Cellular Immune responses to human betaretrovirus in patients with primary biliary cholangitis

By

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Abstract

Human betaretrovirus (HBRV) infection has been characterized in patients with primary biliary cholangitis (PBC). Our lab has documented HBRV proviral integrations in bile ducts of PBC patients. However, serological diagnostics cannot detect HBRV infection in the majority of PBC patients, limiting further confirmation of viral infection. In FACS studies using pooled 17-20 aa peptides derived from the HBRV Gag (n=58) and Env (n=85) proteins, 40% of PBC patients PBMCs were found to make proinflammatory cellular immune responses to HBRV. Then, to characterize immunodominant HBRV epitopes, we screened intra-hepatic lymphocytes (IHL) from PBC patients and control subjects for evidence of IFN-γ production. IHL isolated from liver transplant recipients with PBC (n=8) and other hepatic disorders (n=9) were individually stimulated with 18-mer peptides from HBRV Gag or Env proteins (n=143) or the characterized CD8+ reactive epitope derived from the mitochondrial autoantigen, pyruvate dehydrogenase-E2 (PDC-E2). ELISpot was used to measure spot forming colonies (SFC) producing IFN-γ. 10 HBRV Gag and 12 HBRV Env peptides were found to stimulate IHL. The mean number of SFC producing IFN-γ was higher in PBC patients versus control subjects. Using background cut off level of 1:100 SFC, the individual HBRV Gag and Env peptides provided a high specificity and sensitivity for detecting HBRV infection in PBC patients' IHL. Notably, only one PBC patient had detectable IFN-γ producing IHL following stimulation with the characterized PDC-E2 peptide. These are the first data to demonstrate that the intrahepatic IFN-γ cellular immune responses to HBRV greatly exceed the autoimmune response, suggesting that HBRV infection plays an important role in mediating PBC. The identified HBRV peptides can be evaluated to measure the IFN-γ release in peripheral blood mononuclear cells and construct a diagnostic IFN-γ release assay.

Preface

This thesis is an original document by Hiatem Abofayed. No part of this thesis has been previously published.

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List of Abbreviations

Abbreviation	Meaning
Aac.	Amino acids
AIH	Autoimmune hepatitis
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AMA	Anti-mitochondrial antibody
AST	Aspartate transaminase
BEC	Biliary epithelial cells
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CO_2	Carbon dioxide
DC	Dendritic cells
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
ENV	Envelop
FACS	Flowcytometry
FBS	Fetal bovine serum
FCS	Fetal calf serum
GAG	Group of antigens
HBRV	Human betaretrovirus
HCV	Hepatitis-C virus
HI-FBS	Heat-inactivated fetal bovine serum
HLA	Human leukocyte antigen
IFN-γ	Interferon gamma
IHL	Intrahepatic lymphocyte
MHC	Major histocompatibility complex
MMTV	Mouse mammary tumour virus
NK	Natural killer cells
NOD	Non-obese diabetic

OGDC	Oxoglutarate dehydrogenase complex
PBC	Primary Biliary Cholangitis
PBMC	peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PDC	Pyruvate dehydrogenase complex
PDC-E2	E2 subunit of the pyruvate dehydrogenase complex
PMA	Phorbol 12-myristate 13-acetate
pol	Polymerase
pro	Protease
PSC	Primary sclerosing cholangitis
PVDF	Polyvinylidene fluoride
RBC	Red blood cells
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT-PCR	Real time-polymerase chain reaction
Sag	Superantigen
SFC	Spot-forming cells
ssRNA	single stranded Ribonucleic acid
TCR	T cell receptor
TNF-α	Tumour necrosis factor alpha
UDCA	Ursodeoxycholic acid
HIV	Human immunodeficiency virus
RT	Reverse transcriptase
CART	Combination antiretroviral therapy
FDA	Food and Drug Administration
CMV	Cytomegalovirus
CMI	Cell-mediated immunity
HCMV	Human cytomegalovirus

Chapter 1: Introduction

1.1 Introduction

Primary Biliary Cholangitis, previously referred to as Primary Biliary Cirrhosis, is a chronic liver disease with an unknown etiology. The prevalence and possibly incidence in the number of cases of PBC has been increasing worldwide (1, 2).

Incidence rates range from 0.7 to 56 cases per million and prevalence rates range from 6.7 to 402 cases per million (3). In North America, the prevalence is 1/3000 cases where 9 out of 10 patients are women (4). In Canada alone, 8680 patients were diagnosed with PBC in 2015 translating to a prevalence of 318 cases per million (5). PBC is the named diagnosis of at least 5% of all liver transplants in Canada due to chronic persistent disease (6). Because of the severity of the disease, PBC not only has detrimental effects on patient physiology but also significantly impacts their psychological state and social life (7).

1.2 Primary Biliary Cholangitis

PBC is a progressive cholestatic liver disease of unknown etiology that possesses a well-defined autoimmune component. It is a disease that affects predominantly middle age women (40-60 years), but it has been diagnosed outside of this age range. The histology of the disease is characterized by non-suppurative cholangitis with lymphocytic infiltration and granulomatous destruction of small interlobular bile ducts ranging from 30-80um in diameter (Figure 1). The intrahepatic bile ducts develop progressive ductopenia and cholestasis over time. Ductopenia often leads to fibrosis, cirrhosis and eventually may lead to liver failure (Figure 2). Patients require a liver transplant to prolong life (8, 9).

Up to a half of patients with primary biliary cholangitis are asymptomatic and the disease is found incidentally because of abnormal liver function tests. However, symptoms and signs might develop during any stage of the disease. The symptoms include mild to disabling chronic fatigue, itching of the skin (often on palms or soles of the feet), gradual darkening of the skin, and some people may experience dry mouth and right upper quadrant pain.

Diagnosis of the disease is usually made with cholestatic blood tests and the finding of antimitochondrial antibody (AMA) in the serum of 90-95% of the patients with PBC. The level of serum alkaline phosphatase (ALP) is increased and is derived from damaged bile ducts. The liver enzymes alanine transaminase (ALT) and aspartate transaminase (AST) are monitored and a measure of hepatocytes damage. Also, there are some additional imaging studies helpful for diagnosis of disease such as abdominal ultrasound, liver biopsy, and a FibroScan test. These are used to assess the stage of the disease. Ursodeoxycholic acid, and more recently obeticholic acid, are used to treat PBC and delay the development of disease progression in the liver, but these do not cure PBC.

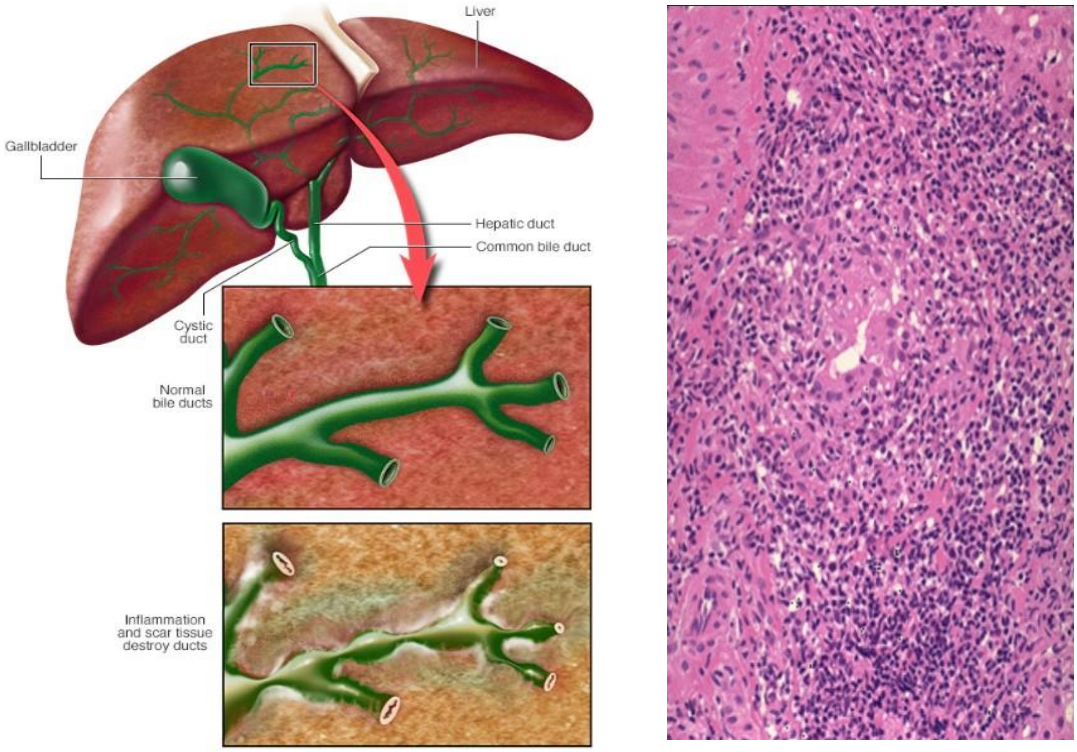

Figure 1: Intermediate bile duct damage in patient with primary biliary cholangitis. When bile ducts became damaged, bile acid can back up into the liver which lead to damage of liver cells. This damage can cause liver failure (*Mayo foundation for Medical Education and Research*).

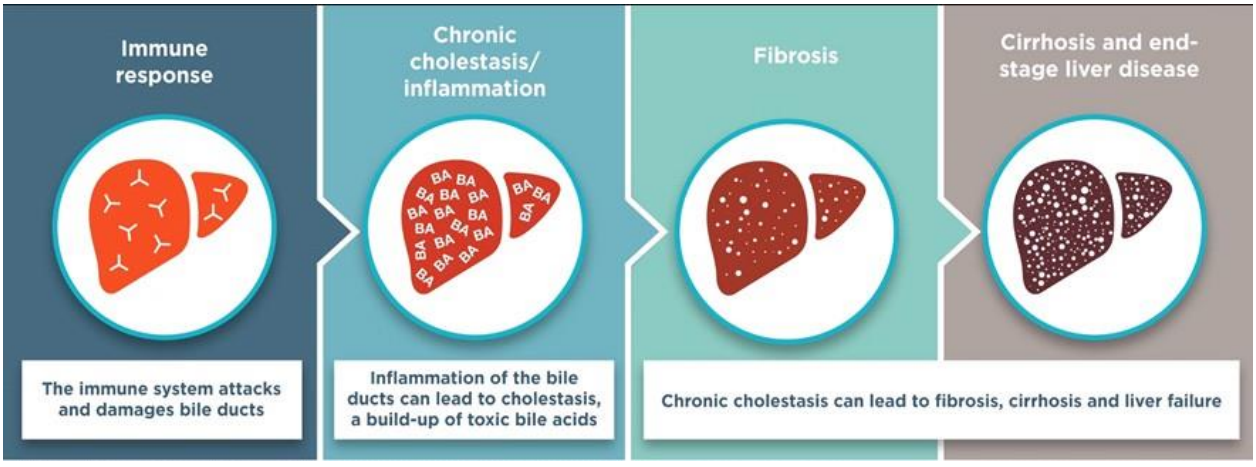

Figure 2 : Progression of primary biliary cholangitis disease (adapted from Intercept Pharma**)**

1.3 Autoimmunity and anti-mitochondrial antibodies

PBC results from autoimmune-mediated destruction of the small intrahepatic bile ducts. The association of autoantibodies with mitochondrial antigens in PBC might suggest an autoimmune disease model. Presence of circulating autoantibodies have been observed that recognize the pyruvate dehydrogenase complex (PDC) enzymes, Oxoglutarate dehydrogenase complex (OGDC) and branched chain Oxoacid Dehydrogenase complex in around 90-95% of PBC patients (10, 11).

These enzymes are usually located as subunits within the inner mitochondrial membrane antigens (Table 1). It is suggested that the majority of AMA activity is directed towards PDC-E2 (12). In healthy individuals, the mitochondrial proteins remain within the inner mitochondrial membrane of all aerobic cells. In PBC patients, these proteins are present on the plasma membrane of biliary epithelial cells of small intrahepatic bile ducts and also observed in perihepatic lymph nodes and damaged salivary glands and this exposure to the immune system is thought to trigger the formation of these autoantibodies (13).

Table 1: Mitochondrial antigens characterized in PBC (*Adapted from Gershwin et al., 2000***)**

Targeted Complex	**Specific Subunit(s) Targeted (Frequency of autoantibodies in PBC)**
Pyruvate Dehydrogenase Complex (PDC)	PDC-E2 (95%) PDC-E1alph (41-66%) PDC-E3BP (95%)
Oxoglutarate dehydrogenase complex (OGDC)	OGDC-E2 (39-88%)
Branched-Chain Oxoacid dehydrogenase complex (BCOADC)	BCOADC-E2 (53-55%)

However, B-cell autoimmunity does not appear to be essential for the development of PBC. Although the presence of AMA enables diagnosis, they may not play a role in disease progress. PBC patients without serum AMA have been shown to experience comparable disease

progress to those that do. Additionally, AMA titers do not correlate with disease severity, while some AMA positive individuals do not develop liver disease at all (14). While reduced AMA titer has been shown to coincide with improved hepatic biochemistry with UDCA therapy (4), there is no firm data to support a causal role for B lymphocyte autoimmunity in PBC.

