Studying the Regulatory Effects of Betaretrovirus infection in the NOD.c3c4 mouse model of Primary Biliary Cholangitis

By

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Abstract

A human betaretrovirus (HBRV) has been previously characterized in patients with primary biliary cholangitis (PBC). HBRV shares between 91% to 99% nucleotide similarity with the mouse mammary tumor virus (MMTV). Retroviruses are known to suppress host immune responses by the presence of immunosuppressive domains (ISD) located in the Envelope (Env) portion. These ISDs have been shown to inhibit lymphocyte proliferation and promote secretion of IL-10, an immunoregulatory cytokine. For example, HBRV Env has been found to harbor two ISDs in the Env protein that inhibit lymphocyte proliferation and trigger IL-10 secretion *in vitro* (M. Rahbari, CDDW, 2015). *In vivo*, we investigated whether MMTV Env protein harbors an ISD with similar immunoregulatory characteristics. Then, we addressed the hypothesis that B cells in the PBC mouse model (NOD.c3c4) are promoted to secret more IL-10 in response to the presence of MMTV infection. To test the characteristics of ISD, splenocytes from healthy BALB/c were stimulated by 85 individual peptides composed from the HBRV Env. Then, ELISA was used to detect the levels of IL-10 secretion. In addition to, T cell proliferation assays were performed to assess the ability of each ISD to inhibit lymphocyte division. To assess the role of B cells in response to MMTV infection, splenocytes from infected NOD.c3c4 and healthy C57Bl/6 were immunostained for the viral proteins, B cell markers and IL-10. These studies showed that the ISD significantly promoted higher secretion of IL-10 compared to the other Env peptides. In the proliferation assays, there was reduction in proliferation by 20% ($p < 0.01$) and 44% ($p < 0.001$) in BALB/c and C57Bl/6 splenocytes respectively. Furthermore, the NOD.c3c4 mice had a significant expansion of B cells secreting IL-10 compared to C57Bl/6 ($p < 0.001$). MMTV ISD modulate the immune response via inhibiting lymphocyte proliferation and promoting IL-10 secretion, which we hypothesize prevents antiviral immune responses and enables disease progression. Moreover, MMTV-infected B cells may play an appreciable role in the development of the PBC-like phenotype in NOD.c3c4 mice. More B cells secreting IL-10 were found in NOD.c3c4 compared to healthy mouse strains. This study reveals the immune modulation of MMTV and potentially enables a better understanding of PBC pathophysiology. Further work may reveal a different role for IL-10 in disease progression.

Preface

This thesis is an original work by Kerolous Messeha. No part of this thesis has been previously published.

Several individuals have participated in the work discussed herein. The original Splenocytes extraction protocol was performed by Ishwar Hosamani. Filip Wysokiniski, Ishwar Hosamani and Mary Labib & Tammi Li aided in splenocytes extraction. For Flow cytometry experiments, I performed the staining protocol and optimizations for both cell surface and intracellular staining using the protocol by Biolegend company, USA. Mandana Rahbari devised the original studies in humans for identifying ISD and inhibition of cellular proliferation. For ELISA experiments to detect IL-10 secretion, Hiatem Abofayed helped in preparing the 85 individual Envelope peptides. Dr. K. Suzuki (UofA) helped in setting up the parameters of Mesoscale Reader at the Molecular Biology and Biochemistry Core Facility, UofA. In regard to the proliferation assay experiments, Bruna Dutra aided in culturing the splenocytes and staining the cells.

I performed statistical analysis on flow cytometry data, ELISA (Mesoscale) as well as proliferation assay and appealingly modified all graphs included.

This study was approved by the University of Alberta, Research Ethics Committee (AUP00000294).

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List of Abbreviations

Abbreviation	Meaning
2-ODCA	2-oxoacid dehydrogenase complexes
ABD	Autoimmune biliary disease
AMA	Antimitochondrial Antibody
BEC	Biliary Epithelial Cell
CFSE	Carboxyfluorescein Succinimidyl Ester
CSB	Cell Staining buffer
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
Gag	Group of antigens
GWAS	Genome Wide association study
HBRV	Human Betaretrovirus
HLA	Human Leukocyte Antigen
IHC	Immunohistochemistry
IL-10	Interleukin - 10
IL-12	Interleukin – 12
ISDs	Immunosuppressive Domains
JAK - STAT	Janus kinase/signal transducers and activators of transcription
MMTV	Mouse Mammary Tumor Virus

NF- kB	Nuclear Factor kB
NOD	Non-obese Diabetic
OCA	Obeticholic acid
PBC	Primary Biliary Cholangitis
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffered saline
PDC-E2	E2 subunit of the pyruvate dehydrogenase complex
PWB	Perm-Wash buffer
SAg	Superantigen
SU	Surface part of the envelop
TM	Transmembrane part of the envelop
TNF – Alpha	Tumor Necrosis Factor Alpha
UDCA	Ursodeoxycholic acid
UTI	Urinary Tract Infection
VFP	Viral Fusion Proteins

Chapter 1: Introduction

1.1 Introduction

The term "Primary Biliary Cirrhosis" (PBC) was first used in 1949, as biliary cirrhosis was the main process associated with disease advancement [1]. In 2014, the term "Cirrhosis" was changed worldwide by advocacy groups to "Cholangitis" and now the current term is Primary Biliary Cholangitis [2]. PBC presents in the early stage with fatigue and pruritus, while patients in the late stage of the disease present with jaundice and liver failure. PBC is known to be an autoimmune disease characterized by autoreactivity to mitochondrial proteins abnormally expressed on the surface of biliary epithelial cells (BEC) surface in patients with PBC. Yet, the relation between PBC and autoimmunity is not clearly understood. Herein, we assess the possible role of one of the environmental factors, Human Betaretrovirus (HBRV), in the disease pathogenesis.

1.2 Primary Biliary Cholangitis

PBC is a chronic liver disease characterized by destruction of small sized intrahepatic bile ducts leading to cholestasis, fibrosis and eventually liver cirrhosis [3] (Figure 1.1). Liver transplant is needed to prolong lives of PBC patients with end stage disease [3]. PBC contributes to 5% of liver transplants in Canada which imposes an economic burden nationwide [4]. PBC has a female predominance with a female: male ratio of 10:1[3]. The prevalence of PBC has demonstrates a geographical diversity ranging from 1.91 to 40.2 per 100,000 people [5]. To date, the exact cause of the disease is not well understood. Nevertheless, there is an understanding that PBC is an autoimmune disease with both genetic and environmental influences [6]. Currently, PBC treatment is mainly directed at alleviating cholestasis. Ursodeoxycholic acid (UDCA) was the first licensed treatment that was used to reduce cholestasis, slowing disease progression.

However, 40% of PBC patients on UDCA develop disease progression. Recently, Obeticholic acid (OCA) has been approved in Canada to treat patients who develop intolerance or non-response to UDCA. However, the downside of using OCA is the development of severe pruritus [7]. So far, liver transplant is the main treatment of advanced stages of PBC. However, PBC has been found to have a high recurrence rate after liver transplants [8].

The administration of more potent immune suppression with tacrolimus, versus the less potent cyclosporine, has been investigated regarding the effect on recurrent PBC. In contrast to what is expected in autoimmune diseases, the use of more potent immune suppressor with tacrolimus is associated with a higher recurrence rate post-transplant [5]. These data are more in favor of an infectious disease process. However to date, PBC is simply known as a complex disease where genetically predisposed individuals develop the disease under the influence of an environmental factor [9].

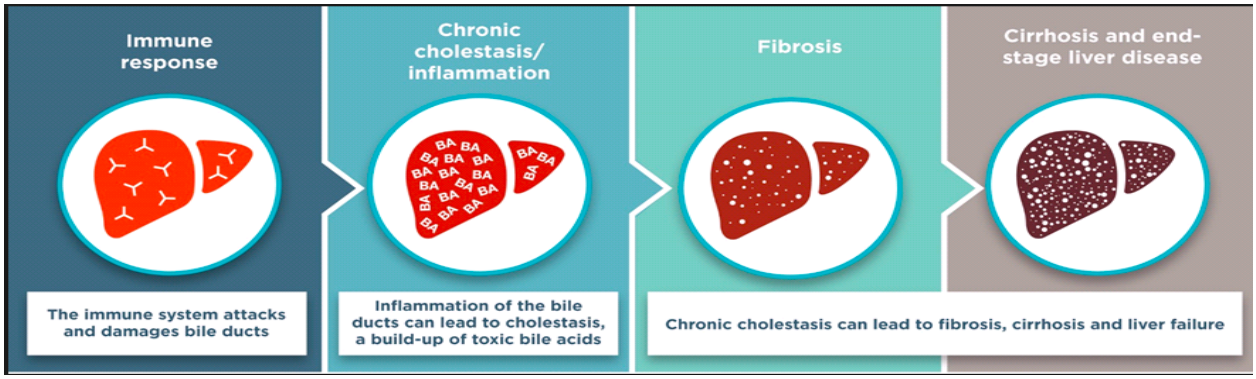

Figure 1.1 *The process of disease progression – adapted from Intercept pharma*

1.3 Genetic predisposition to PBC

There is an increased prevalence of PBC in first-degree relatives with a relative risk of 10.5 and 10.7 in UK and US respectively [10, 11]. Moreover, monozygotic twins have higher occurrence rate compared to dizygotic twins [12]. Additionally, genome wide association studies

(GWAS) have shown that HLA gene variants and genes alleles of the IL-12 immune pathway predispose to the development of PBC [13]. These studies favor the hypothesis that the development of PBC might be a direct cause of immune dysfunction in certain predisposed individuals exposed to an environmental factor.

