# ASSESSING THE IMMUNE COMPETENCE OF DAIRY CATTLE NATURALLY INFECTED WITH BOVINE LEUKEMIA VIRUS

**By**

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Bovine leukemia virus (BLV) is a δ-retrovirus that is the causative agent of enzootic bovine leukosis (EBL). BLV is extremely prevalent in US dairy herds. Over 83% of dairy herds are predicted to be BLV-infected, and with an average within-herd prevalence nearing 50%, it is estimated that over 40% of all dairy cows in the United States are BLV+. Although the prevalence of BLV infection is quite high, it is fairly uncommon for dairy producers to monitor infection within their herds: a recent survey found that less than 13% of dairy producers even test for BLV. It is likely this disinterest is the result of the low incidence of clinical disease. While BLV infection does cause lethal lymphosarcomas, fewer than 5% of infected cattle are expected to develop cancer as a result of BLV infection.

Despite the low incidence of oncogenesis, BLV infection has been associated with decreased milk production and decreased longevity in infected cows. It is now hypothesized that BLV actually impairs immune function in infected cattle and that BLV+ cows are more at risk for contracting secondary infections, and that those secondary infections are responsible for impaired production and health observed in BLV+ cows. Although there was strong evidence for abnormal immune cell populations and functions *in vitro,* there were only a few studies investigating how well the immune system of BLV+ cows functioned *in vivo*. The goal of this dissertation was to investigate the *in vivo* function of the immune system in BLV+ cows.

To investigate the *in vivo* function of the immune system in BLV+ cows, we tracked B and T cell responses to a routine vaccination and to a primary and secondary antigenic exposure. We found that BLV+ cows consistently exhibited lower levels of antigen-specific IgM and equal

levels of IgG1 in comparison to BLV- cows. Based on our data, it is also possible that BLV+ cows produce less antigen-specific IgG2, although our data suggests that altered IgG2 production would not be observed across all antigens and that impaired IgG2 production requires a high level of antigen exposure in BLV+ cows. We also observed reduced circulating effector or memory B and γδ T cell populations in BLV+ cows, as well as abnormal B and T cell responses to antigenic and mitogenic stimulation *in vitro*. Overall, we observed both reduced and abnormal immune function in the B and T cell compartments in BLV+ cows relative to BLV- cows.

When we investigated possible virulence mechanisms by which BLV interferes with IgM production, we found that B cells from BLV+ cows exhibited reduced IGJ, BLIMP1, and BCL6 expression. In addition, markers of BLV transcription were negatively correlated with IgM levels and BLIMP1 and BCL6 expression. In particular, BLIMP1 and BCL6 may be direct targets of BLV miRNAs. Overall, we observed a possible mechanism by which IgM production in BLV+ cows is impaired, although the data would suggest a global, as opposed to IgM-specific, pathway of impaired antibody production.

All together, our data demonstrate that BLV infection in dairy cattle has a much greater effect on the host beyond lymphoma development. Indeed, our data support overall impairment of antibody production and interference with T cell immunity, which likely contributes to an increased risk of secondary infections in BLV+ cattle.

This dissertation is dedicated to the cows. I couldn't have done it without my girls.

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## KEY TO ABBREVIATIONS

ACD: acid citrate dextrose

ACTB: β-actin

AL: aleukemic

BCL6: B-cell CLL/lymphoma 6

BCR: B cell receptor

BHV1: bovine herpesvirus type 1

BLIMP1: B-lymphocyte-induced maturation protein 1

BLV: bovine leukemia virus

BoLA: bovine leukocyte antigen

Bovi-Shield: Bovi-Shield GOLD® FP® 5 L5 HB

BVD1: bovine viral diarrhea virus type 1

BVD2: bovine viral diarrhea virus type 2

Cel-39: *C. elegans* microRNA-39

ConA: concanavalin A

DIM: days in milk

EBL: enzootic bovine leukosis

EDTA: Ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

Env: envelope protein

gp51: BLV glycoprotein 51

HIV: human immunodeficiency virus

HPRT: Hypoxanthine Phosphoribosyltransferase 1

HRP: horseradish peroxidase

HTLV-1: human T cell lymphotropic virus type I

IFNγ: interferon-γ

IgA: immunoglobulin A

IgG: immunoglobulin G

IgM: immunoglobulin M

IL10: interleukin-10

IL12p40: interleukin-12p40

IL2: interleukin-2

IL4: interleukin-4

IL6: interleukin-6

KLH: keyhole limpet hemocyanin

LPS: lipopolysaccharide

LTR: long terminal repeat

MAb: monoclonal antibody

MFI: mean fluorescence intensity

MHCI: major histocompatibility complex type I

MHCII: major histocompatibility complex type II

miRNA: microRNA

MOI: multiplicity of infection

NAb: neutralizing antibody

OD: optical density

P/I: PMA and ionomycin

p24: BLV capsid protein

PAX5: paired box 5

PBMC: peripheral blood mononuclear cell

PBS: phosphate-buffered saline

PHA: phytohaemagglutinin

PL: persistent lymphocytosis

PMA: phorbol myristate acetate

Pol: polymerase

PPIA: peptidylprolyl isomerase A

PVL: proviral load

PWM: pokeweed mitogen

qPCR: quantitative polymerase chain reaction

qRT-PCR: quantitative reverse transcription polymerase chain reaction

RNAPII: RNA polymerase II

RNAPIII: RNA polymerase III

RPMI: Roswell Park Memorial Institute

RT: reverse transcription

SEM: standard error of the mean

SIgM: surface immunoglobulin M

Th1: T-helper 1

Th2: T-helper 2

TLC: total leukocyte count

TMB: 3,3′ ,5,5′ - Tetramethylbenzidine

TNFα: tumor necrosis factor α

Tregs: regulatory T cells

U6: small noncoding RNA U6

WT: wild type

# CHAPTER 1: BOVINE LEUKEMIA VIRUS: A MAJOR SILENT THREAT TO PROPER IMMUNE RESPONSES IN CATTLE

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**Abstract**

Bovine leukemia virus (BLV) infection is widespread in the US dairy industry and the majority of producers do not actively try to manage or reduce BLV incidence within their herds. However, BLV is estimated to cost the dairy industry hundreds of millions of dollars annually and this is likely a conservative estimate. BLV is not thought to cause animal distress or serious pathology unless infection progresses to leukemia or lymphoma. However, a wealth of research supports the notion that BLV infection causes widespread abnormal immune function. BLV infection can impact cells of both the innate and adaptive immune system and alter proper functioning of uninfected cells. Despite strong evidence of abnormal immune signaling and functioning, little research has investigated the large-scale effects of BLV infection on host immunity and resistance to other infectious diseases. This review focuses on mechanisms of immune suppression associated with BLV infection, specifically aberrant signaling, proliferation and apoptosis, and the implications of switching from BLV latency to activation. In addition, this review will highlight underdeveloped areas of research relating to BLV infection and how it causes immune suppression.

Keywords: bovine leukemia virus, retrovirus, immune suppression, apoptosis, latency

## Introduction

Bovine leukemia virus (BLV) is a common infection in US dairy cattle and is the causative agent of enzootic bovine leukosis (EBL). EBL is characterized by three disease stages: asymptomatic or aleukemic (AL), persistent lymphocytosis (PL), and leukemia or lymphoma (Bartlett et al., 2013). Approximately 30% of infected animals will develop PL, while 0.1-10% of infected animals will develop either leukemia or lymphoma (Kabeya et al., 2001). Although BLV has been eradicated in 22 countries worldwide (Bartlett et al., 2014), BLV infection in the United States is widespread. Over 83% of US dairy herds are BLV-infected and the within-herd infection rates typically exceed 30% (USDA-NAHMS, 2007). A growing body of evidence supports that BLV infection negatively impacts dairy production in the US, which has recently been extensively reviewed (Bartlett et al., 2014). However, little is known about how BLV infection affects the risk of other infectious diseases in cattle, which could have a large impact on US dairy production.

Much research has been conducted on how BLV infection alters the host immune system. BLV preferentially infects B cells (Schwartz et al., 1994) and causes a benign, polyclonal expansion of B cells during PL (Meirom et al., 1997). However, evidence supports abnormal functioning of not only B cells, but also T cells and monocytes, including differential cytokine production, surface receptor expression and proliferative and apoptotic capacities. Despite evidence that BLV disrupts normal immune functioning, little research has investigated if this dysregulation impacts bovine immune health and increases the risk of developing other infectious diseases. Although a few studies have suggested that BLV infection increases susceptibility to other infectious diseases (Emanuelson et al., 1992; Erskine et al., 2011a; Trainin et al., 1996), it is essential to explore this risk more fully to best assess if and how the US should

reduce BLV prevalence. This review will discuss research specific to the impact of BLV on immune cells and the immune system as a whole, as well as BLV latency and reactivation and how BLV's replicative strategy may impact immune health.

## Abnormal Immune Function in BLV-Infected Cattle

### Cytokine production and activity

One of the major effector functions of the immune system is production of cytokines, which have varied critical functions including the growth, polarization and responsiveness of various immune cell types and regulation of the strength and duration of immune responses. A large body of evidence indicates that BLV infection alters circulating cytokine levels and production of cytokines in response to stimuli. One study found that freshly isolated peripheral blood mononuclear cells (PBMCs) from PL cattle express less IL2, IL4 and IFNγ mRNA when compared to uninfected cattle, while fresh PBMCs from AL cattle express less IL4 and IFNγ. PL cattle also express less IL2 in comparison to AL cattle and possibly express less IL10 as well (at $p<0.1$) (Amills et al., 2002). Freshly isolated PBMCs from AL cattle have increased IL12p40 mRNA in comparison to uninfected cattle, but PL cattle express less IL12p40 mRNA in comparison to uninfected animals (Pyeon and Splitter, 1998). This reduction in cytokine gene expression in BLV-infected animals may indicate decreased cytokine transcription and, consequently, less cytokine activity in fresh PBMCs. Serum levels of IL6 are also significantly higher in PL cows in comparison to serum from either AL or uninfected cattle (Trainin et al., 1996).

Immune cells from BLV-infected animals also demonstrate aberrant cytokine production in response to various stimulants *in vitro*. PBMCs isolated from PL cattle show increased IL2 activity after culture with concanavalin A (ConA) compared to both AL and uninfected cattle (Sordillo et al., 1994). PBMCs from PL cattle cultured in the presence of either ConA, LPS, or BLV envelope protein gp51 also produce increased IL6 in comparison to both AL and uninfected cattle (Trainin et al., 1996). ConA stimulation of cultured PBMCs isolated from PL and AL

cattle also have increased IL2, IL10, and IFNγ expression when compared to uninfected cattle, although there is no difference in IL4 production. In PBMCs from AL and PL cattle, IL2 and IL10 expression is delayed in culture, although IFNγ and IL4 have the same expression kinetics as in cells from uninfected cattle (Trueblood et al., 1998). Although freshly isolated $CD4^+$ lymphocytes from PL cattle produce less IL2 and IL4, IL2 and IL4 expression is similar between uninfected and PL cattle after culture in the presence of ConA (Amills et al., 2002). These *in vitro* results suggest that PBMCs from PL cattle are more sensitive to stimulation in culture than PBMCs from uninfected cattle.

The reported changes in *ex vivo* and *in vitro* cytokine expression are interesting because they cover a wide range of immune responses. IFNγ, IL2 and IL12 are all proinflammatory cytokines characteristic of a Th1 response (Mosmann et al., 1986), while IL4 is characteristic of a Th2 or humoral response (Bao and Cao, 2014). IL10 regulates and suppresses the proinflammatory immune response (Pestka et al., 2004). IL6 can induce T cell activation and the development of B cells into plasmoblasts. Although there is no correlation between B cell leukemogenesis and IL6 receptor expression on PBMCs, PBMCs from cattle with PL have less binding affinity for IL6 in comparison to PBMCs from uninfected cattle (Droogmans et al., 1994). This reduction in responsiveness could explain the increased circulating IL6 in PL cattle seen by Trainin *et al*., 1996. Due to the wide range of functions most cytokines exhibit, alteration in their expression could have varied implications for clearing infections, response to vaccines, tissue remodeling, and inflammation in BLV-infected cattle.

**B cell abnormalities**

In addition to evidence of abnormal immune signaling in BLV-infected animals, many studies detail extensive dysregulation of the humoral immune response. The most obvious

difference between uninfected and infected animals is the polyclonal B cell expansion that is characteristic of PL animals (Meiron et al., 1985). In cattle, the polyclonal expansion is almost exclusively within the $CD5^+$ B cell subset: 90.5% of B cells from PL cattle express CD5, while only 31% of B cells from uninfected cattle will typically express CD5 (Stone et al., 1995). $CD5^+$ B cells are also the primary targets for BLV proviral integration: 95% of gp51-expressing cells are $CD5^+IgM^+$ (Meirom et al., 1997). In humans and mice, $CD5^+$ B cells represent a distinct non-classical B cell lineage called B-1a cells. These cells originate from the fetal liver, respond to T-cell independent antigens, produce natural IgM (Carsetti et al., 2004) and likely will not form memory B cells (Tung et al., 2006). In sheep, BLV-induced polyclonal expansion occurs in $CD11b^+$ B cells, which are believed to be analogous to B-1 cells in mice (and, consequently, cattle) (Chevallier et al., 1998).

Freshly isolated B cells from PL cattle show no difference in either MHCI or MHCII mRNA expression in comparison to either AL or uninfected cattle (Teutsch and Lewin, 1996). However, surface expression of MHCII is increased on B cells isolated from both AL and PL cattle in comparison to uninfected cattle (Isaacson et al., 1998; Sordillo et al., 1994; Stone et al., 1995), while surface expression of CD25 is decreased (Isaacson et al., 1998). When PBMCs are cultured in the presence of IL2, only B cells from PL cattle increase CD25 surface expression (Stone et al., 1995). However, isolated B cells from PL cattle cultured with IL2 fail to upregulate CD25 surface expression (Trueblood et al., 1998), indicating that PL B cell responsiveness to IL2 stimulation is likely T cell dependent. Pokeweed mitogen (PWM) stimulation of cultured PBMCs increases CD25 surface expression on B cells from both PL and uninfected cattle, but PL cattle show significantly increased CD25 upregulation in comparison to uninfected animals (Stone et al., 1995). Interestingly, in the same study of cultured PBMCs, IL2 stimulation

increased MHCII surface expression on B cells isolated from uninfected cattle, but had no effect on MHCII expression on B cells isolated from PL cattle. In line with the numerous alterations in B cell function in animals infected with BLV, Klener *et al.,* 2006 performed a microarray assessing the differences between ovine B cells cultured in the presence or absence of Tax. They found significant gene expression differences in the signaling pathways DNA transcription and repair, cell cycle regulators, kinases, phosphatases, and small Rho GTPases/GTPase binding proteins (Klener et al., 2006). The microarray data suggests widespread signaling dysfunction in B cells exposed to Tax protein, which could explain abnormal B cell receptor and cytokine expression. All together, these data indicate that resting B cells from BLV-infected cattle present surface receptors typical of an infected cell subset, but that those cells are increasingly reactive to proliferative signals without the expected reactivity to presented antigens. This may be a strategy of BLV to continue replicating while evading immune surveillance.

A crucial and classical B cell function is to produce antibodies in response to an immune challenge and this function is also disrupted in BLV-infected animals. Freshly isolated PBMCs from PL cattle express more Ig-μ mRNA, but less Ig-λ (Teutsch and Lewin, 1996), indicating some level of transcriptional disruption in antibody production. Experimentally infected calves demonstrate equal total serum IgG levels in comparison to uninfected calves, but experience a transient increase and subsequent decrease in total serum IgM (Meiron et al., 1985). BLV-infected cattle also demonstrate defective antibody production in response to specific challenges: when inoculated with synthetic antigens, PL cattle take twice as long as uninfected cattle to produce antigen-specific antibodies, and their antibody production is not consistent (PL animals demonstrate no constant antibody ratio, while uninfected cattle consistently produce IgM:IgG at a 1:10 ratio) (Trainin et al., 1976). More recently, BLV-infected cattle inoculated with the J5 *E.*

*coli* vaccine produced significantly less antigen-specific serum IgG2 when compared to healthy cattle, although the IgM and IgG1 levels were comparable between the two groups (Erskine et al., 2011a). This finding is particularly intriguing because IgG2 antibodies would be most effective as an opsonin against an *E. coli* infection, indicating that the antibody-producing deficiency in cattle could negatively impact protection through vaccination.

**T cell abnormalities**

Although BLV primarily infects B cells, it can infect other cell types (Lavanya et al., 2008), although this is rare. Even without direct infection of other immune cells, BLV infection not only impacts B cell function but also the function of other immune cells, particularly T cells. Freshly isolated T cells from BLV-infected cattle show no difference in either MHCII or CD25 surface expression (Isaacson et al., 1998), and cultured T cells from infected cattle respond similarly to different stimulants in culture when compared to T cells from uninfected cattle. Despite this, freshly isolated T cells from BLV-infected cattle display a distinct phenotype indicating host suppression of T cell activity. The mean fluorescence intensity (MFI) of LAG3, a negative regulator of T cell activation and proliferation, is significantly increased on both $CD4^+$ and $CD8^+$ T cells isolated from both PL and AL cattle (Konnai et al., 2013). Shirai *et al.,* 2011 also found a trending increase (significant at $p<0.1$) in LAG3 mRNA produced by both $CD4^+$ and $CD8^+$ T cells from BLV-infected cattle (Shirai et al., 2011). Freshly isolated $CD4^+$ and $CD8^+$ T cells also express significantly more Tim3, a negative regulator of immune cell activation and proliferation, in both PL and AL cattle, as well as a significant increase in the expression of Gal9, Tim3's ligand, in $CD8^+$ cells (Okagawa et al., 2012). The team confirmed Tim3's suppressive role by adding Tim3-expressing cells to 12 hour cell culture as ligand trappers. This addition significantly increases IFNγ expression from BLV+ PBMCs, indicating that Tim3 does

act to reduce T cell responsiveness. PBMCs from PL cattle also have significantly more B cells expressing PD-L1, a T cell repressor, at the cell surface than cells from uninfected cattle (Ikebuchi et al., 2011). A PD-L1 blockade significantly increases both IL2 and IFNγ in PBMCs from infected cattle, indicating its role in repressing cytokine expression (Konnai et al., 2013). It was also found that PL cattle have a significantly higher relative percent of $CD4^+$ T cells expressing FoxP3, indicating that PL cattle have a higher proportion of regulatory T cells (Tregs). Tregs negatively regulate T cell activation and proliferation. There is a significant positive correlation between percent $CD4^+FoxP3^+$ cells and the number of leukocytes, viral titer, and viral load. The percent $CD4^+FoxP3^+$ population is also negatively correlated with IFNγ expression (Suzuki et al., 2013). The presence of increased LAG3 and Tim3 on T cells, increased PD-L1 on B cells, increased Tregs and decreased IFNγ expression indicates a clear suppression of classical T cell activity in BLV-infected cattle.

BLV-infected animals also display unusual cytotoxic T cell activity. When bovine PBMCs were cultured for 10 to 12 days in the presence of rIL2, PBMCs isolated from AL cattle had significantly increased levels of $\gamma\delta^+$ T cells in comparison to both PL and uninfected PBMC cultures. At the same time, AL cultures had significantly reduced $CD8^+$ T cells compared to both PL and uninfected cattle. To demonstrate the role of $\gamma\delta^+$ T cells, Lundberg and Splitter, 2000 added them to cultures of both autologous fibroblasts and D17 cells expressing BLV Env. Only $\gamma\delta^+$ T cells from AL cattle showed significantly increased lysis of antigen-expressing cells and lysis of D17 cells, demonstrating that lysis can occur in both an MHC-restricted and MHC-unrestricted manner (Lundberg and Splitter, 2000). Although BLV antigens will stimulate perforin release from $CD8^+$ cells in BLV+ animals (Florins et al., 2009), the importance of $\gamma\delta^+$ T cells during the AL stage of EBL may be a result of the suppression of $CD8^+$ cell activation,

although this would not explain the lack of $\gamma\delta^+$ cytotoxicity in PL cattle. This difference may be due to the heterogeneity of $\gamma\delta^+$ T cell populations: some $\gamma\delta^+$ T cells express the coreceptor WC1, and $WC1^+$ $\gamma\delta^+$ T cells can express either WC1.1 or WC1.2. $WC1.1^+$ cells may also express WC1.3 and evidence suggests that $\gamma\delta^+$ T cells expressing different WC1 profiles have different and sometimes opposite functions (Rogers et al., 2005).

**Monocyte and macrophage abnormalities**

Although most BLV research focuses on its impact on the adaptive immune system, some research supports that BLV infection also negatively affects the functioning of monocytes and macrophages. Freshly isolated peripheral blood leukocytes (PBLs) from AL cattle have an increased relative percentage of monocytes expressing CD11b and CD32, but that the MFIs of CD11b, MHCII and CD14 are decreased. These data suggest that while more monocytes are expressing CD11b and MHCII, each cell is expressing less of the two proteins than cells from uninfected cattle. After culture to produce monocyte-derived macrophages, macrophages from AL cattle have a significantly reduced percentage of cells expressing both CD11b and CD14 (Werling et al., 1998). These surface receptors are useful markers for monocyte and macrophage function: CD11b is important for cellular adhesion and migration (Solovjov et al., 2005), CD32 is a B cell coreceptor that is important for regulating antibody levels (Veri et al., 2007), MHCII will present antigens to $CD4^+$ T cells, and CD14 is a coreceptor that detects LPS. The decreased MFIs and prevalence of monocytes or macrophages expressing these surface receptors would indicate a deficiency in their function. In line with this, monocytes isolated from AL cattle have significantly decreased phagocytic activity against *E. coli* when compared to monocytes from uninfected cattle (Werling et al., 1998). However, monocytes cultured in the presence of exogenous BLV do not exhibit altered surface expression of CD11b, CD32 or MHCII

(Altreuther et al., 2001), indicating that deficiencies in monocyte or macrophage function are likely not a result of direct BLV infection within those cell types.

All together, a large body of evidence supports widespread immune dysfunction in BLV-infected animals (summarized in Table 1), although little research has been done to investigate the impact this dysfunction has on bovine health and well-being. This deficit is likely due to the belief that BLV infection is only detrimental in the small proportion of animals that develop either leukemia or lymphoma, a stance that must change in the face of recent studies indicating a decrease in production (Bartlett et al., 2014), economic losses (Ott et al., 2003) and reduced resistance to infectious diseases. Studies that have investigated the risk of infectious disease in BLV-infected cattle indicate that abnormal immune function due to BLV infection does negatively impact immune response to vaccines and infections. Erskine *et al.,* 2011a demonstrated reduced IgG2 antibody production to J5 vaccine in BLV infected cattle and Emanuelson *et al.,* 1992 found a positive correlation between BLV infection and incidence of mastitis, hoof problems, gastroenteritis and bronchitis in dairy herds (Emanuelson et al., 1992). Trainin *et al.,* 1996 found that 12 of 32 BLV-positive cattle naturally infected with ringworm failed to clear the infection after 4 months, while only 1 of 34 BLV-negative cattle failed to clear the infection after four months, strongly suggesting a deficient immune response in the BLV+ cattle (Trainin et al., 1996). These results support that the extensive immune dysfunction does negatively affect BLV-infected cattle, a concept that should be further explored to assess its role in herd production and animal health and well-being.