Cellular immune studies do not support the hypothesis that PBC has an autoimmune etiology either. For example, the precursor frequency of T cells recognizing PDC-E2 ranges from 1 in 10^{-7} to 10^{-8} in PBMC and only 1 in 100,000 intrahepatic lymphocytes (IHL) recognize the mitochondrial autoantigen (15). However, these frequencies are considerably lower than those observed in patients with chronic viral infection. For example, patients' with chronic hepatitis C virus (HCV) infection have a range of 1-2 in 10^{-2} CD8+ T cells that recognize HCV epitopes in PBMC with at least a 30-fold higher reactivity within IHL (16). When we compare chronic hepatitis C virus infection and PBC, the anti-viral T cell responses appear to be two log fold higher than the autoimmune cellular immune responses in PBC.

1.4 Genetic and Environmental predisposition to PBC

1.4.1 Genetic Factors

PBC pathogenesis is probably multifactorial with a complex genetic and environmental interactions at play. The etiology of PBC remains unclear. Nevertheless, there are several elements of data supporting evidence for a genetic predisposition in PBC. Twin studies show that the concordance rate of PBC in monozygotic twins is 63%, which is the highest prevalence for any autoimmune disease (17). The incidence of the disease is high in females; around 9:10 patients are female. These observations indicate a potential role for X chromosome activity in PBC (18). About 6% of patients who suffer from PBC have a first-degree relative with PBC (19).

Of the major histocompatibility complex (MHC) alleles in PBC, a genome wide association study has indicated a major role of HLA DRB1*13 and HLA DRB1*11. HLA DRB1*13 and HLA DRB1*11 alleles confer disease protection in haplotype analyses, whereas HLA DRB1*08 alleles causes disease susceptibility (20, 21).

1.4.2 Candidate environmental causes of PBC

The etiology of PBC remains unresolved. Largely, disease development is thought to be triggered via the complex interaction between environmental and genetic contributions. Geographical prevalence, disease clustering, and seasonality of diagnostic rates suggest a strong environmental factor to PBC. Chemical toxins, xenobiotics, and infectious agents are the likely environmental factors affecting PBC. In genetically susceptible individuals these factors may drive loss of tolerance to mitochondrial antigens. Studying an infectious factor, several epidemiological studies have been conducted to study the risk factors of infectious agents for PBC development. In 1984, a hypothesis of an *E.coli* infection in PBC was made, because of the observation that bacteriuria is more prevalent in women with PBC than in women suffering from other chronic disease (22). A link between PBC pathogenesis and bacterial infection was also studied. Selmi and colleagues reported two bacterial proteins from *N. aromaticivorans* with marked amino acid identity to human PDC-E2. They suggested that PBC patients with antibody responses against mitochondrial antigen recognized the lipoylated bacterial proteins (23). Since, studies into PBC etiology have evolved. More recently, in the last two decades, there have been studies to investigate the pathogenesis of PBC and a possible association with viral infection. A betaretrovirus infection was characterized in PBC patients and co-culture studies showed that viral particles cultivated with cholangiocytes led to an increase in the expression of PDC-E2 on

biliary epithelium cell surface (8). Other investigators have studied the possibility of non-infectious environmental factors (i.e. xenobiotics), which consist of biologically foreign chemical compounds such as drugs, pesticides, cosmetics etc. It has been suggested that xenobiotics modify the native lipoyl moiety of the mitochondrial autoantigen PDC-E2, which may lead to loss self-tolerance and eventually biliary tract lesion (24). Common environmental factors linked to PBC are provided in (Table 2).

Table 2: Reported environmental factors linked with PBC

Infectious	Non-infectious Xenobiotics
E. Coli	Acetaminophen
Novosphingobium aromaticvoran	Cigarette smoking
L. delbrueckii	Hair dyes
Betaretrovirus	Vitamin D deficiency
T. Gondii	Estrogens
Mycobacteria	Waste disposals

1. 5 Characterization of a human betaretrovirus in patients with PBC

The hypothesis that PBC has some infectious elements are supported by reports that PBC occurs in women who have higher related or unrelated family members. The prevalence and incidence of disease clusters in specific geographic areas. Also, the incidence increases when people migrate from a low incidence area to an area of higher incidence. PBC reoccurs in up to 50% of patients after liver transplantation with all the clinical and laboratory manifestations including AMA detection in serum. There is no relation between the level of AMA in serum and intensity of disease progression. Aggressive recurrence in transplant recipients is linked with the potent immunosuppressive therapy.

1.5.1 Prevalence of HBRV infection in PBC patients

Previous serological studies have hinted at a viral association with PBC (25). Our lab subsequently characterized a HBRV infection in patients with PBC (8). In order to verify that there was a higher prevalence of HBRV infection in patients with PBC, RT- PCR and immunohistochemistry were performed using perihepatic lymph nodes removed at the time of surgery. Approximately 75% of PBC patients had HBRV in these lymph nodes. However, HBRV is difficult to find in liver samples, and was only found in 30% of PBC samples by RT-PCR (8) and it is seldom detected in peripheral blood.

1.5.2 HBRV trigger the mitochondrial phenotype

Although antimitochondrial antibodies are associated with PBC, there is no evidence of pathogenicity. Indeed, we found that HBRV was linked with PDC-E2 expression *in vivo* and *in vitro*. Co-culture studies of biliary epithelium cells from normal liver with homogenized PBC peri-hepatic lymph nodes developed an increased expression of PDC-E2 enzymes, whereas the control lymph nodes had no such effects (8). In perihepatic lymph nodes of PBC patients, viral proteins were found in the same cells that expressed AMA reactive proteins, suggesting that the virus provoked AMA formation by bystander activation or "guilt by association" (26). Notably, HBRV isolated from PBC peri-hepatic lymph nodes shares approximately 95 to 98% nucleotide identity with MMTV (27). Therefore, it was interesting to note that the PBC mouse model NOD. c3c4, has evidence of MMTV cholangitis where MMTV proteins and AMA reactive proteins were both localized to the inflamed bile ducts; suggesting that the sponatenous production of AMA in this model is triggered by MMTV infection (28). Taken together, these two studies suggest a potential mechanism for viral induction of autoimmunity in PBC patients (26).

1.6 Is PBC an infectious disease?

Bacteria, viruses, and xenobiotics have been proposed as potential aetiological agents for PBC. Environmental agents have been implicated in PBC with epidemiological studies showing PBC clustering in specific geographic regions such as water supply, coal mines and toxic waste sites in North America. Also, it has been found that the risk of disease increases when children emigrate from low incidence to high incidence areas (29). It has been reported that PBC develops in unrelated members in the same household, such as care givers and spouses. Furthermore, the recurrence and severity of the disease after liver transplant is higher with more potent immunosuppressive regimes including tacrolimus (1, 6). This raises the hypothesis that an inadequate cellular immunity response in patients with PBC leads to an increase of infectious triggers of disease (30). In contrast, cyclosporine A is a less potent immunosuppressive drug and protective against recurrence of the PBC following liver transplantation, which is interesting because it has antiviral activity against betaretrovirus, HIV, and other virus agents (6, 31).

Having characterized a HBRV infection in patients with PBC (8, 25), a major goal was to construct a highly specific and sensitive assay for diagnosing HBRV infection to perform prevalence studies. In the first instance, ligation mediated PCR and next generation sequencing was used to demonstrate HBRV proviral integrations in the majority of PBC patients lymph nodes and bile ducts in the majority of PBC patients tested (32). In addition, several diagnostic tests have been created to detect evidence of HBRV infection in peripheral blood but failed to achieve sufficient specificity and sensitivity to conduct epidemiological studies.

1.7 Prior work showing humoral and cellular immunity to HBRV with PBC patients

1.7.1 Humoral immunity

Prior studies in our lab (Zhang, CDDW 2015), focused on investigating serological evidence of HBRV infection in PBC patients by ELISA assay. HBRV Env gp52 Su protein was expressed in HEK293 cells and the purified supernatants were then used to create an ELISA assay. However, 11.5% of patients with PBC had demonstrable antibody activity to HBRV gp52 Su proteins as compared to 2% of the age and sex matched healthy female controls for the breast cancer patients and 3% of blood donors (Figure 3). The low prevalence of humoral response to HBRV is consistent with observations from mice. Others have reported that neonatal mice with MMTV infection fail to develop neutralizing antibodies or curtail infection due to the virus initiating IL-10 production which leads to tolerization of mice against antiviral responses.

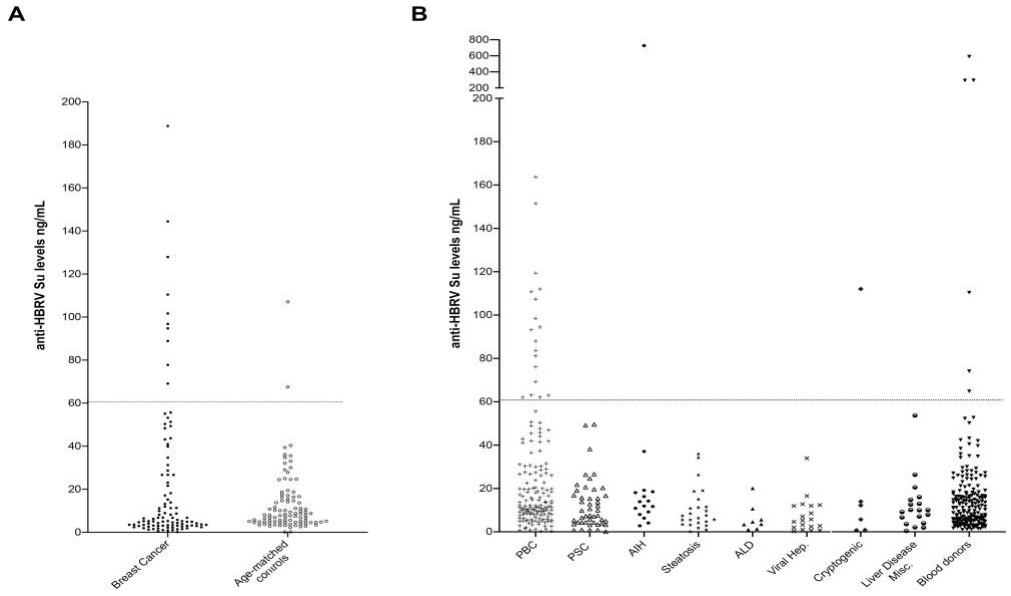

Figure 3: **Seroprevalence studies of HBRV infection using an HBRV Su ELISA.**
(A) A higher percentage of reactivity to HBRV Su was observed in breast cancer patients' sera versus age/sex matched healthy controls (10/98 vs. 2/102; p=0.017). (B) Anti-HBRV reactivity was highest in patients with PBC (18/156) and also found in AIH (1/ 16), cryptogenic liver disease (1/6) and healthy blood donors (6/194); whereas reactivity was not observed in patients with PSC, steatosis (NAFLD), ALD or miscellaneous liver disease, (PBC vs. blood donors 11.5% vs. 3.1%, *p*=0.0024, OR=4.09 [1.66-10.1]).

1.7.2 Cellular immunity

In order to assess the HBRV pro-inflammatory cellular immune responses, our lab adopted a similar technique previously used to characterize immunodominant T cell epitopes for HCV (33, 34). Over-lapping 18mer HBRV peptides were synthesized at Mimotopes (*Mimotopes, Mulgrave Victoria, Australia*), from HBRV Gag (n=58) and Env (n=85) were aggregated into pools and used for stimulation of PBMC. FACS analysis was used to detect intra-cellular production of INF- g and TNF-α (Rahbari, CDDW 2015). Using this assay, 38% of PBC patients had CD8 positive to HBRV Gag pool peptides and 7% to Env pool peptides (Figure 4).

