseline (Table 5). Due to small sample sizes however, no significant difference in frequency of HBV DNA detection was observed between cell subsets. A follow-up blood sample was collected from 5 treatment naïve HBV mono-infected patients that subsequently started anti-HBV therapy (median follow-up of 22.6, range 16-32 months). In all 5 follow-up cases, HBV DNA continued to be detectable in at least one cell subset from each patient and in every cell subset type, even despite suppression of HBV DNA on treatment (Table 5). No significant difference in frequency of HBV DNA detection between cell subsets originating from treatment naïve compared to anti-HBV treated HBV mono-infected patients could be concluded. HBV cccDNA was not found in any subsets despite previously testing positive for HBV DNA from either treatment naïve or anti-HBV treated HBV mono-infected patients (Table 5).

In comparison, using HBV C, S, and P gene specific primers to detect HBV DNA in cell subsets from 6/6 HBV/HIV-1 co-infected patients (5/6 on HAART) resulted in positive signals in at least one cell subset from each patient and all target subset types with the statistically significant exception of the CD4+ T cells subset; P = 0.04 (Table 6). HBV cccDNA was only
detected in CD8+ T cell and CD56+ NK cell subsets from 3/6 HBV/HIV-1 co-infected patients (Table 6).

In summary, with exception of the CD4+ T cell subset where HBV DNA was not detected in any HBV/HIV-1 co-infected patient samples, HBV DNA was detected in all other target PBMC subsets (i.e., CD8+ T cells, CD14+ monocytes, CD19+ B cells, CD56+ NK cells) regardless of treatment or HIV-1 infection status (Table 7). There was no significant difference in detection frequency between subsets. Although PBMC subsets with <80% purity were excluded from analysis, the results of nPCR/NAH testing of the lower purity cell subsets in both HBV monoinfected or HBV/HIV-1 co-infected patients did not change the overall results of our study (data not shown). Furthermore, HBV cccDNA was only detected in CD8+ T cell and CD56+ NK cell subsets from HBV/HIV-1 co-infected patient samples but none of the samples from HBV monoinfected patients, regardless of their treatment status.
Table 5: Summary of HBV genome detection in whole peripheral blood mononuclear cells and subsets isolated from 14 HBV mono-infected patients

<table>
<thead>
<tr>
<th>HBV Mono-infected</th>
<th>PBMC Subset C,S or P and cccDNA</th>
<th>Whole PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID#</td>
<td>CD4+</td>
<td>CD8+</td>
</tr>
<tr>
<td>1A</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>1B</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3A</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3B</td>
<td>N/A</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>N/A</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>8A</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>8B</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>12A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12B</td>
<td>+</td>
<td>N/A</td>
</tr>
<tr>
<td>13A</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>13B</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Rx Naive

<table>
<thead>
<tr>
<th>HBV DNA+</th>
<th>8/12</th>
<th>5/13</th>
<th>5/14</th>
<th>6/13</th>
<th>7/13</th>
<th>12/12</th>
<th>13/13</th>
<th>11/11</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV cccDNA+</td>
<td>0/8</td>
<td>0/5</td>
<td>0/5</td>
<td>0/6</td>
<td>0/7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Follow Up

<table>
<thead>
<tr>
<th>HBV DNA+</th>
<th>2/3</th>
<th>2/3</th>
<th>3/4</th>
<th>1/1</th>
<th>1/1</th>
<th>4/4</th>
<th>4/4</th>
<th>4/4</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV cccDNA+</td>
<td>0/2</td>
<td>0/2</td>
<td>0/3</td>
<td>0/1</td>
<td>0/1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: PBMC - peripheral blood mononuclear cells, HBV - hepatitis B virus, HIV-1 – human immunodeficiency virus type 1, C- Core, S- Surface, P-Polymerase, cccDNA – covalently closed circular DNA, mRNA- messenger RNA. PBMC subsets: CD4+ T cells, CD8+ T cells, CD14+ monocytes, CD19+ B cells, CD56+ NK cells. (+/-) positive/negative HBV DNA signal detected. N/A – samples excluded from summary analysis if verified to be <80% cell purity by FACS or unavailable. The results of PCR testing in less pure subsets did not change overall findings (data not shown). **Bold** font indicates cccDNA stats