1.4 Environmental factors in PBC

Previous studies have hypothesized the involvement of various environmental triggers in the development of PBC; either chemical or infectious agents. Epidemiological studies have shown that there is a geographical clustering of reported PBC patients around specific water supplies, coal mines, and even toxic waste sites [11, 14]

In 1984, molecular mimicry was theorized to explain the link between chemical agents and PBC [15]. The molecular mimicry theory suggested that foreign chemicals excreted in bile reacted with haptens that resembled the lipoic acid domain of 2-ODCA complexes leading to the production of specific antigens that stimulated the immune responses [16] to develop high-titer antibodies toward mitochondrial autoantigens [17].

Regarding transmissible agents, two groups have been suggested; bacterial and viral infections [18]. In addition, various retrospective studies in liver transplant patients have shown that the use of potent immunosuppressive therapies led to recurrent disease rather than halting an immune based disease progression. Taken together, these studies suggest a hypothesis that PBC development might be due to deficiency of the host immune response where patients were unable to contain an infectious disease process [19].

A relation between urinary tract infection (UTI) and PBC has been consistently observed in epidemiological studies [20]. Consequently, this has led to hypothesis that bacterial antigens may be one of the main causative triggers of PBC through molecular mimicry theory. Researchers

suggested that mitochondrial antigens in bacteria, might lead to triggering of the autoimmune response and eventually breakdown the immune tolerance to similar self-mitochondrial proteins [21]. Both *N. aromaticivorans* and *E. coli* have been hypothesized to induce antimitochondrial antibodies (AMA) [21, 22]

A human betaretrovirus (HBRV) has been reported to be a candidate environmental agent involved in the pathogenesis of PBC by Xu et al [23]. On one hand, the presence of virus was linked to PBC both *in vivo* and *in vitro*. Xu et al. detected autoantigen expression in normal BEC cells (a PBC specific phenotype) after co-culture with lymph node homogenates containing HBRV from PBC patients [23]. Xu et al. cloned the virus and showed by RT-PCR as well as immunohistochemistry (IHC) the presence of HBRV in 75% of peri-hepatic lymph nodes from PBC patients collected at the time of liver transplantation [23]. They were able to visualize virus-like particles in biliary epithelial cells by electron microscopy. In BECs from normal subjects after being cocultured with PBC lymph node homogenates and MMTV isolates, they were able to locate an increase in PDC-E2 by immunoblotting with AMA and through immunostaining of BEC membrane. Using RT-PCR, they were not able to show evidence of betaretrovirus in BEC before coculture with lymph nodes. However, after being cocultured with PBC lymph nodes for 7 days, the human betaretrovirus had been detected in 7 of 10 BEC derived from PBC lymph node cocultures compared with 2 of 15 BEC incubated with control lymph node [23]. In subsequent studies, similar observations were made in the NOD.c3c4 mouse with autoimmune biliary disease. The mice developed MMTV related cholangitis and the bile ducts demonstrated both viral proteins and mitochondrial AMA staining[24]. Furthermore, anti-retroviral therapies in NOD.c3c4 mice led to marked improvement on both liver biochemistry as well as histology [25].

Selmi et al. also studied PBC patients' sera, liver specimens as well as peripheral blood lymphocytes for evidence of betaretrovirus infection. They performed western blotting to test sera from PBC patients and controls using antibodies against three different MMTV strains and polyclonal antibodies against MMTV (anti-p27 & anti-gp52). Also, they stained liver specimens by polyclonal antibodies against MMTV in order to visualize viral proteins and performed PCR on DNA extracted from peripheral blood lymphocytes and tissue specimens to identify specific MMTV genomic DNA [26]. Selmi et al. were unable to derive any evidence of MMTV infection, previously reported by Xu et al [23], and concluded that there was neither molecular nor immunohistology evidence of MMTV in PBC. The main drawback in Selmi's experiments is that they failed to analyse samples shown by Xu et al. and others as being the main reservoir for MMTV, such as perihepatic lymph nodes [27]. Moreover they did not study the phenomena of betaretrovirus induction of the disease specific phenotype *in vitro*, with increased and aberrant expression of the mitochondrial phenotype in cholangiocytes co-cultured with the virus [23].

1.5 Mouse mammary tumor virus and Human betaretrovirus:

HBRV shares 91% - 99% genetic similarity with mouse mammary tumor virus (MMTV) [23]. MMTV and HBRV are members of the betaretroviridae family. MMTV is known to have super antigen (SAg) activity which facilitates viral replication as well as the transmission of infection [28]. Retroviral genes encode various structural and enzymatic components; Gag, Pol, Pro, Env and SAg. Gag is a polyprotein that includes matrix, capsid and nucleocapsid. Envelope constitutes surface (SU) and transmembrane (TM) proteins. Protease, Reverse transcriptase and Integrase are encoded by the *pol* gene that harbor enzymes responsible for the viral life cycle. (Figure 1.2) [29].

HBRV Env is a Class I viral fusion protein (VFP). VFP are synthesized in the endoplasmic reticulum and then cleaved in the Golgi apparatus into two subunits; surface (SU) and transmembrane (TM) subunits. SU is responsible for binding to the receptors on the target cells, while TM mediates fusion to the cell membrane. TM is anchored in the viral envelop toward C terminus [30]. SU and TM undergo different conformational changes through binding to the target cell. SU binds to the receptor on the target cell exposing the hydrophobic region of N terminus of fusion peptide (TM). Consequently, TM fuses to the plasma membrane of the target cell [31].

Retroviruses are known to have highly conserved immunosuppressive domains (ISD) in the envelope surface or transmembrane proteins [32]. Through these ISD, the virus has the capacity to mask the host immune response through promoting the secretion of IL-10 as well as inhibiting the proliferation of lymphocytes [33]. Members of our lab (M. Rahbari) investigated the effects of 85 overlapping 18-merpeptides, that cover the full sequence of the Envelope of HBRV for ISDs activity using healthy peripheral blood monocytes (PBMCs). She investigated the effect of each individual peptide for the reactivity of IL-10 secretion from PBMCs. She identified two peptides in the SU and TM of HBRV which had the highest IL-10 secretion compared to the rest of 85 individual peptides. The two peptides were tested for their ability to suppress T lymphocyte proliferation using flow cytometry and CFSE. She was able to identify two peptides that had characteristics of immunosuppressive domains (M. Rahbari, CDDW, 2015). Consequently, I performed similar studies to identify ISDs in mice testing the characteristics of the MMTV peptides to inhibit T lymphocytes proliferation and promote the secretion of IL-10.

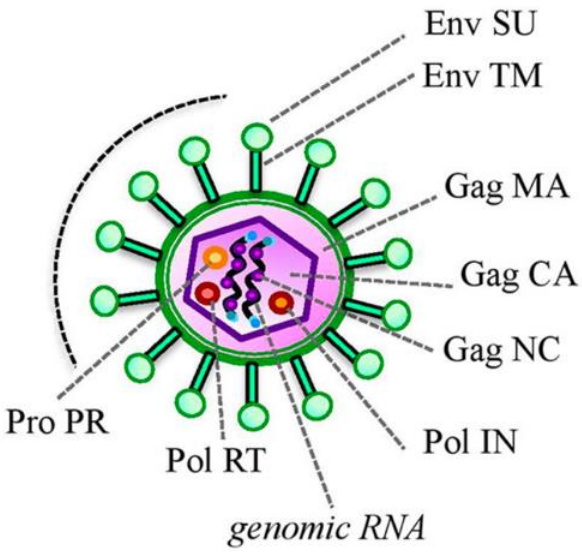

Figure 1.2 Structural and enzymatic components of retroviruses, *from Grandi et al, 2018*

1.6 B cells Secreting IL-10:

Traditionally, B cells have been known for their ability to produce antibodies [34]. However, B cells are well known to have both positive and negative regulatory functions during immune responses [35]. With positive regulation, B cells can act as professional antigen-presenting cells with a $10^3 – 10^4$- fold increased antigen presentation compared to other nonprofessional Ag-presenting cells [35]. Through antigen presentation, B cells can induce $CD4^+$ T cells expansion [36, 37], cytokine secretion and the formation of memory cells [38-40]. Studying the negative regulatory role of B cells, B cells have been reported to have different mechanistic roles in suppressing the host immune response [41, 42]. An important cytokine that negatively regulate the immune response is IL-10 which is an effective anti-inflammatory cytokine that plays a crucial role in preventing inflammatory and autoimmune pathologies [43, 44]. Also, IL-10 is known to have a regulatory function that allow for induced tolerance [43].

IL-10 secreting B cells are the extensively investigated regulatory B cell subset [41, 45-47]. One specific subset is known as B10 cells to emphasize on the immune-regulatory role of this

subset and to characterize it from the other B subsets that negatively modulate the immune response via different mechanisms [47].