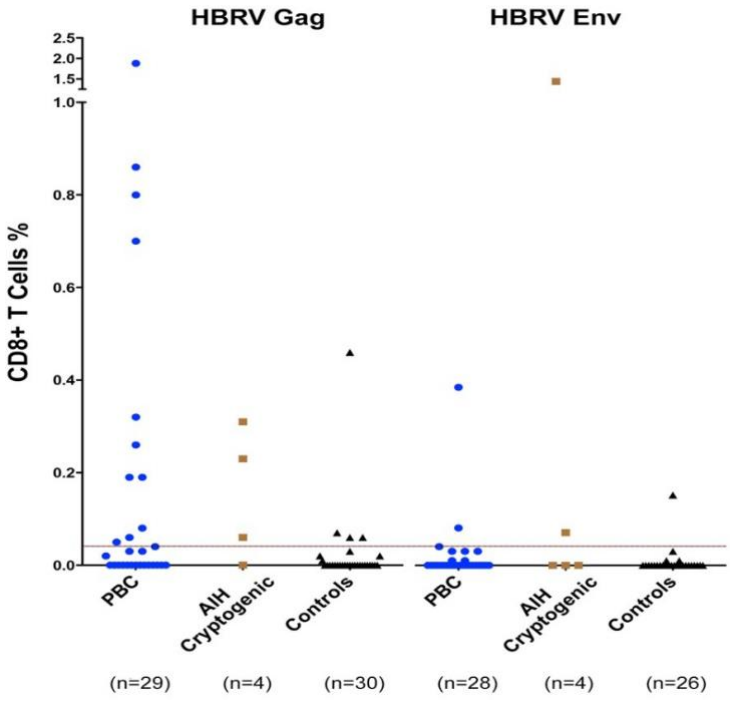

Figure 4: Cellular immune response to HBRV Gag and Env pools with percentage of CD8$^+$ T cells in PBMCs responding to Gag (n=58) and Env (n=85) peptides of HBRV. PBC Patients showed higher response in comparison to other liver diseases and healthy controls

We have already discussed that humoral autoimmunity with AMA production is not necessary to develop PBC (14). Nevertheless, it is commonly thought that PBC has an autoimmune etiology, even though the cellular autoimmune studies do not support this hypothesis either. As discussed, patients with chronic HCV infection have anti-HCV $CD8^+$ T cells in a range of 0.01% to 1.2% of in peripheral blood and 30% higher in the liver (16). Whereas the precursor frequency of PDC-E2 reactive $CD8^+$ T Cells in patients with is in the range of 1 in 10^{-7} to 10^{-8} in peripheral blood and approximately 1 in $1x10^{-5}$ in the liver (15). Could such a low frequency of autoimmune T cells mediate the disease process in PBC?

1.8 Hypothesis

Our current cellular immune data from PBMC stimulated with HBRV peptides shows that the FACS can detect IFN-γ and TNF-α production in 38% of PBC patients (Figure 4). However, none of the PBC patients demonstrated reactivity to PDC-E2 peptides probably due to the inability of the assay to detect such a low precursor frequency (1 in 10^{-7} to 10^{-8} PBMC). From the aforementioned studies, we can hypothesize that (i) study of intrahepatic lymphocytes using a more sensitive assay, such as ELISpot, may provide evidence for HBRV infection in a higher proportion of individuals, if not all patients with PBC, (ii) that the frequency of reactivity will be higher for viral infection versus the autoimmune response and (iii) identification of individual HBRV peptides that stimulates an immune response in PBC patients will enable the production of a cellular immune assay to detect HBRV infection.

1.9 Implications and Importance of investigation

Understanding of the immunity linking HBRV with PBC will provide further insight into disease pathology. The mechanism of developing PBC seems to circle around the role of anti-microbial versus autoimmune pathogenesis of disease and if this question can be address, we can then concentrate on antiviral rather than immune based therapies. Furthermore, if HBRV peptides can be identified that stimulate a cellular immune response, we can construct an assay with superior sensitivity and specificity to detect HBRV infection. Improvement in effectiveness of anti-viral therapies will likely increase the importance of an early diagnosis. Accordingly, a major goal of this research is to identify a sensitive and specific diagnostic tool to diagnose HBRV infection for PBC. Such cytokine release assays to microbial cellular immune responses has proven utility for monitoring and diagnosing disease when other tests are less effective (35, 36).

Chapter 2: Materials and Methods

2.1 Human beta-retrovirus

The beta-retroviruses are simple retroviruses and one of the best-known examples is the mouse mammary tumor virus (MMTV) (37). HBRV was found to share 93% to 98% nucleotides similarity with MMTV. The viral genome is a dimer of linear positive-sense, single stranded RNA (ssRNA). The HBRV genome contains five genes which are group antigens (Gag), protease (Pro), polymerase (Pol), envelop (Env), and superantigen (Sag) (Figure 5) (27). These proteins are collinear with their counterparts in MMTV.

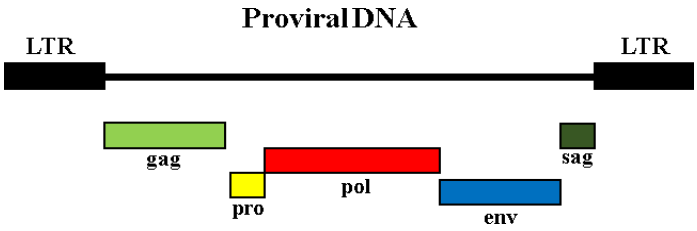

Figure 5: Representation of the human betaretrovirus (HBRV) genome (*adapted from Ovid_fields Virology Ch. 47Retroviridae*).

2.1.1 Gag Peptides

The *gag* gene is located toward the 5-proximal position on all retroviral genomes (37). Gag peptides were synthesized for T cell stimulation studies as crude material on a small 7.8 mg scale by Mimotopes (*Mimotopes, Mulgrave Victoria, Australia*). Peptides were synthesized as overlapping 18- mers which are capable of binding HLA class I alleles. They were synthesized as purified material >95 %. Table 3 shows the number and the sequence of amino acids of each individual Gag peptide.

Table 3: Sequences of amino acids of individual Gag peptides

Peptides number	Gene	Sequences	Peptides number	Gene	Sequences
1/58	Gag	MGVSGSKGQKLFVSVLQRLL	30/58	Gag	KTLKELQLAVKTMGPSAPYT
2/58	Gag	LFVSVLQRLLSERGLHVKES	31/58	Gag	KTMGPSAPYTLQVVDMVASQ
3/58	Gag	SERGLHVKESSAIEFYQFLI	32/58	Gag	LQVVDMVASQWLTPSDWHQT
4/58	Gag	SAIEFYQFLIKVSPWFPEEG	33/58	Gag	WLTPSDWHQTARATLSPGDY
5/58	Gag	KVSPWFPEEGGLNLQDWKRV	34/58	Gag	ARATLSPGDYVLWRTEYEEK
6/58	Gag	GLNLQDWKRVGREMKRYAAE	35/58	Gag	VLWRTEYEEKSKETVQKAAG
7/58	Gag	GREMKRYAAEHGTDSIPKQA	36/58	Gag	SKETVQKAAGKRKGKVSLDM
8/58	Gag	HGTDSIPKQAYPIWLQLREI	37/58	Gag	KRKGKVSLDMLLGTGQFLSP
9/58	Gag	YPIWLQLREILTEQSDLVLL	38/58	Gag	LLGTGQFLSPSSQIKLSKDV
10/58	Gag	LTEQSDLVLLSAEAKSVTEE	39/58	Gag	SSQIKLSKDVLKDVTTNAVL
11/58	Gag	SAEAKSVTEEELEEGLTGLL	40/58	Gag	LKDVTTNAVLAWRAIPPPGV
12/58	Gag	ELEEGLTGLLSTSSQEKTYG	41/58	Gag	AWRAIPPPGVKKTVLAGLKQ
13/58	Gag	STSSQEKTYGTRGTAYAEID	42/58	Gag	KKTVLAGLKQGNEESYETFI
14/58	Gag	TRGTAYAEIDTEVDKLSEHI	43/58	Gag	GNEESYETFISRLEEAVYRM
15/58	Gag	TEVDKLSEHIYDEPYEEKEK	44/58	Gag	SRLEEAVYRMMPRGEGSDIL
16/58	Gag	YDEPYEEKEKADKNEEKDHV	45/58	Gag	MPRGEGSDILIKQLAWENAN
17/58	Gag	ADKNEEKDHVRKVKKVVQRK	46/58	Gag	IKQLAWENANSLCQDLIRPI
18/58	Gag	RKVKKVVQRKEISEGKRKEK	47/58	Gag	SLCQDLIRPIRKTGTIQDYI
19/58	Gag	EISEGKRKEKDQKAFLATDW	48/58	Gag	RKTGTIQDYIRACLDASPAV
20/58	Gag	DQKAFLATDWNDDDLSPEDW	49/58	Gag	RACLDASPAVVQGMAYAAAM
21/58	Gag	NDDDLSPEDWDDLEEQAAHY	50/58	Gag	VQGMAYAAAMRGQKYSTLVK
22/58	Gag	DDLEEQAAHYHDDDELILPV	51/58	Gag	RGQKYSTLVKQTYGGGKGGQ
23/58	Gag	HDDDELILPVKRKVVKKKPQ	52/58	Gag	QTYGGGKGGQGSEGPVCFSC
24/58	Gag	KRKVVKKKPQALRRKPLPPV	53/58	Gag	GSEGPVCFSCGKTGHIKKDC
25/58	Gag	ALRRKPLPPVGFAGAMAEAR	54/58	Gag	GKTGHIKKDCKEEKGSKRAP
26/58	Gag	GFAGAMAEAREKGDLTFTFP	55/58	Gag	KEEKGSKRAPSGLCPRCKKG
27/58	Gag	EKGDLTFTFPVVFMGESDDD	56/58	Gag	SGLCPRCKKGYHWKSECKSK
28/58	Gag	VVFMGESDDDDTPVWEPLPL	57/58	Gag	YHWKSECKSKFDKDGNPLPP
29/58	Gag	DTPVWEPLPLKTLKELQLAV	58/58	Gag	DKDGNPLPPLETNTENSKNL

2.1.2 Env Peptides

The *Env* gene is expressed from a subgenomic mRNA in all retroviruses (37). The peptides derived for Env proteins of 18 overlapping amino acid length were produced from Mimotopes Company (*Mimotopes, Mulgrave Victoria, Australia*). All Env peptides used in this study had more than 90% purity. Table 4 shows the number and sequence of each Env peptide.

Table 4: Sequences of amino acids of individual Env peptides.

Peptides number	Gene	Sequences	Peptides number	Gene	Sequences
1/ 85	Env	MPNHQSGSPTGSSDLLLS	36/85	Env	SVDQSDQIKSKKDLFGNY
2/ 85	Env	PTGSSDLLLSGKKQRPHL	37/85	Env	KSKKDLFGNYTPPVNKEV
3/ 85	Env	LSGKKQRPHLALRRKRRR	38/85	Env	NYTPPVNKEVHRWYEAGW
4/85	Env	HLALRRKRRREMRKINRK	39/85	Env	EVHRWYEAGWVEPTWFWE
5/85	Env	RREMRKINRKVRRMNLAP	40/85	Env	GWVEPTWFWENSPKDPND
6/85	Env	RKVRRMNLAPIKEKTAWQ	41/85	Env	WENSPKDPNDRDFTALVP
7/85	Env	APIKEKTAWQHLQALIFE	42/85	Env	NDRDFTALVPHTELFRLV
8/85	Env	WQHLQALIFEAEEVLKTS	43/85	Env	VPHTELFRLVAASRYLIL
9/85	Env	FEAEEVLKTSQTPQTSLT	44/85	Env	LVAASRYLILKRPGFQEH
10/85	Env	TSQTPQTSLTLFLTLLSV	45/85	Env	ILKRPGFQEHDMIPTSAC
11/85	Env	LTLFLTLLSVLGPPPVTG	46/85	Env	EHDMIPTSACATYPYAIL
12/85	Env	SVLGPPPVTGESYWAYLP	47/85	Env	ACATYPYAILLGLPQLID
13/85	Env	TGESYWAYLPKPSILHPV	48/85	Env	ILLGLPQLIDIEKRGSTF
14/85	Env	LPKPSILHPVGWGNTDPI	49/85	Env	IDIEKRGSTFHISCSSCR
15/85	Env	PVGWGNTDPIRVLTNQTI	50/85	Env	TFHISCSSCRLTNCLDSS
16/85	Env	PIRVLTNQTIYLGGSPDF	51/85	Env	CRLTNCLDSSAYDYAAII
17/85	Env	TIYLGGSPDFHGFRNMSG	52/85	Env	SSAYDYAAIIVKRPPYVL
18/85	Env	DFHGFRNMSGNVHFEGKS	53/85	Env	IIVKRPPYVLLPVDIGDE
19/85	Env	SGNVHFEGKSDTLPICFS	54/85	Env	VLLPVDIGDEPWFDDSAI
20/85	Env	KSDTLPICFSLSFSTPTG	55/85	Env	DEPWFDDSAILTFRYATD
21/85	Env	FSLSFSTPTGCFQVDKQV	56/85	Env	AILTFRYATDLIRAKRFV
22/85	Env	TGCFQVDKQVFLSDTPTV	57/85	Env	TDLIRAKRFVAAIILGIS
23/85	Env	QVFLSDTPTVDNNKPGGK	58/85	Env	FVAAIILGISALIAIITS
24/85	Env	TVDNNKPGGKGDKRRMWE	59/85	Env	ISALIAIITSFAVATTAL
25/85	Env	GKGDKRRMWELWLTTLGN	60/85	Env	TSFAVATTALVKEMQTAT
26/85	Env	WELWLTTLGNSGANTKLV	61/85	Env	ALVKEMQTATFVNNLHRN
27/85	Env	GNSGANTKLVPIKKKLPP	62/85	Env	ATFVNNLHRNVTLALSEQ
28/85	Env	LVPIKKKLPPKYPHCQIA	63/85	Env	RNVTLALSEQRIIDLKLE
29/85	Env	PPKYPHCQIAFKKDAFWE	64/85	Env	EQRIIDLKLEARLNALEG
30/85	Env	IAFKKDAFWEGDESAPPR	65/85	Env	LEARLNALEGVVLELGQD
31/85	Env	WEGDESAPPRWLPCAFPD	66/85	Env	EGVVLELGQDEANLKTRM
32/85	Env	PRWLPCAFPDQGVSFSPK	67/85	Env	QDEANLKTRMSTRCHANY
33/85	Env	PDQGVSFSPKGTLGLLWD	68/85	Env	RMSTRCHANYDFICVTPL
34/85	Env	PKGTLGLLWDFSLPSPSV	69/85	Env	NYDFICVTPLPYNASESW
35/85	Env	WDFSLPSPSVDQSDQIKS	70/85	Env	PLPYNASESWERTKAHLL
71/85	Env	SWERTKAHLLGIWNDNEI	79/85	Env	FIFIGVGALLLVIVLMIF
72/85	Env	LLGIWNDNEISYNIQELA	80/85	Env	LLLVIVLMIFPIVFQCLA
73/85	Env	EISYNIQELANLISDMSK	81/85	Env	IFPIVFQCLAKSLDQVQS
74/85	Env	LANLISDMSKQHIDTVDL	82/85	Env	LAKSLDQVQSDLNVLLLK
75/85	Env	SKQHIDTVDLSGLAQSFA	83/85	Env	QSDLNVLLLKKKKGGNAA
76/85	Env	DLSGLAQSFANGVKALNP	84/85	Env	LKKKKGGNAAPAAEMVEL
77/85	Env	FANGVKALNPLDWTQYFI	85/85	Env	GNAAPAAEMVELPRVSYT
78/85	Env	NPLDWTQYFIFIGVGALL			