1Patient ID# 12B – follow up patient sample taken at a treatment naïve time point (not included in % HBV DNA positive (N) analysis), 2Follow up patient samples collected from 4 HBV mono-infected patients (ID# 1B, 3B, 8B, 13B) taken at a second time point following initial antiviral treatment.
Table 6: Summary of HBV genome detection in whole peripheral blood mononuclear cells and subsets isolated from 6 HBV/HIV-1 co-infected patients

<table>
<thead>
<tr>
<th>HBV/HIV-1 Co-infection</th>
<th>PBMC Subset</th>
<th>Whole PBMC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C, S or P and cccDNA</td>
<td></td>
</tr>
<tr>
<td>ID#</td>
<td>CD4+</td>
<td>CD8+</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>4¹</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>6</td>
<td>N/A</td>
<td>-</td>
</tr>
</tbody>
</table>

| HBV DNA+ | 0/5 | 3/6 | 3/6 | 2/5 | 4/5 | 2/2 | 2/2 | 2/2 |
| HBV cccDNA+ | 0/0 | 2/3 | 0/3 | 0/2 | 3/4 |

Abbreviations: PBMC - peripheral blood mononuclear cells, HBV - hepatitis B virus, HIV-1 - human immunodeficiency virus type 1, C - Core, S - Surface, P - Polymerase, cccDNA - covalently closed circular DNA, mRNA - messenger RNA. PBMC subsets: CD4+ T cells, CD8+ T cells, CD14+ monocytes, CD19+ B cells, CD56+ NK cells. (+/-) positive/negative HBV DNA signal detected, N/A - samples excluded from summary analysis if verified to be <80% cell purity by FACS or unavailable, **bold** font indicates HBV cccDNA results. ¹Patient not always compliant with highly active antiretroviral therapy
Table 7: Summary comparison of HBV genome detection in peripheral blood mononuclear cell subsets isolated from 14 HBV mono-infected (treatment naïve), 4 HBV mono-infected (ART follow-up) and 6 HBV/HIV-1 (on HAART) co-infected patients

<table>
<thead>
<tr>
<th>Group</th>
<th>PBMC Subset C, S or P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4+</td>
</tr>
<tr>
<td>HBV mono-infected (Rx naïve)</td>
<td>8/12</td>
</tr>
<tr>
<td>HBV mono-infected (Rx ART)</td>
<td>2/3</td>
</tr>
<tr>
<td>HBV/HIV-1 co-infected (Rx HAART)</td>
<td>0/5</td>
</tr>
</tbody>
</table>

HBV genomes in CD4+ T cell, CD8+ T cell, CD14+ monocytes, CD19+ B cells and CD56+ NK cells were detected by nested polymerase chain reaction (PCR) followed by nucleic acid hybridization to a digoxigenin (DIG) labelled PCR probe labelled with surface (S), core (C) and polymerase (P) primers. The size of the expected amplicon is indicated to the right of the panel. Water from second round of amplification (NW) and first round of amplification carried into second round (DW) and a mock nucleic acid extraction using water served as negative controls. An HBV genotype A plasmid served as the positive control. The data shows clean negative controls and positive signalling of the positive control in all three PCRs. A positive signal for HBV DNA was detected in CD4+ T cells and CD19+ B cells using C primers and CD56+ NK cells using P primers. No signalling for HBV DNA was detected using S primers. (ID: 8A)
3.4 Detection of HBV DNA, Messenger RNA and Covalently Closed Circular DNA in Whole Peripheral Blood Mononuclear Cells