Regarding identifying B10 cells phenotypically, many cell surface markers have been suggested [41, 42] but none of them were found to be unique for B10 cells identification. Therefore, the only way to characterize B10 cells is to functionally identify them through performing intracellular staining for IL-10 following stimualtion [48].

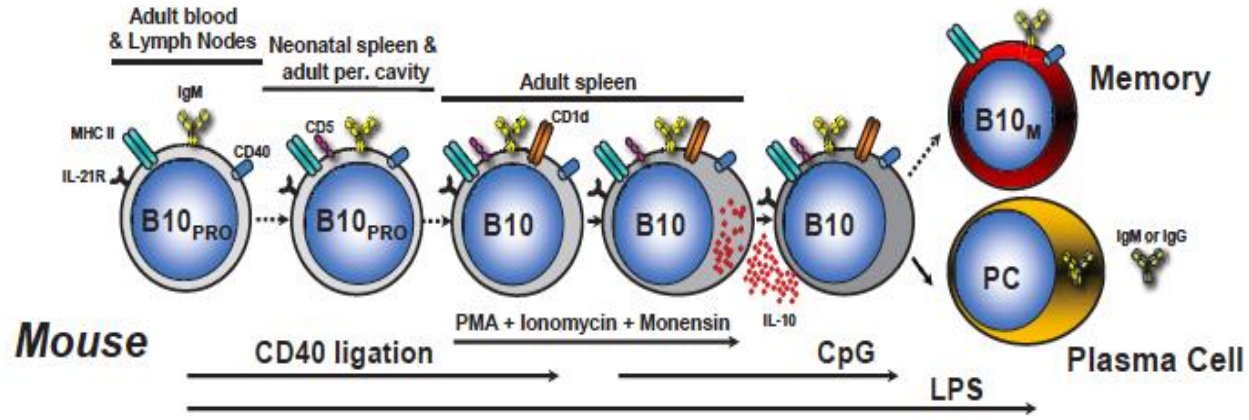

Figure 1.3 Development and differentiation of IL-10 secreting B10 Cells in mice*, from Kalampokis et al, 2013*

1.7 Mouse Mammary Tumor Virus and B cells:

Mouse Mammary Tumor Virus (MMTV) is a B-type retrovirus that causes the development of breast carcinoma in infected mice [49]. MMTV is transmitted through breast milk to pups within the first two weeks of life. MMTV infects B cells in Peyer's patches in the ileum of the small intestine [50]. This in turn, promotes the proliferation of infected B cells under the feedback effect of superantigen-reactive T cells that amplify the B cell population [51]. The

lymphocytes then carry the virus around the body and may end up in breast epithelium to cause mammary tumors [49].

1.8 PBC mouse model (NOD.c3c4):

NOD.c3c4 mouse is a congenic mouse constructed on non-obese diabetic (NOD) background. The NOD.c3c4 strain develops autoimmune biliary disease (ABD) that shows similarities with PBC. For example, NOD.c3c4 mice spontaneously produce antimitochondrial antibodies against pyruvate dehydrogenase complex (PDC-E2) between 8-10 weeks of age, before the development of histological features of cholangitis which occurs between 9-12 weeks of age [52-54]. Similar to PBC, NOD.c3c4 develop intrahepatic bile duct inflammation and fibrosis in late stages of the disease (Figure 1.4). Unlike the human disease, NOD.c3c4 develops inflammation of extrahepatic bile ducts as well as biliary cysts [55]. Our lab has shown that not only do the NOD.c3c4 mice have evidence of MMTV related cholangitis, they also express the mitochondrial antigens in bile ducts[24]. Accordingly, NOD.c3c4 show similarity to PBC and therefore this mouse model was chosen to study various immunological, virological and histological aspects of the disease pathogenesis.

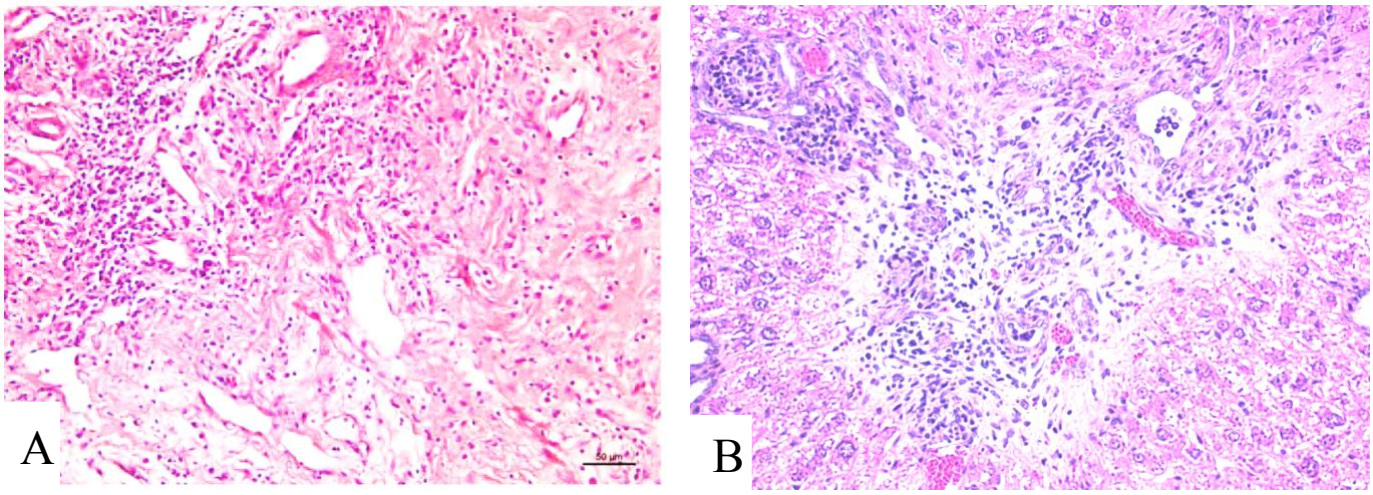

Figure 1.4 Histological similarity between (A) PBC in humans and (B) ABD in NOD.c3c4 mice.

1.9 Hypothesis:

The pathogenesis of PBC is poorly understood. The NOD.c3c4 mouse model spontaneously develop a picture that resembles PBC and the mice expressed an endogenous MMTV infection. As discussed, previous work has been done to identify ISD in HBRV. Therefore, we hypothesized that MMTV resides in B cells, and through the mechanism of conserved ISD in the Envelope protein, promotes the secretion of IL-10 and inhibits antiviral immune response.

Therefore, the aim of our study is to identify ISDs in MMTV Env using the NOD.c3c4 mouse model by testing the ability of the potential ISD to promote IL-10 secretion and inhibit T lymphocyte proliferation and then tests whether MMTV infected B cells existed in the NOD.c3c4

1.10 Importance and implications of the investigations:

Our lab has been able to link HBRV to the development of PBC, and show that HBRV can induce cell surface PDC-E2 expression by co-culturing lymph nodes homogenates from PBC

patients with BECs from control subjects [23]. We also have found that PBC patients respond to combined antiretroviral therapy.

It is known that PBC mouse model (NOD.c3c4) has MMTV residing in B cells and also found in bile ducts. Furthermore, mice with ABD respond to combined antiretroviral therapy[56] . Our lab has synthesized 85, 18mer peptides that were used to identify immunosuppressive domains in HBRV by studying healthy PBMCs for the secretion of IL-10 and inhibit the proliferation of lymphocytes.

Therefore, we plan to establish the role of highly conserved ISD in MMTV Env in modulating the host immune response and enabling the disease progression in NOD.c3c4 mouse model.

We plan to provide a better understanding for PBC pathogenesis by showing that HBRV blocks antiviral immune response by secreting IL-10.

Chapter 2: Materials and Methods

2.1 Splenocyte preparation

2.1.1 Harvesting spleens from NOD.c3c4

Spleens were harvested from NOD.c3c4 between 14– 20 weeks of age from the NOD.c3c4 colony maintained by our laboratory in the animal facility at University of Alberta (protocol # 294). NOD.c3c4 mice were euthanized by CO_2 according to the mouse euthanasia standard operating procedure (SOP). Then, they were assessed for death as per the mouse determination of death SOP approved by the ACUC of the university of Alberta.

2.1.2 Splenocytes extraction from NOD.c3c4 spleens

After harvesting, the spleens were maintained in 2 ml cold phosphated buffered saline (PBS, Gibco, Waltham, MA, USA) for 45 minutes until processing. Spleens were macerated and then filtered through 40um Nylon mesh (Fisher Scientific, CA) into 15 ml conical tubes and topped up to 10 ml with phosphate buffered saline (PBS, Gibco, Waltham, MA, USA). The suspension was centrifuged at 200g for 10 mins at 4^{o}C. The supernatant was removed, and the pellet was resuspended in 1x PBS (Gibco, Waltham, MA, USA). Red blood cells were lysed using 1ml of 10x PBS (Gibco, Waltham, MA, USA) and 9 ml pure distilled water (Gibco, Waltham, MA, USA). Tubes were agitated for 5 – 10 seconds then the suspensions were centrifuged at 200g for 10 mins at 4^{o}C. Supernatant was removed and the pellet was resuspended in 10ml PBS (Gibco, Waltham, MA, USA) and cells were counted in a dilution of 1:2 in trypan blue (Gibco, Waltham, MA, USA) and stored in a freezing media (45% RPMI 1640) with L-glutamine (Gibco, UK), 45% fetal bovine serum (FBS) (Gibco, UK) & 10% Dimethyl sulfoxide (DMSO) (Sigma, CA). Cells were stored in freezers at -80^{0}C for 48 hours and then stored in liquid Nitrogen (N_2).