Research into the effect of BLV infection on the function of the immune system has primarily focused on the immune cells without investigating the broader consequences of s

**Table 1.1. Summary of altered immune functioning in BLV-infected animals.**

| BLV Infection Effect | BLV Status | References |
|---|---|---|
| Altered transcriptome | Tax-expressing | Klener *et al.*, 2006 |
| ↓ IL2, IL4, IFNγ expression | AL, PL | Amills *et al.*, 2002 |
| ↑ IL2, IL10, IFNγ expression | AL, PL | Trueblood *et al*., 1998 |
| ↑ IL2 activity | PL | Sordillo *et al*., 1994 |
| Delayed IL2, IL10 expression | PL | Trueblood *et al*., 1998 |
| Altered IL12p40 expression | AL, PL | Pyeon and Splitter, 1998 |
| ↑ serum IL6 | PL | Trainin *et al*., 1996 |
| ↑ LAG3 expression in T cells | AL, PL | Shirai *et al*., 2011; Konnai *et al*., 2013 |
| ↑ Tim3, Gal9 expression in T cells | AL, PL | Okagawa *et al.,* 2012 |
| ↑ PD-L1 expression | PL | Ikebuchi *et al.,* 2011 |
| ↑ $CD4^+$ FoxP3 expression | PL | Suzuki *et al.,* 2012 |
| ↑ MHCII surface expression on PBMCs | PL | Sordillo *et al*., 1994 |
| ↑ MHCII surface expression on B cells | AL, PL | Stone *et al.,* 1995; Isaacson *et al*., 1998 |
| ↓ MHCII upregulation in response to stimulation | PL | Stone *et al*., 1995 |
| Altered CD25 surface expression on B cells | AL, PL | Stone *et al*., 1995; Isaacson *et al.*, 1998 |
| ↑ CD25 upregulation on B cells | PL | Stone *et al*., 1995 |

| **Table 1.1 (cont'd)** | | |
|---|---|---|
| Altered antibody immunoglobulin expression | PL | Teutsch and Lewin, 1996 |
| Delayed antibody production | PL | Trainin *et al*., 1976 |
| ↓ antibody production | BLV+ | Meiron *et al*., 1985; Erskine *et al*., 2011a |
| ↑ monocytes expressing CD11b, CD32 | AL | Werling *et al*., 1998 |
| ↓ CD11b, CD32, CD14 MFI | AL | Werling *et al*., 1998 |
| ↓ phagocytic activity | AL | Werling *et al*., 1998 |
| Positive correlation with mastitis, hoof problems, gastroenteritis, bronchitis | BLV+ | Emanuelson *et al*., 1992 |
| Delayed clearance of ringworm infection | BLV+ | Trainin *et al*., 1996 |

abnormalities. Clearly, the immune system is essential for protection against infectious agents, but only the three research studies highlighted above have investigated the susceptibility of BLV-infected cattle to other infectious diseases. Addressing this question is essential to fully determine the production and economic impacts of BLV infection on the US dairy industry. Mastitis is a common problem that plagues dairy operations. The average cost of clinical mastitis in dairy cattle is $179 per case (Bar et al., 2008) and a recent study of Canadian dairy herds reported an incident rate of clinical mastitis to be 26.3 cases per 100 cow-years. This study also found a negative correlation between the strength of an antibody-mediated immune response and the incident rate of clinical mastitis (Thompson-Crispi et al., 2013). A more clear understanding of BLV infection as a risk factor for other infectious diseases is critical for optimal dairy production and animal welfare.

The immune system is not only essential for protection against infectious agents, but in dairy cattle it is important to maintain a healthy reproductive capacity. Dairy cattle insemination efficiency is quite low and a major cause is the large proportion of abortion within the first 20 days of gestation. Uterine infections are a contributing factor to this low rate of successful pregnancy in dairy cattle. Unresolved uterine infections, which are common following parturition, can cause metritis or endometritis (Evans and Walsh, 2011). Endometritis, in particular, can impede the establishment and maintenance of a healthy pregnancy in multiple ways (Gilbert, 2011). BLV infection may not only affect decreased milk production and an increased culling rate (Bartlett et al., 2013), but may also have an effect on the reproductive efficiency, which could also significantly contribute to economic losses in a BLV-infected herd.

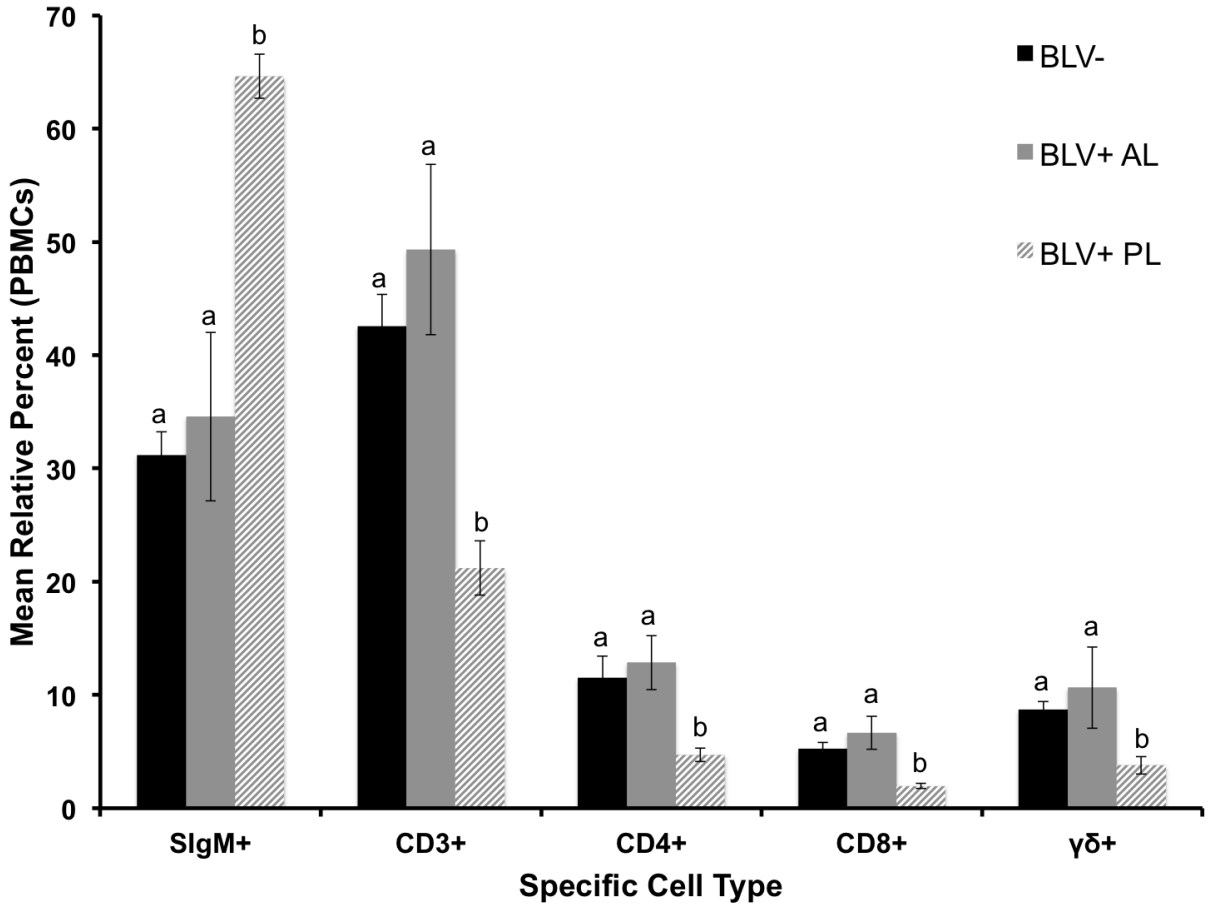
## Alterations of Cell Survival in BLV-Infected Animals

Animals infected with BLV will often show abnormal ratios of cell populations in comparison to healthy animals. In both AL and PL cattle, fresh PBMCs isolated from BLV-infected cattle have significantly higher relative B cell percentages and significantly lower relative T cell percentages, including significantly less percent CD3, CD4 and CD8 positive cells (Figure 1) (Erskine et al., 2011b; Sordillo et al., 1994; Stone et al., 1995; Stone et al., 1996; Van den Broeke et al., 2010). BLV-infected cattle also tend to exhibit a higher ratio of CD4:CD8 ($p<0.1$) cells than in uninfected controls (Stone et al., 1995). While polyclonal proliferation of B cells is likely one factor leading to dramatic alterations in the relative percent of other cell types in PBMCs, there are other factors that must be considered, such as the rate of proliferation and apoptosis in various immune cells. Evidence in the literature suggests that both cellular proliferation and apoptosis rates are altered in BLV infected cattle, though there is not a general agreement on the direction of these changes (Klener et al., 2006).

### B cells

Experimental evidence disagrees on whether or not B cells from BLV-infected animals have increased or decreased rates of proliferation. *In vitro* experiments using ovine B cells demonstrate increased rates of B cell proliferation in Tax-expressing clones and that BLV-expressing B cell lines have increased B cells in the G2/M phase of the cell cycle when compared to uninfected B cells (Szynal et al., 2003). However, another group found no significant difference in cell cycle distribution between infected and uninfected ovine PBMCs (Debacq et al., 2002) and suggest that B cells isolated from PL cattle are less likely to be in S/G2/M than B cells from uninfected or AL cattle (Debacq et al., 2003). *In vivo* experiments in sheep show that B cells in BLV-infected sheep have significantly higher rates of proliferation

**Figure 1.1. Relative cell percentages in BLV- and BLV+ cattle.** PBMCs were isolated from whole blood using Percoll density centrifugation. After 44h culture in RPMI complete media, cells were harvested and labeled for surface receptors using the following antibodies: SIgM for B cells (PIG45A2), $CD3^+$ for T cells (MM1A), $CD4^+$ for Th cells (ILA11A), $CD8^+$ for CTLs (BAQ111A) and TCR1 δ chain for $\gamma\delta^+$ T cells (GB21A). BLV+ cattle were determined via BLV serum ELISA. AL and PL cattle were assigned based upon the relative abundance of $SIgM^+$ cells that were three standard deviations above the mean of relative abundance of $SIgM^+$ cells in BLV- cattle. BLV- n=19; AL n=5; PL n=8. Error bars represent the standard error of the mean. Means within each marker with different superscripts are significantly different using Student's T test ($p<0.05$).



than B cells in uninfected sheep (Debacq et al., 2002; Florins et al., 2009). In contrast, *in vivo* experiments in cattle show a significant reduction in B cell proliferation in PL cattle compared to both uninfected and AL cattle (Debacq et al., 2003).

Altered rates of cellular apoptosis may also be affecting the relative percent of various cell populations in PBMCs from BLV infected cattle. Due to the classical expansion of B cells seen in persistent lymphocytosis, most research has focused on B cell apoptosis, although some evidence does indicate atypical T cell apoptosis in BLV-infected animals. The majority of studies have utilized cultured cells, although one study found that freshly isolated B cells do not have significantly different rates of apoptosis between BLV-infected and uninfected animals (Erskine et al., 2011b). Bovine PBMCs cultured without stimulant show no difference in B cell apoptosis rates. However, bovine PBMCs from PL cattle cultured in the presence of either phorbol myristate acetate (PMA) and ionomycin (P/I) or crosslinking IgM antibodies have significantly reduced B cell apoptosis rates when compared to cells from uninfected cattle (Cantor et al., 2001; Debacq et al., 2003). BrdU incorporation in cattle *in vivo* also demonstrates decreased B cell apoptosis in PL cattle in comparison to both uninfected and AL cattle (Debacq et al., 2003).

Cultured ovine PBMCs also demonstrate significantly lower rates of apoptosis in cells from BLV-infected sheep (Debacq et al., 2002), which is unaffected by the addition of either cyclohexamide or a PKC inhibitor (Chevallier et al., 1998). Ovine B cells transfected with a Tax-expressing plasmid have lower rates of apoptosis compared to control B cells (Szynal et al., 2003). Cultured ovine PBMCs from infected animals also have lower expression of activated caspases in comparison to cultured PBMCs from healthy sheep (Takahashi et al., 2005).

Other research has demonstrated opposite results suggesting that BLV infection increases rates of B cell apoptosis. In these studies, PBMCs from PL cattle cultured without stimulant show significantly increased B cell apoptosis, although the kinetics of apoptotic rates was equivalent between PL, AL and uninfected cattle (Dequiedt et al., 1999). *In vivo* injection of CFSE-labeled PBMCs also showed significantly increased B cell apoptosis rates in BLV-infected sheep in comparison to uninfected sheep (Florins et al., 2009). The disagreement concerning how BLV affects apoptosis may be due to myriad factors, including the biological differences between cattle and sheep and their immune response to BLV; small sample sizes and the natural variation seen in outbred species; and unique environmental challenges that would vary between studies and could affect immune responses. It is also possible that BLV may differentially affect apoptosis depending on the circumstances within the same animal. Regardless, the research strongly suggests that BLV can alter cellular homeostasis and typical immune cell growth and death rates.

**T cells**

$CD4^+$ T cells from BLV-infected cattle also exhibit different proliferative rates in cell culture: $CD4^+$ cells from PL cattle proliferate in the presence of metabolically active irradiated autologous PBMCs, but the proliferative response is contact dependent and mediated by both IL2 and MHCII expression and does not occur in $CD4^+$ cells from AL or BLV-negative cattle. Interestingly, the proliferation is not mediated by BLV expression and $CD4^+$ cells from PL cattle do not proliferate in response to exogenous BLV, although $CD4^+$ cells from AL cattle proliferate in response to exogenous BLV in a dose-dependent fashion (Stone et al., 2000). Another group found that PBMCs from AL cattle proliferate in culture with supernatant from a BLV-expressing cell line and that $CD4^+$ cells comprise the majority of proliferating cells; however, as in studies

by Stone *et al.*, 2000, PBMCs from PL cattle did not proliferate in response to BLV antigens (Orlik and Splitter, 1996).

In respect to other immune cells, very little research has investigated altered apoptosis rates. However, one study found that freshly isolated PBMCs from PL cattle had significantly higher apoptotic or dead T cells in comparison to AL cattle, although there was no significant difference between PL cattle and uninfected cattle. Despite the difference in apoptosis, there were no differences in either caspase 9 activity or Bax expression between the cohorts in this study (Erskine et al., 2011b). Due to the decline of the T cell compartment during the expansion of the B cell compartment seen during PL, it is important going forward that apoptosis rates are examined in other cell types besides B cells.

**Mechanisms of Apoptosis Dysregulation**

While it is not clear precisely how BLV affects apoptosis, many potential mechanisms have been explored for decreased apoptosis in BLV-infected animals. PBMCs from both AL and PL cattle have significantly elevated Bcl2 expression and PL cattle have significantly increased Bcl2L1 expression, both of which inhibit apoptosis (Erskine et al., 2011b). Ovine cell lines transfected with Tax also have significantly increased Bcl2 expression, although there is no change in either Bax or Bcl-xl. The Tax-expressing clones also have significantly decreased apoptotic cells in response to DNA damage and NFκB inhibition in comparison to Tax-free clones (Szynal et al., 2003). In contrast to ovine cell lines, cultured primary ovine PBMCs from infected sheep exhibit increased expression of the anti-apoptotic Bcl-xL gene, compared to uninfected controls (Takahashi et al., 2005). BLV-infected cell lines transfected with Tax have increased micronuclei and ssDNA from DNA damage, but no difference in apoptosis compared to any apoptotic treatment or other oncogenic viral infection. The cells transfected with Tax also show a defect in base excision repair in response to oxidative DNA damage (Philpott and Buehring, 1999). Telomerase activity was also detected in 25 of 29 bovine tumor samples (Suzuki et al., 2008).

PBMCs from PL cattle also have significantly more $TNFRII^{+}IgM^{+}$ and $TNFRII^{+}CD5^{+}$ cells than uninfected cattle, although there is no difference in TNFRII expression on $CD4^{+}$, $CD8^{+}$ or $WC1^{+}$ T cells, nor is there any significant difference in TNFRI expression between BLV-infected and uninfected cattle (Konnai et al., 2005). This is interesting because the majority of TNFα signaling occurs through TNFRI, which is expressed on many cell types. TNFRII is only expressed on a few cell types, including T cells and B cells, and can only respond to membrane-bound TNFα. Signaling through TNFRII can stimulate both cellular proliferation and

cytotoxicity, depending on the level of TNFα stimulation and the induction of cooperative signaling with TNFRI (Cabal-Hierro and Lazo, 2012; Rodriguez et al., 2011; Weiss et al., 1997). In bovine PBMCs, there is a significant positive correlation between the TNFα-induced proliferative response and the level of TNFRII expression and a significant reduction in apoptosis in the PL PBMCs in response to TNFα. The BLV proviral load also has a significant positive correlation with TNFRII expression (Konnai et al., 2005).

All together, the existing data supports that BLV infection disrupts both B and T cell homeostasis and alters both the proliferative and apoptotic responses in these cells. Although it is still unknown exactly how BLV alters cellular growth cycles, evidence suggests that BLV can affect both growth and death signaling pathways. The observed variability between studies could be a result of BLV interfering with multiple signaling cascades at different times following infection or be related to availability of co-factors that alter how exactly BLV interferes with gene expression and/or signaling. The observed differences may also be due to differences in the viral activation state: BLV may differentially regulate or induce apoptosis depending on being latent or active within a host cell. These discrepancies in apoptosis within infected animals highlight the need for more research into which signaling pathways BLV interferes with. Apoptosis rates in infected animals are critical for understanding how well the immune system functions within infected animals. Focusing on the activity of BLV within an infected cell and how it affects apoptosis will help clarify how BLV alters the life cycle of infected cells. More research is also needed to understand altered cell cycles of uninfected cells, since uninfected immune cells may also have altered cell cycles and would be essential for proper immune function in infected cattle.

**Viral Latency, Reactivation and its Implications**

While the BLV provirus is presumed to be transcriptionally silent in the majority of infected cells, many scientists hypothesize that BLV genes are constantly being expressed in a small subset of infected cells that are then rapidly cleared by the host immune system. This is a commonly used explanation for the extreme difficulty in detecting BLV proteins or mRNA in freshly isolated PBMCs, as well as the constant production of anti-BLV antibodies by the host (Florins et al., 2007). This idea is certainly interesting from the perspective of viral reactivation, due to the supposition that cells expressing BLV proteins would be rapidly eliminated by other host immune cells. Unsurprisingly, a diverse body of research has been done to investigate both what stimuli reactivate BLV expression, as well as the cellular mechanisms supporting reactivation.

**Activators of BLV Transcription**

Both exogenous and endogenous cellular factors have been shown to activate BLV transcription. NFκB heterodimers p49/p65 and p50/p65 are both capable of stimulating BLV transcription, even in the absence of Tax, in a cell culture system (Brooks et al., 1998). Infected bovine PBMCs treated with IL2 increase Tax, Pol, and p24 expression (Pyeon and Splitter, 1999; Trueblood et al., 1998). In contrast, recombinant bovine IFNγ reduces both reverse transcriptase activity and syncytium formation in cultures of cells expressing BLV, but does not impede growth of the cell line (Sentsui et al., 2001). Infected bovine PBMCs cultured with anti-inflammatory IL10 show a decrease in both Tax and Pol expression. Culture with IL12 shows no effect on BLV expression (Pyeon and Splitter, 1999).

In addition, common immune mitogens have also been demonstrated to activate BLV transcription. Cultured ovine PBMCs from infected sheep express increased BLV capsid protein in the presence of LPS (Kidd and Radke, 1996). LPS also stimulates bovine PBMCs in culture to express increased gp51 (Jensen et al., 1992) and increases both the number of BLV-expressing PBMCs and the amount of BLV RNA expressed per cell (Lagarias and Radke, 1989). These effects are also seen when bovine PBMCs are stimulated with either PMA, phytohaemagglutinin (PHA) or PWM, all of which are common T cell and B cell mitogens (Jensen et al., 1992; Lagarias and Radke, 1989). Crosslinking the B cell receptor (BcR) with anti-IgM antibodies also activates BLV transcription (Lagarias and Radke, 1989).

**Host proteins that bind to BLV LTR**

Research has also elucidated which cellular factors bind directly to regions within the BLV LTR promoter. A wide range of host cell proteins can bind to the BLV LTR, including CREB, ATF1, ATF2 (Adam et al., 1996) and CREMτ (Nguyen et al., 2007). Phosphorylated

USF1 and USF2 will both bind to the enhancer sequence Ebox4 motif in the LTR and this binding is increased in response to P/I stimulation and can stimulate BLV transcription in the absence of Tax (Calomme et al., 2002; Colin et al., 2011). Both IRF1 and IRF2 will bind to an IRSE-like motif in the LTR, but the LTR is not responsive to IFNα (Kiermer et al., 1998). NFκB also binds to the LTR and has increased binding affinity in the presence of Tax (Szynal et al., 2003). The BLV LTR can also directly bind both PU.1 and Spi-B; this binding promotes Tax-independent transcription, but is not necessary for Tax-dependent transcription (Dekoninck et al., 2003).

These activators of BLV transcription and host proteins that can directly bind the BLV LTR share a common feature in that they can either stimulate an immune response or are active in intracellular signaling in response to stimuli and would likely be present in activated B cells. For example, infected cells would be exposed to soluble IL2 or IFNγ during any inflammatory response and to LPS during specific bacterial infections. CREB is a transcription factor that activates constitutive transcription of MHCII within B cells (van der Stoep et al., 2002). ATF1 and ATF2 are transcription factors that can dimerize with other proteins to form the transcription factor AP-1, which can be activated in response to BcR stimulation. ATF1 and CREB have also been shown to bind to the promoter of MHCII (van der Stoep et al., 2002). CREM is a transcription factor with multiple isoforms: CREMτ activates transcription (Sassone-Corsi, 1995) and has been associated with cyclin A and cell cycle progression (Desdouets et al., 1995). USF1 and USF2 are transcription factors that bind to E box motifs. USF activates transcription of the J chain, which is essential for the assembly and secretion of pentameric IgM from plasma cells (Wallin et al., 1999). IRF1 can activate transcription of IFNα and IFNβ and IRF2 classically is a competitive inhibitor of IRF1 function, but has also been shown to activate specific loci. Both

IRF1 and IRF2 can bind to the MHCII promoter to activate MHCII expression in B cells in response to IFNγ (Piskurich et al., 2006). Like AP-1, NFκB is activated in response to BcR stimulation and promotes B cell differentiation, activation and proliferation (Hostager and Bishop, 2013). PU.1 and Spi-B are members of the Ets family of transcription factors and are highly expressed in B cells. They are also essential proteins for B cell development and are critical for germinal center formation and BcR signal transduction in mature B cells (Garrett-Sinha et al., 1999). All together, these data indicate that host cell proteins can activate BLV transcription and that activation of an infected B cell could cause reactivation of BLV expression. A major unanswered question is how reactivation following B cell stimulation, with subsequent clearance of such cells by the immune system, would affect response to vaccines or other common infections.

**Advantages of latency to BLV survival**

In regards to survival of the virus, both direct and indirect evidence points to the advantage of latent BLV infection over active infection with cells expressing BLV proteins. There is general agreement that most BLV proviruses exist latently *in vivo*, due to the nonexistent to extremely low expression of BLV in freshly isolated blood, as well as the observation that BLV expression can be induced simply by cell culture at 38°C (Lagarias and Radke, 1989). BLV proviral insertion tropism also suggests selection for latent proviral survival: in two case studies, all transformed cells contained infection-deficient proviral clones (Merimi et al., 2007). Large-scale analysis of proviral integration in bovine tumor samples demonstrated that 19 of 55 unique integration sites were in repetitive sequences, which are generally considered to be transcriptionally silent. While 24 of 55 integration sites were within a transcriptional unit, only 1 integration site was within an exon, and none were integrated into a

protein-coding sequence (Murakami et al., 2011). Analyzing proviral integration after experimental infection of cows, it was found that while primary infection is characterized by integration into transcriptionally active sites, after seroconversion the majority of proviral clones are integrated into transcriptionally silent regions (Gillet et al., 2013). This research suggests that specific integration sites provide one mechanism to promote latency and that latent clones have a selective advantage during chronic infection.