To confirm HBV presence within whole PBMC despite absence of detection within specific subsets, nPCR/NAH and qPCR assays were conducted on whole PBMC from the same patients when available. HBV DNA (using P primers) and replicative-indicative HBV mRNA were detected in all whole PBMC samples using sensitive nPCR from 12/12 HBV treatment naïve mono-infected patients (11/11 for HBV mRNA), 5/5 HBV follow up patients, and 2/2 HBV/HIV-1 co-infected patients (Table 5 and 6). Using qPCR we were able to quantify HBV cccDNA copy numbers in whole PBMC originating from the same patients (Figure 9). In general, cccDNA copy numbers did not differ significantly between the HBV/HIV-1 co-infected cohort and the HBV mono-infected cohort regardless of treatment status (P=0.21). HBV cccDNA copy numbers were highest in HBV mono-infected treatment naïve patients and lowest in HBV ART follow-up (Figure 10). In the 11 HBV treatment naïve mono-infected patients the mean cccDNA copies/cell were 1.7 x 10\(^{-1}\) (range 5.3 x 10\(^{-1}\) – 2.8 x 10\(^{2}\)). In the 4 HBV follow-up patients on ART, the mean copy number was lower (6.5 x 10\(^{-2}\) cccDNA copies/cell, range 4.5 x 10\(^{2}\) - 8.7 x 10\(^{2}\)), compared to treatment naïve patients, albeit not significant (P=0.09). In the 2 HBV/HIV-1 co-infected patients on HAART the mean copy number was 7.7 x 10\(^{-2}\) cccDNA copies/cell (range 7.6 x 10\(^{2}\) – 7.8 x 10\(^{2}\)). In summary, HBV DNA and replicative-indicative HBV mRNA/cccDNA were detected in all whole PBMC assayed from both HBV mono-infected (treatment naïve and ART follow-up) and HBV/HIV-1 co-infected patients.
Figure 9: Quantitation of HBV covalently closed circular DNA levels in whole peripheral blood mononuclear cells by quantitative polymerase chain reaction

HBV covalently closed circular DNA were detected in 11 HBV treatment naïve mono-infected (initial visit (A), follow-up on antiviral therapy (B), follow up not on antiviral therapy (B*), and 2 HBV/HIV-1 co-infected (**) patients on highly active antiretroviral therapy.
Figure 10: The comparison of mean HBV covalently closed circular DNA copies in peripheral blood mononuclear cells in HBV mono-infected (before and after treatment) and HBV/HIV-1 co-infected patients on HAART.

The mean HBV covalently closed circular (ccc)DNA copies/peripheral blood mononuclear cell (PBMCs) for 11 HBV mono-infected treatment naïve, 4 HBV mono-infected on follow-up antiviral therapy (ART) and 2 HBV/HIV-1 co-infected patients treated on highly active antiviral therapy (HAART) were determined by quantitative PCR using a TaqMan probe and normalized to a housekeeping gene (i.e., β-galactosidase). The mean cccDNA copies/PBMC was lower in HBV ART treated compared to untreated patients albeit non-significant ($P=0.09$), and did not differ between HBV ART vs HBV/HIV-1 HAART treated cohorts.
Chapter Four: Literature Review

4.1 Discussion on Main Findings

The aim of this study was to characterize the presence of HBV DNA and HBV replicative intermediates in isolated PBMC subsets (i.e., CD4+ T cells, CD8+ T cells, CD14+ monocytes, CD19+ B cells, and CD56+ NK cells) from HBV mono-infected as compared to HBV/HIV-1 co-infected patients. We further assayed whole PBMC samples from some patients taken at the same time points when available. In the current study we found HBV genomes and replicative intermediates in whole PBMC isolated from HBV mono-infected (both treatment naïve and after starting anti-HBV therapy) and in HBV/HIV-1 co-infected patients. In summary, HBV genomes were detected in all PBMC subset types from HBV mono-infected and in HBV/HIV-1 patients on HAART with the exception of the CD4+ T cell subpopulation in HBV/HIV-1 co-infected patients (Table 7). The median CD4+ T cell count of our HBV/HIV-1 cohort was 240 cells/mm³ (113-800 cells/mm³) and although CD4+ T cell counts were not determined in the HBV mono-infected cohort, the expected normal range in HIV-1 uninfected individuals is ~500-1,000 cells/mm³ (99). No significant difference was noted in HBV genome detection between PBMC subset types either before or after subsequent initiation of anti-HBV therapy in HBV mono-infected samples. In addition, there is variability in HBV DNA detection in PBMC subsets from individual patient samples (i.e., HBV DNA was not detected in every subset from each patient) which supports our conclusions that the subset samples are not contaminated. Target subset fractions were enriched and input HBV DNA template for nPCR/NAH assays was standardized; it is therefore unlikely that the inability to detect HBV genomes in CD4+ T cells from all HIV-1 co-infected patients tested is influenced by the lower levels of CD4+ T cells in HIV-1 co-infection compared to HBV mono-infection (106) or the
effect of suppressive ART. Overall the data suggests that concomitant HIV-1 infection affects HBV lymphotropism, specifically in the CD4+ T cell subset.