2.1.3 Splenocytes from C57Bl/6 & BALB/c

Frozen splenocytes from C57Bl/6 and BALB/c were obtained from IQ Bioscience, California, USA. Splenocytes for both strains were harvested from mice between 14 weeks – 20 weeks of age and used as healthy controls for the experiments.

2.2 HBRV peptides

85 customized peptides derived from HBRV Envelope were purchased from Mimotopes (Minneapolis, USA) with a purity around 95%. Each peptide was 18 amino acids long with 10 amino acids overlap between two consecutive peptides (illustrated in Table 1). Peptides were purchased in a cleaved format (powder form). In order to solubilize peptides in solution, we dissolved by reconstitution in 10% acetic acid/water to reach a target peptide concentration of 1mg/ml. For the insoluble peptides, 10% Acetic acid was added to the peptides and they were sonicated until they dissolved. The stock solutions for individual peptides were stored at a concentration of 100ug/ vial. Individual peptides were lyophilised to remove the acetic acid and water. Finally, all tubes were stored in -20^{o} C freezer for further usage (Table 1).

Peptide	Peptide Sequence	Peptide	Peptide Sequence
ENV 1	MPNHQSGS**PTGSSDLLLS** MPKHQSGSLTDSSDLSLS (MMTV)	ENV 2	**PTGSSDLLLS**GKKQRPHL
ENV 3	LSGKKQRPHLALRRKRRR	ENV 4	HLALRRKRRREMRKINRK
ENV 5	RREMRKINRKVRRMNLAP	ENV 6	RKVRRMNLAPIKEKTAWQ
ENV 7	APIKEKTAWQHLQALIFE	ENV 8	WQHLQALIFEAEEVLKTS
ENV 9	FEAEEVLKTSQTPQTSLT	ENV 10	TSQTPQTSLTLFLTLLSV TSQTPQTSLTLFLALLSV (MMTV)
ENV 11	LTLFLTLLSVLGPPPVTG LTLFLALLSVLGPPPVTG (MMTV)	ENV 12	SVLGPPPVTGESYWAYLP
ENV 13	TGESYWAYLPKPSILHPV	ENV 14	LPKPSILHPVGWGNTDPI LPKPPILHPVGWGNTDPI (MMTV)

ENV 15	PVGWGNTDPIRVLTNQTI	ENV 16	PIRVLTNQTIYLGGSPDF
ENV 17	TIYLGGSPDFHGFRNMSG	ENV 18	DFHGFRNMSGNVHFEGKS
ENV 19	SGNVHFEGKSDTLPICFS	ENV 20	KSDTLPICFSLSFSTPTG KSDTLPICFSFSFSTPTG (MMTV)
ENV 21	FSLSFSTPTGCFQVDKQV FSFSFSTPTGCFQVDKQV (MMTV)	ENV 22	TGCFQVDKQVFLSDTPTV
ENV 23	QVFLSDTPTVDNNKPGGK	ENV 24	TVDNNKPGGKGDKRRMWE
ENV 25	GKGDKRRMWELWLTTLGN	ENV 26	WELWLTTLGNSGANTKLV
ENV 27	GNSGANTKLVPIKKKLPP	ENV 28	LVPIKKKLPPKYPHCQIA
ENV 29	PPKYPHCQIAFKKDAFWE	ENV 30	IAFKKDAFWEGDESAPPR
ENV 31	WEGDESAPPRWLPCAFPD	ENV 32	PRWLPCAFPDQGVSFSPK
ENV 33	PDQGVSFSPKGTLGLLWD PDQGVSFSPKGALGLLWD (MMTV)	ENV 34	PKGTLGLLWDFSLPSPSV PKGALGLLWDFSLPSPSV (MMTV)
ENV 35	WDFSLPSPSVDQSDQIKS	ENV 36	SVDQSDQIKSKKDLFGNY
ENV 37	KSKKDLFGNYTPPVNKEV	ENV 38	NYTPPVNKEVHRWYEAGW
ENV 39	EVHRWYEAGWVEPTWFWE	ENV 40	GWVEPTWFWENSPKDPND
ENV 41	WENSPKDPNDRDFTALVP	ENV 42	NDRDFTALVPHTELFRLV
ENV 43	VPHTELFRLVAASRYLIL	ENV 44	LVAASRYLILKRPGFQEH
ENV 45	ILKRPGFQEHDMIPTSAC	ENV 46	EHDMIPTSACATYPYAIL EHDMIPTSACVTYPYAIL (MMTV)
ENV 47	ACATYPYAILLGLPQLID ACVTYPYAILLGLPQLID (MMTV)	ENV 48	ILLGLPQLIDIEKRGSTF
ENV 49	IDIEKRGSTFHISCSSCR	ENV 50	TFHISCSSCRLTNCLDSS
ENV 51	CRLTNCLDSSAYDYAAII	ENV 52	SSAYDYAAIIVKRPPYVL
ENV 53	IIVKRPPYVLLPVDIGDE	ENV 54	VLLPVDIGDEPWFDDSAI
ENV 55	DEPWFDDSAILTFRYATD DEPWFDDSAIQTFRYATD (MMTV)	ENV 56	AILTFRYATDLIRAKRFV AIQTFRYATDLIRAKRFV (MMTV)
ENV 57	TDLIRAKRFVAAIILGIS	ENV 58	FVAAIILGISALIAIITS
ENV 59	ISALIAIITSFAVATTAL	ENV 60	TSFAVATTALVKEMQTAT
ENV 61	ALVKEMQTATFVNNLHRN	ENV 62	ATFVNNLHRNVTLALSEQ

ENV 63	RNVTLALSEQRIIDLKLE	ENV 64	EQRIIDLKLEARLNALEG EQRIIDLKLEARLNALEE (MMTV)
ENV 65	LEARLNALEGVVLELGQD LEARLNALEEVVLELGQD (MMTV)	ENV 66	EGVVLELGQDEANLKTRM EEVVLELGQDVANLKTRM (MMTV)
ENV 67	QDEANLKTRMSTRCHANY QDVANLKTRMSTRCHANY (MMTV)	ENV 68	RMSTRCHANYDFICVTPL
ENV 69	NYDFICVTPLPYNASESW	ENV 70	PLPYNASESWERTKAHLL
ENV 71	SWERTKAHLLGIWNDNEI	ENV 72	LLGIWNDNEISYNIQELA LLGIWNDNEISYNIQELT (MMTV)
ENV 73	EISYNIQELANLISDMSK EISYNIQELTNLISDMSK (MMTV)	ENV 74	LANLISDMSKQHIDTVDL
ENV 75	SKQHIDTVDLSGLAQSFA	ENV 76	DLSGLAQSFANGVKALNP
ENV 77	FANGVKALNPLDWTQYFI	ENV 78	NPLDWTQYFIFIGVGALL
ENV 79	FIFIGVGALLLVIVLMIF	ENV 80	LLLVIVLMIFPIVFQCLA
ENV 81	IFPIVFQCLAKSLDQVQS	ENV 82	LAKSLDQVQSDLNVLLLK
ENV 83	QSDLNVLLLKKKKGGNAA	ENV 84	LKKKKGGNAAPAAEMVEL
ENV 85	GNAAPAAEMVELPRVSYT		

Table 1 *Amino acid sequences for 85 individual peptides derived from HBRV Envelop synthesized for the peptide mapping studies. MMTV amino acid sequences are presented in Blue with differences from HBRV in red.*

2.3 ELISA assay for detection of IL-10 secretion

2.3.1 Splenocytes culture

Two hundred thousand splenocytes per well from BALB/c mice were cultured in 200 ul of 1640 RPMI with 10% FBS (Gibco, UK) in 96 well plates and 10% antibiotic/ antimycotic solution (1000 units penicillin/10mg streptomycin/25ug amphotericin/ml; Sigma-Aldrich). Individual envelop peptides (Table1) were then added to 96 well plates using no stimulant (Nils; splenocytes + 30% DMSO) as negative control for comparison as well as a

positive stimulated control [splenocytes in addition to 2ul Lipopolysaccharides (LPS – 10ug/ml)]. Cultured splenocytes were maintained at 37^0C, 5% CO_2, 95% air/O_2 for 24H.

2.3.2 Assay preparation

The plate was centrifuged, and 25 ul of the supernatant was removed and placed in ELISA plate (Mesoscale – MSD, Rockville, US) for measuring IL-10 secretion levels according to the manufacturer's instructions.