The HBRV Gag and Env peptides libraries from Mimotopes (*Mimotopes, Mulgrave Victoria, Australia*) were supplied in cleaved format as a powder. To obtain peptides in solution, they were dissolved to obtain a homogeneous solution, aliquoted and stored. For dissolving peptides, we reconstituted Gag and Env peptides in 0.1% acetic acid/water to give a target peptide concentration of 1mg/ml. For any insoluble peptides, we added pure acetic acid to bring the concentration of acetic acid to 10% and sonicated the undissolved peptides. After dissolving all Gag and Env peptides, we made all our peptide stock solution for individual peptides as well as pools at concentration of 100µg per tubes. Then, we lyophilized samples using a Lobcano™ lyophilizer to remove the acetic acid and water. Finally, all tubes were stored in -20 °C freezer.

2.2 Intrahepatic Lymphocytes

The liver is a unique organ. Every minute, around 30% of the total blood passes through the liver, carrying about 10^8 peripheralblood lymphocytes in 24 hours (38, 39). The liver's structural organization has profound implication for its immune function. The pathogenesis of liver disease is commonly considered to be associated with the presence of lymphocytes. Moreover, hepatic infiltration by lymphocytes is well described and documented in inflammatory conditions such as autoimmune and viral liver disease (40). The intrahepatic lymphocytes are a more complicated mixture, containing T cells (TCR$\alpha\beta^+$ cells and TCR$\gamma\delta$ cells), natural killer cells (NK), and dendritic cells (Figure 6). The ratio of CD4/CD8 in the liver is the opposite than that of the peripheral blood and lymph nodes; there are more $CD8^+$ cells in the liver (CD4:CD8 ratio in the liver of 1:3.5 as compared to 2:1 in peripheral blood)(41). This mix of intrahepatic

lymphocyte population produces elevated amounts of several cytokines such as IFN-*g*, TNF-*a*, IL-15, and IL-10 in comparison with peripheral blood mononuclear cells (42).

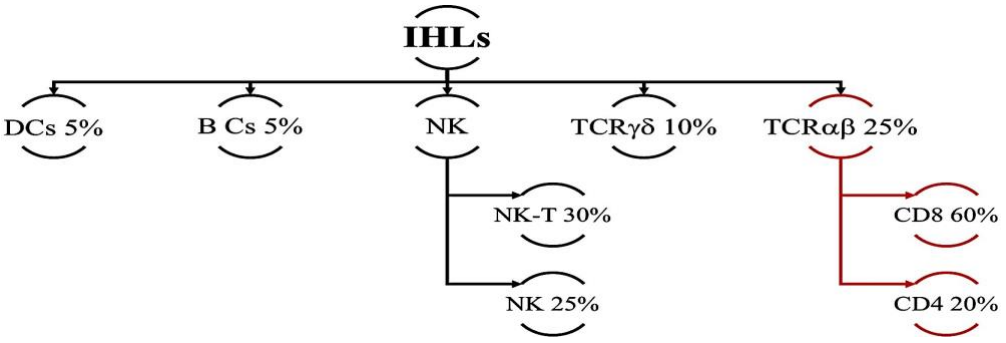

Figure 6: Approximate composition of intrahepatic lymphocytes subsets in humans (*Adapted from Mehal, et al., 2001***)**

2.2.1 Flushing Intra-Hepatic Lymphocytes(IHLS) from hepatectomy specimens

After obtaining informed consent, we collected intrahepatic lymphocytes from diseased organs explanted during liver transplantation. For this study, we flushed 17 recipient livers; 8 from PBC patients and 9 from other liver diseases, carried out under complete aseptic conditions in the operating room. The recipient liver was cannulated via the portal veins by Foley catheter which was kept in place by the inflated balloon (Figure7, A). The liver is flushed by one liter of normal saline in order to remove the RBCs and PBMCs. Then, we collected the second flushing liter from hepatic veins in a sterile bottle. This kind of technique for isolation of intrahepatic lymphocytes is simpler and more convenient compared with enzymatic digestion methods.

2.2.2 IHL isolation

Using a protocol modified from established methods, the one liter of perfusate was distributed into 20 x 50 ml Falcon tubes (*ThermoFisher Scientific, Waltham, MA, USA*) and centrifuged at (1800 RPM for 7 minutes 25 °C) to pellet the cells and achieve a clear supernatant. Then, the supernatants were aspirated by a sterile glass pipette, and the pellets were resuspended in one ml of phosphate buffered saline (PBS, Gibco, Waltham, MA, USA) to each of the 50 ml Falcon tubes (*ThermoFisher Scientific, Waltham, MA, USA*). Tubes were suspended slowly to transfer the cells to two 50 ml Falcon tubes (*ThermoFisher Scientific, Waltham, MA, USA*), and then 20 ml of the lymphoprep buffer (*Stemcell, Vancouver, BC, CA*) was added to each 50 ml Falcon tube (*ThermoFisher Scientific, Waltham, MA, USA*) and added gently to 30 ml of cells solution. After layering, the tubes were centrifuged at (2100 RPM for 21 minutes 25 °C), without the deceleration. After centrifugation, the buffy coat layer was visualized at the interface between the original PBC layer and lymphoprep layer. The buffy coat layer was placed in 50 ml Falcon tube (*ThermoFisher Scientific, Waltham, MA, USA*) with PBS (*Gibco, Waltham, MA, USA*) to top up the tubes to 50 ml. The tube was then centrifuged at (1700RPM, 7min., 25°C) (Figure7, B). The cell pellet was resuspended in 50 ml PBS (*Gibco, Waltham, MA, USA*). 20 µl of solution was transferred into 1.5 ml Eppendorf tube and mixed with Trypan Blue to count live cells. Then, cells were stored at a concentration of 10 million cells per 1 ml in a cryopreservation solution with -50% FBS (HI-FBS, *Gibco, Waltham, MA, USA*), 40% RPMI, (*Gibco, Waltham, MA, USA*), and 10%DMSO (*Sigma-Alorich, Oakville, ON, Canada*) - and then stored in 1 ml cryotubes and preserved in a liquid nitrogen tank.

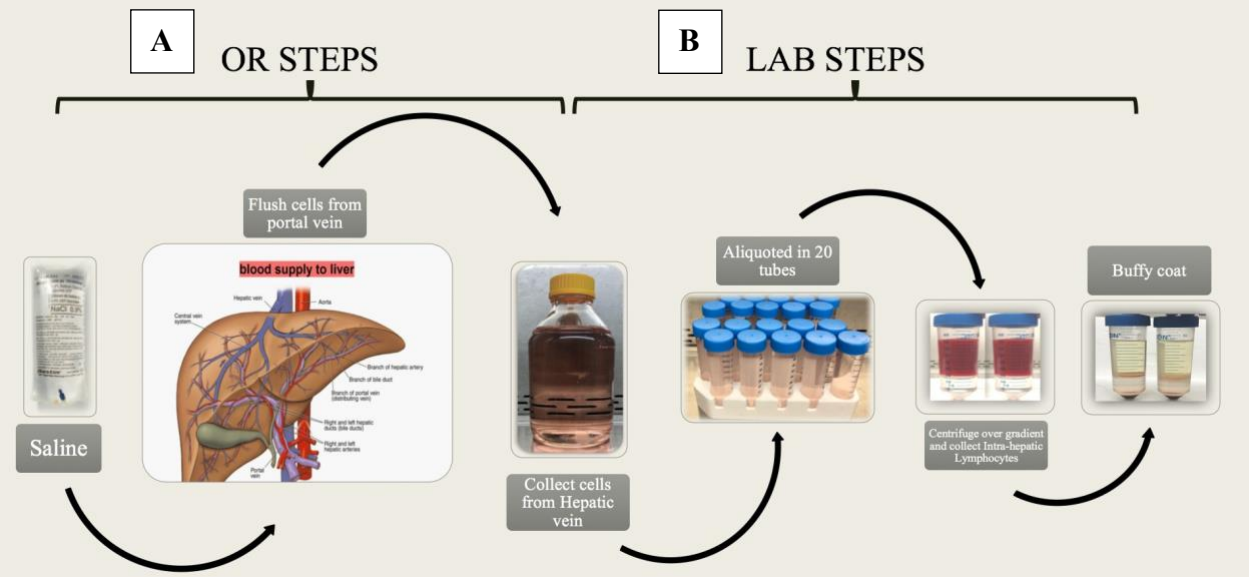

Figure 7: Illustrates Intrahepatic Lymphocytes extraction (A) At operation room: Perfusion of recipient liver with Foley catheter- inserted into the portal vein. (B) At Lab.: one litre perfusate is divided into 20 (50ml) tubes. 50 ml tubes were centrifuged and buffy coat layers (lymphocytes) were collected.

2.3 Peripheral Blood Monocytes

2.3.1 Blood Drive

Venous blood was collected in heparin containing tubes (*Greiner bio-one, Monroe, NC, USA*) from liver disease patients at the Zediler clinic and healthy controls after signing informed consent.

2.3.2 PBMCs isolation

Blood samples were collected in heparinized tubes (*Greiner bio-one, Monroe, NC, USA*) and processed within 1-4 hours according to established modified protocol. For lymphocyte extraction. SepMate column (*Stemcell, Vancouver, BC, CA*) was filled with 15 ml of lymphoprep density gradient (*Stemcell, Vancouver, BC, CA*). Then, 15 ml of blood was diluted with 15 ml of sterile PBS (*Gibco, Waltham, MA, USA*) in 50 ml Falcon tube (*ThermoFisher Scientific,*

Waltham, MA, USA). The 30 ml of blood and sterile PBS (*Gibco, Waltham, MA, USA*) was added slowly to the SepMate tube (*Stemcell, Vancouver, BC, CA*) containing lymphoprep buffer (*Stemcell, Vancouver, BC, CA*). All SepMate tubes (*Stemcell, Vancouver, BC, CA*) were centrifuged for 20 min. at 1300 x g in 25°C. We aspirated the buffy coat in the 1ml (PBMCs) formed in the interface between RBCs at the bottom and plasma layer above. The PBMCs were pipetted into new Falcon tube (*ThermoFisher Scientific, Waltham, MA, USA*) and topped up to 45 ml with sterile PBS (*Gibco, Waltham, MA, USA*). The tubes were centrifuged at 500xg for 15 min., 25°C. The supernatant was removed until 5 ml of fluid remained. We resuspended the PBMCs in the remaining 5 ml of sterile PBS (*Gibco, Waltham, MA, USA*) and added 25ml of sterile PBS (*Gibco, Waltham, MA, USA*) to obtain a volume of 25 ml. 20 µl of PBMCs were taken and added to 20 µl trypan blue for counting the cells. We centrifuged the tube as the before and resuspended the pellets with storage medium (50% FBS (HI-FBS, *Gibco, Waltham, MA, USA*), 40%RPMI (*Gibco, Waltham, MA, USA*), and 10%DMSO (*Sigma-Alorich, Oakville, ON, Canada*). We preserved the PBMCs in the liquid nitrogen tank.