Mechanisms of retroviral latency may be evolutionarily conserved because of the survival advantage it confers: BLV, HTLV-1 and HIV-1 LTRs all contain E box motifs, which function to repress viral transcription. Within an individual infected with HIV, all quasispecies will maintain E box motifs and the presence of E box motifs is specific to certain subspecies (only HIV-1 subtypes E, F and G contain E box motifs in the LTR). In HTLV-1, only groups HTLV-1a and HTLV-1e contain E box motifs. HTLV-1a is the most widely distributed group in infected individuals and 97% of HTLV-1a isolates contain the E box motif (Terme et al., 2009).

**Consequences of BLV reactivation on viral survival**

Other experiments have directly assessed the effect of active BLV transcription on its host cell. Treating infected ovine PBMCs with increasing concentrations of valproate significantly increases BLV p24 concentration in culture medium, indicating an increase in mature virions in a dose-dependent manner. Concomitantly, increasing concentrations of valproate decreases the percentage of live cells expressing p24 and increases the percentage of B cells undergoing apoptosis (Achachi et al., 2005). Thus, it appears that activated B cells expressing p24 are rapidly cleared in PBMC cultures. Similarly, cells transfected with BLV have an increased percentage of cells expressing activated caspases in comparison to controls; when cells were transfected with mutant BLV that had increased transactivation potential and

increased BLV expression, the percentage of cells expressing activated caspases is significantly higher in comparison with cells transfected with WT BLV (Takahashi et al., 2005).

*In vivo* experiments in sheep also support a selective advantage for latent BLV. Merezak *et al.,* 2001 created a mutant BLV strain that contained perfect CRE consensus sequences as opposed to WT BLV CRE sequences. *In vitro* experiments demonstrated that perfect CRE sequences increase CREB, ATF1 and ATF2 binding to the BLV LTR and that this increased binding is accompanied by increased basal transcription of BLV. When sheep were infected with the mutant BLV, they had significantly reduced proviral loads at both 3 months and 6 months post infection in comparison to sheep infected with WT BLV (Merezak et al., 2001). Florins *et al.,* 2012 collected PBMCs from infected and control sheep, cultured them to induce BLV expression, fluorescently labeled the cells and reinjected them into the sheep. While proliferation rates of injected cells were the same between both groups, BLV-expressing cells die at a significantly higher rate in infected sheep versus B cells in control sheep (Florins et al., 2012). When Tajima *et al.,* 2003 injected a mutant strain of BLV with increased replicative and infectious capacity into sheep, they saw no difference in the degree of BLV replication or infection *in vivo*. However, the mutant strain of BLV was transcriptionally silenced *in vivo* just like the WT BLV strain, indicating that a Tax-independent mechanism preserved viral latency (Tajima et al., 2003).

It is interesting to note that studies focusing on BLV reactivation demonstrate that an active BLV infection promotes apoptosis of the infected cell, while studies of apoptosis rates in BLV-infected animals have mostly demonstrated a decreased rate of apoptosis compared to uninfected animals. This difference is likely due to latency or activation of the BLV virus. It is possible that latently infected cells or cells expressing low levels of BLV have increased

protection against apoptosis, which would promote viral survival and is a common viral strategy to promote survival and replication (Upton and Chan, 2014). However, reactivated virus with high expression would make the host cell a target for the immune system, which would result in rapid destruction of infected cells. This distinction becomes important when considering the normal functioning of the host immune system: what implications would viral reactivation have if a collection of BLV-infected cells recognized a particular pathogen and was required for efficient clearance of such an infection or for protection during response to a vaccine?

To better understand how BLV impacts infected cells, it is essential to characterize the intracellular life of BLV. Very little is known about BLV's activity within an infected cell. BLV contains four genes outside of essential retroviral genes: Tax, Rex, R3 and G4 (Aida et al., 2013). Tax is a transactivator of BLV transcription and Rex exports viral mRNA from the nucleus. However, little is known about some of Tax's described functions, including its oncogenic potential. The functions of R3 and G4 are also unknown: they are dispensable for infectivity, but are important for the pathogenicity of BLV, as well as maintaining a high proviral load (Gillet et al., 2007). To understand BLV infection and its effect on the host immune system, it is crucial to understand how viral proteins interfere with normal host cell signaling. Transcriptomic and proteomic studies, as well as characterizing host binding partners of viral proteins, would be a great advance for both the virology of BLV and the immunology of BLV infection.

## Conclusion

BLV can cause immune suppression in affected animals through multiple mechanisms, including 1) disruption of proper immune cell signaling molecules and cytokine production, 2) disturbance of immune cell proliferation and apoptosis and typical lymphocyte ratios, and 3) possible destruction of activated, infected cells. Although immune suppression and disruption have been demonstrated comprehensively, very little research has been conducted to assess the impact BLV infection may have on cattle health and well-being, especially in regards to vaccine protection and susceptibility to other infectious diseases. Much of the available research on BLV and immune function is 10 to 15 years in the past, given recent advances in our general understanding of immunology, the availability of reagents to study bovine immunology in particular, and advances such as NextGen sequencing technologies, it is perhaps time to reconsider this important and widespread pathogen. Future research into how BLV impacts responses to vaccines and other pathogens, including those causing long-term chronic infections is critical to assess the true economic impact of BLV infection in the US dairy industry.

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**Specific Aims**

The goal of this dissertation was to investigate whether BLV infection negatively affects the immune function of naturally infected dairy cows. In addition, if BLV infection was found to impair immunity in infected cattle, this dissertation aimed to elucidate possible mechanisms of viral interference in normal immune functions. BLV infection is extremely prevalent in US dairy herds and its effect on herd health and production may be underestimated. It was hypothesized that BLV+ cows would exhibit abnormal immune responses to immune stimulation. Once it was consistently observed that BLV+ cows produced less IgM, it was also hypothesized that BLV microRNAs might impair the transcription of IGJ, the gene that encodes the J chain. Thus, BLV would negatively impact the ability to assemble and secrete pentameric IgM. The specific aims of this dissertation were designed to test these hypotheses.

**Specific aim 1:** To investigate whether BLV infection interfered with healthy immune reactivity *in vivo* in naturally infected dairy cattle.

**Subaim 1.1:** To assess B and T cell responses to routine vaccination in adult, lactating Holstein cows infected with BLV.

**Subaim 1.2:** To characterize B and T cell responses to a primary and secondary antigenic exposure in adult, lactating Holstein cows infected with BLV.

**Specific aim 2:** To investigate a potential mechanism by which BLV infection interfered with IgM production in naturally infected adult, lactating Holstein cows.

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## CHAPTER 2: REDUCED HUMORAL IMMUNITY AND ATYPICAL CELL-MEDIATED IMMUNITY IN RESPONSE TO VACCINATION IN COWS NATURALLY INFECTED WITH BOVINE LEUKEMIA VIRUS

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**Abstract**

Bovine leukemia virus (BLV) is a retrovirus that is widely distributed across US dairy herds: over 83% of herds are BLV-infected and within-herd infection rates can approach 50%. BLV infection reduces both animal longevity and milk production and can interfere with normal immune health. With such a high prevalence of BLV infection in dairy herds, it is essential to understand the circumstances by which BLV negatively affects the immune system of infected cattle. To address this question, BLV- and BLV+ adult, lactating Holstein dairy cows were vaccinated with Bovi-Shield GOLD® FP® 5 L5 HB and their immune response to vaccination was measured over the course of 28 days. On day 0 prior to vaccination and days 7, 14 and 28 post-vaccination, fresh PBMCs were characterized for T and B cell ratios in the periphery. Plasma was collected to measure titers of IgM, IgG1 and IgG2 produced against bovine herpesvirus 1 (BHV1), *Leptospira hardjo* and *L. pomona*, as well as to characterize neutralizing antibody titers produced against BHV1 and bovine viral diarrhea virus types 1 and 2. On day 18 post-vaccination, PBMCs were cultured in the presence of BHV1 and flow cytometry was used to determine IFNγ production by $CD4^+$, $CD8^+$ and γδ T cells and to investigate CD25 and MHCII expression on B cells. BLV+ cows produced significantly lower titers of IgM against BHV1, *L. hardjo* and *L. pomona* and produced lower titers of IgG2 against BHV1. γδ T cells from BLV+ cows displayed a hyper reactive response to stimulation *in vitro*, although no differences were observed in $CD4^+$ or $CD8^+$ T cell activation. Finally, B cells from BLV+ cows exhibited higher CD25 expression and reduced MHCII expression in response to stimulation *in vitro*. All together, data from this study support the hypothesis that BLV+ cows fail to respond to vaccination as strongly as BLV- cows and, consequently, may have reduced protective immunity when compared to healthy BLV- cows.

Keywords: BLV, vaccine, antibody, T cell, IFNγ, MHCII

## Introduction

Bovine leukemia virus (BLV) is a δ-retrovirus that is the causative agent of enzootic bovine leukosis (EBL) (Gillet et al., 2007). BLV most commonly infects B cells (Meirom et al., 1997) and, in approximately 30% of infected cows, BLV infection causes a non-malignant, polyclonal expansion of the B cell compartment, termed persistent lymphocytosis (PL) (Kabeya et al., 2001). Until recently, BLV infection was considered a relatively benign infection with minimal impact on herd health. Indeed, fewer than 5% of infected cows will develop lymphosarcoma/lymphoma, which leads to rapid death of the animal (Schwartz and Levy, 1994). Despite tumors associated with BLV being the number one cause of dairy cow carcass condemnation at slaughter (Bartlett et al., 2014; White and Moore, 2009), the low risk of tumor development was not seen as sufficient reason to test or control for the transmission of BLV within dairy herds in the US.

With no effort to control the spread of BLV, the virus is widely prevalent in US dairy herds. In 2007, it was estimated that over 83% of herds are BLV-infected, and the within-herd prevalence is estimated to be between 23%-46% (USDA-NAHMS, 2007), but the current prevalence may be even higher. Previous estimates of the economic losses associated with BLV found that infection is associated with an annual $500 million loss nationwide (Ott et al., 2003). More recently, researchers found that BLV infection is associated with reduced longevity and reduced milk yield in dairy cows (Bartlett et al., 2013; Erskine et al., 2012; Norby et al., 2016).

The economic impact of BLV infection is very likely the result of its negative effect on the host immune system. A large body of evidence demonstrates that immune cells isolated from BLV-infected cattle have abnormal phenotypes or responses to stimulation *in vitro*, as reviewed by Frie and Coussens (2015). Few studies have directly investigated if host immune health is

compromised by BLV infection, which would lead to increased susceptibility to secondary infections with subsequent production losses. However, it has been shown that BLV infection was positively correlated with the incidence of mastitis, gastroenteritis and bronchitis (Emanuelson et al., 1992); that BLV-infected cows exhibited delayed clearance of ringworm infection (Trainin et al., 1996); and, finally, that BLV-infected cows produced lower titers of IgG2 in response to the J5 *E. coli* mastitis vaccine (Erskine et al., 2011a).

Preventing infectious diseases within dairy herds is a critical component of production and welfare maintenance. Vaccines are especially important in dairy farming to protect herd health; dairy cattle can receive vaccines that are protective against upwards of 20 different pathogens, including *Brucella abortus, Clostridium perfringens,* and bovine viral diarrhea virus. Many of these pathogens can cause devastating effects including severe milk loss, abortion and death. As a result of the importance of vaccination in dairy farming and the evidence of immune dysregulation in BLV-infected cattle, we investigated whether BLV-infected cows would demonstrate a similar immune response to their uninfected herdmates after receiving a boost vaccine of Bovi-Shield GOLD® FP® 5 L5 HB. The memory immune response is essential for providing vaccine protection, so both humoral and cell-mediated immune responses were characterized over 28 days following vaccination. Overall, this study was designed to provide insight as to whether BLV infection in adult cows interfered with routine vaccination boosts.

## Methods

### Animals

Three cohorts of adult, lactating Holstein cows were enrolled over the course of the study for a total of 40 cows (Table 1). Animals in cohorts 1 and 2 were housed at the Michigan State University Dairy Teaching and Research Center in East Lansing, MI, while animals in cohort 3 were housed at a local commercial dairy operation in mid-Michigan. In each cohort, BLV-infected cows were initially screened on the basis of positive milk ELISA testing. BLV+ cows were then selected on the basis of subsequent serum ELISA testing (positive result indicated by OD > 1.0) (AntelBiosystems, Inc) and an expanded peripheral B cell population (as determined by immunostaining and flow cytometry analysis of fresh PBMCs); BLV- cows were then age- and lactation- matched with selected BLV+ cows and serum ELISA tested to confirm BLV- status. To ensure BLV status did not change throughout the study, animals were retested for BLV on the final day of collection. All protocols were reviewed and approved by the Michigan State University Animal Use and Care Committee (04/16-061-11).

### Vaccination

Cows were vaccinated intramuscularly in the left flank with a single 2mL dose of Bovi-Shield GOLD® FP® 5 L5 HB (Bovi-Shield) (Zoetis). The vaccine consisted of a preparation of freeze-dried modified live virus strains of bovine herpesvirus type 1 (BHV1), bovine viral diarrhea virus (BVD) types 1 and 2, bovine parainfluenza 3 ($PI_3$) and bovine respiratory syncytial virus (BRSV) and a liquid fraction containing inactivated and adjuvanted *Leptospira canicola, L. grippotyphosa, L. hardjo, L. icterohaemorrhagiae,* and *L. pomona.* Enrolled cows had previously received Bovi-Shield vaccinations according to herd management practices: briefly, all cows received their initial Bovi-Shield vaccination and first boost between 2 and 5 months of age.

**Table 2.1. Animal enrollment summary.** Cows were enrolled in 3 separate cohorts over a 1-year period. Day 0 refers to the date of vaccination boost. The table lists means and (range).

| Cohort | Vaccination Date | Farm | N | | Percent B cell Prevalence | | Age d0 (months) | | Days in Milk d0 | | Lactation Number | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | BLV- | BLV+ | BLV- | BLV+ | BLV- | BLV+ | BLV- | BLV+ | BLV- | BLV+ |
| 1 | 3-17-15 | MSU Dairy | 5 | 5 | - | 76.9 (61.7-90.0) | 68.6 (55-103) | 72.8 (59-101) | 141.6 (35-245) | 238.2 (206-265) | 3.8 (3-6) | 3.8 (3-6) |
| 2 | 9-18-15 | MSU Dairy | 6 | 6 | - | 62.8 (36.8-75.6) | 63 (50-101) | 59.7 (50-68) | 266.5 (132-462) | 225 (90-419) | 3 (2-6) | 3.2 (2-4) |
| 3 | 1-28-16 | Commercial Dairy | 9 | 9 | - | 75.4 (62.9-83.0) | 65.4 (56-84) | 67.7 (56-89) | 265.3 (244-310) | 286.2 (235-313) | 3.6 (3-5) | 3.6 (3-5) |

Cows in cohorts 1 and 2 (Michigan State University) then received subsequent boosts every six months upon entering the milking herd; cows in cohort 3 (commercial dairy operation) received a second boost at 9 months, a third boost when moved into the breeding pen and then received additional boosts at 21-27 days in milk for each subsequent lactation. For all three cohorts, cows in the current study were vaccinated on day 0 between 6 and 12 months after their most recent Bovi-Shield boost.

**Plasma and PBMC isolation**

Whole blood was collected from the coccygeal vein on day 0 prior to vaccination and on days 7, 14, 18 and 28 post-vaccination. Blood was collected in acid-citrate-dextrose (ACD) Vacutainer Blood Collection Tubes (Becton Dickinson) and centrifuged at room temperature for 20 minutes at 930 x *g*. Plasma aliquots were collected and sodium azide was added for a final concentration of 0.1%. Plasma aliquots were stored at -80°C until use. Peripheral blood mononuclear cells (PBMCs) were isolated by Percoll density centrifugation (density 1.084 g/mL). Buffy coats were layered over 10 mL of Percoll in 20 mL of 1X phosphate buffered saline (PBS) and centrifuged at room temperature for 41 minutes at 365 x *g*. PBMCs were harvested from the Percoll/PBS interface and washed twice with 30 mL of PBS and centrifuged at room temperature for 8 minutes at 625 x *g*. PBMCs were suspended in PBS at approximately $2\times10^7$ cells/mL. PBMCs were then counted using a hemocytometer for subsequent use.

**Antigen-specific antibody quantification**

Bovine diagnostic kits for BHV1 and *Leptospira* infection (Biovet) were modified to measure the relative quantities of antigen-specific antibodies against BHV1, *L. hardjo* and *L. pomona*. All biological samples were run in duplicate and duplicate blanks for each isotype and antigen were included to detect nonspecific binding activity. To detect anti-BHV1 IgM, IgG1

and IgG2, plasma was diluted at 1:200, 1:400 and 1:800, respectively. 100µL diluted plasma was incubated with immobilized antigens in 96-well plates (Biovet) for 1 hour at 37°C and then washed 5 times with wash buffer. 100µL anti-bovine IgM-, IgG1- or IgG2-horseradish peroxidase (HRP) conjugate (Thermo Fisher Scientific) was diluted at 1:10,000 and incubated for 1 hour at 37°C. Wells were then washed 5 times with wash buffer and incubated with 100µL 3,3',5,5'-Tetramethylbenzidine (TMB) for 15 minutes at room temperature in the dark. 100µL stop solution (2M $H_2SO_4$) was added to all wells and the optical density (OD) was measured at 450nm using a SpectraMax 190 microplate reader (Molecular Devices). To detect anti-*L. hardjo* IgM, IgG1 and IgG2, plasma was diluted at 1:200, 1:50 and 1:100, respectively. To detect anti-*L. pomona* IgM, IgG1 and IgG2, plasma was diluted at 1:100, 1:50 and 1:100, respectively. 100µL diluted plasma was incubated with immobilized antigens in 96-well plates (Biovet) for 30 minutes at room temperature and then washed 3 times with wash buffer. 100µL anti-bovine IgM-, IgG1- or IgG2-HRP conjugate was diluted at 1:10,000 and incubated for 30 minutes at room temperature. Wells were then washed 3 times with wash buffer and incubated with 100µL TMB for 15 minutes at room temperature in the dark. 100µL stop solution was added to all wells and the OD was measured at 450nm using a SpectraMax 190 microplate reader.

**Neutralizing antibody quantification**

Quantification of neutralizing antibody (NAb) production against BHV1, BVD1 and BVD2 was performed using a modified microtiter assay (Spatz et al., 1994) and completed by the Maes laboratory at the Diagnostic Center for Population and Animal Health (Michigan State University). Briefly, plasma was heat inactivated at 56°C for 30 minutes. Two-fold serial dilutions of plasma were made in EMEM (Sigma Aldrich), containing 10% FBS (Atlanta Biologicals), 10mM glutamine (Gibco), 10 µg/ml of ciprofloxacin (Sigma Aldrich) and 50

μg/ml of gentamycin (Sigma Aldrich). The Colorado strain of BHV1, BVD1 and BVD2 (Animal and Plant Health Inspection Service, National Veterinary Services Laboratory, Ames, IA, USA) were added to each dilution at a concentration of 500 $TCID_{50}$ per well. A cell-control, to which no virus was added, was included for each plasma dilution tested. Virus-serum mixtures were incubated at 37°C at 5% $CO_2$ for 1 hour. Bovine turbinate (BT) cells were used as the indicator cells. The number of BT cells added to each well was 15,000 in a volume of 100μL. The test plates were incubated as described above for 4 days. Absence or presence of cytopathic effect (CPE) was assessed microscopically. VN titers were expressed as the reciprocal of the highest serum dilutions that completely inhibited the typical CPE effect induced by BHV1, BVD1 or BVD2.

**Immunostaining of freshly isolated PBMCs**

Details of the following procedure are summarized in Table 2 and denoted "Fresh." $8\times10^5$ PBMCs were fixed in 100μL 4% paraformaldehyde for 20 minutes at 4°C. Samples were then labeled with mouse anti-bovine MAbs at a dilution of 0.5μg in 50μL staining buffer (PBS with 2% heat inactivated horse serum, 10% ACD and 0.09% sodium azide) for 30 minutes at 4°C in the dark. PBMCs were washed with 100μL wash buffer (PBS with 10% ACD and 0.09% sodium azide) and then centrifuged at 4°C for 5 minutes at 435 x *g*. Samples were then labeled with anti-mouse secondary antibodies and incubated at a dilution of 0.1μg in 50μL staining buffer for 30 minutes at 4°C in the dark. Samples were washed with 100μL wash buffer and centrifuged at 4°C for 5 minutes at 435 x *g*. PBMCs were suspended in 100μL wash buffer and stored at 4°C in the dark until flow cytometry analysis.

**Table 2.2. Antibodies used for immunostaining experiments.** All primary MAbs were purchased from the Washington State University Monoclonal Antibody Center except IFNγ (Bio-Rad) and BLV gp51 (VMRD). All secondary antibodies were purchased from Thermo Fisher Scientific except IgM PE-Cy7 (eBioscience) and IgG2b PE-Cy7 (Abcam). * indicates antibodies used for intracellular labeling after PBMC permeabilization.

| Stain | 1° MAb Target | Target Phenotype | Clone | 2° Ab Target |
|---|---|---|---|---|
| Fresh | γδ TcR | Gamma delta T cells | CACTB81A | IgG1 AF488 |
| | CD4 | Helper T cells | IL11A | IgG2a PE |
| | CD8 | Cytotoxic T cells | BAQ111A | IgM PE-Cy7 |
| | SIgM | B cells | BAQ155A | IgG2b AF647 |
| T cell | γδ TcR | Gamma delta T cells | GB21A | IgG2b AF488 |
| | CD4 | Helper T cells | IL11A | IgG2a PE |
| | CD8 | Cytotoxic T cells | BAQ111A | IgM PE-Cy7 |
| | IFNγ* | Proinflammatory cytokine | CC302 | IgG1 AF647* |
| B cell | CD25 | IL2 receptor, α-chain | LCTB2A | IgG3 AF488 |
| | MHCII | Antigen presentation | TH16A | IgG2a PE |
| | SIgM | B cells | PIG45A2 | IgG2b PE-Cy7 |
| | BLV gp51* | BLV surface glycoprotein | | IgG1 AF647* |

### *In vitro* stimulation of PBMCs

$2\times10^6$ PBMCs were plated per well in 24-well polystyrene culture plates (Corning) in 2mL Roswell Park Memorial Institute (RPMI) complete media (RPMI plus 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% fungizone, pH 7.4) and cultured for 18h at 38°C and 5% $CO_2$. Unstimulated (nil) PBMCs were cultured in RPMI alone; BHV1-stimulated PBMCs were cultured for 18h with live BHV1 (courtesy of Dr. Roger Maes, Michigan State University) at a multiplicity of infection (MOI) of 1; positive control PBMCs were cultured for 18h with 5ng/mL phorbol 12-myristate 13-acetate (PMA) and 100ng/mL ionomycin (P/I) or for the final 6h with 20μg/mL concanavalin A (ConA). Unstimulated, BHV1- and ConA-stimulated cultures were treated with 20ng/mL brefeldin A at 12h to inhibit IFNγ secretion.