2.3.3 Analysis

The plates were processed using Mesoscale plate reader (Molecular Biology and Biochemistry core facility, Alberta Diabetes Institute, University of Alberta) and the data were analysed by assessing the mean + 2 SD variation with two tailed t test, using prism software (V.8)

2.4 Proliferation Assay – Carboxyfluorescein Succinimidyl Ester (CFSE)

2.4.1 Splenocytes staining and culture

Splenocytes from C57Bl/6 and BALB/c mice were cultured in media with DMEM 1x (Gibco, UK) + 10% FBS (Gibco, UK), with 10% antibiotics and antimycotic (1000 units penicillin/10mg streptomycin/25ug amphotericin/ ml) (Sigma-Aldrich), 1% non-essential amino acids (MEM NEAA 100X – Gibco, USA) and 1.5ul 2-Mercaptoethanol (Sigma).

Ten million splenocytes from C57Bl/6 and BALB/c were cultured in 1 ml prepared media in 15 ml conical tubes (Falcon, Mexico). Cells were centrifuged at 350g for 10 mins at 24^0C, supernatant was removed, and the cells resuspended in 1 ml PBS (Gibco, Waltham, MA, USA). 0.3ul CFSE (Biolegend, USA) was added and mixed with 10 million splenocytes. Splenocytes

were incubated for eight minutes, in the dark at room temperature and then centrifuged at 350g for 10 mins at 24^0C. Then the cells were washed twice using 10 ml media and then resuspended in media. Splenocytes were divided equally into 5 wells (2 million cells/100ul media) as follows:

1) NIL (unstained cells and no peptides added)

2) Unstimulated splenocytes + 2ul DMSO 30% (same concentration used to reconstitute peptides)

3) Stimulated splenocytes [2ul PMA- 5ng/ml (Sigma- Aldrich), 1ul Ionomycin (sigma- Aldrich), 0.75ul/ml anti-CD3e (BD pharmagin), 0.25ul/ml anti-CD28 (BD pharmagin)] + 2ul DMSO 30%

4) Stimulated splenocytes + Envelope peptide 46 (Mimotopes, Minneapolis, USA)

5) Stimulated splenocytes + Envelope peptide 65 (Mimotopes, Minneapolis, USA).

Splenocytes were incubated for 5 days in the incubator (37^0C, 5% CO_2) to allow for proliferation. Splenocytes were spun down at 350g for 10 mins at 24^0C, resuspended into 1ml cell staining buffer (CSB) (Biolegend, USA) and the surface T cell marker (anti-CD3e – Biolegend, USA) was added at a dilution of 1:1000. The cells were then maintained on ice (4^0C) in dark for 20 mins. Cells were centrifuged at 350g for 10 mins at 4^0C and washed in 2ml CSB twice. Pellets were resuspended into 100ul PBS and 1ul Live/Dead stain (Zombie Aqua Fixable Viability Kit – Biolegend, USA). The cells were incubated at room temperature for 10 mins in dark, washed twice in 2ml cold CSB and resuspended in 500ul cold CSB for analysis by flowcytometry (Flow core- University of Alberta).

2.4.2 Assessing lymphocytes proliferation using flow cytometry

Splenocytes were analysed by flow cytometry (Flow core, University of Alberta). Gating was performed in sequential manner: gating for single cells, live cells, CD3e positive cells and then for cell proliferation using CFSE stain.

2.4.3 Analysis

Facs files (.fcs) were analysed using Flowjo software (V.10). Graphs and statistics were performed using prism software (V.8)

2.5 Flow cytometry for detection of MMTV- infected B cells and IL-10 secretion

2.5.1 Cell staining

Splenocytes from NOD.c3c4 and C57Bl/6 mice were thawed in 1640 RPMI media with 10% FBS, 10% antibiotic and antimycotic and then spun down at 350g for 5 mins at room temperature. The pellets were resuspended in 100ul 1x PBS and 1ul of Live/Dead stain (Zombie Aqua), incubated in the dark for 10 mins and then washed twice using cooled cell staining buffer (CSB – Biolegend, USA). Then the cells were resuspended in 1ml CSB and 1ul of pan-B cell surface marker (B220/CD45R- Biolegend, USA) was added, incubated at 4^0C in dark for 20 mins and consequently centrifuged and pellets were washed twice in 2ml CSB. The splenocytes were resuspended in 500ul of fixation buffer (Biolegend, USA). Cells were incubated in dark at room temperature for 20 mins and washed twice in 1x perm wash buffer (PWB – Biolegend, USA). To permeabilize the cells, they were incubated in 1000ul of 1x PWB (Biolegend, USA) for 20 mins. Then, washed twice incubated with 1ul of rabbit anti-p27 (MMTV Capsid protein – Rockland, USA) with 1ml of 1x PWB in a dilution of 1:1000 and kept at room temperature in dark for 20 minutes. Then they were washed twice in 1x PWB. The pellets were resuspended in 1ml 1X PWB in addition to anti rabbit Alexa flour 647, as a secondary antibody for anti-p27, in addition to anti-IL-10 (PE – Biolegend, USA) was added to detect intracellular IL-10 secretion, both added in a dilution of 1:1000. The cells were incubated in dark for 20 mins at room temp. Finally, they were

washed twice in 2 ml CSB and the pellet was resuspended in 500ul CSB. The flowcytometry was done within 24 hours after.

2.5.2 Analysis

The FACS files (.fcs) were analysed using Flowjo software (V.10). The gating was performed in the following sequence: splenocytes, single cells, live cells, B cells and MMTV-infected B cells secreting IL-10. Graphs and statistics were performed using prism software (V.8)

Chapter 3: Results

Section 1: Characterizing Immunosuppressive domains in MMTV Env

3.1: Increased secretion of IL-10 in response to MMTV Env peptide stimulation

Retroviruses are known to modulate the host immune response via immunosuppressive domains (ISD) harbored within the Envelop protein [57]. Previously, HBRV Env peptides were synthesized in our laboratory by Mandana Rahbari, who characterized ISD using the HBRV Env peptides for activity in healthy human PBMC.

For these studies, 85 HBRV Env peptides were synthesized as overlapping 18-mer amino acids (Table 1), which were then tested for their ability to (i) trigger IL-10 secretion in PBMC and (ii) inhibit the proliferation of T lymphocytes (M. Rahbari, CDDW, 2015). Two ISD were characterized within the Surface and Transmembrane proteins of HBRV, peptides 45 and 64 respectively (CDDW, 2015). Using alanine mutagenesis, the conserved amino acids in the transmembrane peptide (Env 64) were further characterized as being essential for maintaining the immunosuppressive function with IL-10 production (M. Rahbari, CDDW, 2015).

In order to characterize ISD in MMTV Env, the 85 customized Env peptides of MMTV/HBRV were tested individually to identify those with characteristics of ISD that specifically promoted IL-10 secretion. Accordingly, splenocytes from healthy BALB/c mice without evidence of MMTV infection were incubated with individual peptides for 24 hours and then 25 ul of the supernatant were collected for ELISA detection of IL-10 on the Mesoscale plate. The data were normalized by dividing individual values by the mean value of 85 individual peptides and then IL-10 levels for individualized peptides were plotted on a heatmap and compared to the responses observed in humans. Peptides promoting IL-10 production > mean + 2x standard deviations (2.1 pg/ml) were considered significantly elevated. In the BALB/c mice, Env

46 and Env 65 as were identified as promoting the highest production of IL-10 (Table 2).

Interestingly, Env 46 and Env 65 mice found to have an overlap with the ISD characterized in humans, Env 45 and Env 64 (Figure 3.1).

ENV	BALB/c	Human	ENV	BALB/c	Human
1	1.8	0.4	43	0.3	0.3
2	0.5	0.4	44	0.7	0.3
3	0.0	1.1	*45	1.7	24.9
4	0.0	0.0	*46	2.5	0.6
5	0.0	0.6	47	0.8	0.9
6	0.0	0.8	48	0.5	0.6
7	0.0	0.6	49	0.4	0.0
8	0.0	2.0	50	0.8	0.4
9	1.8	0.0	51	0.1	0.5
10	0.0	0.9	52	0.6	1.8
11	0.0	0.0	53	0.4	0.7
12	1.4	0.6	54	0.4	0.0
13	1.0	0.4	55	0.7	0.4
14	0.4	1.2	56	0.6	0.6
15	0.9	2.2	57	0.8	0.8
16	0.1	0.0	58	0.8	0.4
17	0.0	0.7	59	0.8	0.4
18	0.0	1.0	60	1.5	1.0
19	0.1	0.8	61	1.8	1.7
20	1.6	0.0	62	0.6	3.0
21	1.4	0.8	63	1.3	0.9
22	0.0	1.1	*64	1.5	5.2
23	1.6	0.0	*65	2.5	0.5
24	2.0	0.5	66	1.5	1.4
25	1.0	1.8	67	1.2	0.5
26	0.1	0.5	68	1.8	0.6
27	0.5	0.6	69	1.6	0.6
28	0.0	0.6	70	0.4	1.2
29	0.0	0.0	71	1.7	0.6
30	0.1	0.4	72	1.4	0.6
31	0.2	1.5	73	1.8	0.9
32	0.6	0.4	74	1.4	0.6
33	0.5	0.8	75	0.5	1.3
34	0.8	1.4	76	1.5	0.8
35	0.5	0.5	77	1.0	0.4
36	0.0	0.0	78	0.8	0.9
37	1.5	1.0	79	1.6	1.2
38	0.5	2.7	80	1.2	1.3
39	0.2	0.6	81	0.7	17.4
40	0.1	1.1	82	1.5	0.7
41	0.6	0.8	83	1.2	1.9
42	0.5	0.0	84	0.7	0.5
			85	0.0	0.5