2.4 ELISpot assay

2.4.1 The Enzyme-linked Immuno-Spot (ELISpot) assay

ELISpot or enzyme linked immunospot, is a method that was developed for the detection of secreted proteins same as cytokines and growth factors in single cells. This technique is a highly sensitive immunoassay that detects the frequency of cytokine-secreting cells at the single cell level. It provides both qualitative (type of immune protein) and quantitative (number of responding cells) information. The assay is considered as one of most sensitive cellular assays available, with a detection limit of 1 in 100,000 cells. Furthermore, the assay is useful for studies of a small number of cells which are found in specific immune responses (MABTECH Company).

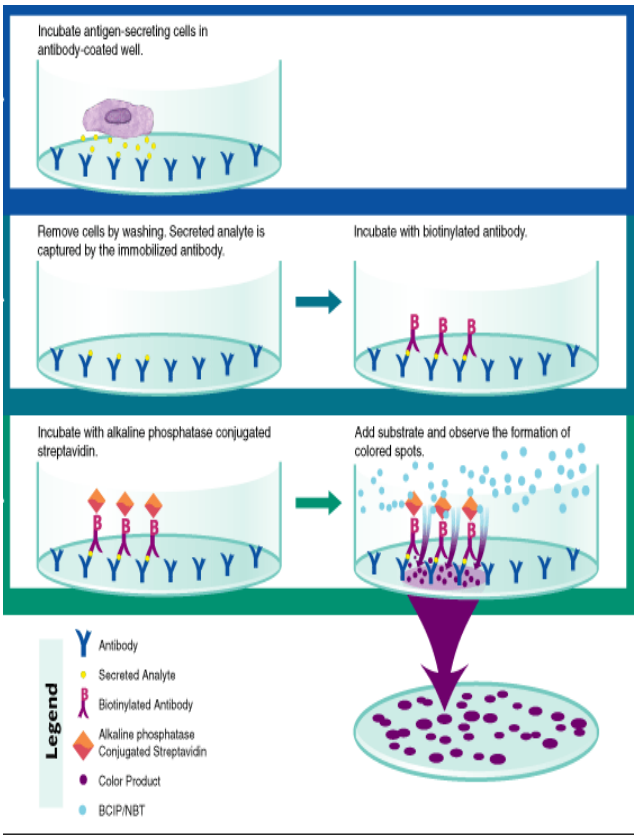

Figure 8: the ELISpot Assay workflow

2.4.2 The principle of the ELISpot Assay

Elispot assays work through the sandwich enzyme-linked immunosorbent assay (ELISA) technique. It works through either monoclonal or polyclonal antibodies, pre-coated onto a polyvinylidene difluoride (PVDF) - backed microplate. The stimulated cells are pipetted into the wells and the plate is incubated into a humidified 37°C, 5% CO2 for 12-24 hours. During incubation, the immobilized antibody binds to the secreted proteins. After incubation, the wells are washed to remove any cells or unbound substances, then a biotinylated polyclonal antibody specific for the chosen analyte is added to the wells. The wells are the washed, and alkaline-phosphatase conjugated to streptavidin is added. Subsequently, washes are performed to remove any unbound enzymes. The plate is allowed to dry. A blue-black precipitate appears as spots at the sites of cytokine localization. Each individual spot represents an individual secreting cell. Spots are then counted using an automated ELISpot plate reader (R&D systems a biotechne brand) (Figure 8).

2.4.3 Design of the experiments

Intrahepatic lymphocytes from PBC patients (n=8) and other liver diseases (n=9) were seeded at concentration of 100,000 cells per well (table 5). PBC IHLs were stimulated by 58 individual HBRV Gag and 85 individual HBRV Env peptides for 24 hours, and were also stimulated by PDC-E2 pool peptides, PMA+ Ionomycin as a positive control, and NIL as a negative control. The interferon-gamma responses were measured by ELISpot assay.

Table 5: IHLs diagnosis used for Elispot assay

Patient Number	Diagnosis	Patient Number	Diagnosis
IHL 432	Primary Biliary Cholangitis	IHL 430	Nonalcoholic Steatohepatitis
IHL 434	Primary Biliary Cholangitis	IHL 431	Primary Sclerosing Cholangitis
IHL 438	Primary Biliary Cholangitis	IHL 433	Alcoholic Liver Disease
IHL 440	Primary Biliary Cholangitis	IHL 436	Primary Sclerosing Cholangitis
IHL 443	Primary Biliary Cholangitis	IHL 445	Nonalcoholic Steatohepatitis
IHL 450	Primary Biliary Cholangitis	IHL 447	Primary Sclerosing Cholangitis
IHL 451	Primary Biliary Cholangitis	IHL 449	Primary Sclerosing Cholangitis
IHL 461	Primary Biliary Cholangitis	IHL 453	Alcoholic Liver Disease
		IHL 455	Nonalcoholic Steatohepatitis

2.4.4 ELISpot Protocol

To determine whether HBRV Gag or Env peptides were recognized by IHL cells from PBC and non-PBC patients, we assayed responses by using ELISpot assay. We used PVDF-plates, type MSIP from MultiScreen company (*Millipore, Etobicoke, ON, Canada*). First of all, the coating antibody (1-D1K) was diluted to 15µg/ml in sterile PBS PH 7.4 (*Gibco, Waltham, MA, USA*). The plate was treated with 15 µl 35% ethanol per well for up to 1 min., then washed 5 times with 200µl sterile water per well. After that 100µl of coating antibody solution was added per well and incubated overnight at 4◦C.

Subsequently, excess antibody solution was removed and the plate was washed 5 times with 200µl sterile PBS (*Gibco, Waltham, MA, USA*) per well. We then added 200 µl per well of medium containing 10% FBS (HI-FBS, *Gibco, Waltham, MA, USA*), antibacterial/antimycotic (*1000 units penicillin/10mg streptomycin/25ug amphotericin/ml*) for 30 min. at room temperature. 100,000 IHLs were resuspended in 200µl media and added to each well of the 96 well plate. Then 2µl (2µg/ml) of individual peptides 85-Env and 58-Gag were added to each well. 2µl (2µg/ml) of PDC-E2 was added to 100,000 cells resuspended in 200µl media in separate wells. For controls of experiments, 100,000 cells were added to 200µl media + 2µl (30% DMSO and 70% PBS) (*Sigma-Alorich, Oakville, ON, Canada*) as negative control. For positive control, 2µl (5ng/ml) PMA (*Sigma-Alorich, Oakville, ON, Canada*) + 1µl (1µg/ml) Ionomycin (*Sigma-Alorich, Oakville, ON, Canada*) were added to 100,000 cells/200µl media. Each plate was incubated at 37◦C in a humidified incubator with 5% CO2 for 24 hours. All the steps were performed in sterile conditions.

After the 24 hours of incubation, we removed the cells from the plates and washed them 5 times with 200 µl sterile PBS per well. The detection antibody was diluted in PBS containing 0.5% fetal calf serum (PBS-0.5%FCS) (PBS, *Gibco, Waltham, MA, USA*) at 1µg/ml concentration. Then, 100µl of detection antibody solution was added per well and incubated for 2 hours at room temperature. The plates were washed 5 times with sterile PBS 200µl/well. Then streptavidin-ALP was diluted (1:1000) in PBS-0.5% FCS and added 100µl per well. We incubated the plates for 1 hour at room temperature. The plates were washed as described previously and the 100µl of substrate solution (BCIP/NBT) was added per well. After adding the substrate, we carefully monitored for spot formation. Then, we stopped the reaction before over saturation by color development by gently washing the plate with tap water. We allowed the membranes to dry at room temperature and inspected and counted spots in an ELISpot reader the following day.

Chapter 3: Results

Section 1:

3.1 Screening of PBC IHL by individual Gag and Env peptides vs PDC-E2 pool peptides

3.1.1 Mapping analysis of individual HBRV Gag peptides in IHLs from PBC patients

3.1.2 Mapping analysis of individual HBRV Env peptides in IHLs from PBC patients

Section 2:

3.2 Screening of positive Gag and Env peptides in non-PBC (Other liver diseases) IHLs

Section 3:

3.3 Magnitude of response to HBRV Gag and Env peptides in PBC patients versus non-PBC liver disease controls

3.4 Comparison of T cell response to HBRV pool and PDC-E2 pool in PBC patients

Section 1:

3.1 Screening of PBC IHL by individual Gag and ENV peptides vs PDC-E2 pool peptides

3.1.1 Mapping analysis of individual HBRV Gag peptides using IHL from PBC patients

To determine whether the HBRV Gag peptides were recognized by T cells, we assayed responses to 58 individual HBRV Gag peptides (Table 3). IHL (100,000) from 8 patients with PBC were stimulated for 24 hours and the IFN-γ response was measured by the ELISpot assay, enabling quantification of responsive cells. We observed that the majority of HBRV Gag peptides did not respond to IHL. Notably, 11 HBRV Gag peptides stimulated more than 100 IHL cells to produce IFN-γ, and 20 HBRV Gag peptides produced > 50 spot forming cells. However, only one PBC patient out of 8 responded to PDC-E2 pool peptides and formed just 65 Spot forming cells (Figure 9). In this study, our target was to identify HBRV Gag peptides that have high sensitivity to IHLs from PBC patients and compare the result of HBRV Gag peptides to PDC-E2 pool peptides. 20 of these peptides were selected for further analysis.

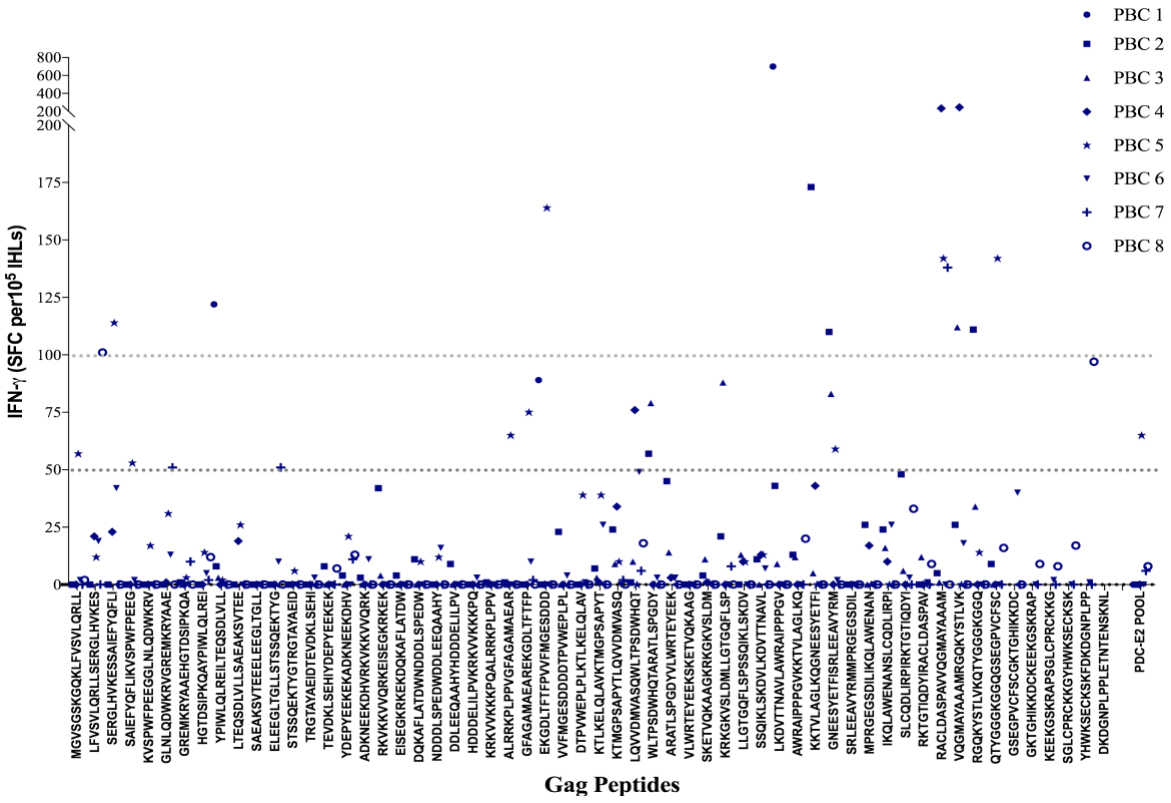

Figure 9: Mapping analysis of 58 Gag peptides of HBRV and PDC-E2 peptides. Stimulations of IHLs were performed using 18-merpeptides for 24 hours to 8 different PBC patients. 100,000 IHLs were stimulated/ sample. ElISpot for detecting INF gamma responses were performed. All 8 samples were found to be highly sensitive to 20 Gag peptides. All 20 Gag peptides formed >50 SFC.