### Immunostaining of cultured PBMCs

Live PBMCs were labeled with 2 4-color stains. Unstimulated, BHV1-stimulated and ConA-stimulated PBMCs were labeled with a 4-color stain as previously described and detailed in Table 2, denoted "T cell". A fixation/permeabilization kit (eBioscience) was used according to manufacturer's instructions to fix and permeabilize PBMCs. PBMCs were subsequently labeled with mouse anti-bovine IFNγ at a dilution of 0.5μg in 50μL permeabilization buffer for 45 minutes at 4°C in the dark. Samples were washed with 100μL permeabilization buffer and then samples were labeled with anti-IgG1 at a dilution of 0.2μg in 50μL permeabilization buffer for 30 minutes at 4°C in the dark. Samples were suspended in 100μL wash buffer and stored at 4°C in the dark until flow cytometry analysis. Unstimulated, BHV1-stimulated and P/I-stimulated PBMCs were labeled as described with a 4-color B cell stain detailed in Table 2 and denoted "B cell".

**Immunostaining analysis**

Data were acquired using an Accuri C6 flow cytometer with CSampler (Becton Dickinson). Immunostaining data were initially compensated using BD CSampler Software (Becton Dickinson) and exported for analysis using FCS Express 4 (De Novo Software). PBMCs were selected for analysis using forward and side scatter gating to exclude debris. PBMCs were then initially gated on specific T or B cell populations. Expression of IFNγ, CD25 and MHCII was then measured within specific cell subsets. Data are expressed in either mean relative percent or mean fluorescence intensity (MFI). The mean relative percent describes the population of cells within gated PBMC populations that are positive for a specific marker, while the MFI describes the average expression density per cell for a specific marker.

**Statistical analysis**

All statistical analyses were done using Statistical Analysis Software 9.4 (SAS Institute). For both flow cytometry and ELISA data, data were processed using a repeated measures ANOVA general linear model. Post-hoc Bonferroni correction was used for pair-wise comparisons. Grubbs' test was used to detect outliers, which were excluded from analysis. The study performed was designed as a matched study, so data analysis was only performed on complete sample sets for all collection times for matched BLV- and BLV+ cows. Significance was determined at $p < 0.05$.

## Results

### BLV+ cows demonstrate an increase in circulating SIgM$^+$ B cells

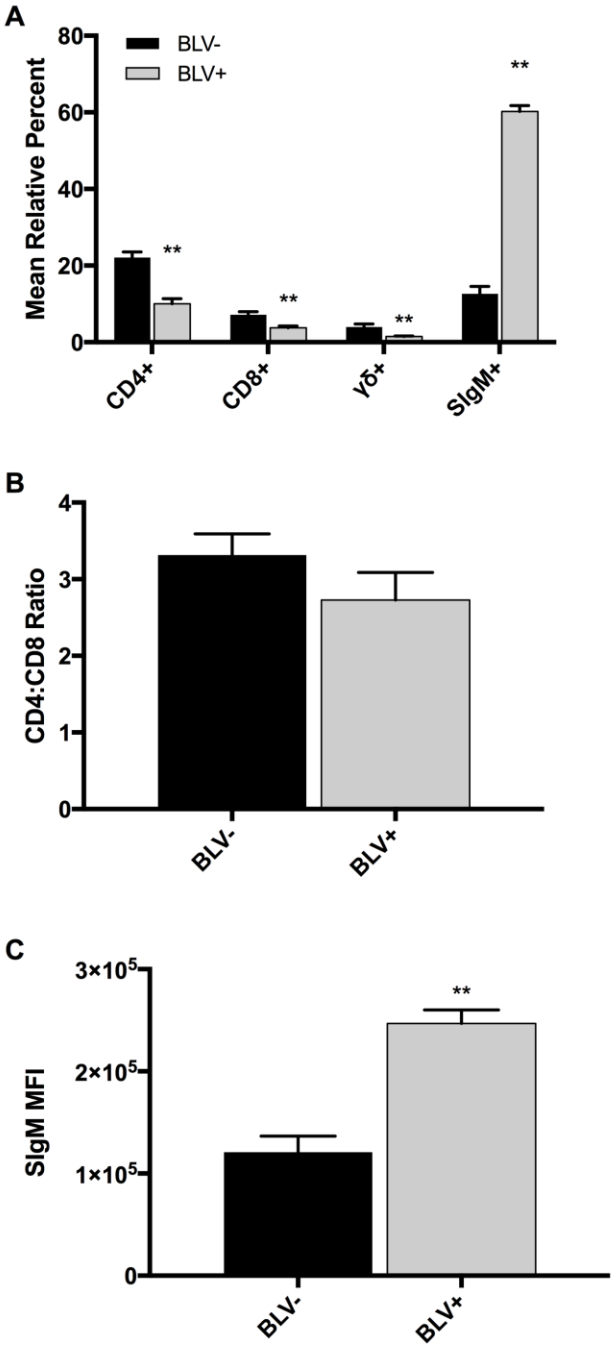
Relative proportions of B cells and T cell subsets were measured by 4-color flow cytometry on freshly isolated PBMCs from cohort 3 cows on day 0 prior to vaccination and on days 7, 14 and 28 post-vaccine boost. On day 0 prior to vaccination, BLV+ cows demonstrated lower mean relative percentages of CD4$^+$, CD8$^+$ and γδ T cells in comparison to BLV- cows. Conversely, BLV+ cows had an elevated proportion of surface IgM$^+$ (SIgM$^+$) B cells (Figure 2.1A). These trends were consistent across all collection times and there was no difference in the population kinetics between BLV- and BLV+ cows (data not shown). Although the ratio of T:B cells was altered in BLV+ cows, no difference was observed in the ratio of CD4:CD8 T cells between BLV- and BLV+ cows (Figure 2.1B). The average expression (MFI) of SIgM was also doubled at all collection times in BLV+ cows in comparison to BLV- cows (Figure 2.1C). The proportions of T and B cell populations observed in BLV+ cows were expected based on the selection criteria for cows with elevated B cell populations in peripheral blood.

### BLV+ cows produce lower antibody titers after vaccination against both viral and bacterial pathogens

Plasma from cows in cohorts 1, 2, and 3 was collected on day 0 before vaccination and on days 7, 14 and 28 after vaccination and relative quantities of IgM, IgG1 and IgG2 antibodies against *L. hardjo, L. pomona* and BHV1 were measured to assess if BLV- and BLV+ cows exhibited comparable humoral immune responses to vaccination. For all isotypes and all antigens measured, both BLV- and BLV+ cows showed a significant temporal increase in antibody titer after vaccination, as expected.

Interestingly, BLV+ cows consistently produced less IgM compared to BLV- cows:

**Figure 2.1. Relative abundance of B and T cell subsets in freshly isolated PBMCs.** The relative abundance of B and T cell subsets was characterized by flow cytometry on freshly isolated PBMCs from BLV- and BLV+ cows on day 0 prior to vaccination. **(A)** BLV+ cows (grey bars) exhibited an increase in the mean relative percent of B cells and a decrease in the mean relative percent of T cells relative to BLV- cows (black bars). $n = 9$/group, $^{**}p < 0.05$. **(B)** The CD4:CD8 ratio was not altered between BLV- and BLV+ cows. $n = 9$/group, $p = 0.27$. **(C)** The SIgM MFI was increased in BLV+ cows relative to BLV- cows, $n = 9$/group, $^{**}p < 0.05$. Data represent the mean ± SEM.



BLV+ cows had lower initial titers of IgM against *L. hardjo, L. pomona* and BHV1 and this persisted throughout the collection times (Figure 2.2). BLV+ cows also demonstrated consistent IgG1 production against all three antigens: BLV+ cows produced IgG1 titers comparable to BLV- cows against *L. hardjo, L. pomona* and BHV1 (Figure 2.3). Surprisingly, IgG2 production by BLV+ cows was not consistent across all antigens. BLV+ cows produced equal titers of IgG2 against both *L. hardjo* and *L. pomona* when compared to BLV- cows (Figure 2.4A, B). However, this trend was not evident when measuring IgG2 produced against BHV1. In cohorts 1 and 2, BLV+ cows produced trending ($p = 0.06$) less IgG2 in comparison to BLV- cows (Figure 2.4C). In contrast, cohort 3 BLV+ cows produced equal titers of IgG2 against BHV1 (Figure 2.4D).

Neutralizing antibody (NAb) titers from cohort 1 cows were also measured to determine if BLV+ cows produced lower NAb titers against BHV1, BVD1 and BVD2 after vaccination. Both BLV- and BLV+ cows produced NAbs in response to vaccination, but there was no significant overall difference between BLV- and BLV+ cows in the amount of NAb produced (Figure 2.5). However, BLV+ cows did exhibit a lower NAb titer against BVD1 at day 7 in comparison to BLV- cows (Figure 2.5B).

**SIgM$^+$ B cells from BLV+ cows display altered expression of activation markers in response to BHV1 stimulation *in vitro***

PBMCs from cohort 3 were isolated on day 18 and cultured for 18h with BHV1- or P/I-stimulation to assess B cell activation. Activation was measured by the surface expression of CD25 (the IL2 receptor α-chain) or MHCII, which presents antigen to CD4$^+$ T cells. Our culture conditions were unable to detect any BLV gp51 protein expression. However, differences between B cell activation in BLV- and BLV+ cows were observed. As expected, B cells from both BLV- and BLV+ cows showed an increase in the proportion of B cells expressing CD25 in

**Figure 2.2. Relative quantities of IgM antibodies produced against *L. hardjo, L. pomona* and BHV1.** Plasma was collected from BLV- (black circles) and BLV+ (grey squares) cows on days 0, 7, 14, and 28. Relative quantities of IgM produced against *L. hardjo, L. pomona* and BHV1 were measured. **(A)** BLV+ cows produced less IgM against *L. hardjo*. $n = 20$/group, $^{**}p < 0.05$. **(B)** BLV+ cows produced lower titers of IgM against *L. pomona*. $n = 18$/group, $^{**}p < 0.05$. **(C)** BLV+ cows produced less IgM against BHV1. $n = 17$/group, $^{**}p < 0.05$. Data represent the mean ± SEM.

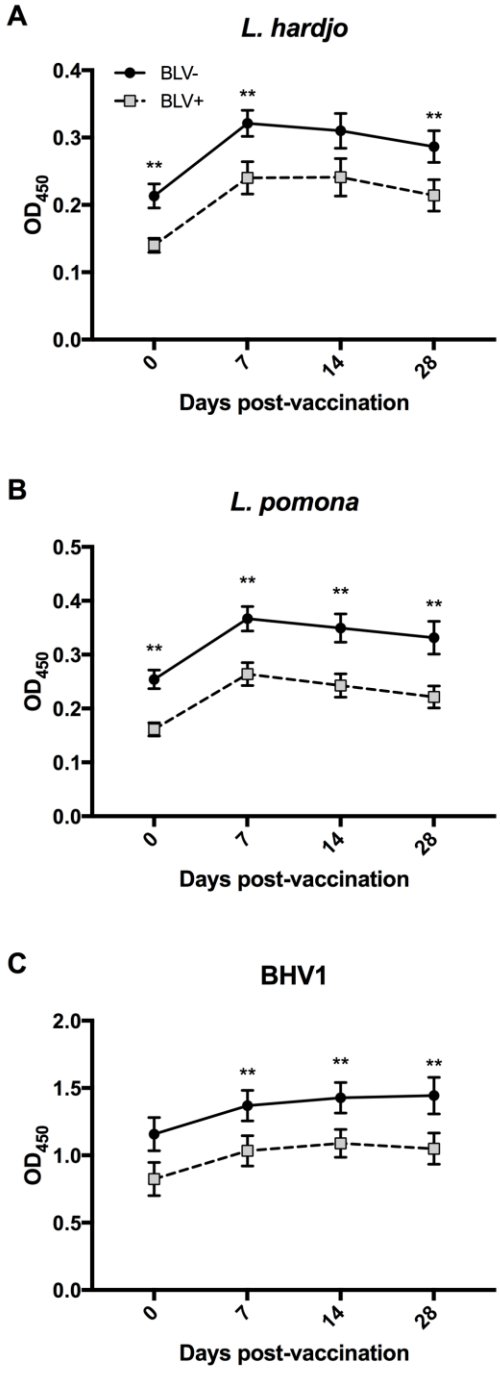


**Figure 2.3. Relative quantities of IgG1 antibodies produced against *L. hardjo, L. pomona* and BHV1.** Plasma was collected from BLV- (black circles) and BLV+ (grey squares) cows on days 0, 7, 14, and 28. Relative quantities of IgG1 produced against *L. hardjo, L. pomona* and BHV1 were measured. **(A)** BLV- and BLV+ cows generated equal quantities of IgG1 against *L. hardjo.* $n$ = 10/group, $p$ = 0.78. **(B)** BLV- and BLV+ cows demonstrated equal levels of IgG1 against *L. pomona.* $n$ = 10/group, $p$ = 0.80. **(C)** BLV+ cows demonstrated no deficiency in IgG1 production against BHV1. $n$ = 18/group, $p$ = 0.65. Data represent the mean ± SEM.

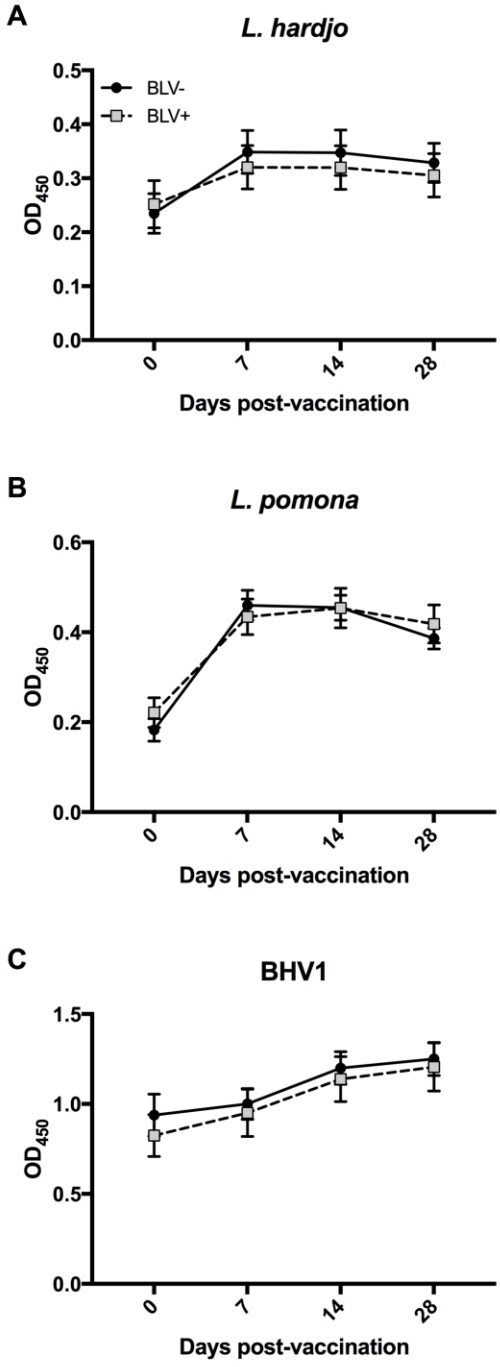


**Figure 2.4. Relative quantities of IgG2 antibodies produced against *L. hardjo, L. pomona* and BHV1.** Plasma was collected from BLV- (black circles) and BLV+ (grey squares) cows on days 0, 7, 14, and 28. Relative quantities of IgG2 produced against *L. hardjo, L. pomona* and BHV1 were measured. **(A)** BLV- and BLV+ cows showed similar titers of IgG2 against *L. hardjo*. . $n$ = 18/group, $p$ = 0.65. **(B)** BLV- and BLV+ cows exhibited equivalent IgG2 titers against *L. pomona*. $n$ = 17/group, $p$ = 0.84. **(C)** BLV+ cows in cohorts 1 and 2 produced trending ($p$ = 0.06) less IgG2 against BHV1. $n$ = 9/group, $^{**}p < 0.05$. **(D)** BLV- and BLV+ cows from cohort 3 exhibited no significant difference in plasma IgG2. $n$ = 8/group, $p$ = 0.53. Data represent the mean ± SEM.

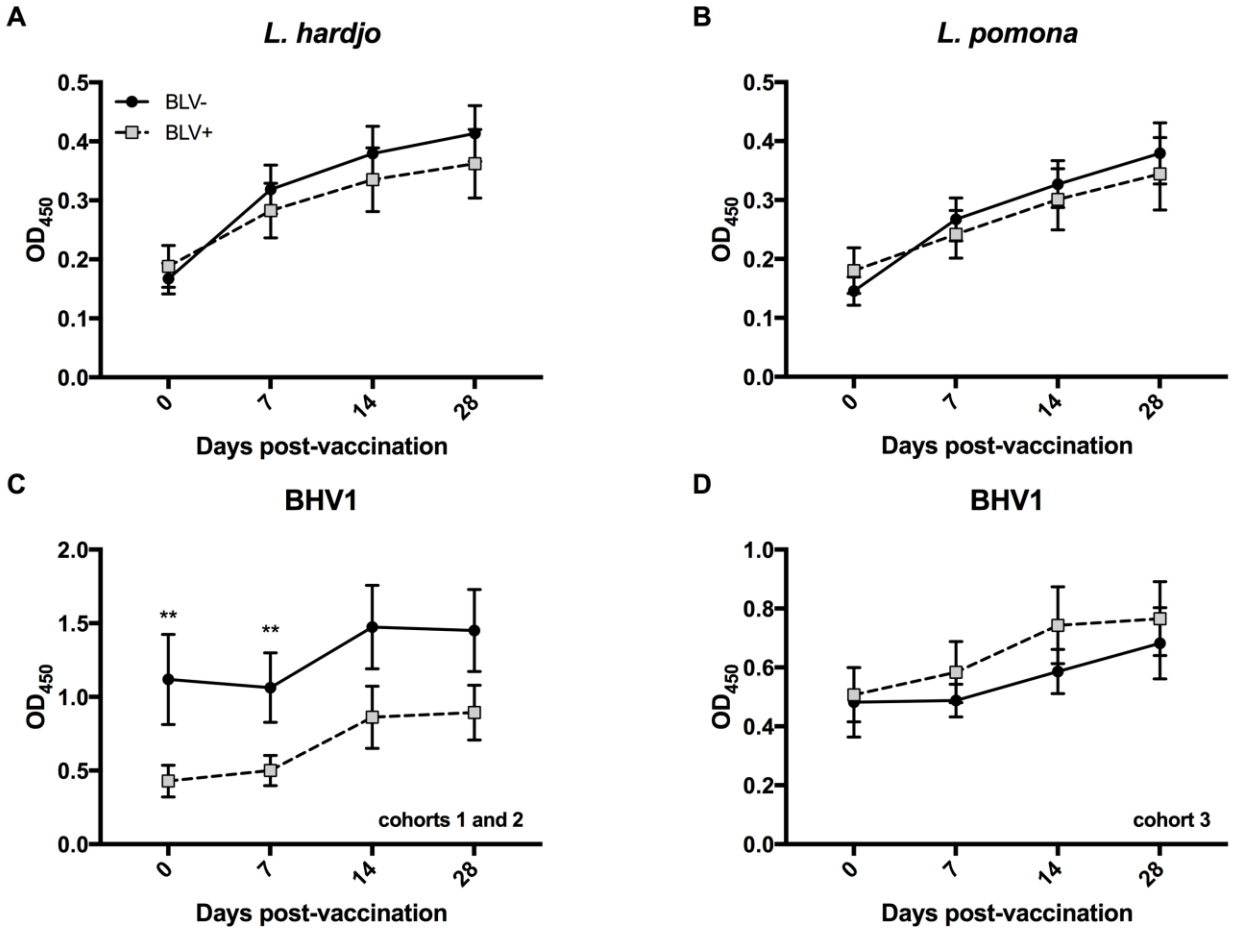
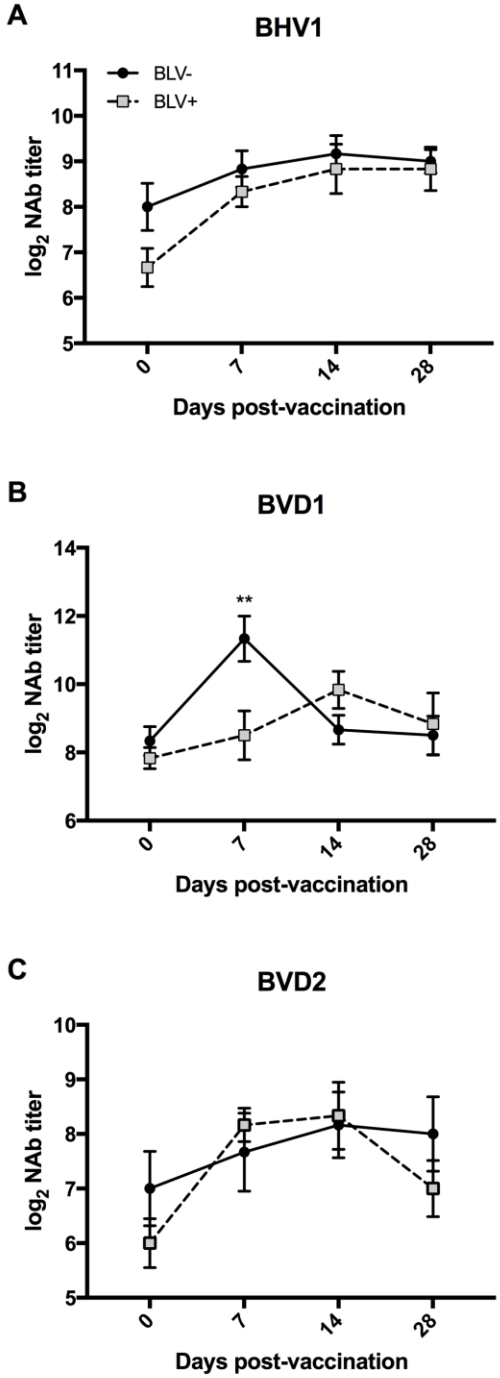


**Figure 2.5. Neutralizing antibody titers against BHV1, BVD1 and BVD2.** Plasma was collected from BLV- (black circles) and BLV+ (grey squares) cows on days 0, 7, 14, and 28 and the neutralizing antibody titers against BHV1, BVD1 and BVD2 were assessed. **(A)** BLV- and BLV+ cows generated equal titers of NAbs against BHV1. $n$ = 5/group, $p$ = 0.16. **(B)** BLV- and BLV+ cows produced equivalent NAb titers against BVD1 overall ($p$ = 0.40), although the NAb titer from BLV+ cows was reduced on day 7. $n$ = 5/group, $^{**}p < 0.05$. **(C)** BLV- and BLV+ cows exhibited no differences in the production of NAbs against BVD2. $n$ = 5/group, $p$ = 0.63. Data represent the mean ± SEM.



response to both BHV1- and P/I-stimulation. BLV+ cows did show an overall trending ($p = 0.06$) difference in CD25 expression: in BHV1-stimulated cultures, B cells from BLV+ cows showed a higher proportion of $CD25^+$ B cells in comparison to BLV- cows (Figure 2.6A). The MFI of CD25 was also measured; while both BLV- and BLV+ cows showed increased MFI in response to stimulation, there was no difference between BLV- and BLV+ cows (data not shown).