The mechanisms by which HIV-1 infection can potentially impact HBV infection in specific PBMC subsets remain unclear. Increased T-cell turnover and proinflammatory cytokines observed during HIV-1 infection (91) might ultimately affect the cytokine milieu in which HBV interacts with PBMC subsets. In a study by Stacey et al., plasma cytokine profiles of HIV-1 positive individuals were compared to HBV mono-infected individuals. Notably, there were sustained increases of IFN-α and monocyte chemotactic protein 1, lower increases of proinflammatory factors such as IFN-γ and late increases in immunoregulatory cytokines like IL-10 in HIV-1 positive individuals when compared to HBV mono-infected individuals (107). Such cytokine cascades in HIV-1 positive persons may alter the susceptibility of PBMCs to HBV infection. For example, IFN-γ activates macrophages and is a major contributor for viral clearance while increases in IL-10 have been correlated to persistence of HBV infection (108). Alternatively, a more direct interspecific competition between HIV-1 and HBV may be possible. For example HBV surface proteins and HIV gp160 envelope glycoprotein are both membrane-associated proteins that are translated at the ER membrane, and competition for host cellular machinery associated with secretion may occur (106). This, however, does not fully explain why HBV DNA was not detected in the CD4+ T cell subset isolated from our HBV/HIV-1 co-infected patients, as the frequency of HIV infection in CD4+ T cells, even during chronic infection, is relatively low (~ 1.4%) (109).

Earlier studies offer mixed support for our data. HBV genomes – but not replicative intermediates – were detected in assayed HBV monoinfected patient PBMC subsets with no significant difference in frequency (20, 85). Pasquinelli et al. detected HBV genomes but no
replicative forms in CD4+ and CD8+ T cells and B lymphocytes using a $^{32}$P-labelled PCR probe hybridization assay (sensitivity $3\cdot4 \times 10^8$ virus copies/µg HBV DNA, compared to $1 \times 10^3$ virus copies/µg HBV DNA in our assay) (85). Yoffe et al. observed that HBV DNA was most frequently found in monocytes and B cells yet was absent from the T and NK cell subset (20). The discrepancy between previous reports and the current study may be due to differences in PBMC subset isolation and enrichment techniques, the enhanced sensitivity of our nPCR/NAH and qPCR assay and use of multiple HBV primers to detect HBV genomes (110).

Studies in HBV/HIV-1 co-infection have found HBV DNA in unsorted PBMC even despite the absence of HBV DNA detection in the plasma of those same patients (25). Additionally, some HBV/HIV-1 co-infected unsorted PBMC samples were positive for HBV cccDNA (25). In our study, HBV cccDNA was detected in CD8+ T and CD56+ NK cells of HBV/HIV-1 co-infected patients, and in total PBMC from 2/2 HBV/HIV-1 co-infected patients, 11/11 HBV treatment naïve and 5/5 on follow-up (4/5 on ART) mono-infected patients. Further, HBV mRNA was detected in all HBV mono-infected (treatment naïve and anti-HBV treated) and HBV/HIV-1 co-infected patients. HBV cccDNA can represent ~1.81% of total DNA in whole PBMCs while one cccDNA molecule can produce more than 100 pgRNA transcripts (22). Hence, the detection of HBV mRNA within unsorted PBMCs despite undetectable HBV cccDNA is indicative of active HBV replication (22).

The statistical significance of the current study was limited by the number of HBV/HIV-1 co-infected and HBV (treatment naïve and ART follow up) mono-infected patients recruited. Additionally, the use of FACS to exclude cell subsets with <80% purity as well as use of qPCR further enhanced our ability to detect low level cccDNA template. Nonetheless our data suggests
that despite anti-HBV therapy and low level HBV DNA in plasma, PBMCs are a site of ongoing extrahepatic replication. The absence of HBV genome in CD4+ T cells in HBV/HIV-1 co-infected cases tested suggests that concomitant HIV-1 may impact specific PBMC subsets infected by the HBV. Further work is needed to understand the impact of HIV-1 on HBV infection in PBMC and the immune abnormalities that persist in HBV/HIV-1 co-infected patients who remain at risk of liver-related mortality despite HAART (2, 4, 30).