3.1.2 Mapping analysis of individual HBRV Env peptides using IHL from PBC patients

In order to detect which individual HBRV Env peptides were recognized by PBC patients, we preformed stimulation studies using a panel of 85 overlapping 18-mer peptides (Table 4) using 8 PBC patients. IHL (n=100,000) were stimulated by individual Env peptides for 24 hours, and the IFN-γ production from the cells were measured by ELISpot assay. Most of the HBRV Env peptides did not react with IHL from PBC patients. However, 12 HBRV Env peptides stimulated more than 100 IHL cells to produce IFN-γ, and 27 HBRV Env peptides produced > 50 spot forming cells (Figure 10). We found that two different patients sharing the same HLA reacted with the same Env peptide # 49 (IDIEKRGSTFHISCSSCR).

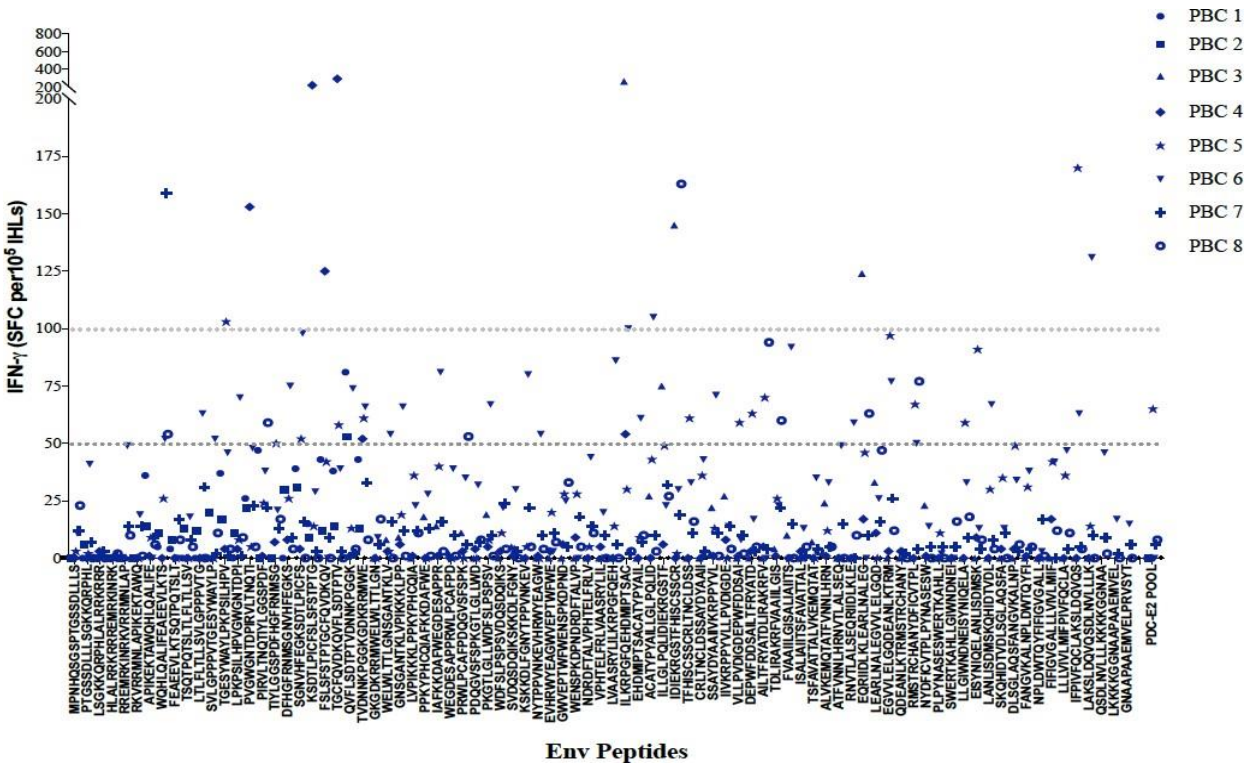

Figure 10: Mapping analysis of 85 Env peptides of HBRV and PDC-E2 peptides. Stimulations of IHLs were done using 18-merpeptides for 24 hours to 8 different PBC patients. 100,000 IHLs were stimulated/ sample. Elispot for detecting INF gamma responses were performed. All 8 samples were found to be highly sensitive to 27 Env peptides. All 27 Env peptides formed > 50 SFC.

Taken together, our data shows that 20 of 58 HBRV Gag and 27 out of 85 HBRV Env peptides stimulated IFN-γ cellular immune response in PBC patients' IHL to produce > 50 SFC. We originally decided to use 50 SFC as our cut off in order to screen smaller proportion of individual Gag and Env with IHLs from other liver disease to detect the specificity of reactivity with IFN-γ production for each of the peptides peptides. Using these criteria, all PBC patients demonstrated cellular immunity to react to HBRV, while just one patient reacted to PDC-E2 pool. The 47 individual Gag and Env HBRV peptides were then selected for further analysis.

Section 2:

3.2 Screening of Positive Gag and Env peptides by NON-PBC (Other liver diseases) IHLs

Intrahepatic lymphocytes (IHLs) were collected from non-PBC recipient livers (n=9). These IHLs were stimulated by 47 individual HBRV Gag and Env peptides for 24 hours, using PMA and ionomycin as positive control and no stimulant as the negative control. The peptide specificity was assessed by IFN-γ ELISpot technique using these negative controls. The majority of peptides demonstrated minimal reactivity to the IHLs from non-PBC patients. Just four HBRV Gag peptides stimulated the IHLs and formed more than 50 spot forming cells. Four of the HBRV Env peptides were produced more than 50 spot forming cells (Figure 11).
Using a cut-off of 100 SFC per peptide which is (mean+2 standard deviations), collectively the ENV peptides provided 100% sensitivity and specificity for detection of cellular immune response to HBRV. Not surprisingly, one of Gag peptides reacts with Non-PBC IHLs, which is Gag number 50 (VQGMAYAAAMRGQKYSTLVK) because this epitope may be shared with other viral agents (Figure 12). The identification of 11 and 12 HBRV Gag and Env peptides respectively that stimulate PBC IHL with high sensitivity and specificity is an important finding, because these finding will provide better understanding of the immunology of PBC disease.

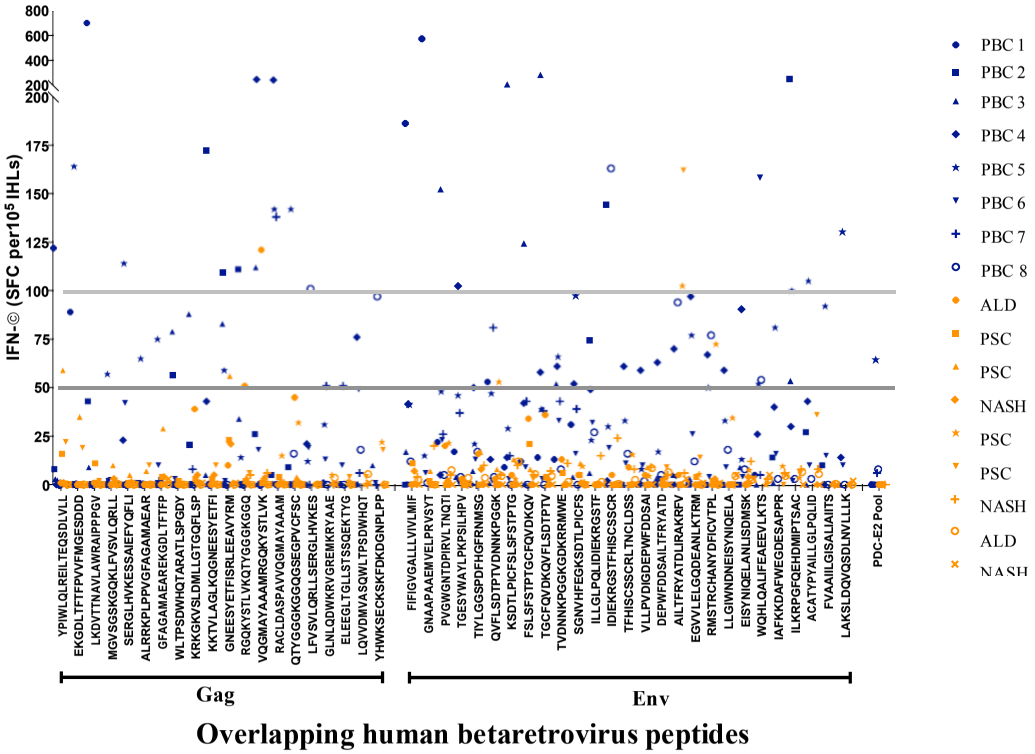

Figure 11: Mapping analysis of 47 HBRV Gag and Env peptides. Using the > 50 SFC cut-off, we identified 47 HBRV peptides stimulating IFN-γ secretion in 8 PBC patients. The IHL from the 9 non-PBC controls with other end stage liver disease demonstrated reactivity to 4 Gag and 4 Env HBRV peptides. One PBC sample showed response to PDC-E2 pool peptides.

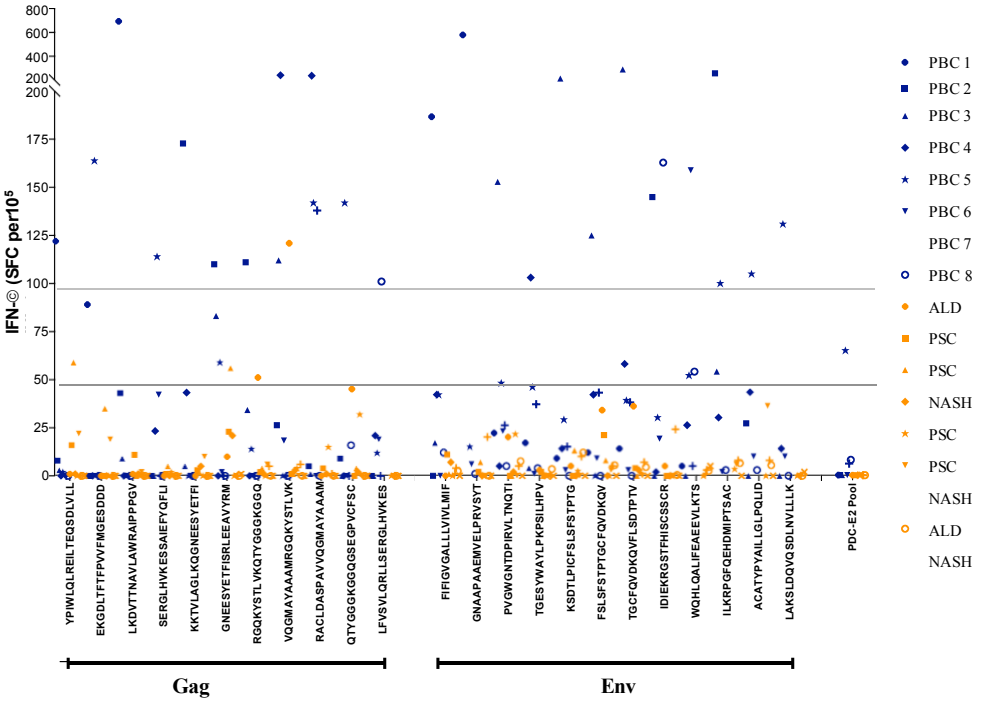

Figure 12: Mapping analysis of 23 HBRV Gag and Env peptides. Using a cut-off > 100 SFC, we identified 23 HBRV peptides that stimulated IFN-γ secretion in 8 PBC patients. Using the IHL from the 9 non-PBC liver disease controls, we observed that only one patient was reactive to Gag peptide # 50. demonstrating 100% sensitivity and 89% specificity for reactivity to any HBRV peptide with the cut off > 100 SFC.

Section 3:

3.3 Magnitude of response to HBRV Gag and Env peptides in PBC versus non-PBC liver disease controls

In this section, we show the cumulative IHL response to Gag and Env peptides. We combined the total SFCs formed per patient in response to 20 unique Gag peptides and 27 unique Env peptides. Samples were taken from patients with PBC and other liver disease.

We were able to demonstrate the range of frequency of IHL response in PBC versus controls to identified peptides (Gag and Env) from HBRV. Additionally, we found an improved response of IHLs from PBC patients to HBRV pool compared to PDC-E2 pool.

We analyzed the total Gag peptide SFC responses for each patient together in order to compare the IFN-γ responses for all the patients. We observed that the number of cells producing IFN-γ was increased in IHL from PBC patients compared to liver disease controls, with a mean frequency of 455 versus 90 SFC/100,000 (P= 0.0012) in PBC versus control patients (Figure 13).