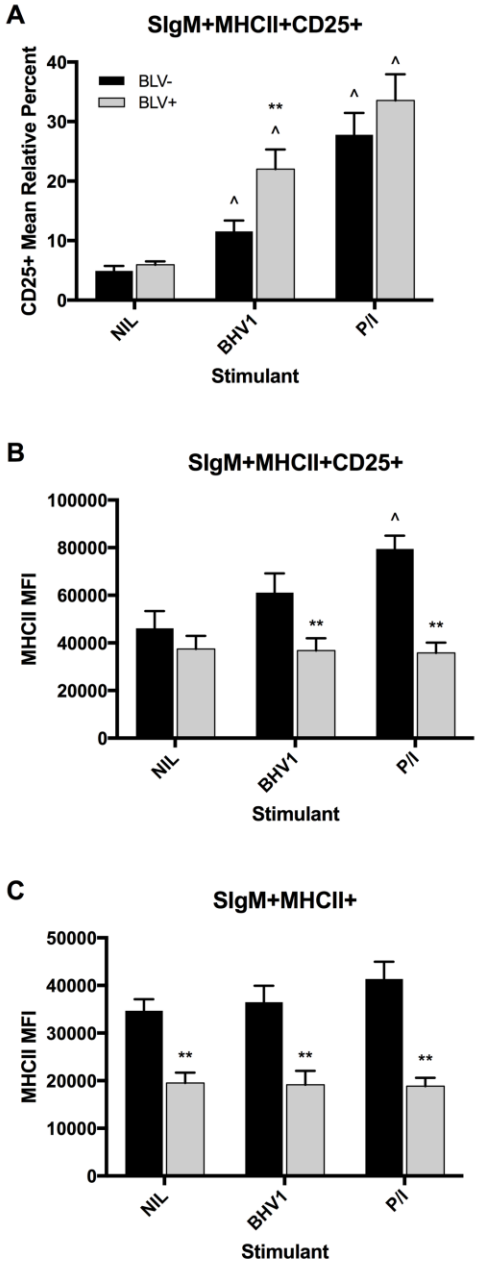
As a second measure of B cell activation, MHCII expression was also assessed. The mean relative percent of B cells expressing MHCII was not different between BLV- and BLV+ cows and did not change in response to stimulation; over 95% of B cells expressed MHCII (data not shown). Interestingly, differences between BLV- and BLV+ cows became apparent when analyzing the MFI of MHCII. MHCII MFI on the total B cell population did not change in response to stimulation, but it was greatly reduced on B cells from BLV+ cows in all culture conditions (Figure 2.6B). MHCII expression on B cells became even more intriguing when looking specifically at activated $CD25^+$ B cells. MHCII MFI only increased in response to stimulation on $CD25^+$ B cells from BLV- cows; in addition, MHCII MFI in BHV1- and P/I-stimulated cultures was much higher in $CD25^+$ B cells from BLV- cows when compared to BLV+ cows (Figure 2.6C).

The MFI of SIgM did not change in response to stimulation and was not different between BLV- and BLV+ cows (data not shown). This is surprising, considering that freshly isolated B cells from BLV+ cows exhibited a much higher surface expression of IgM in comparison to B cells from BLV- cows (Figure 2.1C).

**BLV+ cows exhibit higher reactivity of γδ T cells in response to stimulation *in vitro***

PBMCs from cohorts 2 and 3 were isolated on day 18 and cultured for 18h with BHV1- or ConA-stimulation to assess antigen-specific T cell activation. Activation was measured by the

**Figure 2.6. B cell activation in response to BHV1 stimulation *in vitro*.** PBMCs were isolated on day 18 post-vaccination and cultured in the presence of BHV1- or P/I-stimulation and B cell activation was measured by flow cytometry. **(A)** The mean relative percent of $MHCII^+$ B cells expressing CD25 was reduced in BLV- cows (black bars) in comparison to BLV+ cows (grey bars). The mean relative percent of $MHCII^+$ B cells expressing CD25 increased in BLV- and BLV+ cows in response to BHV1- and P/I-stimulation. $n$ = 8/group, $^{**}p < 0.05$ compared to BLV-, $^{\wedge}p < 0.05$ compared to nil. **(B)** The MHCII MFI on all B cells was higher in BLV- cows, but did not change in response to stimulation. $n$ = 8/group, $^{**}p < 0.05$ compared to BLV-. **(C)** The MHCII MFI on $CD25^+$ B cells was higher in BLV- cows and only increased in response to P/I stimulation in BLV- cows. $n$ = 8/group, $^{**}p < 0.05$ compared to BLV-, $^{\wedge}p < 0.05$ compared to nil. Data represent the mean ± SEM.

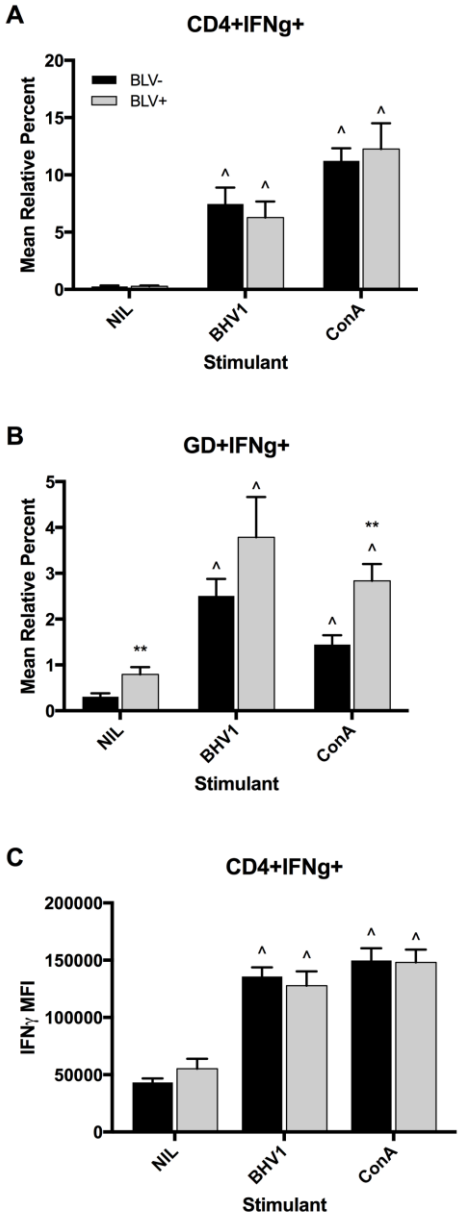


production of proinflammatory cytokine IFNγ from $CD4^+$ helper T cells, $CD8^+$ cytotoxic T cells and γδ T cells. Surprisingly, BLV- and BLV+ cows exhibited equal levels of stimulation for both $CD4^+$ and $CD8^+$ classical T cells: the mean relative percent of $CD4^+$ T cells expressing IFNγ increased in response to both BHV1- and ConA- stimulation in both BLV- and BLV+ cows (Figure 2.7A). $CD8^+$ T cells demonstrated the same *in vitro* T cell activation profile as $CD4^+$ T cells (data not shown).

In contrast to classical T cell activation, γδ T cells from BLV+ cows demonstrated a different pattern of activation in comparison to BLV- cows. γδ T cells from both BLV- and BLV+ showed a positive reaction to BHV1- and ConA-stimulation. However, γδ T cells from BLV+ cows exhibited a higher degree of activation versus BLV- cows in both unstimulated and ConA-stimulated cultures (Figure 2.7B).

Finally, the MFI of IFNγ was measured as a second indication of T cell activation. In $CD4^+$ T cells from both BLV- and BLV+ cows, IFNγ MFI increased in response to both BHV1- and ConA-stimulation, as expected (Figure 2.7C), and no difference between BLV- and BLV+ activation was observed. This trend was also observed in $CD8^+$ and γδ T cells (data not shown).

**Figure 2.7. IFNγ production from T cells stimulated with BHV1 *in vitro*.** PBMCs were isolated on day 18 post-vaccination and cultured in the presence of BHV1- or ConA-stimulation and IFNγ production by T cells was measured using flow cytometry. **(A)** The mean relative percent of $CD4^+$ T cells producing IFNγ was similar between BLV- (black bars) and BLV+ (grey bars) cows ($p = 0.97$). The mean relative percent of $CD4^+$ T cells producing IFNγ from both BLV- and BLV+ cows increased in response to BHV1- and ConA-stimulation. $n = 11$/group, ^$p < 0.05$ compared to nil. **(B)** The mean relative percent of γδ T cells producing IFNγ was elevated in BLV+ cows. The mean relative percent of γδ T cells producing IFNγ from BLV- and BLV+ cows rose in response to BHV1- and ConA-stimulation. $n = 11$/group, **$p < 0.05$ compared to BLV-, ^$p < 0.05$ compared to nil. **(C)** The IFNγ MFI increased in $CD4^+$ T cells in both BLV- and BLV+ cows in response to BHV1 and ConA stimulation. $n = 11$/group, ^$p < 0.05$ compared to nil. Data represent the mean ± SEM.



**Discussion**

In this study, we sought to determine if BLV- and BLV+ cows were capable of mounting equivalent immune responses to a boost vaccination with Bovi-Shield. This commercial vaccine was chosen as the stimulus because it is a multivalent vaccine that protects against both viral and bacterial pathogens, and because it is commonly used in commercial dairy operations to vaccinate adult lactating cows. Our results demonstrated that BLV+ cows produced lower antibody titers in response to vaccination and that both B and γδ T lymphocytes from BLV+ cows reacted abnormally to antigen-specific and mitogenic stimulation *in vitro*. Considering that BLV+ cows exhibited abnormalities in both B and T cell compartments, these data support the hypothesis that BLV-infected dairy cows have impaired immunity after vaccination, although this impairment is not an overall suppression of B and T cell activity.

Cows enrolled in our study demonstrated consistently less secreted IgM and similar IgG1, as well as potentially less IgG2 in BLV+ animals, which is consistent with previous reports of aberrant antibody production in BLV-infected cattle. Previous research demonstrated that $IgM^+$ B cells isolated from BLV+ cows with persistent lymphocytosis (PL) expressed equal *Ig-μ* (heavy chain) mRNA levels, but expressed lower *Ig-λ* (light chain) mRNA than both BLV- and BLV+ cows in the alymphocytic (AL) stage of infection (Teutsch and Lewin, 1996). At the protein level, calves experimentally infected with BLV demonstrated a decline in total serum IgM when compared to healthy calves (Meiron et al., 1985), and PL cows produced IgM and IgG1 more slowly and at inconsistent ratios after exposure to a synthetic antigen (Trainin et al., 1976). More recently, it was shown that BLV+ cows produced lower titers of IgG2 in response to the J5 *E. coli* mastitis vaccine (Erskine et al., 2011a), although IgM and IgG1 titers were unaffected by BLV status; in contrast, another study found that BLV+ cows produced lower IgM

and IgG1 titers in response to foot-and-mouth disease virus (FMDV) vaccination, while IgG2 titers were equivalent between BLV- and BLV+ cows (Puentes et al., 2016).

The observed differences in antibody deficiencies reported by these three studies are likely the result of different vaccine antigens and/or adjuvants. Bovi-Shield is multivalent and protective against both viral and bacterial pathogens, while Erskine et al. and Puentes et al. measured antibodies produced only against a single bacterial or viral species, respectively. The observed differences may also be a result of the nuanced differences in immune protection against bacterial versus viral pathogens: *Leptospira* are spirochetes (Levett, 2001) and *E. coli* are gram-negative bacteria; BHV1 is an enveloped, double-stranded DNA virus (Muylkens et al., 2007), while FMDV is a non-enveloped single-stranded RNA virus (Gao et al., 2016). In addition, the J5 vaccine was used in dry cows, while our study utilized lactating cows; the FMDV vaccine response was measured over a long period of time, while our study focused on the early vaccine response, and these differences could also affect the results seen in our study. Finally, Puentes et al. cattle received their primary vaccination when BLV infection was already established; our study could not control for when cows were first infected with BLV in relation to how many booster vaccines they received and it is currently unknown how a primary antigenic exposure before or after BLV infection affects the immune system over the lifespan of the animal.

Although our results corroborate previous research and support the hypothesis that BLV+ cows exhibit impaired humoral immunity development in response to vaccination, it is unclear if the observed differences would result in increased susceptibility to the infectious diseases vaccines are meant to protect against. Answering that question requires experimental pathogenic challenge, which was beyond the scope of this project. However, we can make some inferences

with the given dataset. While class-switched IgG1 and IgG2 are considered pillars of the memory humoral immune response, memory IgM B cells have been documented in humans and mice and likely have functional importance (Kurosaki et al., 2010), so it is entirely possible that lower IgM in BLV+ cows would negatively affect their ability to combat BHV1 and *Leptospira* infections.

The observed IgG production by BLV+ cows also has intriguing implications for immunity in BLV-infected cattle. While the functions of IgG1 versus IgG2 antibodies are well characterized in humans and mice (Vidarsson et al., 2014), these roles are less defined in cattle: while both bovine IgG1 and IgG2 promoted opsonization by macrophages, only IgG2 promoted opsonization by neutrophils *in vitro* (Howard, 1984), and IgM, IgG1 and IgG2 isotypes have all been implicated as exhibiting neutralizing functions depending on the infectious agent (Lin et al., 2001; Pega et al., 2013). As a result, it is possible that our equivalent NAb titers between BLV- and BLV+ cows are reflective of the equal IgG1 titers produced by BLV- and BLV+ cows. Although we only observed trending ($p = 0.06$) lower IgG2 titers against BHV1 in cows from cohorts 1 and 2, it is possible that the divergent IgG2 data from cohort 3 resulted from different farm management, particularly the difference in vaccination schemes. If BLV+ cows do produce lower titers of IgG2, it appears to be related to the type of antigenic stimulus, considering *Leptospira* antigens consistently induced similar IgG2 titers from BLV- and BLV+ cows. Overall, our results suggest that BLV infection would not interfere with the ability to class-switch, but instead would specifically affect class-switching to IgG2, although at this point it is unclear by what mechanism this would occur.

To investigate potential abnormal cellular phenotypes contributing to reduced antibody production by BLV+ cows, we also investigated B cell activation in response to BHV1 *in vitro*

using CD25 and MHCII as markers of activation. B cells isolated from BLV+ cows exhibited higher proportions of $CD25^+$ B cells *in vitro*. CD25 is the high affinity α chain of the IL2 receptor and can be used as a marker of lymphocyte activation for both B and T cells. Previous research supports our data that B cells from BLV+ cows may be hyper-responsive to certain activation signals; when PBMCs from BLV+ PL cows were cultured in the presence of IL2 or pokeweed mitogen (PWM), the proportion of CD25-expressing B cells was higher in comparison to BLV- cows (Erskine et al., 2011b; Stone et al., 1995). However, sorted B cells from PL cows cultured with IL2 failed to increase CD25 expression (Trueblood et al., 1998), suggesting that B cell reactivity in BLV+ cows depends on signaling from other cell types, likely T cells or monocytes.

In sharp contrast to the CD25 results, MHCII expression on B cells from BLV+ cows was lower and hypo-responsive to stimulation *in vitro*. While CD25 is part of a receptor that integrates external growth and activation signals, MHCII is expressed on antigen presenting cells (APCs) like B cells and functions to present antigen and activate cognate $CD4^+$ T cells. When MHCII on B cells presents antigen to $CD4^+$ T cells, it also participates in the critical signal cascade that causes B cell isotype switching. In addition, similarly to CD25, MHCII expression on B cells increases in response to stimulation (Reith et al., 2005). When looking at the total B cell population, MHCII surface expression was clearly reduced in all culture conditions on B cells from BLV+ cows. When we analyzed activated $CD25^+$ B cells, BLV- and BLV+ cows showed no difference in MHCII surface expression in unstimulated cultures. However, while B cells from BLV- cows showed a consistent increase in MHCII expression in response to stimulation, MHCII expression on B cells from BLV+ cows remained unchanged. Previous research found that MHCII expression was actually higher on freshly isolated B cells from PL

cows (Sordillo et al., 1994; Stone et al., 1995; Trueblood et al., 1998), which is in direct opposition to our results. Indeed, in our own study we saw contrasting results in SIgM expression between freshly isolated versus cultured B cells. However, supporting our results, B cells from PL cows cultured with IL2 failed to increase MHCII expression like B cells from BLV- cows (Stone et al., 1995).

Our *in vitro* B cell activation data demonstrated that B cells isolated from BLV- and BLV+ cows exhibited distinct phenotypes and responses to stimulation. Although we did not measure the total leukocyte count over a period of 3 months to establish PL in enrolled BLV+ cows (Panei et al., 2013), we specifically selected BLV+ cows exhibiting elevated B cell proportions in PBMCs and the high percentage of peripheral B cells (Table 1) strongly suggests that the BLV+ cows in our study are PL, which likely contributed to the observed results. Both CD25 and MHCII data support possible BLV strategies for survival within the host. Increased sCD25 expression could be a mechanism to promote proliferation of infected B cells and, consequently, BLV replication; prior research demonstrated that lymphocytes from BLV+ cows exhibit phenotypes to promote longevity (Erskine et al., 2011b; Frie and Coussens, 2015). In addition, interference with MHCII expression is a common viral evasion mechanism and is utilized by Epstein Barr virus, which also persistently infects B cells (Keating et al., 2002). It is unclear if BLV actively interferes with these pathways, or if its tropism for $CD5^+$ B cells (Meirom et al., 1997) is a result of a distinct activation phenotype unique to $CD5^+$ B cells. $CD5^+$ B cells are innate-like lymphocytes that produce natural IgM and are thought to not class-switch or differentiate into memory cells (Carsetti et al., 2004; Tung et al., 2006), and they are the class of B cells that polyclonally expands during PL (Stone et al., 1995). The expanded proportion of

B cells exhibited by our enrolled BLV+ cows suggests that our study animals exhibited PL, which would also suggest a higher proportion of $CD5^+$ B cells in cultures from BLV+ cows.

Finally, we investigated T cell activation in response to BHV1 *in vitro*. To measure T cell activation, we used flow cytometry to detect IFNγ production from $CD4^+$, $CD8^+$ and γδ T cells. We were interested in IFNγ production specifically because it preferentially induces IgG2 production over IgG1 production in cattle (Estes et al., 1994), in addition to its role as a major proinflammatory cytokine produced by T cells during the course of infection. To our surprise, we did not observe any differences in the percentage of classical $CD4^+$ or $CD8^+$ T cells expressing IFNγ, nor did we observe any difference in the MFI of IFNγ in any T cell subset. Previous work has found that fresh PBMCs from PL cows express less IFNγ than BLV- cows (Amills et al., 2004), while cultured PBMCs stimulated with ConA from PL cows exhibited higher IFNγ activity than PBMCs from BLV- cows (Trueblood et al., 1998). The differences in results are likely attributed to whole PBMC IFNγ production versus T cell subset IFNγ production.

In contrast to classical αβ T cells, γδ T cells did exhibit significantly different activation profiles between BLV- and BLV+ cows. γδ T cells from BLV+ cows exhibited a higher overall prevalence of activated γδ T cells in both unstimulated and ConA-stimulated cultures. Little research has investigated the role of γδ T cells during the course of BLV infection. However, one study found that γδ T cells from AL cows only, not PL cows, displayed heightened cytotoxicity against virally-infected fibroblasts in comparison to BLV- cows (Lundberg and Splitter, 2000), although it's not clear how IFNγ production from γδ T cells relates to their cytotoxicity.

It was surprising that our cell culture showed no differences in $CD4^+$ or $CD8^+$ T cell activation, considering the evidence suggesting increased negative regulation of T cell activity in BLV+ cows. Classical T cells from BLV+ cows expressed higher levels of LAG3 (Konnai et al.,

2013; Shirai et al., 2011), Tim3 and Gal9 (Okagawa et al., 2012), all of which are negative regulators of T cell activation. In addition, BLV+ cows have been shown to have higher proportions of $FoxP3^+$ regulatory T cells (Tregs) (Suzuki et al., 2013) and that these Tregs express higher levels of negative T cell regulator CTLA4 (Suzuki et al., 2015). It is possible that this level of negative regulation is not high enough to impede T cell activation in our culture system; it is also possible that BLV infection negatively interferes with T cell activity through mechanisms independent of IFNγ production.

While γδ T cell activity has not been well characterized in the context of BLV infection, bovine γδ T cell activity in the context of many other infections has been investigated. Cattle are considered to have a high proportion of γδ T cells in the periphery, which is suggestive that they are of particular importance to cattle immunity (Baldwin and Telfer, 2015). Indeed, evidence suggests that γδ T cells in cattle can form memory populations in response to vaccination (Blumerman et al., 2007). In addition, $WC1^+$ γδ T cells in cattle can produce IFNγ in response to stimulation, and this may skew immune polarization to a Th1 phenotype (Baldwin and Telfer, 2015). However, at this point it is unclear why γδ T cells from BLV+ cows have increased reactivity to stimulation *in vitro*, although it is possible that BLV+ cows have a higher ratio of $WC1^+$: $WC1^-$ γδ T cells, which could explain the higher proportion of IFNγ-producing γδ T cells observed. It is also important to note that short term *in vitro* PBMC culture conditions can induce BLV protein expression (Frie and Coussens, 2015), so it is possible that increased reactivity of γδ T cells isolated from BLV+ cows is the result of γδ T cells that are reactive against BLV antigens, not the selected culture stimulant. However, under these culture conditions we were unable to detect BLV gp51 protein expression.

In this study, vaccination was used to determine if BLV+ cows mount a weaker immune response to stimulation. BLV+ cows tended to produce lower antigen-specific IgM and IgG2 antibody titers and displayed atypical B and T cell responses to *in vitro* stimulation after boost vaccination. These data support the hypothesis that BLV+ cows develop abnormal immune responses to stimulation and likely have compromised protection against other infectious diseases. The plasma antibody titers in conjunction with *in vitro* B and T cell activation data also warrant further investigation to understand the mechanism by which BLV infection interferes with antibody production. Further understanding of BLV's effect on the bovine immune system is critical to ensure that the US dairy industry is equipped to assess and address the threat of BLV infection to dairy herds nationwide.

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# CHAPTER 3: DAIRY COWS NATURALLY INFECTED WITH BOVINE LEUKEMIA VIRUS (BLV) EXHIBIT ABNORMAL B AND T CELL PHENOTYPES AFTER PRIMARY AND SECONDARY EXPOSURE TO KEYHOLE LIMPET HEMOCYANIN

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**Abstract**

Bovine leukemia virus (BLV) is a retrovirus that is highly prevalent in US dairy herds: over 83% are BLV-infected and the within-herd infection rate can be almost 50% on average. While BLV is known to cause lymphosarcomas, only 5% or fewer infected cattle will develop lymphoma; this low prevalence of cancer has historically not been a concern to dairy producers. However, more recent research has found that BLV+ cows without lymphoma produce less milk and have shorter lifespans than uninfected herdmates. It has been hypothesized that BLV infection interferes with normal immune function in infected cattle, and this could lead to reduced dairy production. To assess how naturally infected BLV+ cows responded to a primary and secondary immune challenge, 10 BLV+ and 10 BLV- cows were injected subcutaneously with keyhole limpet hemocyanin (KLH) and DDA. B and T cell responses were characterized over the following 28 days. 56 days after primary KLH exposure, cows were re-injected with KLH and B and T cell responses were characterized again over the following 28 days. BLV+ cows produced less KLH-specific IgM after primary immune stimulation; demonstrated fewer $CD45R0^+$ B cells, altered proportions of $CD5^+$ B cells, altered expression of CD5 on $CD5^+$ B cells, and reduced MHCII surface expression on B cells *ex vivo*; exhibited reduced B cell activation *in vitro*; and displayed an increase in BLV PVL after KLH exposure. In addition, BLV+ cows had a reduced $CD45R0^+$ $\gamma\delta^+$ T cell population in the periphery; and demonstrated a greater prevalence of IL4-producing T cells *in vitro*. All together, our results demonstrate that both B and T cell immunity is disrupted in BLV+ cows, and that antigen-specific deficiencies can be detected in BLV+ cows even after a primary immune exposure.