Table 2 IL-10 production in BALB/c splenocyte following stimulation with MMTV/HBRV Env peptides as compared to human PBMCs. *Splenocytes from BALB/c were cultured with 85 individual peptides. Supernatants were assessed by ELISA to determine IL-10 production in response to individual peptides. Normalization was performed by dividing individual values (pg/ml) by nil. The cut-off point was determined based on the mean plus two standard deviations. Values in BALB/c mice were compared with those derived from human PBMCs in our laboratory by M. Rahbari.*

ILKRPGFQEHDMIPTSAC *(ENV 45 - Human)*
EHDMIPTSACATYPYAIL *(ENV 46 - Mouse)*

EQRIIDLK**LEARLNALEG** *(ENV 64 - Human)*
LEARLNALEGVVLELGQD *(ENV 65 - Mouse)*

Figure 3.1 Overlap between ISDs in humans (45, 64) and mice (46, 65). *The overlap in amino acids sequence between two consecutive peptides.*

3.2: Inhibition of lymphocytes proliferation in response to Immunosuppressive domains

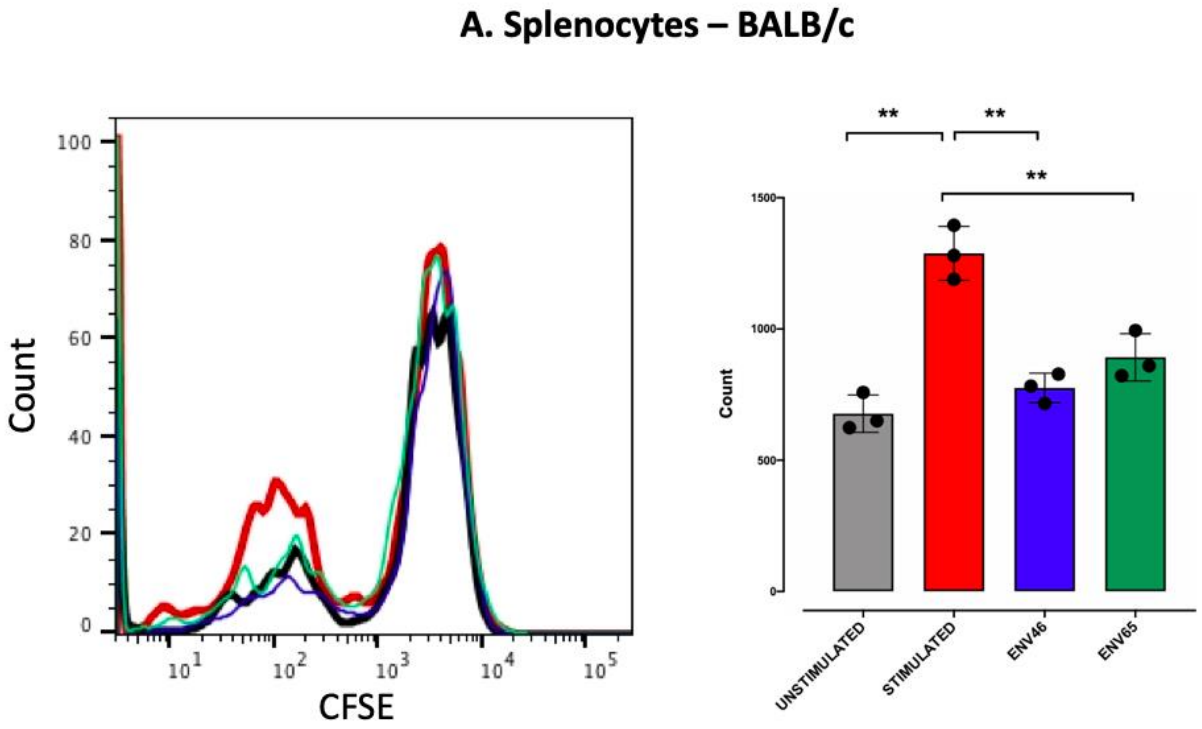

Figure 3.2 (A) Inhibition of lymphocyte proliferation in response to Immunosuppressive peptides in BALB/c. *The graph showed marked reduction in proliferation lymphocytes of BALB/c (n=3) in response to both Env 46 (SU) and Env 65 (TM). Proliferation assay was performed by flowcytometry after 5 days of incubation with the two identified ISD. **p < 0.001, by two-tailed t-test, using GraphPad (prism v.8) software.*

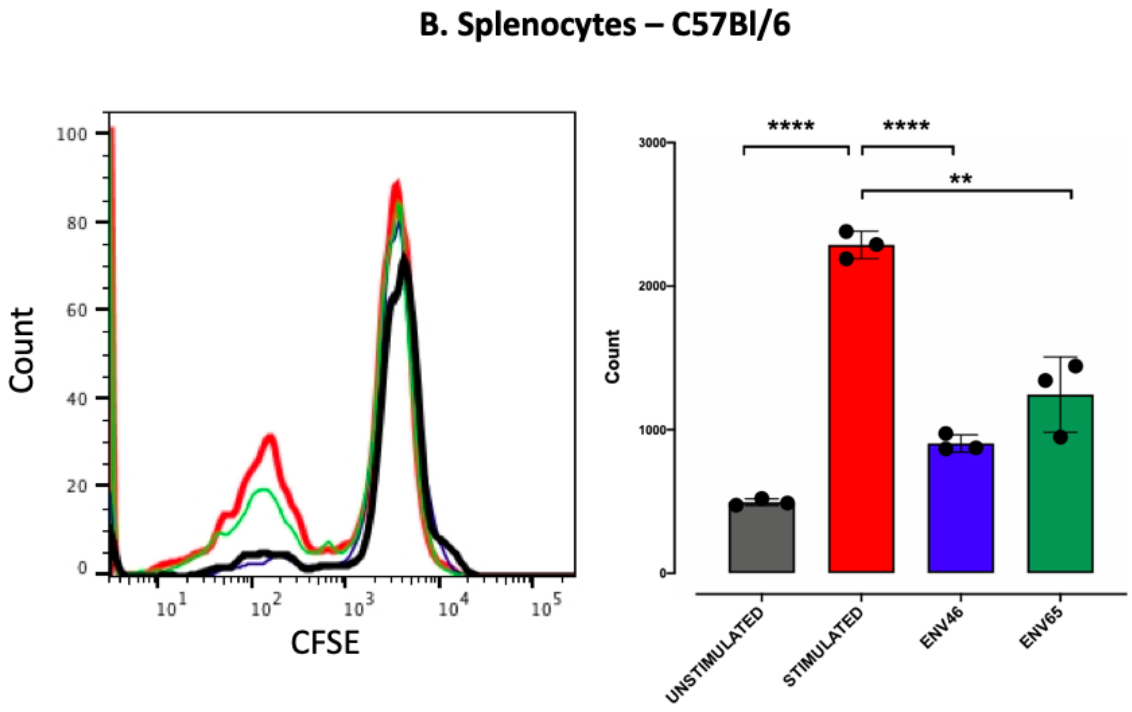

Figure 3.2 (B) Inhibition of lymphocyte proliferation in response to Immunosuppressive peptides in C57Bl/6. *The graph shows marked reduction in proliferation of lymphocytes in C57Bl/6 (n=3) in response to both Env 46 (SU) and Env 65 (TM). Proliferation assay was done by flowcytometry after 5 days of incubation with the two identified ISDs. **p < 0.001, ****p < 0.0001, by two-tailed t-test, using GraphPad (prism v.8) software.*

Section 2: Identifying the effect of MMTV on IL-10 producing B cells

3.3 Comparison of B cells from MMTV infected NOD.c3c4 to healthy C57Bl/6

Whole Splenocytes from C57Bl/6 (n=3) and NOD.c3c4 (n=3) were prepared for immune staining using pan B surface marker (B220/CD45R) to compare the percentage of B cells in splenocytes the NOD.c3c4 versus C57Bl/6.

3.4 Identifying MMTV- Infected B cells

In order to identify MMTV- infected B cells, splenocytes were intracellularly stained by anti- MMTV p27 Capsid antibody and the B cells were gated using flow cytometry to identify the

anti-p27 positive population. C57Bl/6 splenocytes lacked evidence of MMTV infection, while approximately 2% of the NOD.c3c4 B cells had evidence of infection. [Figure 3.3 (A,B)]

3.5 Comparing IL-10 producing B cells in the NOD.c3c4 mouse model of PBC to healthy control C57Bl/6 mice

IL-10 producing B cells are a subpopulation of lymphocytes that have regulatory properties but no specific surface marker. In order to detect the IL-10 secreting B cells, immunostaining was performed using anti-IL-10 for flow cytometry in both NOD.c3c4 and C57Bl/6 splenocytes. IL-10 populations were sub-divided into anti-MMTV p27 positive infected cells and negative cells, in the B cell populations.