4.2 Technical Issues

Specific technical issues arose regarding the purity of our subsets verified by FACS, our methods of DNA isolation and consequently our ability to detect cccDNA by nPCR assay. Initially, we employed a commercial nucleic acid isolation kit in order to simultaneously collect DNA, RNA as well as protein from the first 6 HBV mono-infected and 5 HBV/HIV-1 co-infected patient samples. We were however unsuccessful in obtaining satisfactory concentrations of DNA (~15-100 ng/µl) and RNA (~ < 25 ng/µl) from the commercial kit which impacted the total amount of input template available for RT-PCR and nPCR using P, S and C primers for DNA (standardized input ~1-2 µg) and cccDNA (~5 µg). Following a test extraction, we switched to phenol-chloroform ethanol precipitation (subsets and whole PBMCs) and TRIzol® reagent (in whole PBMCs only) to improve the quality of our DNA and RNA respectively. Although, the amount of input template increased, we were unable to detect cccDNA within most subsets indicating that cccDNA levels remained below the sensitivity threshold of our nPCR assay (sensitivity ~10^2 virus copies/mL). HBV mRNA was detected in total RNA extracted from whole PBMCs using TRIzol® reagent. HBV cccDNA was also
detected in whole PBMCs from 11 treatment naïve and 4 ART follow-up HBV mono-infected and 2 HBV/HIV-1 co-infected using a more sensitive qPCR assay (3.7 x 10⁻¹ copies/mL) which provided a record of cccDNA amplification in real-time.

Overall, 30% (17/57) subsets isolated from 13 patients were found to have <80% purity as determined by FACS analysis. Trippler et al. previously determined that the cell purity obtained from direct FACS as compared to the faster method of MACS beads for subset enrichment were comparable at >95% (21). We therefore chose MACS beads for cell sorting. We did not however, reach the same levels of purity as reported by Trippler et al. This is likely attributable to factors such as user experience and less than optimal MACS beads to cells ratio; as cells were counted prior to, and not again following, separation into different fractions for incubation. In addition there may have been lower efficiency for antibody binding to different target subsets as evidenced by more than half of stained CD19+ B cells from HBV mono-infected patients resulting in <80% purity (Table 4). Additionally, samples for FACS analysis were not always available to be taken (3/6 HBV/HIV-1 co-infected, 7/14 HBV treatment naïve mono-infected, 2/6 HBV ART follow up) thus we were unable to provide a subset purity analysis for all patient samples in our study.

The maximum possible contamination with cellular debris and other lymphocytes or monocytes in subsets included after FACS analysis was <20%. The extracellular debris is however, later removed by Trypsin/DNase digestion and washing steps (77). The HBV DNA detected is unlikely to be due to contamination by other cell types as we were still unable to detect HBV DNA in subsets with very high purity in certain cases. Thus, positive PCR results attributable to the <20% fraction of other cell types and debris is unlikely.
4.3 Significance of Research and Future Directions

4.3.1 HBV Infection in Peripheral Blood Mononuclear Cells

This study extends and confirms previous investigations of HBV infection and replication within PBMCs, which remains controversial despite a number of studies which have successfully detected HBV replicative intermediates in PBMCs (16, 20, 85, 111). As discussed in Section 1.3.2, such extra-hepatic viral replication sites would potentially facilitate HBV re-infection of hepatocytes, intrauterine transmission and HBV escape from the immune response (21, 22). A recent study by Loustaud-Ratti et al. showed immune status dependant distribution of HBV DNA and cccDNA in PBMCs of HIV-1 positive patients with varying HBV serological markers (i.e., HBsAg positive/negative, anti-HBe positive with occult HBV infection) (22). The authors found that in patients with high viral loads and HBsAg positive active infection, only low levels of HBV cccDNA was detected in PBMC (22). Conversely, HBV cccDNA was often found in PBMCs from patients with occult HBV infection and undetectable HBV DNA in their serum (22, 25). The authors proposed that in occult HBV infection where viral loads are suppressed, PBMCs serve as an active reservoir for HBV replication whereas in patients with high viral loads such as HBsAg positive patients with active infection, PBMCs may play a more passive role as hepatocytes are the main source of plasma viremia (22). Within our own study, it was observed that cccDNA was detected in PBMC subsets from all HBV/HIV-1 co-infected patients on HAART (median HBV DNA 313 IU/mL, range <55 – 690 IU/mL). HBV cccDNA however, was not found in specific PBMC subsets in our HBV mono-infected cohort, even on ART (median HBV DNA $3.6 \times 10^2$ IU/mL, range $<10 – 1.7 \times 10^3$ IU/mL), lending support to the potential of a more active viral replication role within PBMCs during overall decreased HBV viral loads. The effect of ART treatment on HBV persistence in PBMCs remains poorly
understood (112), however, studies in the WHV animal model of hepatitis B indicates a possible disposition to infect the lymphatic system over hepatocytes at low levels of HBV infection (76).