Keywords: Bovine leukemia virus, memory, $CD5^+$ B cells, gamma delta T cells, CD45R0, IFNγ, IL4, antibody

## Introduction

Bovine leukemia virus (BLV) is a δ-retrovirus (Gillet et al., 2007) that infects over 83% of dairy herds in the United States; as many as 40% of all US dairy cattle are infected (Bartlett et al., 2014). BLV is the causative agent of enzootic bovine leukosis (EBL), where BLV+ cattle develop malignant lymphoma or leukemia (Kabeya et al., 2001). Although lymphoma is deadly and results in carcass condemnation at slaughter (Bartlett et al., 2014), it's estimated that fewer than 10% of infected cattle will eventually develop EBL (Kabeya et al., 2001). Unfortunately, recent research suggests that BLV infection has more negative impacts on herd health than previously appreciated.

BLV infection reduces both milk production (Bartlett et al., 2014) and longevity (Bartlett et al., 2013) in infected dairy cows. While it is unclear how BLV interferes with milk production and lifespan, one hypothesis is that BLV causes immune dysregulation, which could put BLV+ cattle at an increased risk for other infections. BLV most commonly infects B cells (Meirom et al., 1997) and can cause persistent lymphocytosis, a chronic, benign, polyclonal expansion of the B cell compartment that occurs in 30% of infected cattle (Kabeya et al., 2001). *In vitro* experiments have demonstrated abnormalities in both innate and adaptive immune cells isolated from BLV+ cattle (Frie and Coussens, 2015). In addition, a few studies have found positive correlations between BLV and other infectious diseases (Emanuelson et al., 1992; Trainin et al., 1996) and a reduction in vaccine immunity in BLV+ cattle (Erskine et al., 2011a; Frie et al., 2016; Puentes et al., 2016). However, when investigating immunity in naturally infected BLV+ cattle, many studies were unable to control for how much antigen exposure occurred before or after BLV infection.

The current study was designed to address that specific problem. BLV+ and BLV- cows were exposed to an immunostimulatory antigen, keyhole limpet hemocyanin (KLH), to mimic a primary immune response. At 56 days after primary exposure, cows were re-exposed to KLH to mimic a secondary memory immune exposure. To characterize both primary and secondary adaptive immune responses, B and T cell responses were tracked using ELISAs to measure antibody production against KLH, flow cytometry to measure the dynamics of freshly isolated B and T cell subsets, and cell culture to investigate B and T cell responses to KLH and mitogenic stimulation *in vitro*. Specifically, CD5 and CD45R0 expression on *ex vivo* B cells and CD45R0 expression on *ex vivo* $CD4^+$, $CD8^+$, $\gamma\delta^+$ T cells was characterized. BLV and CD25 expression was characterized in B cells and IFNγ and IL4 production was characterized in T cells after *in vitro* stimulation. Abnormalities in both B and T cell subsets were detected in BLV+ cattle during both primary and secondary immune responses, providing further support that BLV infection causes immune dysregulation.

## Methods

### Animals and KLH inoculation

10 BLV- and 10 BLV+ lactating Holstein dairy cows were enrolled in the current study (Table 3.1). BLV+ cows (as determined by the producer's BLV milk ELISA results) were not confirmed to have persistent lymphocytosis, but were selected for elevated total leukocyte counts (as determined using a Beckman Coulter counter) and an elevated proportion of circulating B cells (as determined by immunostaining for surface IgM on freshly isolated PBMCs) one week prior to the study's initiation. BLV+ cows all had a high proviral load on d0 (Panei et al., 2013). BLV- cows were then age- and lactation-matched to the 10 selected BLV+ cows. Both BLV- and BLV+ cows were also re-screened for BLV infection using a commercial serum ELISA (NorthStar Cooperative) one week prior to the study start. BLV serum ELISAs and endpoint PCR (on DNA extracted from whole blood) to detect BLV provirus were also used on samples collected on the first and last days of the study to confirm BLV status. One BLV- cow seroconverted in between enrollment diagnostics and the start of the study; this cow and her matched BLV+ cow were excluded from the final data analysis.

Upon study initiation on day 0 (d0), all cows received the primary KLH inoculation consisting of 1.5mL KLH cocktail injected subcutaneously into the left side of the neck. The KLH cocktail was composed of 200μg KLH (Sigma) in 0.75mL 1X phosphate-buffered saline (PBS) containing 5% bovine serum albumin and 0.75mL 20mg/mL adjuvant dimethyldioctadecylammonium bromide (DDA) (Sigma) in 1X PBS (Nguyen et al., 2009). On d56, cows received the secondary KLH inoculation with 1.5mL KLH cocktail subcutaneously injected into the right side of the neck. All protocols were reviewed and approved by the Michigan State University Institutional Animal Use and Care Committee (AUF# 04/16-061-00).

**Table 3.1. Cow enrollment characteristics.** Data are presented from d0. Data are represented as the mean and (range).

| | BLV- cows | BLV+ cows |
|---|---|---|
| Age | 4y11mo (3y7mo – 6y11mo) | 4y11mo (3y7mo – 7y5mo) |
| Days in milk | 203.5 (140 – 350) | 197.7 (127 – 293) |
| Lactation number | 3.3 (2 – 5) | 3.2 (2 – 5) |
| Reproduction status | All pregnant | All pregnant |
| %B cell | 37.89 (29.65 – 48.59) | 55.81 (40.96 – 72.86) |
| PVL per 100,000 cells | N/A | 95,879 (62,247 – 123,429) |

**Whole blood, plasma and PBMC isolation**

Whole blood was collected by coccygeal venipuncture on d0 before primary inoculation and on days 7, 14, 18, 21, 28, 54 and 56 after primary inoculation; after blood collection on d56, the secondary KLH inoculation was administered and blood was collected on days 60, 67, 70, 77 and 84 after primary inoculation (days 4, 11, 14, 21 and 28 after secondary inoculation). Blood for proviral load quantification was collected in Vacutainer blood collection tubes containing the anticoagulant EDTA (Becton Dickinson); 1mL aliquots of whole blood were stored at -80°C. Blood for antibody quantification and PBMC immunostaining was collected in Vacutainer blood collection tubes containing the anticoagulant ACD (Becton Dickinson). Plasma and PBMCs were isolated as previously described (Frie et al., 2016). Briefly, aliquots of plasma with 0.1% sodium azide were stored at -80°C and PBMCs were isolated using Percoll density centrifugation.

**BLV proviral load quantification**

DNA was extracted from whole blood using the DNeasy Blood and Tissue kit (Qiagen) using a modified protocol. Briefly, 200µL whole blood, 40µL proteinase K, 218µL buffer AL and 218µL 100% ethanol were used instead of the recommended kit volumes. Extracted DNA was quantified using a Nanodrop-1000 and A260/280 ratios were used to assess sample purity. DNA was diluted to 30ng/µL in elution buffer for proviral load (PVL) quantification. BLV PVL was determined using the Coordination of Common Motifs (CoCoMo) –qPCR to amplify the long terminal repeat (LTR) of the BLV provirus. To normalize genomic DNA input, the single-copy Bola-DRA gene was also amplified (Jimba et al., 2010). In brief, 30 ng of genomic DNA were assayed using TaqMan Gene Expression Master Mix (Applied Biosystems, California, USA) on the 7500 FAST Real-time PCR System (Applied Biosystems, California, USA). BLV

copy number and BoLA-DRA copy number were calculated using 10 to 1 x $10^5$ copies of the standard plasmid, which contained a copy of BLV-LTR and BoLA-DRA. Each value was calculated using the algorithm suggested by the manufacturer. PVL was the ratio of BLV copy number to BoLA-DRA copy number multiplied by 100,000 (Jimba et al., 2012). PVL was expressed as BLV copy number/100,000 cells.

**Anti-KLH antibody quantification**

Anti-KLH antibodies were quantified from plasma collected on days 0, 7, 14, 21, 28, 56, 60, 67, 70, 77, and 84 after primary inoculation. For the KLH ELISA, flat-bottomed 96-well ELISA plates (Thermo Fisher Scientific) were incubated with 100µL of 1µg/mL KLH in 50mM carbonate/bicarbonate buffer overnight at 4°C. Plates were washed 3X with wash buffer (0.05% Tween-20 in 1X PBS) and blocked with 2% heat-inactivated horse serum in wash buffer for 1h at 37°C. Plates were then washed 5X and 100µL of plasma (diluted 1:50 in blocking buffer) was added to wells and incubated for 1h at room temperature. Plates were washed 5X and then incubated for 1h at room temperature with 100µL anti-bovine IgM, IgG1 or IgG2 conjugated to horseradish peroxidase (Thermo Fisher Scientific) diluted 1:10,000 in blocking buffer as previously described (Frie et al., 2016). Plates were washed 5X and incubated for 15m at room temperature in the dark with 100µL TMB substrate (Sigma). 100µL stop solution (2M $H_2SO_4$) was added and the optical density (OD) was measured at 450nm using a SpectraMax M5 microplate reader. All samples were run in duplicates and each plate included blank and naïve (not exposed to KLH) controls.

**Immunostaining of freshly isolated PBMCs**

Immunostaining of freshly isolated PBMCs was performed as previously described (Frie et al., 2016) on days 0, 7, 14, 21, 28, 56, 60, 67, 70 and 77 after primary inoculation. Briefly, 5 ×

$10^5$ PBMCs were fixed in 4% paraformaldehyde and then labeled via indirect immunofluorescence first with mouse anti-bovine primary antibodies and second with goat anti-mouse secondary antibodies. The freshly isolated B cell population was characterized using the stain labeled "fresh B" in Table 3.2 and the freshly isolated T cell population was characterized using the stain labeled "fresh T" in Table 3.2.

***In vitro* stimulation of PBMCs**

To investigate T cell activation, $2 \times 10^6$ PBMCs were cultured at 38°C and 5% $CO_2$ in 1mL Roswell Park Memorial Institute (RPMI) complete media (RPMI plus 10% fetal bovine serum, 1% penicillin/streptomycin, and 1%fungizone, pH 7.4) in 24-well culture plates (Corning). PBMCs were either cultured in medium alone (NIL) for 18h, with 200μg/mL KLH for 18h, or with 20μg/mL positive control concanavalin A (CONA) for the final 6 hours. All samples were treated with 20ng/mL brefeldin A at 12h to prevent cytokine secretion. T cell activation was measured on days 7, 14, 56, 67 and 77.

To investigate B cell activation, $5 \times 10^6$ PBMCs were cultured at 38°C and 5% $CO_2$ in 3mL RPMI complete media in 12-well culture plates (Corning) with medium alone (NIL), 200μg/mL KLH, or with positive control 20ng/mL phorbol 12-myristate 13-acetate (PMA) and 400ng/mL ionomycin (P/I) for 18h. B cell activation was measured on days 18, 54 and 70.

**Immunostaining of cultured PBMCs**

PBMCs were labeled with 4-color stains to investigate IFNγ production by T cells ("IFNγ" in Table 3.2), IL4 production by T cells ("IL4" in Table 3.2), and BLV expression in B cells ("CD45R0" and "CD25" in Table 3.2). Immunostaining of cultured PBMCs was performed as previously described (Frie et al., 2016). Briefly, indirect immunostaining for surface receptors on live PBMCs was done as described for freshly isolated PBMCs. PBMCs were then fixed and

**Table 3.2. Antibodies used for immunostaining experiments.** All primary MAbs were purchased from Washington State University Monoclonal Antibody Center except IFNγ and IL4 (Bio-Rad) and BLV gp51 (VMRD). All secondary antibodies were purchased from Thermo Fisher Scientific except IgM PE-Cy7 (eBioscience) and IgG2b PE-Cy7 (Abcam). [a] indicates antibodies used for intracellular labeling.

| Stain | 1°MAb target | Target Phenotype | Clone | 2° Ab Target |
|---|---|---|---|---|
| Fresh B | CD45R0 | Effector/memory lymphocytes | ILA116A | IgG3 AF88 |
| | MHCII | Antigen presentation | TH16A | IgG2a PE |
| | SIgM | B cells | PIG45A | IgG2b PE-Cy7 |
| | CD5 | $CD5^+$ B cells | CACT105A | IgG1 AF647 |
| Fresh T | CD45R0 | Effector/memory lymphocytes | ILA116A | IgG3 AF88 |
| | CD4 | Helper T cells | IL11A | IgG2a PE |
| | CD8 | Cytotoxic T cells | BAQ111A | IgM PE-Cy7 |
| | γδ TcR | Gamma delta T cells | GB21A | IgG2b AF647 |
| IFNγ | γδ TcR | Gamma delta T cells | GB21A | IgG2b AF88 |
| | CD4 | Helper T cells | IL11A | IgG2a PE |
| | CD8 | Cytotoxic T cells | BAQ111A | IgM PE-Cy7 |
| | IFNγ[a] | Th1 cytokine | CC302 | IgG1 AF647[a] |
| IL4 | γδ TcR | Gamma delta T cells | GB21A | IgG2b AF88 |
| | CD4 | Helper T cells | CACT138A | IgG1 PE |
| | CD8 | Cytotoxic T cells | BAQ111A | IgM PE-Cy7 |
| | IL4[a] | Th2 cytokine | CC303 | IgG2a AF647[a] |
| CD45R0 | CD45R0 | Effector/memory lymphocytes | ILA116A | IgG3 AF88 |
| | MHCII | Antigen presentation | TH16A | IgG2a PE |
| | SIgM | B cells | PIG45A | IgG2b PE-Cy7 |
| | BLV gp51[a] | BLV-expressing B cells | | IgG1 AF647[a] |
| CD25 | CD25 | IL2 receptor, α chain | LCTB2A | IgG3 AF88 |
| | MHCII | Antigen presentation | TH16A | IgG2a PE |
| | SIgM | B cells | PIG45A | IgG2b PE-Cy7 |
| | BLV gp51[a] | BLV-expressing B cells | | IgG1 AF647[a] |

permeabilized using a fixation/permeabilization kit according to manufacturer's instructions (eBioscience) and PBMCs were labeled with a primary mouse anti-bovine or anti-BLV monoclonal antibody and subsequently labeled with a goat anti-mouse secondary antibody. Labeled PBMCs were stored at 4°C overnight until flow cytometry analysis.

**Immunostaining analysis**

Data were acquired using an Accuri C6 flow cytometer equipped with CSampler (Becton Dickinson). Data were compensated using Accuri C6 software (Becton Dickinson) and then exported for analysis using FCS Express 4 (De Novo Software). PBMCs were initially selected using forward and side scatter gating to exclude debris, and cells were identified gating on lineage-specific markers (CD4, CD8, γδ, or surface IgM {SIgM}). Expression of IFNγ, IL4, CD45R0, MHCII, CD5, and CD25 were then subsequently characterized within specific cell types, and marker expression was expressed as either the mean relative percent or the mean fluorescence intensity (MFI). Unless noted, all referenced B cells are $SIgM^{+}MHCII^{+}$. CD5 labeling on fresh PBMCs exhibited 3 populations; the center population was denoted "$CD5^{dim}$" and the far right population was denoted "$CD5^{bright}$".

**Statistics**

Statistical analysis was performed using SAS 9.4 (SAS Institute). Antibody and fresh PBMC data were analyzed using repeated measures ANOVA with BLV and time as fixed effects and post hoc Bonferroni correction for pairwise comparisons. Cultured PBMC data were analyzed using repeated measures ANOVA with BLV and stimulant as fixed effects and post hoc Bonferroni correction for pairwise comparisons. A Tukey test was used to analyze the difference in MHCII or CD25 MFIs between different B cell subsets. Outliers were detected using Grubbs

test; both outliers and their matched cow were eliminated from analysis. Significance was determined as $p<0.05$.

## Results

### BLV+ cows exhibit reduced IgM production *in vivo*

To characterize the strength of the humoral immune response in BLV+ cows, the relative quantities of KLH-specific IgM, IgG1 and IgG2 were measured. As expected, both BLV+ and BLV- cows produced KLH-specific IgM, IgG1 and IgG2 after primary and secondary KLH inoculation ($p<0.001$ for all isotypes). Interestingly, BLV+ cows had less IgM ($p=0.0045$) after primary KLH inoculation at all time points, including d0. However, BLV+ cows made IgM at levels equal to BLV- cows after the secondary KLH inoculation ($p=0.7742$) (Figure 3.1A). In contrast to IgM production, BLV+ and BLV- cows produced equivalent levels of IgG1 ($p=0.6715$) (Figure 3.1B) and IgG2 ($p=0.9437$) (Figure 3.1C) after both primary and secondary KLH inoculation. These data support recent evidence that BLV infection interferes with IgM antibody production after both a primary immune challenge and a common vaccine booster injection (Frie et al., 2016).

### BLV+ cows demonstrate abnormal circulating B cell populations *in vivo*

To investigate how circulating B cell populations changed in response to immune stimulation, freshly isolated PBMCs were immunostained to examine both CD45R0 and CD5 B cell populations in BLV+ and BLV- cows. Unless noted in the text, all B cells are $SIgM^+MHCII^+$. Surprisingly, the $SIgM^+$ B cell population from BLV+ cows did steadily increase over time ($p<0.05$) after both primary and secondary KLH inoculations; however, this trend was not observed in BLV- cows. As expected, BLV+ cows had significantly more circulating B cells than uninfected, age-matched cows ($p<0.0001$) (Figure 3.2C). Some B cell populations did not shift in response to KLH inoculation: in both BLV+ and BLV- cows, the mean relative percent of $MHCII^+$ $SIgM^+$ B cells and $CD45R0^+$ B cells, as well as the CD5 MFI on B cells, all remained

**Figure 3.1. Anti-KLH antibodies produced by BLV+ and BLV- cows.** Anti-KLH antibodies from BLV+ and BLV- cows were relatively quantified. **A)** IgM production. **B)** IgG1 production. **C)** IgG2 production. *$p<0.05$. $n$=7-9/group. Arrows denote KLH inoculations. Data represent the mean ± SEM.

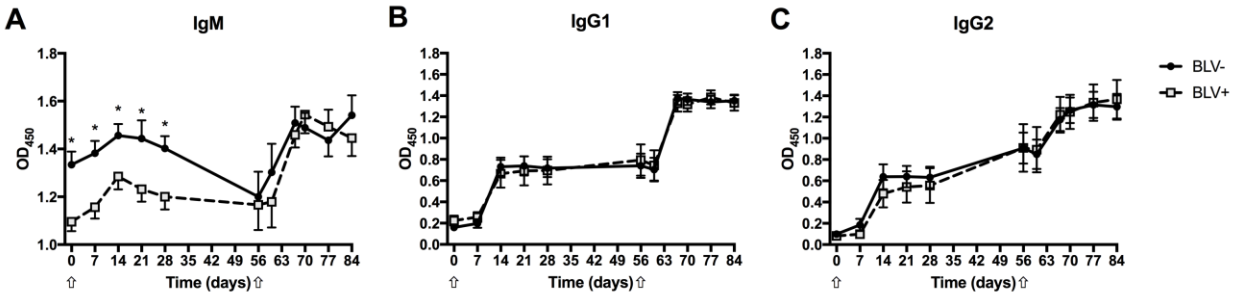
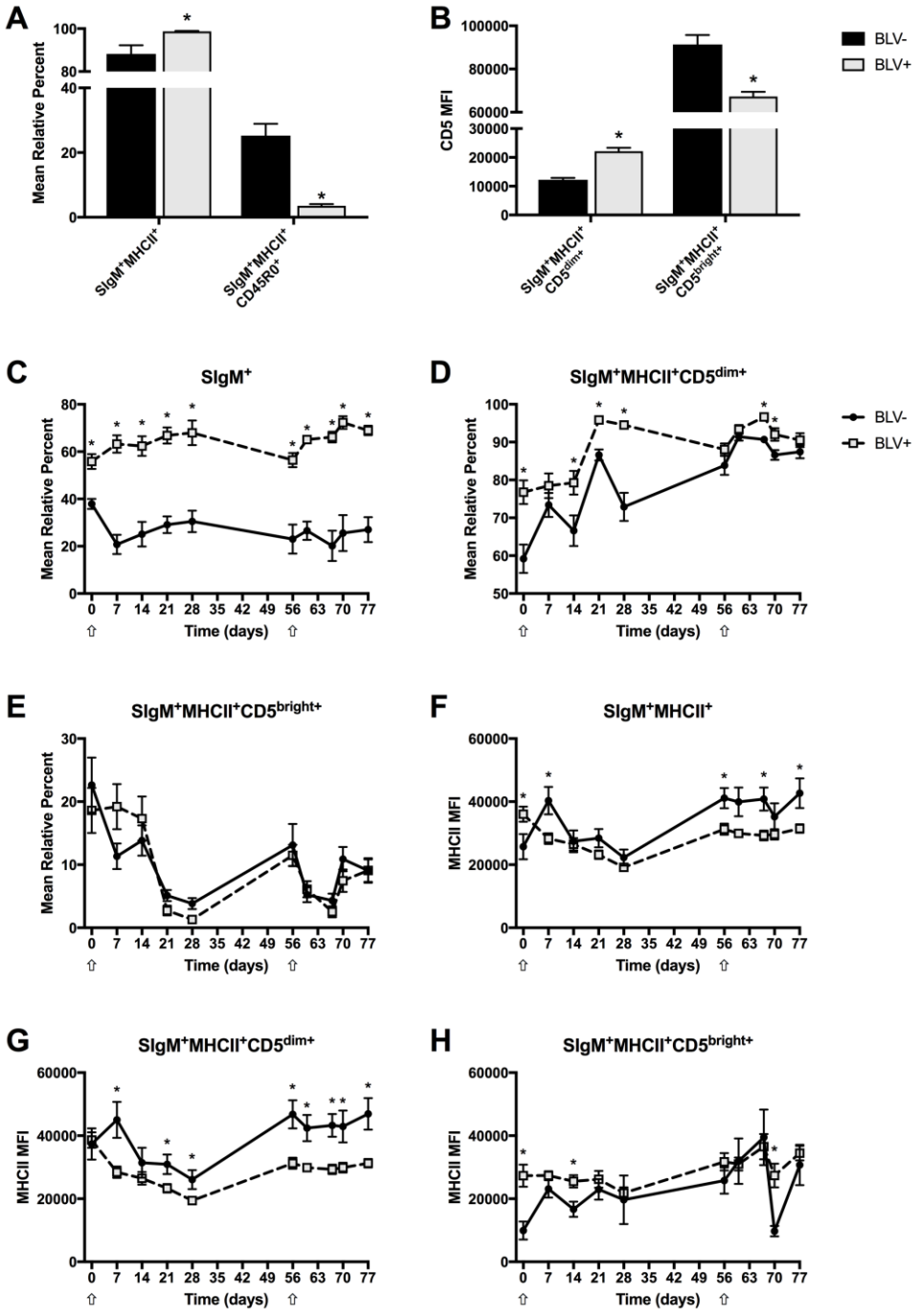


**Figure 3.2. Circulating B cell populations in BLV+ and BLV- cows.** Freshly isolated PBMCs from BLV+ and BLV- cows were immunostained to characterize the circulating B cell population. **A)** Mean relative percentages of B cell populations on d0. **B)** MFIs of B cell populations on d0. **C)** Mean relative percent of $SIgM^+$ PBMCs. **D)** Mean relative percent of $CD5^{dim+}$ $SIgM^+MHCII^+$ B cells. **E)** Mean relative percent of $CD5^{bright+}$ $SIgM^+MHCII^+$ B cells. **F)** MHCII MFI on $SIgM^+MHCII^+$ B cells. **G)** MHCII MFI on $SIgM^+MHCII^+CD5^{dim+}$ B cells. **H)** MHCII MFI on $SIgM^+MHCII^+CD5^{bright+}$ B cells. *$p<0.05$. $n$=6-9/group. Arrows denote KLH inoculations. Data represent the mean ± SEM.



constant. However, these populations were all altered in BLV+ cows. While BLV+ cows exhibited a higher proportion of $MHCII^+$ $SIgM^+$ B cells ($p$=0.0084), they also demonstrated a large reduction in $CD45R0^+$ B cells compared to uninfected, age-matched controls ($p$<0.0001) (Figure 3.2A). When analyzing CD5 expression on B cells, two distinct positive populations were observed, which we denoted $CD5^{dim}$ and $CD5^{bright}$ (Figure 3.3). Interestingly, B cells from BLV+ cows had higher CD5 expression on $CD5^{dim+}$ B cells ($p$=0.0002), but lower CD5 expression on $CD5^{bright+}$ B cells ($p$=0.0017) (Figure 3.2B).