A significant difference in the percentage of B cell populations between NOD.c3c4, PBC mouse model versus C57Bl/6 healthy control was observed with a mean percentage of 45% & 38% respectively ($^{*}p < 0.01$) [Figure 3.3 (C)]. The presence of MMTV (anti-p27 + ve) in NOD.c3c4 mice played an important role in the proportion of B cell population in NOD.c3c4 compared to C57Bl/6. Where 2% of NOD.c3c4 B cells have evidence of MMTV infection, while 0.021% of C57Bl/6 B cells only express MMTV p27 protein from endogenous retrovirus ($^{**}p \leq 0.001$) [Figure 3.4 (A)]. Furthermore, there was a noteworthy increase in the proportion of B cells secreting IL-10 in NOD.c3c4 compared to C57Bl/6 with a mean value of 1.38% & 1.82% respectively.

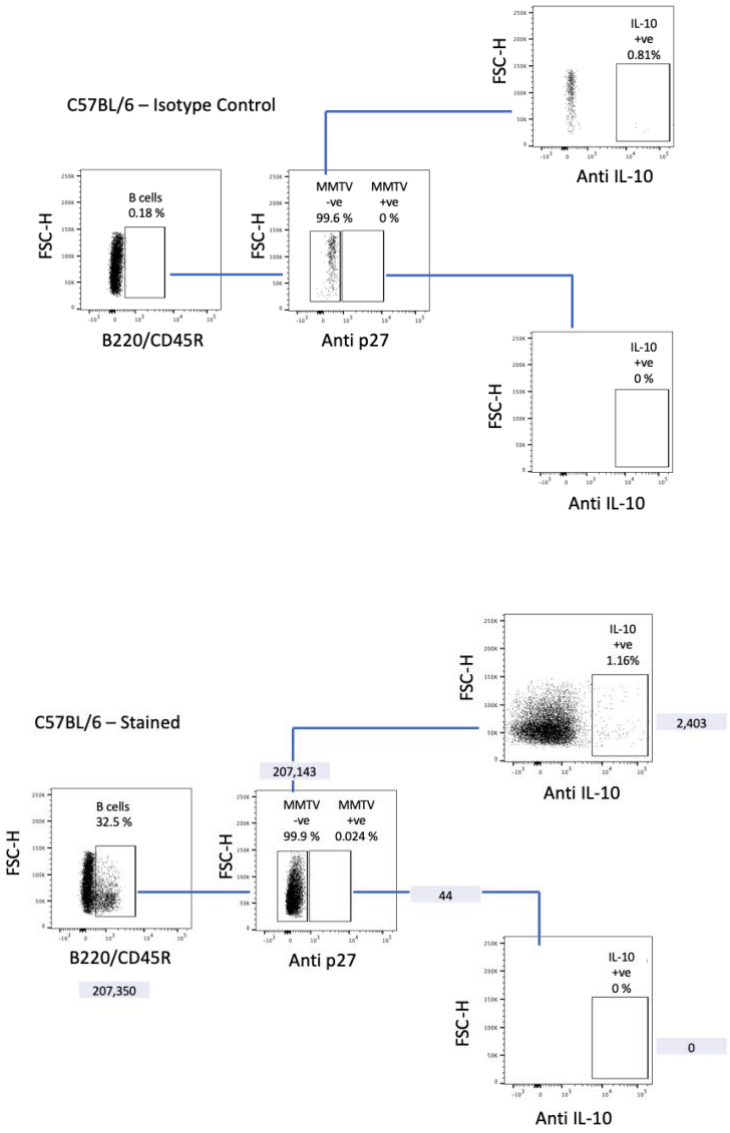

Figure 3.3 (A) Identification of capsid p27 +ve B cells in C57Bl/6 mice as well as B cells secreting IL-10*. Gating strategy using an Isotype control splenocytes from C57Bl/6 (n=3) were used for gating. Splenocytes from C57Bl/6 were stained for B cells (B220/CD45R) and cells were fixed and permeabilized for intracellular staining of MMTV using capsid anti-p27) & IL-10 cytokine. The percentage of Il-10 positive B cells in C57Bl/6 was 1.16 $\pm$ 0.5%*

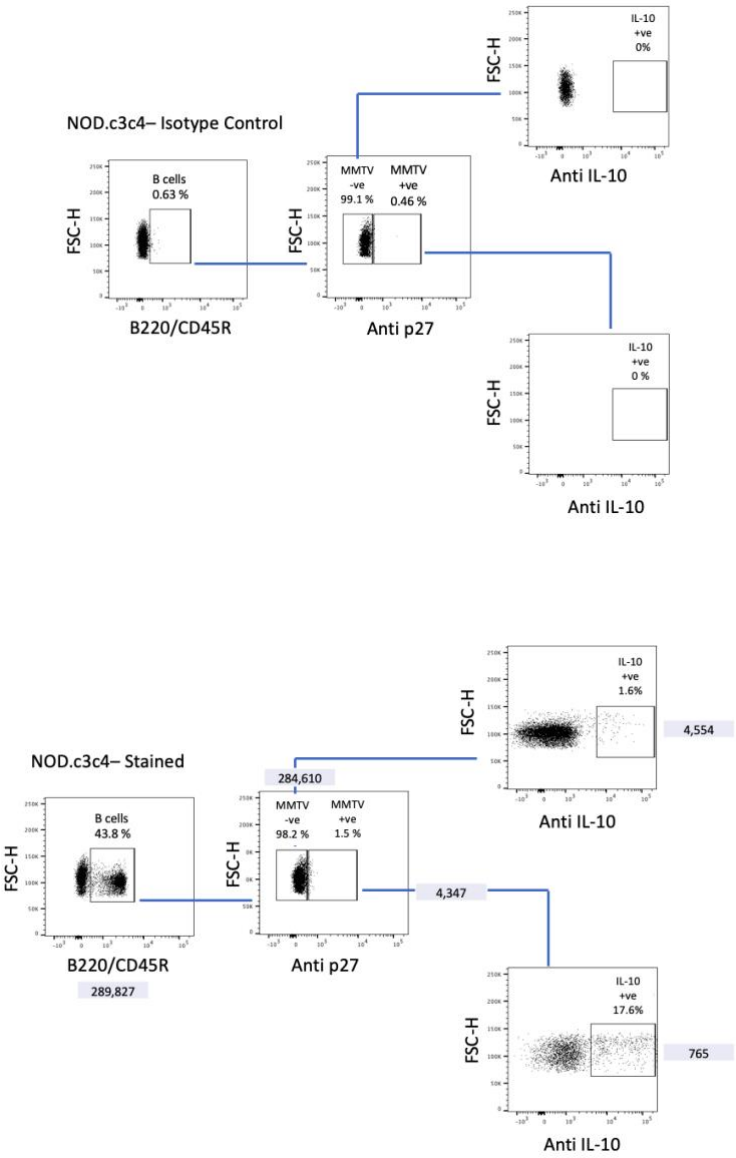

Figure 3.3 (B) Identification of MMTV p27 Capsid +ve B cells in NOD.c3c4 mice as well as B cells secreting IL-10*. Gating strategy using an Isotype control splenocytes from NOD.c3c4 (n=3) were used for gating. Splenocytes from NOD.c3c4 were stained for B cells using pan B cell surface marker (B220/CD45R), MMTV using capsid anti-p27 (primary Antibody) and Alexaflour 647 (secondary Antibody) and cells were fixed and permeabilized for intracellular staining of IL-10. The percentage of B cells secreting IL-10 in MMTV negative B Cells in NOD.c3c4 ranged from 0.8% - 1.6% while for MMTV- infected B cells, the percentage of IL-10 secreting cells was 17% - 22%*

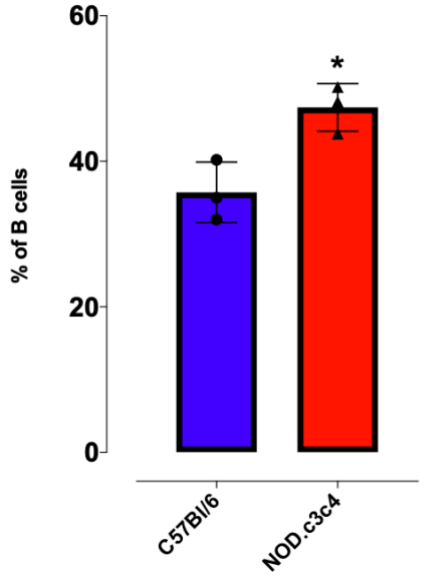

Figure 3.3 (C) Percentage of B cell population in NOD.c3c4 compared to healthy C57Bl/6. *NOD.c3c4 mice have ~ 15% more B cells in the spleen (340,646 B cells) compared to C57Bl/6 (218,880 B cells). * $p < 0.01$, by two-tailed t-test, using GraphPad (prism v.8) software.*

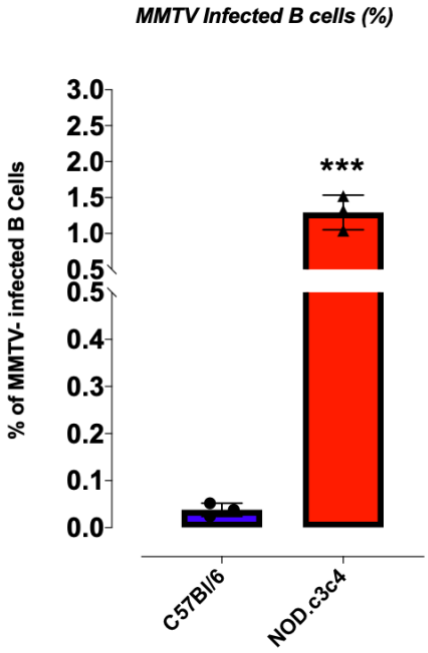

Figure 3.4 A subpopulation of B cells in NOD.c3c4 have evidence consistent with MMTV infection*. Almost 1.5 percent of NOD.c3c4 B cells had evidence of anti-MMTV p27 Capsid (4,347 B cells), whereas C57Bl/6 B cells only express MMTV p27 protein from endogenous retrovirus*