The status of HBV within particular PBMC subsets deserves consideration due to the implications for disease pathogenesis. Monocytes that contain replicating forms of HBV DNA can act as the intermediary in transmitting the HBV to hepatocytes when migrating to the liver. HBV infection may affect specific antibody production associated with B cells (20) and impair the ability of NK cells to mediate antiviral cytotoxicity (113). Furthermore, in CHB carriers, impaired CD4+ and CD8+ T cell responses have been observed despite strong T cell responses during initial infection (113). Our study did not show a significant difference between HBV detection in a specific target PBMC subset. Overall, HBV genomes were detected in all target subsets in HBV mono-infected and in HBV/HIV-1 co-infected patients, except the CD4+ T cells from HBV/HIV-1 co-infected patients. Further understanding of HBV persistence and how the virus targets specific PBMC subsets, will lead to a better appreciation of its potential effect on the host immune response. In future, recruitment of treatment naïve HBV/HIV-1 co-infected patients can further differentiate the role ART plays versus that of HIV-1 on HBV PBMC subset targeting. The advancement of novel genomic technologies, such as that of microfluidic single-cell qRT-PCR, offers the potential to look for gene expression on a single-cell basis. Such novel studies may further elucidate the presence of HBV DNA and replicative forms in specific PBMC subsets even when samples have a limited numbers of cells available (114).
4.3.2 The Viral Interaction of HBV and HIV-1

The most notable observation from our research is the absence of HBV genomes in HBV/HIV-1 co-infected CD4+T cells compared to detection in all CD4+ T cell subsets of HBV monoinfected either treatment naïve or on suppressive anti-HBV NA therapy (Table 7). In HBV/HIV-1 co-infected patients, higher serum HBV DNA and lower rates of serum HBeAg seroconversion and CD4+ T cell counts is often observed (1). Noonan et al. found that the HBV/HIV-1 co-infected infected patients with AIDS had a higher prevalence of HBV DNA in their PBMCs than HBV mono-infected patients (24). The mechanisms of viral interaction between HBV and HIV-1 are still relatively unknown. It has been suggested by Loustaud-Ratti et al. that co-infection with HIV-1 might down-regulate HBV replication (22). This could be due to direct competition between ER translated viral proteins (i.e., the HIV gp160 envelope glycoprotein and HBV surface proteins) for host cellular machinery associated with secretion (106). It may be possible that within CD4+ T cells, a primary target for HIV-1 (90, 115), HIV gp160 envelope glycoprotein out competes HBV surface proteins for host cellular machinery. However, as discussed in Section 4.1, this does not fully explain why HBV DNA was not detected in the CD4+ T cell subset isolated from our HBV/HIV-1 co-infected patients, as the frequency of HIV infection in CD4+ T cells, even during chronic infection, is relatively low (~1.4%) (109). Viral interactions can also lead to upregulation of viral proteins. Infection of HBV positive hepatic cell lines with HIV-1 has shown a significant effect on increasing HBsAg production without affecting HBV DNA synthesis (106). High levels of chemokines involved in directing T cells to the liver are expressed in untreated HBV/HIV-1 co-infected patients (116) and HIV-1 has been known to induce impairment of HBV specific CD4+ and CD8+ T cells responses (1). In the setting of HIV-1 infection, changes in the microenvironment such as
increases in IL-10 and decreases in IFN-γ (107), can down-regulate macrophage mediated HBV clearance during HIV-1 co-infection. It has been suggested that HIV-1 co-infection impairs the quantity and quality of the innate and adaptive immune response to HBV (1). For example, HIV-1 impairs HBV specific CD4+ and CD8+ T cell response (1). On the other hand, HBV infection in PBMCs may alter susceptibility of cells to HIV-1 virus infection (24). Previous studies have also shown that similar genetic factors and immune impairments, such as alteration of chemokine receptor 5 and impaired regulatory T cell function, increase the risk of both HIV-1 and HBV disease progression related outcomes (1). A greater understanding of the bidirectional interactions of HBV and HIV-1 during dual infection of PBMC will enhance our ability to target viral replication sites and ultimately eradicate the viruses from chronically infected persons.