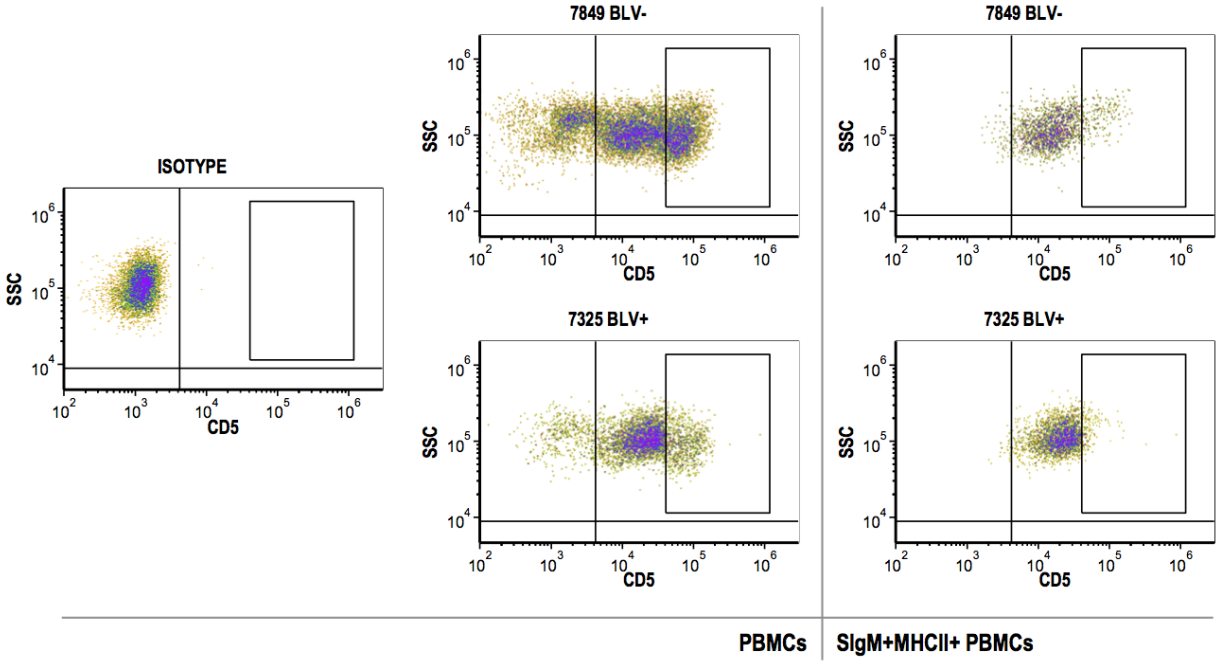
Although the surface expression of CD5 did not change over time, both $CD5^{dim+}$ and $CD5^{bright+}$ B cell populations responded to KLH inoculation ($p$<0.05); while the $CD5^{dim+}$ B cell population steadily increased after inoculation, the $CD5^{bright+}$ B cell population sharply declined. In addition, BLV+ cows displayed a lower $CD5^{dim+}$ B cell population ($p$=0.0043) (Figure 3.2D), but the $CD5^{bright+}$ B cell population was equal between BLV+ and BLV- cows ($p$=0.9611) (Figure 3.2E).

We were also interested in the effect of BLV infection on MHCII surface expression. Surprisingly, MHCII surface expression actually fluctuated in response to KLH exposure on all measured B cell types ($p$<0.05). BLV+ cows presented lower MHCII surface expression on $CD5^{dim+}$ B cells ($p$=0.0168) (Figure 3.2G) and a trending lower MHCII surface expression on all B cells ($p$=0.0722) (Figure 3.2F). In contrast, BLV+ and BLV- cows exhibited equivalent MHCII surface expression on $CD45R0^+$ ($p$=0.7919) (data not shown) and $CD5^{bright+}$ B cells ($p$=0.5662) (Figure 3.2H).

**B cells from BLV+ cows develop atypical phenotypes after *in vitro* stimulation**

To determine if KLH stimulation *in vitro* induced BLV expression in infected B cells, PBMCs were cultured in the presence of KLH or P/I positive control stimulation and BLV

**Figure 3.3. Gating strategy to determine CD5$^{dim+}$ and CD5$^{bright+}$ B cells.** To identify CD5$^{+}$ dim and bright populations, the upper right quadrant was used to denote CD5$^{+}$ cells. A rectangle gate was used to select CD5$^{bright+}$ cells, and cells inside the quadrant gate and outside the rectangle gate were determined to be CD5$^{dim+}$ cells. Representative plots are from fresh labeling on D56 and feature samples from representative BLV- and BLV+ cows.



expression (by immunostaining for viral protein BLV gp51) and B cell activation (by immunostaining for CD25) were characterized. While B cell culture was done on d18, 54 and 70, there was no difference in results from different time points. Thus, results presented are from d70.

There was no overall difference between BLV+ and BLV- cows when comparing the mean relative percent of $CD25^+$ B cells ($p$=0.5849), and both KLH and P/I stimulation increased the proportion of $CD25^+$ B cells. However, B cells from BLV+ cows exhibited only a trending ($p$=0.053) rise in $CD25^+$ B cells after KLH stimulation; in fact, the proportion of $CD25^+$ B cells in KLH-stimulated cultures from BLV+ cows was significantly lower than KLH-stimulated cultures from BLV- cows (Figure 3.4A).

In contrast to the mean relative percent of activated B cells, the surface expression of CD25 on B cells was significantly affected by BLV status ($p$=0.0044). Only B cells from BLV- cows demonstrated higher CD25 MFI after P/I stimulation; B cells from BLV+ cows actually decreased CD25 surface expression after KLH stimulation, and the surface expression of CD25 was lower on B cells from BLV+ cows, in comparison to B cells from BLV- cows, after both KLH and P/I stimulation (Figure 3.4B).

PBMCs were also cultured to investigate if KLH stimulation could induce BLV expression in B cell populations from BLV+ cows. KLH failed to trigger BLV expression in any measured B cell subset. Surprisingly, KLH stimulation actually reduced the proportion of BLV-expressing $CD45R0^+$ B cells. However, CD45R0 expression on B cells did not have a significant effect on BLV expression overall ($p$=0.8236) (Figure 3.5A). In contrast, CD25 expression on B cells did have a significant effect on BLV expression ($p$=0.0013); $CD25^+$ B cells under all culture

**Figure 3.4. B cell activation in BLV+ and BLV- cows after *in vitro* stimulation.** PBMCs from BLV+ and BLV- cows were cultured in the presence of no (NIL), KLH or positive control (P/I) stimulation and activation was measured by CD25 expression. **A)** Mean relative percent of $CD25^+$ $SIgM^+MHCII^+$ B cells. **B)** CD25 MFI on $SIgM^+MHCII^+CD25^+$ B cells. *$p$<0.05 compared to BLV-, ◆◆$p$<0.05 compared to nil, ◆$p$<0.1 compared to nil. $n$=8-9/group. Data represent the mean ± SEM.

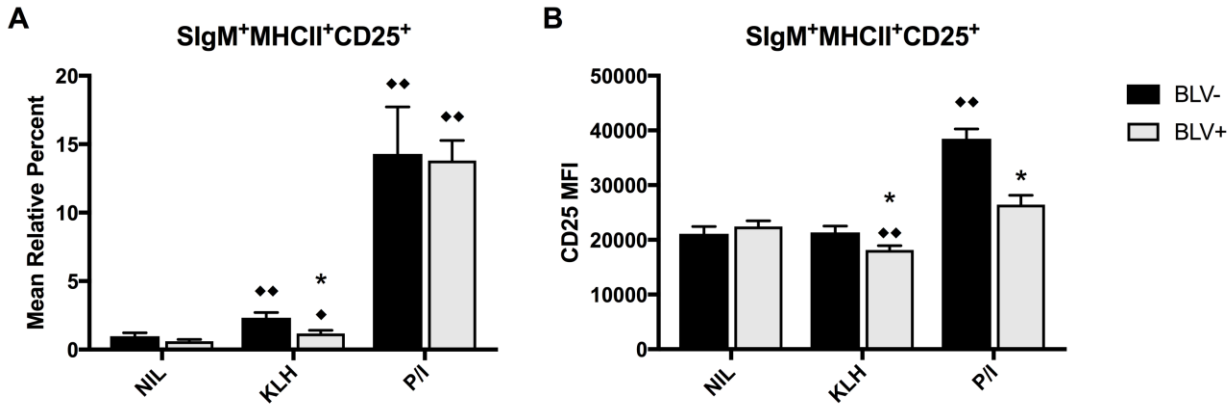
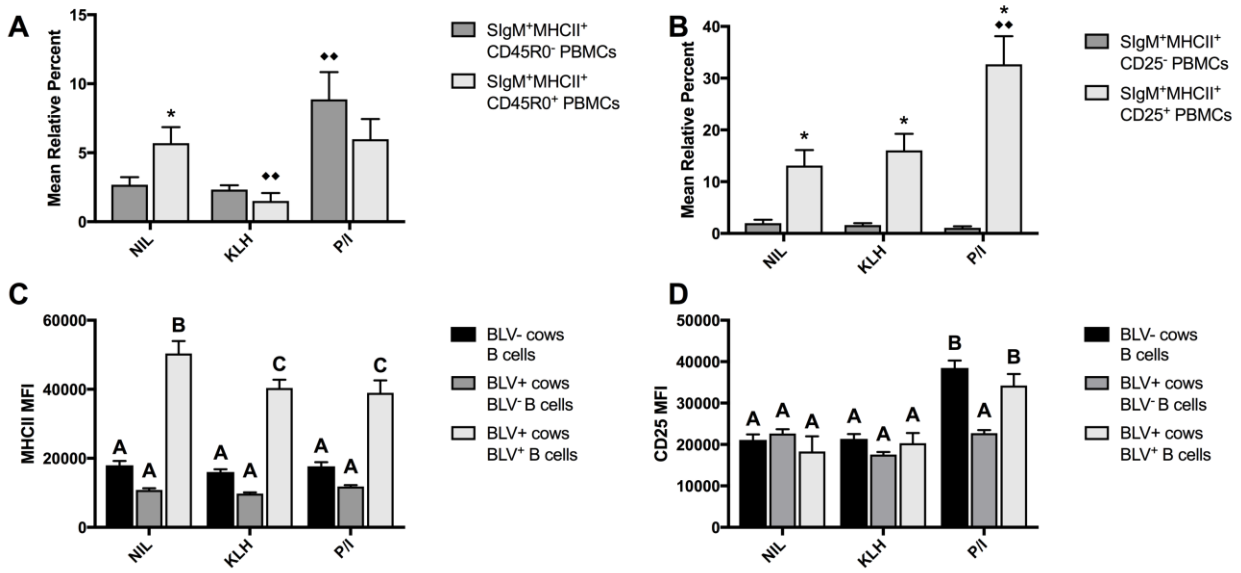


**Figure 3.5. BLV expression *in vitro*.** PBMCs from BLV+ and BLV- cows were cultured in the presence of no (NIL), KLH, or positive control (P/I) stimulation. **A)** Mean relative percent of $BLV^+$ $SIgM^+MHCII^+CD45R0^-$ or $BLV^+$ $SIgM^+MHCII^+CD45R0^+$ B cells from BLV+ cows. **B)** Mean relative percent of $BLV^+$ $SIgM^+MHCII^+CD25^-$ or $BLV^+$ $SIgM^+MHCII^+CD25^+$ B cells from BLV+ cows. **C)** MHCII MFI on $SIgM^+MHCII^+$ B cells from BLV- cows, $SIgM^+MHCII^+BLV^-$ B cells from BLV+ cows and $SIgM^+MHCII^+BLV^+$ B cells from BLV+ cows. **D)** CD25 MFI on $SIgM^+MHCII^+$ B cells from BLV- cows, $SIgM^+MHCII^+CD25^+BLV^-$ B cells from BLV+ cows and $SIgM^+MHCII^+CD25^+BLV^+$ B cells from BLV+ cows. *$p$<0.05 compared to $CD45R0^-$ or $CD25^-$ B cells, ◆◆$p$<0.05 compared to nil. Different letters denote significant differences. $n$=8-9/group. Data represent the mean ± SEM.



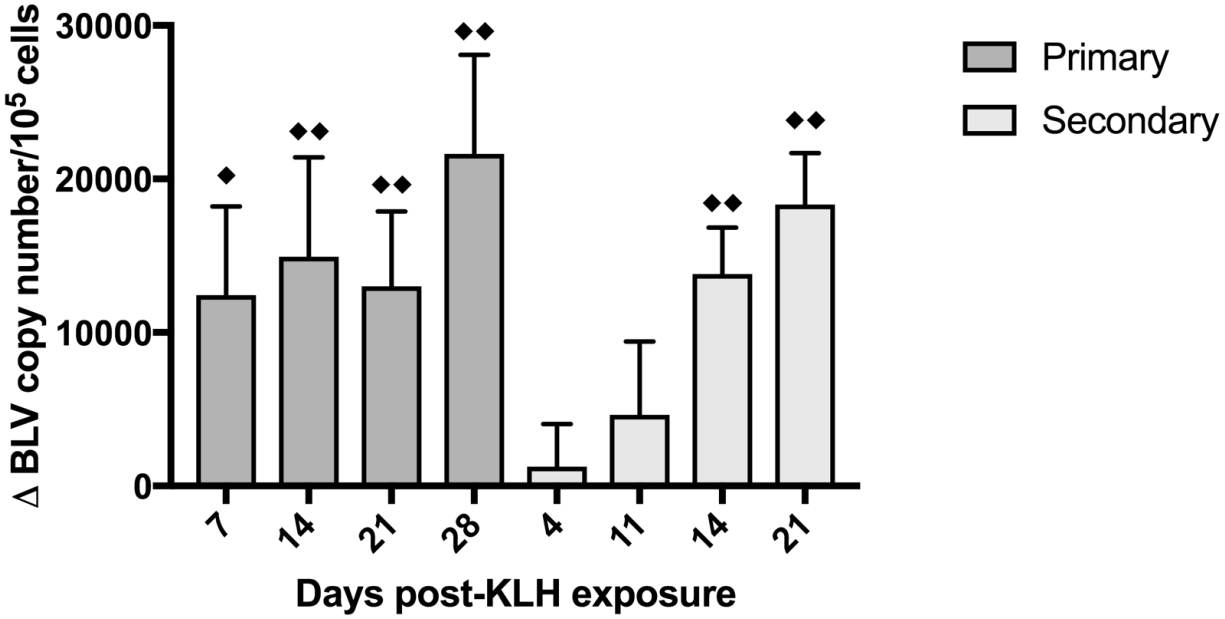
conditions exhibited a much greater proportion of BLV expression in comparison to $CD25^-$ B cells (Figure 3.5B).

We also explored how BLV expression affected B cell phenotypes *in vitro*. When comparing B cells from BLV- cows to B cells from BLV+ cows that did ($BLV^+$) or did not ($BLV^-$) express BLV protein gp51, $BLV^+$ B cells expressed much higher MHCII in all culture conditions in comparison to both $BLV^-$ B cells and B cells from BLV- cows (Figure 3.5C). When investigating the effect of BLV expression on CD25 surface expression, CD25 MFI increased in P/I-stimulated cultures as expected, but only on B cells from BLV- cows and $BLV^+$ B cells from BLV+ cows; $BLV^-$ B cells from BLV+ cows failed to increase the CD25 MFI (Figure 3.5D). Taken together, these results suggest that B cells from BLV+ cows are less reactive to *in vitro* stimulation than B cells from BLV- cows; BLV expression *in vitro* is more prevalent in activated B cells; and B cells from BLV+ cows demonstrate different phenotypes in comparison to B cells from BLV- cows, both in $BLV^-$ and $BLV^+$ B cell subsets.

**BLV proviral load *in vivo* increases after KLH inoculation**

In order to assess how the BLV PVL changed over time after KLH+DDA exposure, DNA was extracted from whole blood from BLV+ cattle and qPCR was used to measure PVL. The average BLV PVL on d0 before inoculation was almost 96,000 copies/$10^5$ cells; after both primary and secondary KLH inoculations, the PVL sequentially increased over time ($p<0.0001$) (Figure 3.6). BLV+ cows exhibited the largest increase in PVL on the final day of collection (28 days post-exposure for primary and 21 days post-exposure for secondary); in both cases, the PVL increased by over 18,000 copies/$10^5$ cells.

**Figure 3.6. BLV proviral load in whole blood in BLV+ cows.** DNA was extracted from whole blood collected from BLV+ cows after KLH inoculation and the BLV proviral load was measured. The change in proviral load after either primary (d0) or secondary (d56) KLH+DDA injection was determined. ◆◆$p$<0.05 compared to d0 (for primary) or to d56 (for secondary), ◆$p$<0.1 compared to d0 or d56. $n$=10. Data represent the mean ± SEM.



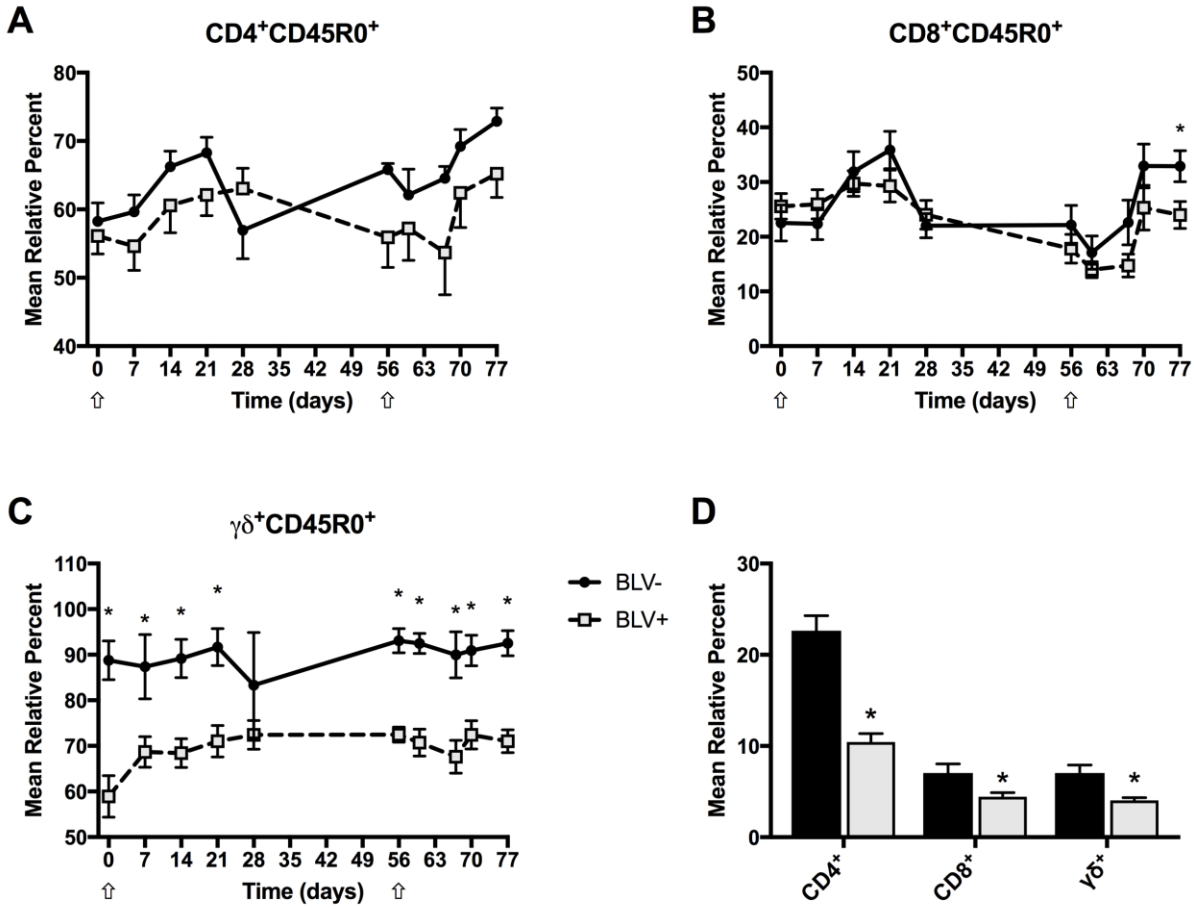
**BLV+ cows have reduced proportions of circulating T cell populations *in vivo***

Although BLV most commonly infects B cells, previous research has suggested that T cell immunity is also compromised in BLV+ cows (Frie and Coussens, 2015). To investigate the circulating effector and memory T cell population in BLV+ cows after KLH inoculation, we labeled freshly isolated PBMCs to measure CD45R0 expression on $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T cells. BLV+ cows consistently exhibited lower relative proportions of $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T cells ($p<0.05$) (Figure 3.7D), although these did not change over time. However, both $CD4^+CD45R0^+$ (Figure 3.7A) and $CD8^+CD45R0^+$ (Figure 3.7B) T cell populations significantly responded to primary and secondary KLH inoculation ($p<0.05$): both populations increased after inoculation, although there was no difference between $CD4^+CD45R0^+$ (p=0.3816) or $CD8^+CD45R0^+$ (p=0.4237) populations in BLV+ and BLV- cows. In contrast to classical T cells, the $\gamma\delta^+CD45R0^+$ population did not significantly change over time after KLH exposure; however, BLV+ cows demonstrated a greatly diminished $\gamma\delta^+CD450^+$ T cell population ($p<0.0001$) (Figure 3.7C). These data indicate that while BLV infection does not appear to affect the classical effector/memory T cell compartment, BLV infection may negatively impact the γδ effector/memory T cell compartment.

**T cells from BLV+ cows produce more IL4 after stimulation *in vitro***

In addition to investigating the circulating effector/memory T cell compartment, we examined whether T cells from BLV+ cows generated IFNγ or IL4 in response to stimulation *in vitro*. While *in vitro* T cell activation was measured on d7, 14, 56, 67 and 77, there was no difference between time points; thus, the IFNγ data presented in Figure 8 is from d56. Overall, KLH stimulation failed to increase the proportion of $IFN\gamma^+$ in any T cell subset, and actually decreased the proportion of $IFN\gamma^+$ $CD8^+$ T cells from both BLV+ and BLV- cows. There was no

**Figure 3.7. Circulating T cell populations in BLV+ and BLV- cows.** Freshly isolated PBMCs from BLV+ and BLV- cows were immunostained to characterize the circulating T cell population. **A)** Mean relative percent of $CD45R0^+$ $CD4^+$ T cells. **B)** Mean relative percent of $CD45R0^+$ $CD8^+$ T cells. **C)** Mean relative percent of $CD45R0^+$ $\gamma\delta^+$ T cells. **D)** Mean relative percent of $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T cell populations on d0. **p*<0.05. *n*=6-9/group. Arrows denote KLH inoculations. Data represent the mean ± SEM.



overall difference between BLV+ and BLV- cows in the proportion of IFNγ$^+$ CD4$^+$ ($p$=0.150) (Figure 3.8A) or CD8$^+$ T cells ($p$=0.112) (Figure 3.8B). Unlike classical T cells, γδ$^+$ T cell IFNγ production was significantly higher in BLV+ cows ($p$=0.0007) (Figure 3.8C).