*expression (44 B cells). *** P ≤ 0.0001, by two-tailed t-test, using GraphPad (prism v.8) software.*

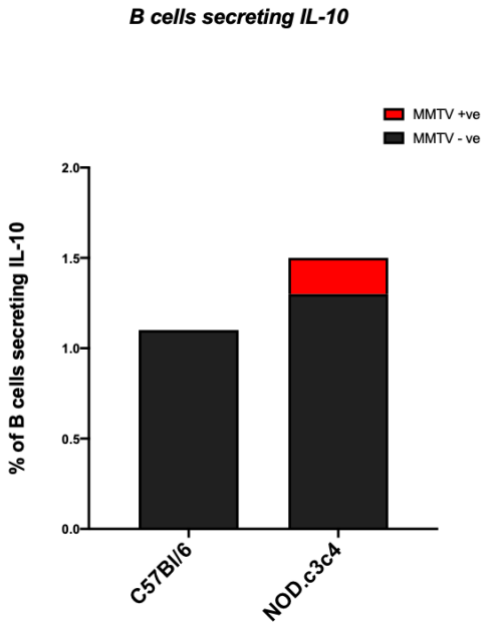

Figure 3.5 A proportion of B cells secreting IL-10 in the NOD.c3c4 have MMTV infection. *A higher proportion of B cells in NOD.c3c4 mouse splenocytes produce IL-10 with a mean percentage of 1.5% compared to 1.1% in C57Bl/6 and a subpopulation have evidence of MMTV infection of a mean percentage of 0.5% in NOD.c3c4 compared to 0% in C57Bl/6*

Chapter 4: Discussion

4.1 Introduction

In this study, we investigated the effect of one of the environmental factors involved in the pathogenesis of PBC. Previous work performed in our laboratory was able to identify a betaretrovirus in patients with PBC [56], closely related to MMTV in mice [23]. The NOD.c3c4 mouse model with autoimmune biliary disease also demonstrates evidence of MMTV infection and this permitted studies to determine how viral infection may modulate innate immune responses.

Herein, we provide evidence that autoimmune biliary disease in mice, similar to PBC in humans, is associated with an increased percentage of B cells secreting IL-10. Reportedly, this regulatory phenotype favors both viral replication as well as modulating the host immune response, leading to disease progression[58].

The key findings of this study are:

a) IL-10 secretion in splenocytes is triggered in response to two MMTV Env peptides that demonstrate characteristics of ISD (Table 2).
b) Similar ISD have been characterised in HBRV in human PBMC (Figure 3.1).
c) The ISDs have the capacity to inhibit lymphocytes proliferation in different strains of mice (Figure 3.2 A, B).
d) These data are consistent with reports that IL-10 secreting B cells infected with MMTV have a role in modulating the host immune response [58].

In the following sections, I will highlight the importance of these findings in relation to previous studies and discuss the implications of this study for future research.

4.2 The relation between MMTV, B cells and IL-10 secretion

In 1994, Beutner et al. studied the role of B cells for transmission of MMTV in B cell knock out models. They found that in the absence of B cells, MMTV transmission was significantly impaired, breast cancer rarely occurred and they concluded that B cells played a pivotal role in the transmission of MMTV [59]. Subsequently, Golovkina's laboratory reported that weanling pups developing MMTV infection from the mothers' milk, secreted IL-10 in the gut associated lymphoid tissue that resulted in a lack of neutralizing antibody formation. They concluded that, MMTV binds to LPS that in turn binds TLR4 and promotes the secretion of IL-10. Under the influence of IL-10, IFN-gamma type I inflammatory cytokines were blocked, thus preventing cells from making neutralizing anti-MMTV Env antibodies [60].

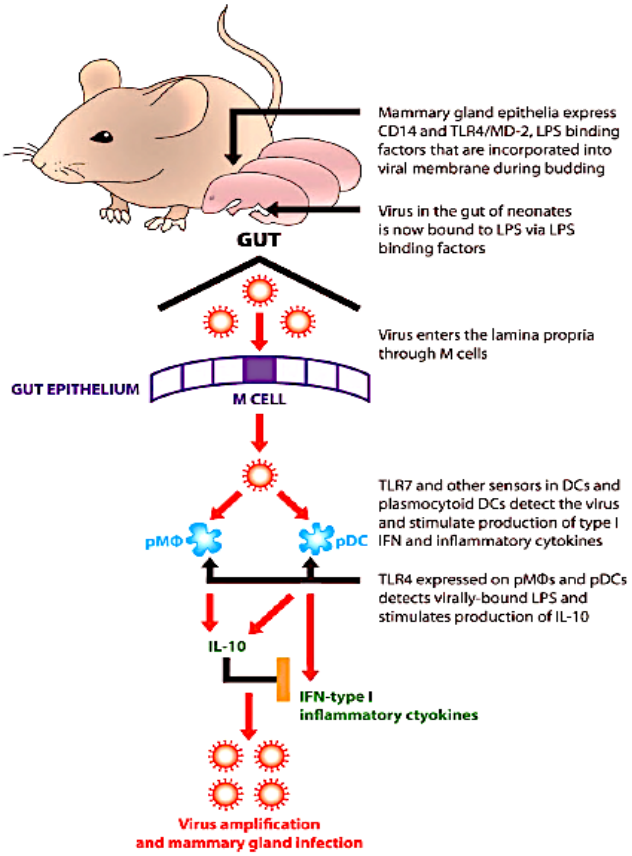

Figure 4. The hypothesis derived from prior studies in the Golovkina lab[58, 60].

While we agree with the general model that IL-10 secretion prevents immune response to the virus, our study suggests another potential mechanism. In our study, we found increased B cell population in NOD.c3c4 compared to C57Bl/6, with increased IL-10 secretion and a proportion of these B cells had evidence of MMTV infection. These findings are in agreement with prior studies regarding the effect of MMTV on both the expansion of B cells and IL-10 secretion, that in turn led to lack of neutralizing anti-MMTV Env antibodies [56].

4.3 Identification of immunosuppressive domains in MMTV in mouse models.

MMTV is a betaretrovirus that reportedly have conserved regions in their Env protein that modulates the host immune response due to the presence of ISD [61]. Taken together with the finding of IL-10 secretion with MMTV infection, we hypothesized that MMTV might have the ability to promote IL-10 secretion through ISD in the Env protein. Also, it is known that ISD inhibit lymphocyte proliferation. To investigate the hypothesis that MMTV Env harbored ISD, we used 85 HBRV Envelop peptides and characterized two peptides, Env 46 and Env 65 in the Surface and Transmembrane respectively with ISD activity. These ISDs had overlapping amino acids with previously identified HBRV ISD in humans, which has been previously reported for ISD activity in different species (M. Rahbari, CDDW, 2015).

4.4 The role of IL-10 producing B cells in modulating the host immune response and the development of autoimmune biliary disease in response to MMTV Envelop

As mentioned previously, Kane et al emphasized that MMTV promotes IL-10 secretion to tolerize the neonatal immune response against MMTV [58]. In order to investigate the effect of MMTV on IL-10, we investigated the presence of MMTV in the NOD.c3c4 ABD mouse model and C57Bl/6 healthy controls. Through intracellular staining and flow cytometry, we were able to identify the presence of the MMTV p27 Capsid protein in NOD.c3c4 splenocytes. Whereas there was minimal signal of MMTV p27 Capsid in C57Bl/6 splenocytes less than 1 in 1000 cells as reported by our laboratory previously [56]. Looking into IL-10 secretion between MMTV-infected and noninfected B cells in NOD.c3c4 mouse, we were able to find a significant increase in the percentage of MMTV-infected B cells secreting IL-10 in NOD.c3c4 mice compared to healthy control C57Bl/6. In this study, we were able to highlight the effect of ISDs harbored within the MMTV and we hypothesized that this effect was responsible for the IL-10 producing B cells.

The novelty of this study was to link MMTV to these B cells secreting IL-10, which is a new hypothesis different from virus-bounded LPS.

4.5 Future directions

This study provides an insight into immunological understanding for the pathogenesis of primary biliary cholangitis through the role of IL-10 secretion. Further work has to be done in NOD.c3c4 to test the *in vivo* effect of blocking IL-10 on both viral replication as well as disease progression.

4.6 Conclusion and Implications of research

The novelty of the study was to investigate the possible effect of immunosuppressive domains in MMTV on the host immune response. We identify ISD in MMTV Env and characterized a MMTV infected B cell population secreting IL-10.

Usually it is assumed that autoimmune diseases lack regulatory cells secreting IL-10. Whereas, our studies suggest that the IL-10 secretion in B cells is in part responsible for chronic MMTV infection. Accordingly, this hypothesis can be tested in further studies.

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