Future studies to elucidate the specific viral interactions may include infection of cultured healthy PBMC subsets with either HBV or HIV-1 alone, or with both HBV and HIV-1 to determine if there is a similar frequency of HBV DNA and cccDNA detection within and among particular subsets. Of particular interest would be whether the absence of HBV DNA within CD4+ T cells, co-infected with HBV/HIV-1, is also found in a cultured cell assay. Furthermore, the expression of specific cytokine production in vitro can be investigated in PBMCs and subsets using enzyme-linked immunosorbent assay, and may provide additional information regarding HBV and HIV-1 viral interactions during dual infections. Finally, the degree of cell apoptosis (programmed cell death) in HBV mono-infected versus HBV/HIV-1 co-infected cultured PBMC subsets could also provide insight into the potential mechanisms and effect of dual infection on HBV lymphotropism (see Section 1.4.5).
4.4 Summary and Conclusion

In this study, HBV genomes, including replicative intermediates (HBV cccDNA and mRNA), were detected in whole PBMCs originating from HBV treatment naïve and, anti-HBV-treated patients as well as HBV/HIV-1 co-infected patients on HAART. The study provides additional evidence in support of the potential role of PBMCs to act as a viral reservoir for the HBV. Within specific PBMC subsets (i.e., CD4+ T cells, CD8+ T cells, CD14+ monocytes, CD19+ B cells, and CD56+ NK cells), HBV DNA can be detected in all HBV mono-infected patients. It is notable that HBV DNA was not detected within CD4+ T cells of all HBV/HIV-1 co-infected patients tested on HAART. Subsequent treatment of HBV mono-infected patients with anti-HBV therapy and suppression of HBV replication did not change the overall distribution of HBV within specific cell subsets when compared to HBV treatment naïve patients. Thus the data suggests that antiviral therapy does not impact HBV targeting of CD4+ T cells, and the absence of HBV in CD4+ T cells may be due to a specific viral interaction between HBV and HIV-1. Analysis of treatment naïve HBV/HIV-1 co-infected patients may confirm the results of our investigations but the data current demonstrates that HBV has a propensity to target certain PBMC subsets over others in the setting of concomitant HIV-1 infection.

HBV cccDNA was only intermittently detected in different PBMC subsets from HBV/HIV-1 co-infected patients, and none was observed in the subsets from the HBV mono-infected cohort. This agrees with other studies showing enhanced HBV replication in HIV-1 co-infected (Section 1.4). Within whole unsorted PBMC tested however, both cccDNA and mRNA HBV replicative intermediates were detected. Thus very low copy numbers of cccDNA within individual PBMC subsets, below the detection threshold of our assay, cannot be ruled out.
Future studies with larger sample sizes, as well as use of more sensitive assays such as qPCR, may improve detection in PBMC subsets despite low HBV cccDNA copy numbers.

The current study suggests a potential role of HIV-1 in affecting HBV lymphotropism and extrahepatic HBV persistence in HBV/HIV-1 co-infection. Improved understanding of factors affecting HBV persistence may lead to the development of more effective therapies to eradicate the HBV cccDNA pool. Further study of HBV and HIV interactions during co-infection will enhance our ability to screen, monitor disease progression and optimize patient management for the millions of HBV/HIV-1 co-infected patients worldwide that remain at risk of HBV related end-stage liver disease.
References


69. **Coffin CS, Fung SK, Ma MM, Canadian Association for the Study of the L.** 2012. Management of chronic hepatitis B: Canadian Association for the Study of the Liver consensus guidelines. Canadian journal of gastroenterology = Journal canadien de gastroenterologie **26:**917-938.


73. **McMahon BJ.** 2010. Natural history of chronic hepatitis B. Clinics in liver disease **14:**381-396.

74. **Tujios SR, Lee WM.** 2013. Update in the management of chronic hepatitis B. Current opinion in gastroenterology **29:**250-256.


76. **Michalak TI, Mulrooney PM, Coffin CS.** 2004. Low doses of hepadnavirus induce infection of the lymphatic system that does not engage the liver. Journal of virology **78:**1730-1738.


82. **Shen HD, Choo KB, Wu TC, Ng HT, Han SH.** 1987. Hepatitis B virus infection of cord blood leukocytes. Journal of medical virology **22:**211-216.

83. **Roisman FR, Castello A, Fainboim H, Morelli A, Fainboim L.** 1994. Hepatitis B virus antigens in peripheral blood mononuclear cells during the course of viral infection. Clinical immunology and immunopathology **70:**99-103.