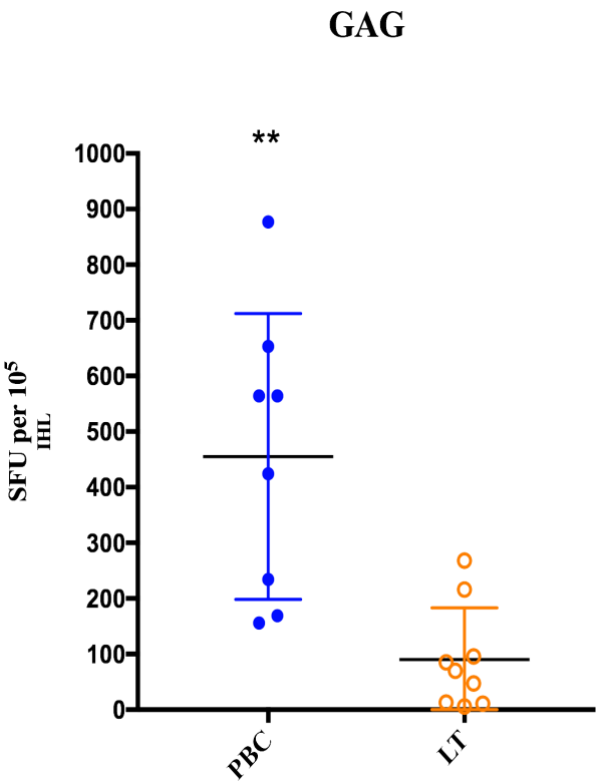

Figure 13: Comparison of IFN-γ production (SFC/100,000 IHLs) in response to HBRV Gag peptides stimulation among patients with PBC (n=8) or without PBC (n=9). Each dot indicates the total number of the Gag peptides spot forming cells from each patient, the mean number of spots in PBC patients (455 SFC) is higher almost 4-fold comparing to control (90 SFC). With a p value of = 0.0012.

In order to analyze responses to the Env peptides, the SFC responses for each patient were combined. We observed that the number of cells producing IFN-γ with Env peptide stimulation

was increased in IHL from PBC patients compared to liver disease controls, with a mean frequency of 785 versus 135 SFC/100,000 (P= 0.0002) in PBC versus control patients (Figure 14).

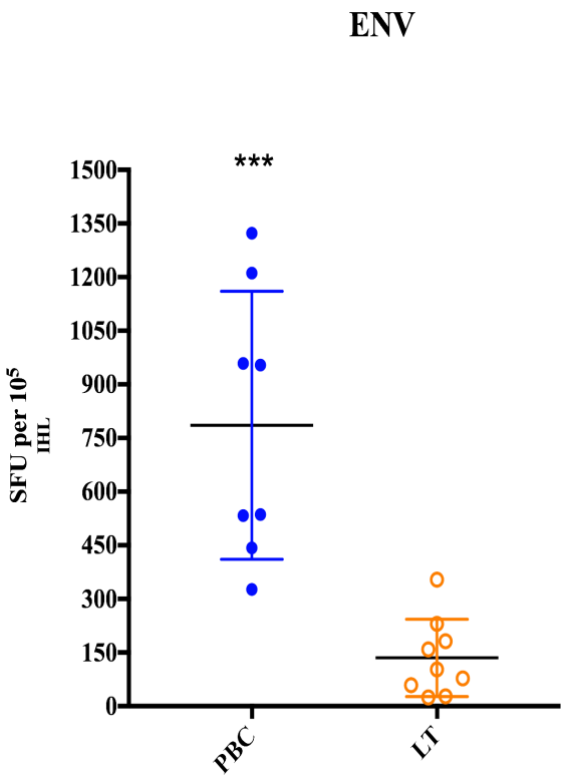

Figure 14: Evaluation of IFN-γ expression levels (SFU/100,000 IHL) among the patients with PBC (n=8) or without PBC (n=9) to stimulation by HBRV Env peptides. Representation of the mean of IFN-γ expression levels in the IHLs from PBC patients is 5-fold higher when compared with IHL from non PBC patients (p value of 0.0002).

SFC responses from all the 8 PBC patients and 9 liver disease controls were then evaluated for precursor frequencies to the HBRV Gag and Env peptides. The mean for the precursor for the PBC versus control liver diseases was 1240 vs. 225 SFC/100,000 (P= 0.0002) as shown in (Figure 15).

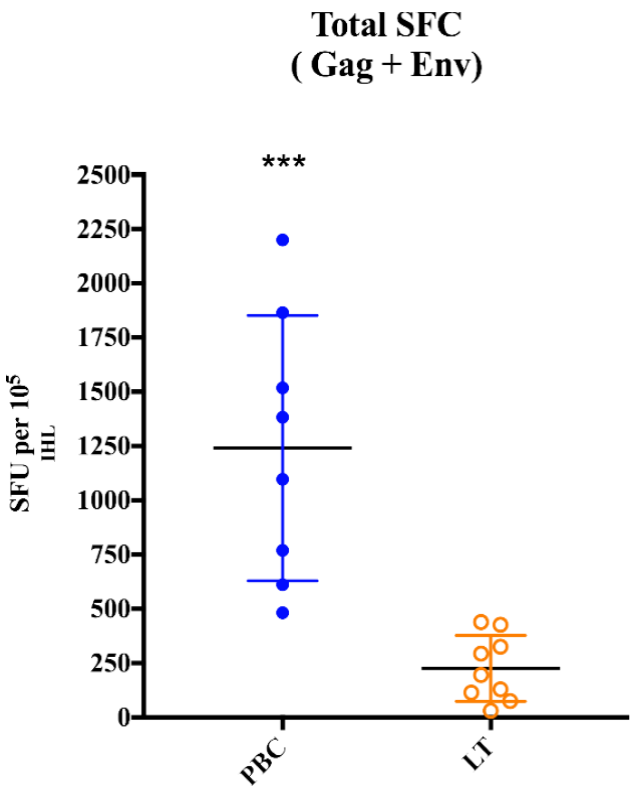

Figure 15: Total SFC response to HBRV (Gag and Env peptides) by stimulation of IHL from PBC patients (n=8) and control (n=9). Total SFC responses were assessed by ELISpot. Our data shows a significant increase in production of SFC in IHLs from PBC patients when compared to control (P value = 0.0002)

3.4 Comparison of T cell response to HBRV pool and PDC-E2 pool in PBC patients

The total number of HBRV specific T cells producing IFN-γ after stimulation of IHL with HBRV Gag and Env peptides was markedly higher than the mean production of SFC using the PDC-E2 peptide pool. Based on the response profiles of SFC, we found a marked difference when comparing the mean SFC of HBRV and PDC-E2 pool, 1240 to 20 SFC/100,000 IHL respectively (p value < 0.0001, Figure 16). Accordingly, we showed that the cellular immune response to the HBRV exceeds the autoimmune response in PBC patients.

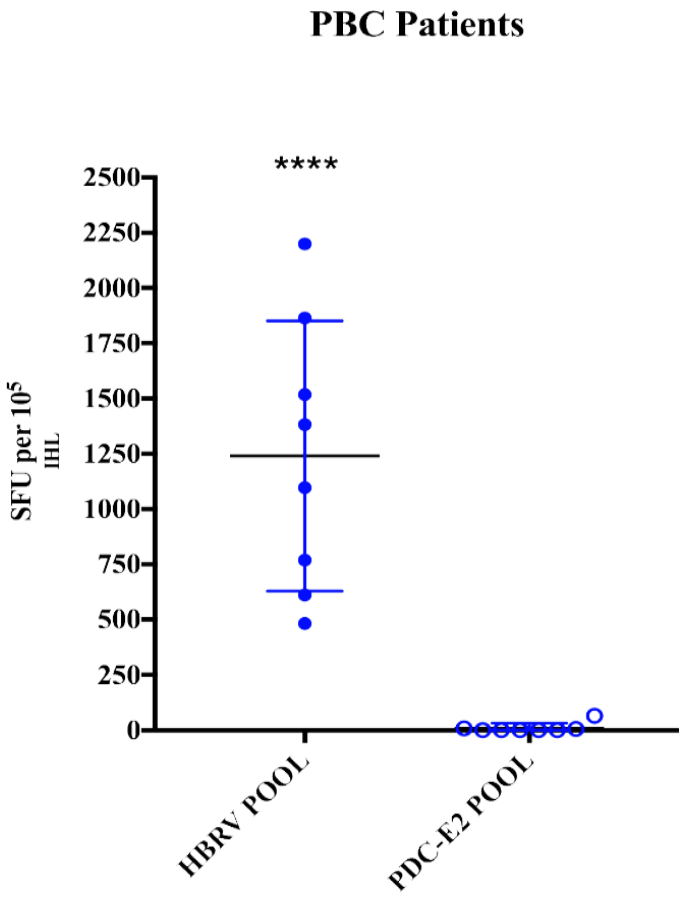

Figure16: Elispot assay analysis of IFN-γ production responses to whole HBRV pool peptides and PDC-E2 pool in Patients with PBC (n=8). Each spot represented the total number of SFC resulted from HBRV pool & PDC-E2 pool for each patient. ($p < 0.0001$).

Chapter 4: Discussion

4.1 Introduction

Given the general consensus that the nature of the immune attack on bile ducts in patients with PBC is an autoimmune process, we sought to further characterize the cellular immunity to HBRV as well to compare the different antigenic stimuli. We also sought to evaluate the proinflammatory cellular immune responses to HBRV in PBC patients for a potential diagnostic utility in detecting HBRV infection. The present study highlights that all PBC patients with end stage liver disease had significantly higher cellular immune responses to HBRV antigens as compared to control liver transplant diseases. In fact, we observed that the precursor frequency of anti-HBRV intrahepatic lymphocytes is comparable to that seen in HCV in the order of 1 in 50 to 200 IHL. In addition, the intrahepatic lymphocytes from PBC patients had negligible reactivity to PDC-E2 with only one in 8 patients reactive to PDC-E2.

The key findings of this study are:

a) PBC IHL show significantly elevated levels of cellular immune response to HBRV antigens as compared to other liver diseases.

b) PBC patients have markedly elevated reactivity to HBRV peptides versus characterized PDC-E2 auto-antigens by 8-fold (and far greater as 7 patients had no reactivity).

In the following sections I will discuss the importance of these findings in relation to previous studies, as well as provide potential explanation for the data. Lastly, I will describe the implications of our study in understanding of the disease and future studies on PBC.

4.2 Is PBC an infectious disease?

The incidence and prevalence of autoimmune diseases have been increasing worldwide. Several hypotheses have been suggested to explain how a breakdown in the balance between auto-regulatory immune pathways and pathogenic auto-reactivity generate autoimmunity (43). Several hypotheses have attempted to define how a disruption in the balance between auto-regulatory immune pathways and pathogenic auto-reactivity results in the development of autoimmunity (43). Retroviruses have been implicated in several autoimmune disease pathogeneses such as Sjögren's syndrome, multiple sclerosis, immune mediated diabetes, and primary biliary cirrhosis (44). There has been a growing interest in human endogenous retroviruses (HERVs) as potential triggers towards autoimmune diseases over the last 30 years (43). HERVs are believed to be pathogenic in several autoimmune diseases especially the rheumatic disorders, such as rheumatoid arthritis (45, 46).

We know that retroviruses have been implicated in the generation of various autoimmune diseases. In the early 1980s, the pathogenesis of acquired immunodeficiency syndrome was not fully understood and although HIV had not been identified at the time, it was commonly believed that AIDS was a communicable disease. Nevertheless, patients presented with diseases that were thought to be autoimmune in nature, such as autoimmune thrombocytopenic purpura, and because it was not yet proven that the condition was infectious in nature, these patients were treated with corticosteroids for what was essentially a retroviral infection and immunodeficiency syndrome (47). The discovery of the etiologic as a lymphotropic retrovirus, HIV, has had a marked impact on diagnosis and treatment of disease.

Currently, PBC is considered as a model autoimmune liver disease with both humoral and cellular immune response to self-proteins, however the etiology of PBC is not known. There is agreement that PBC is a complex disease where susceptible people are exposed to an unknown agent which trigger the breakdown of self-tolerance to mitochondrial proteins. Our lab has studied the viral pathogenesis of PBC since 2003, when we first characterized HBRV with a 93% to 97% nucleotide homology with the mouse betaretrovirus, MMTV (8, 25). HBRV has now been isolated from PBC patient samples, proviral integrations have been demonstrated at the site of disease in biliary epithelium in the majority of PBC patients (32). Also, we have shown that the infection with MMTV and HBRV trigger the generation of autoantigens on the cell surface *in vivo* and *in vitro,* in mice and human respectively (8, 28, 48). The humoral and cellular immune response to HBRV in patients with PBC have been characterized to a degree (14) and further expanded in this thesis. Furthermore, anti-retroviral treatment using reverse transcriptase inhibitors and combination antiretroviral therapy has led to biochemical and histological improvement in randomized controlled trials for PBC patients (49, 50). The same anti-retroviral regimen used in the NOD.c3c4, PBC mouse model also produced histological, biochemical and virological improvement (51). These data raise the possibility that the HBRV may play a central role in the pathogenesis of PBC; however, further work is required to establish this hypothesis.

Our study shows that 23 HBRV Gag and Env peptides stimulate IHL from PBC patients to release IFN-γ. The mean precursor frequency for HBRV reactive lymphocytes in intrahepatic lymphocytes was directly comparable to the frequency of specific $CD8^+$ cells in the liver for reactive to HCV (16). Interestingly, when we stimulated IHL from PBC patients with PDC-E2 peptides, we observed that all PBC patients reacted to HBRV peptides whereas just one patient reacted to PDC-E2 peptides. Indeed, the precursor frequency of PDC-E2 was much lower thatn

the anti-viral responses and approximately 1 x10^{-5} reacted with the autoantigen, in keeping with previous studies (15). These results are very important to emphasize the role of betaretroviral infection and development of PBC disease and also open new avenues for diagnosis and management of PBC patients.

4.3 Consideration for formulating a diagnostic tool for detection of HBRV infection

4.3.1 QuantiFERON assays

In 2001, a new diagnostic test (QuantiFERON-TB) IFN-γ release assay was approved by the Food and Drug Administration (FDA) as diagnostic tool for latent *M. tuberculosis* infection. This assay has used for monitoring and diagnosing infectious diseases, where serological and other immune testing are found to be less effective. This test is an *in vitro* diagnostic tool which measures a component of *M. tuberculosis* reactivity to cell-mediated immunity (52). Moreover, it is used to measure the release of IFN-γ in heparinized whole blood in response to stimulation by purified *M. tuberculosis* peptides (35). Approximately 20-30 *M. tuberculosis* peptides are required to create IFN-γ assay capable of stimulating cellular immune response in patients with different HLA haplotypes (35, 36). Each kits includes three tubes containing (i) a negative control with no stimulants, (ii) a positive mitogen control that stimulates IFN-γ a production, and (iii) the *M. tuberculosis* peptide pool that triggers a proinflammatory T cell response. Following the collection of one ml of blood, all their tubes must be incubated for 16-24 hours at 37°C. Lastly, the plasma in the three tubes are assessed individually using an ELISA method for measuring IFN-γ production Figure 17 (Qiagen, Blood Collection Training Guide 2017). Similar Quantiferon assays have been constructed for viral diagnostics. For example, human cytomegalovirus is one of the primary opportunistic pathogens which is associated with

increased morbidity and mortality in transplant recipients (53, 54). A IFN-γ release assay has been adapted to assess cytomegalovirus cell-mediated immunity for detecting active infection (55). This assay has become a valuable diagnostic tool for the detection of HCMV infection and monitoring the immune response in immunosuppressed patients during therapy. Other IFN-γ release assays in development focus on detection of immune responses to viruses such as JC virus that may be activated to cause disease in patients on Natalizumab.

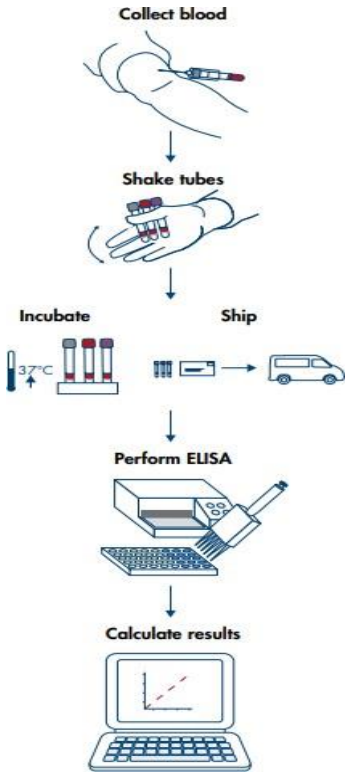

Figure 17*: adapted from Qiagen, Blood Collection Training Guide 2017*, illustrates the steps of IFN-γ release assay, collect 1 ml of blood by venipuncture in each tube. Immediately shake tubes 10 times. Blood tubes must be incubated as soon as possible upright at 37ºC for 16-24 hours. Measure the IFN-γ production from the plasma by ELISA test and calculate the result.

4.4 Future directions

In order to drive a diagnostic assay for PBC, we will focus on developing an IFN-γ release assay using PBMC. Our initial attempt in formulating an IFN-γ release assay was conducted using 2 μg of mixture of 36 HBRV peptides (identified from 4 PBC IHL using a cut off of 50 SFC) to stimulate 2 million PBMC for 24 hours at 37°C. The Mesoscale ELISA was used to measure IFN-γ production levels in 50 ml supernatant. Only 50% of PBC patients met the industry criteria of having an IFN- γ >14pg/ml, while all the negative controls did not respond to HBRV pool and the positive control with breast cancer made a good IFN- γ response (Figure 18). In this study, the breast cancer patient used as a positive control because a proportion of patients are reactive to anti-HBRV Env. Interestingly, the IFN-γ release correlated with disease activity as gauged the alkaline phosphatase levels in PBC patients' blood samples. For example, patient PBC4 had the highest IFN-γ values (135pg/ml) and had also a progressive PBC with alkaline phosphatase of 834 U/L, which is considered to be in the very high range in patients unresponsive to standard therapy. To sum up, this is the preliminary data for somewhat approving our concept in order to create an IFN-γ release assays for follow up of PBC patients.

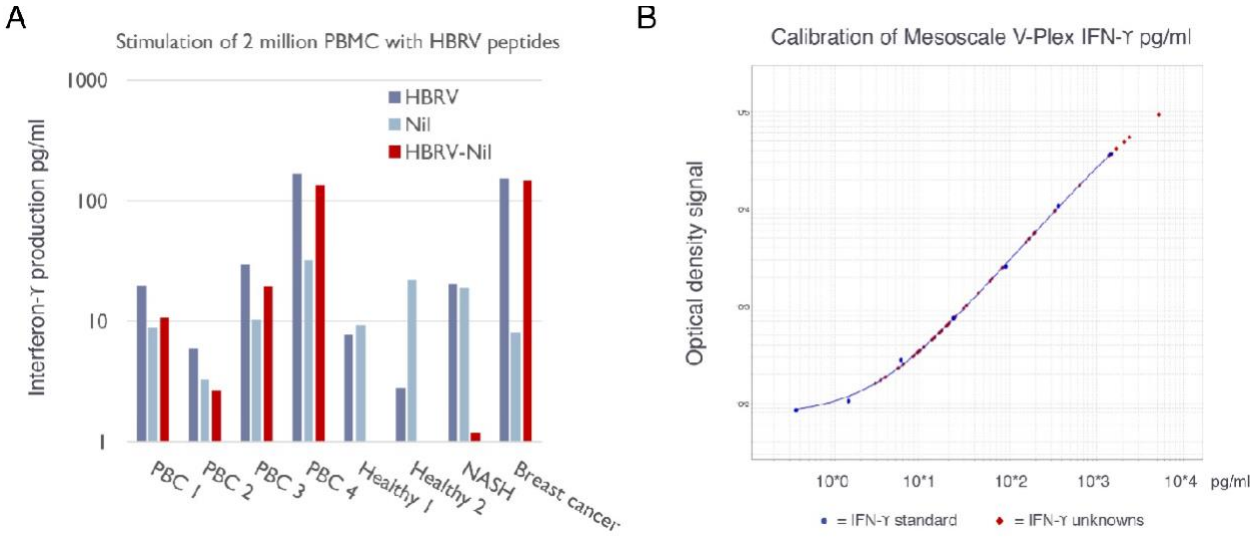

Figure 18: Initial IFN-g release assay using frozen PBMC from PBC patients and relevant control samples. (A) Following stimulation of 2x10⁶ PBMC with 2 ∝g of 36 HBRV Gag and Env peptides pool, The IFN-*g* released in supernatant was assessed using Mesoscale ELISA analysis. (B) THE Mesoscale V-Plex ELISA was chosen for its reproducibility and wide linear range for detection.

4.4.1 Development of IFN- γ release assay for HBRV with PBC patients.

The identification of 22 overlapping Gag and Env HBRV peptides provided 100% sensitivity and specificity for detection of the cellular immune response by using cut off of 100 SFC per peptide (Figure 19). This is an important finding because it may provide a foundation to develop a novel diagnostic IFN-γ release assay to monitor the HBRV specific T cell response in PBC patients. For this proposal, a pilot study will be conducted to establish an optimal cut off for the new IFN-γ assay. A mixture of 22 HBRV Gag and Env peptides pool will be used to stimulate PBMC and whole blood for INF-γ production using PBC patients, healthy individual; other liver diseases and known positive control. Similar studies have been shown in the literature for establishing QuantiFERON assays.

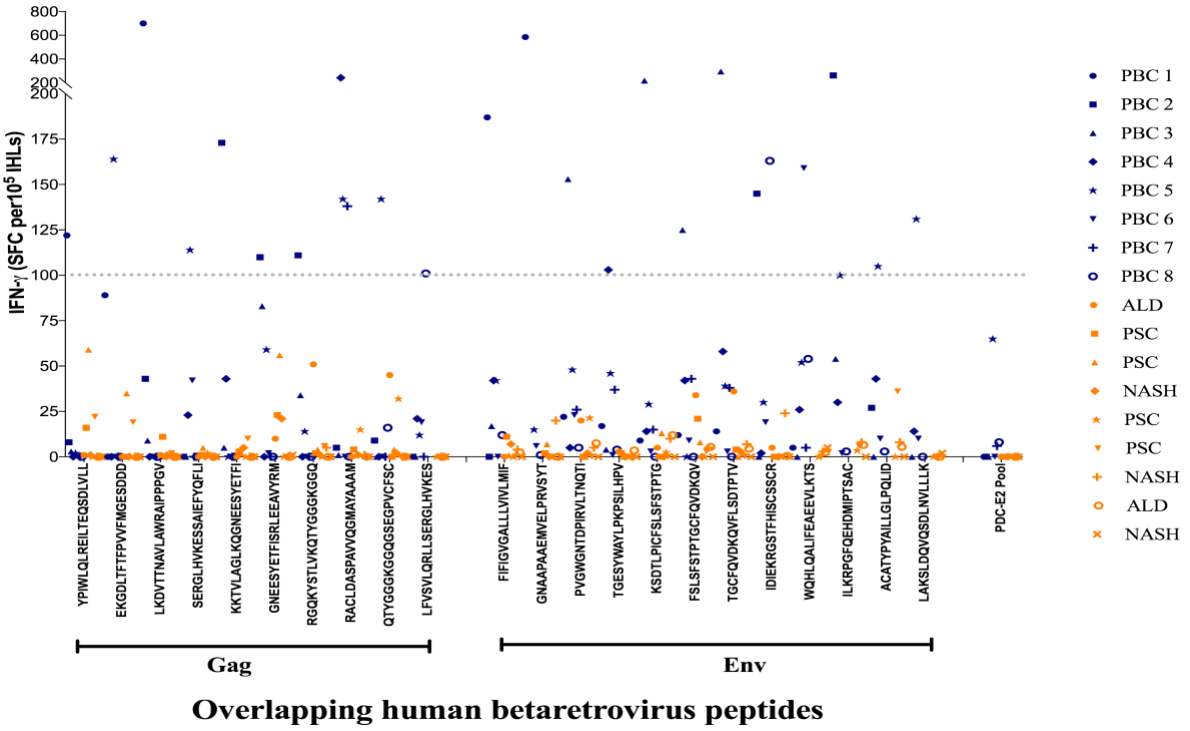

Figure 19: 22 peptides from Gag and Env HBRV peptides. Cut-off was set for ≥ 100 SFC. We were able to identify 22 (Gag & Env) HBRV peptides that were able to show marked IFN-γ secretion in 8 PBC patients and 9 Controls (other liver diseases). All IHLs from PBC samples demonstrated 100% sensitivity as well as specificity to HBRV Gag and Env peptides. Controls were not reactive to HBRV peptides. None of PBC samples showed response to PDC-E2 pool peptides stimulation.

4.5 Conclusion

Given the evidence implicating HBRV infection in primary biliary cholangitis pathogenesis, our study was undertaken with the intention to further study the cellular immune response in IHL from PBC patients with HBRV infection. These data illustrate that the intrahepatic IFN-γ cellular immune response to HBRV greatly exceeds the autoimmune response in PBC patients, which indicates HBRV infection plays an important role in mediating PBC. Moreover, our data provides an alternative view of PBC as an infectious trigger disease as well as potential platform to develop an HBRV diagnostic assay.

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