We also measured the IFNγ MFI as a measure of reactivity to *in vitro* stimulation. While there was no difference in IFNγ MFI from CD4$^+$ or γδ$^+$ T cells between BLV+ and BLV- cows (data not shown), BLV status did have a significant effect on IFNγ MFI from CD8$^+$ T cells ($p$=0.044): reactive CD8$^+$ T cells from BLV+ cows were actually producing more IFNγ than reactive CD8$^+$ T cells from BLV- cows (Figure 3.8D).

We also studied IL4 production by T cell subsets in BLV+ cows. Similar to the *in vitro* IFNγ results, *in vitro* IL4 production was not different between time points; thus, the IL4 data presented in Figure 8 is from d77. Surprisingly, *in vitro* IL4 production was not similar to what was observed with *in vitro* IFNγ production. BLV status had a significant or trending significant effect on the proportion of IL4-producing cells within CD4$^+$ ($p$=0.0009), CD8$^+$ ($p$=0.0006), and γδ$^+$ T cell populations ($p$=0.0879). In all three T cell populations, BLV+ cows consistently exhibited a higher proportion of IL4-producing T cells (Figures 3.9A-C). However, reactive CD8$^+$ T cells from BLV+ cows actually exhibited lower IL4 expression in comparison to reactive CD8$^+$ T cells from BLV- cows ($p$<0.0001) (Figure 3.9D). The IL4 MFI from CD4$^+$ and γδ$^+$ T cells was not different between BLV+ and BLV- cows (data not shown). Taken together, these results imply that T cells from BLV+ cows are capable of producing both IFNγ and IL4 after *in vitro* cell culture; however, the balance in BLV+ cows may favor a greater proportion of IL4-producing T cells.

**Figure 3.8. IFNγ production by T cells from BLV+ and BLV- cows.** PBMCs from BLV+ and BLV- cows were cultured with no (NIL), KLH, or positive control (CONA) stimulation and IFNγ production by T cell subsets was measured. **A)** Mean relative percent of $IFN\gamma^+$ $CD4^+$ T cells. **B)** Mean relative percent of $IFN\gamma^+$ $CD8^+$ T cells. **C)** Mean relative percent of $IFN\gamma^+$ $\gamma\delta^+$ T cells. **D)** IFNγ MFI on $CD8^+$ $IFN\gamma^+$ T cells. **p*<0.05 compared to BLV-, ◆◆*p*<0.05 compared to nil, ◆*p*<0.1 compared to nil. *n*=8-9/group. Data represent the mean ± SEM.

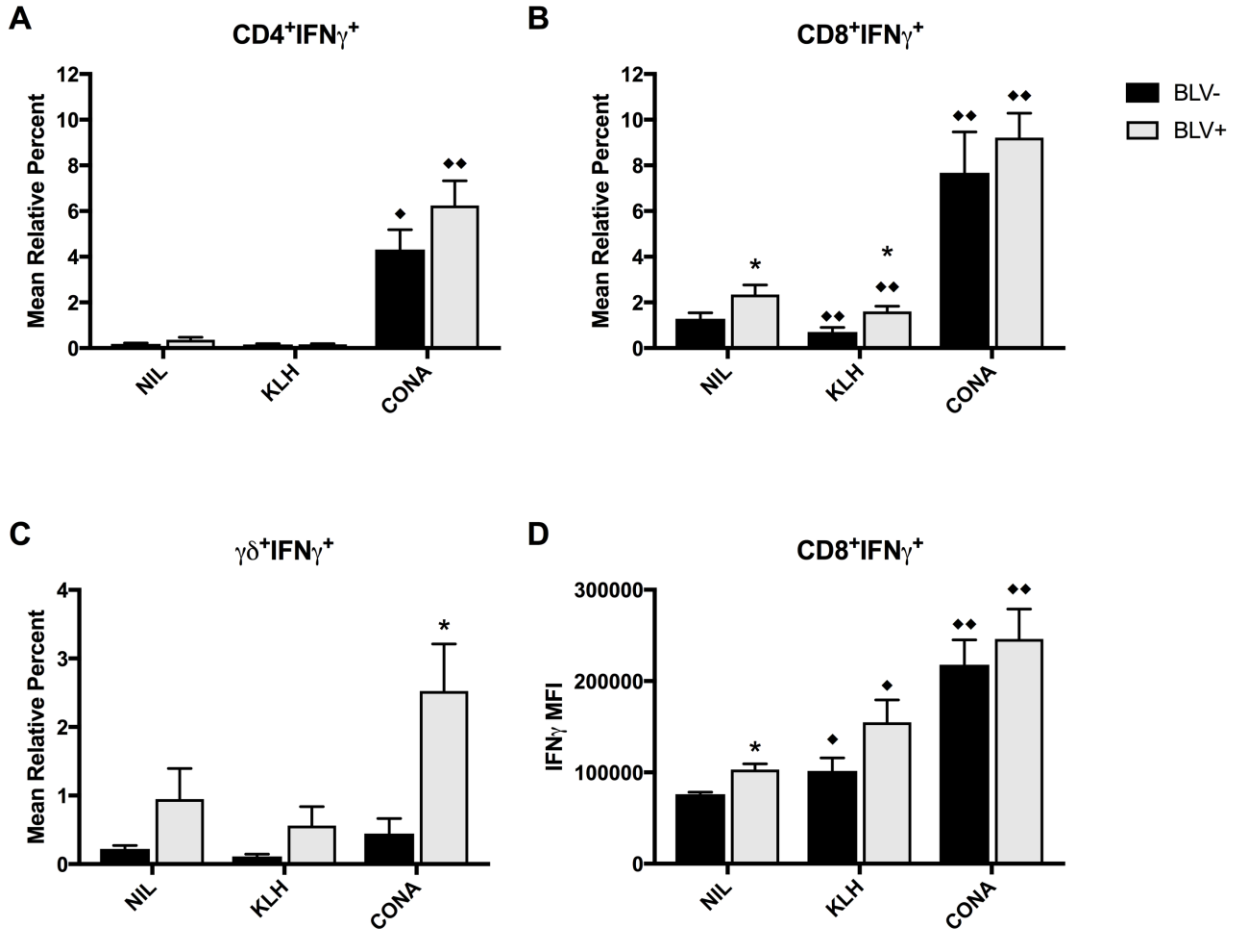
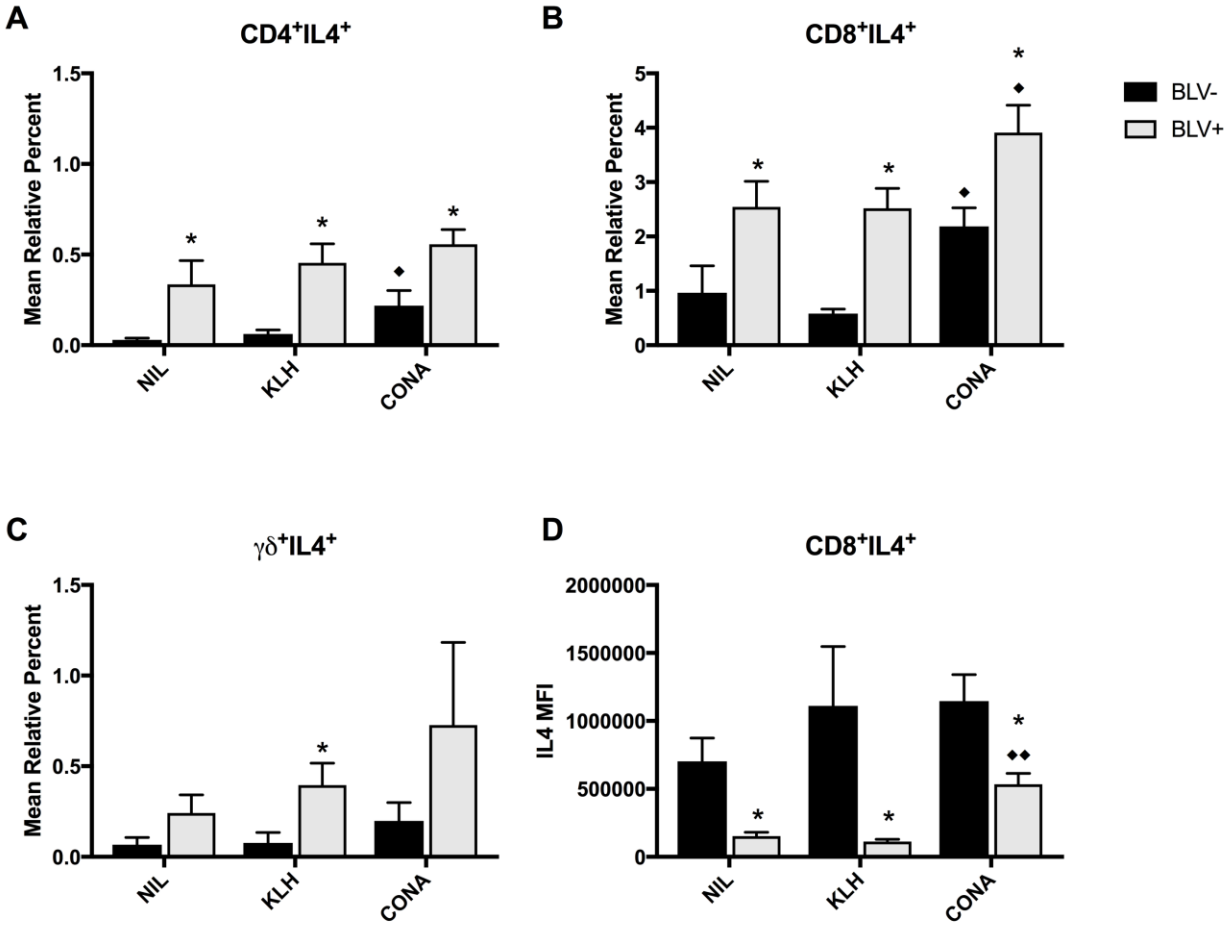


**Figure 3.9. IL4 production by T cells from BLV+ and BLV- cows.** PBMCs from BLV+ and BLV- cows were cultured with no (NIL), KLH, or positive control (CONA) stimulation and IL4 production by T cell subsets was measured. **A)** Mean relative percent of $IL4^+$ $CD4^+$ T cells. **B)** Mean relative percent of $IL4^+$ $CD8^+$ T cells. **C)** Mean relative percent of $IL4^+$ $\gamma\delta^+$ T cells. **D)** IL4 MFI on $CD8^+IL4^+$ T cells. *$p<0.05$ compared to BLV-, ◆◆$p<0.05$ compared to nil, ◆$p<0.1$ compared to nil. $n$=7-8/group. Data represent the mean ± SEM.



**Discussion**

In this study, we investigated how BLV+ dairy cows respond to both a primary and a secondary immune stimulation. After exposing BLV+ and BLV- cows to a noninfectious antigen (KLH), both B and T cell responses were tracked over a three-month period to measure both the primary and secondary immune responses. Alterations were observed in both B and T cell immunity in BLV+ cows relative to uninfected herdmates: BLV+ cows produced lower titers of KLH-specific IgM after primary immune stimulation; exhibited fewer $CD45R0^{+}$ B cells, increased $CD5^{dim+}$ B cells with higher CD5 expression, reduced CD5 expression on $CD5^{bright+}$ B cells, and reduced MHCII surface expression on B cells in circulation; displayed reduced B cell activation *in vitro*; and exhibited an increase in BLV PVL. In addition, BLV+ cows demonstrated a reduced $CD45R0^{+}$ $\gamma\delta^{+}$ T cell population in the periphery; and demonstrated a greater prevalence of IL4-producing T cells *in vitro*. Taken together, these results suggest that BLV+ cows do have abnormal immune responses even upon a primary immune stimulation. Atypical immune responses may make BLV+ cows more vulnerable to other infections of economic importance. This risk is readily apparent at first exposure to a novel antigen as well as after repeated exposure to a common vaccine (Frie et al., 2016).

Similar to results seen in previous studies (Frie et al., 2016; Puentes et al., 2016), BLV+ cows demonstrated less antigen-specific IgM after primary stimulation. Although BLV+ cows produced equivalent levels of IgM after secondary stimulation, this appears to be the result of IgM levels declining in BLV- cows between primary and secondary stimulation. In contrast, no differences were observed in either IgG1 or IgG2 production against KLH. While other studies have found altered IgG levels in BLV+ cattle (Erskine et al., 2011a; Puentes et al., 2016), it is possible that any impairment of IgG production is only detectable after repeated antigen

exposure. While IgG antibodies have higher affinity for antigens, IgM is particularly important during a primary immune response as the first secreted isotype and is especially important for activating complement, which provides critical protection against bacterial infections.

It was interesting to note that the overall circulating B cell population in BLV+ cows alone increased after both primary and secondary KLH stimulation. 30% of BLV+ cattle develop persistent lymphocytosis (PL), which is characterized by a chronic, polyclonal expansion of B cells in peripheral blood (Kabeya et al., 2001). While the mechanisms by which BLV causes PL are not well understood, B cell population dynamics are clearly dysregulated during polyclonal expansion (Frie and Coussens, 2015). Because the prevalence of circulating B cells in BLV+ cows did not remain elevated between primary and secondary stimulations, it suggests that the prevalence of circulating B cells in the periphery was the result of KLH+DDA exposure. It was especially intriguing that the BLV PVL significantly increased over time after both the primary and secondary KLH inoculations. BLV primarily infects B cells (Meirom et al., 1997) and prior research found that 66% of $CD5^+$ B cells carried the provirus (Mirsky et al., 1996), so these results could indicate that BLV-infected B cells proliferate in response to immune stimulation.

In addition to the total B cell population, two subpopulations of circulating B cells were tracked. Although $CD45R0^+$ B cells did not change over time, BLV+ cows consistently demonstrated a substantial reduction in the prevalence of $CD45R0^+$ B cells. While CD45R0 expression is better characterized on αβ T cells, its expression on B cells likely indicates either 1) a memory B cell or 2) a differentiating B cell that may become either a memory B cell or a plasma cell (McHeyzer-Williams and McHeyzer-Williams, 2005). The smaller $CD45R0^+$ B cell population in BLV+ cows at all time points could indicate a deficiency in either developing or maintaining B cell memory, which would be especially detrimental for any immune response

dependent on antibody production. Another concerning result was that the overall B cell population, and the $CD5^{dim+}$ B cell population in particular, in BLV+ cows had lower MHCII surface expression, which could also impair humoral immunity development that is required for isotype switching. Although in this study we did not observe reduced IgG1 or IgG2 antibody production in BLV+ cows, this may be because we only tested a secondary antigen exposure. A previous study has found reduced IgG2 antibody production in BLV+ cows after repeated vaccination (Erskine et al., 2011a).

When examining the $CD5^{+}$ B cell compartment, we detected two distinct $CD5^{+}$ B cell populations, which we denoted $CD5^{dim+}$ and $CD5^{bright+}$. While bovine B cells have been found to contain both a $CD5^{dim}$ and a $CD5^{bright}$ population (Stabel and Khalifeh, 2008), most BLV studies have only focused on CD5 expression in total, where $CD5^{+}$ B cells are the type of B cell that expands in PL cattle (Meirom et al., 1997). However, we kept the $CD5^{dim+}$ and $CD5^{bright+}$ B cell populations distinct because the two populations showed different kinetics after KLH inoculation and there were significant differences in these two cell populations between BLV+ and BLV- cows. While the $CD5^{dim+}$ B cell population increased after KLH inoculation, the $CD5^{bright+}$ B cell population sharply declined. When comparing BLV+ and BLV- cows, BLV+ cows had an elevated $CD5^{dim+}$ population, but the $CD5^{bright+}$ populations were equivalent. Finally, while $CD5^{dim+}$ B cells from BLV+ cows exhibited higher mean CD5 surface expression, $CD5^{bright+}$ B cells from BLV+ cows exhibited lower mean CD5 surface expression. It is unclear what functional differences may exist between $CD5^{dim+}$ and $CD5^{bright+}$ B cells. CD5 expression on B cells may be a lineage marker for innate-like B cells that produce natural IgM (Carsetti et al., 2004), but CD5 expression on B cells may also be induced after stimulation through the B cell receptor (Haas and Estes, 2000), and it is possible that the $CD5^{dim+}$ and $CD5^{bright+}$ populations

reflect these different patterns of CD5 expression. Our data suggests that the distinction is relevant in the context of BLV infection, but our study was unable elucidate the function of these distinct B cell subtypes.

We also investigated how B cells responded to KLH stimulation *in vitro.* While B cells from both BLV+ and BLV- cows showed a minor but significant increase in $CD25^+$ B cells after KLH stimulation, the proportion of $CD25^+$ B cells from BLV+ cows was significantly lower. In addition to the relative percent of $CD25^+$ B cells, $CD25^+$ B cells from BLV+ cows exhibited significantly lower CD25 surface expression after both KLH and P/I stimulation, suggesting that BLV+ cows display less antigen-specific or mitogenic B cell activation when compared to healthy, BLV- cows. We also questioned whether KLH stimulation would induce BLV protein expression. While P/I stimulation induced BLV gp51 expression as expected (Jensen et al., 1992), KLH stimulation did not. However, it was clear that BLV expression was most common in $CD25^+$ B cells, indicating that BLV gp51 expression is related to B cell activation. BLV expression was also related to different patterns of MHCII and CD25 surface expression. Under all culture conditions, BLV-expressing B cells had a dramatically increased MHCII surface expression, which was in contrast to MHCII expression observed on B cells *ex vivo*. Active BLV transcription is rarely detected *ex vivo*, while even short term *in vitro* culture can induce detectable BLV protein production (Florins et al., 2007), which suggests that the BLV protein expression is inducing elevated MHCII expression. In contrast, BLV expression did not affect CD25 surface expression in unstimulated or KLH-stimulated cultures. After P/I stimulation, CD25 surface expression increased only on B cells from BLV- cows or on BLV-expressing B cells from BLV+ cows; the CD25 surface expression on $BLV^-$ B cells from BLV+ cows remained unchanged. Overall, our B cell culture experiments suggest that B cells from BLV+

cows have a reduced responsiveness to antigenic and mitogenic stimulation, and this reduced responsiveness is not necessarily dependent on active BLV protein expression.

While the potential effect of BLV infection on B cells is obvious, it is less clear how BLV infection affects the T cell compartment, although T cell irregularities have been previously observed (Frie and Coussens, 2015). We measured the circulating $CD45R0^+$ T cell populations to investigate effector/memory T cells (Farber et al., 2014). Both the $CD4^+$ and $CD8^+$ $CD45R0^+$ T cell populations increased over time after KLH+DDA stimulation, although the abundance of the cell types was equivalent between BLV+ and BLV- cows. While the $\gamma\delta^+CD45R0^+$ T cell population remained mostly constant after KLH+DDA exposure, BLV+ cows exhibited a large overall reduction in their $\gamma\delta^+CD45R0^+$ T cell population. Although $\gamma\delta^+CD45R0^+$ T cells are not a well-characterized cell population, CD45R0 expression likely indicates current or prior activation, although it is unclear whether this activation would be innate or adaptive or both (De Maria et al., 1993).

We were also interested in investigating IFNγ and IL4 production in response to KLH stimulation *in vitro*. While we were unable to detect increased cytokine production in KLH-stimulated cell culture, we found that both $CD4^+$ and $CD8^+$ T cell populations from BLV+ and BLV- cows were equally responsive to positive control stimulation when measuring IFNγ production. Similar to previous results (Frie et al., 2016), a significantly higher proportion of $\gamma\delta^+$ T cells from BLV+ cows produced IFNγ in cell culture. Surprisingly, while the amount of IFNγ produced by $CD4^+$ and $\gamma\delta^+$ T cells was not different between BLV+ and BLV- cows, $CD8^+$ T cells from BLV+ cows did produce significantly higher amounts of IFNγ. When we similarly investigated IL4 production *in vitro,* we observed directly opposite results. $CD4^+$ and $CD8^+$ T cell populations from BLV+ cows contained higher proportions of IL4-producting cells under all cell

culture conditions; conversely, $\gamma\delta^+$ T cells produced IL4 in equivalent proportions between BLV+ and BLV- cows. Finally, while BLV+ cows contained higher proportions of IL4-producing $CD8^+$ T cells *in vitro*, reactive $CD8^+$ IL4-producing cells from BLV- cows actually produced more IL4 under all culture conditions. This difference in IL4-producing cells versus IL4 expression on a per-cell basis could be a result of suppressed activation in $CD8^+$ T cells from BLV+ cows. While BLV+ cows have a higher proportion of $CD8^+$ T cells producing IL4, it is possible that BLV infection interferes with the degree of activation after $CD8^+$ T cell stimulation, although this was not observed with IFNγ production in $CD8^+$ T cells from BLV+ cows. While IL4 production by $CD8^+$ T cells in cattle is not well-studied, evidence from humans and mice suggest that $CD8^+$ T cells may also be polarized based upon their cytokine secretion and that this cytokine secretion can contribute to the overall balance of a cell-mediated versus humoral immune response (Betts and Kemeny, 2009; Kalinski et al., 2006).

While our experiment was unable to detect any differences in antigen-specific activation in T cells from BLV+ cows, we did detect overall differences in circulating $\gamma\delta^+$ T cells and in αβ and $\gamma\delta^+$ T cell cytokine production *in vitro*. The considerable reduction in circulating $CD45R0^+$ $\gamma\delta^+$ T cells in BLV+ cows could suggest an impairment of effector or memory $\gamma\delta^+$ T cells in BLV+ cows; considering the hypothesized importance of $\gamma\delta^+$ T cells in bovine immunity (Guzman et al., 2012), an impairment in this T cell subtype could have serious consequences on both innate and adaptive immunity in cattle, including reduced responsiveness to vaccination and less immune protection from pathogens including *Mycobacterium bovis* and *Leptospira borgpetersenii* serovar Hardjo (Baldwin and Telfer, 2015). Our *in vitro* experiments suggest that αβ T cells in BLV+ cattle could be more predisposed to producing IL4 as compared to αβ T cells from healthy, BLV- cattle. If BLV+ cattle immunity is more skewed towards IL4 over IFNγ

production, this could have profound effects upon infections that depend on Th1 versus Th2 immunity for effective pathogen clearance.

This study was conducted to investigate B and T cell responses in BLV+ cows to a primary and secondary antigenic immune challenge. While evidence demonstrates that BLV-infected cattle have atypical immunity in comparison to BLV- cows (Frie and Coussens, 2015), little research has investigated whether BLV+ cows would exhibit abnormal immune responses to a primary challenge, or if abnormal adaptive immunity in BLV+ cattle was the cumulative effect of multiple antigenic challenges. Our study did find antigen-specific deficiencies in B cell immunity during a primary immune response, indicating that BLV infection can interfere with antigen-specific immunity without many re-exposures to antigen. While we were unable to detect antigen-specific T cell responses *in vitro*, we did detect abnormalities in circulating $\gamma\delta^+$ T cells in BLV+ cows, as well as a potential bias for IL4-producing $\alpha\beta$ T cells. These data demonstrate that BLV infection can have a detectable impact on immune stimulation even upon a primary antigen exposure, which would likely mean that negative impacts of BLV infection on herd health could occur immediately upon a secondary infection.

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**Conflict of Interest Statement**

The authors declare no conflict of interest. NorthStar Cooperative is a for-profit animal agriculture diagnostic company and conducted all diagnostic testing for this study free of charge in exchange for biological samples.

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# CHAPTER 4: MICRORNAS ENCODED BY BOVINE LEUKEMIA VIRUS ARE ASSOCIATED WITH REDUCED EXPRESSION OF B CELL TRANSCRIPTIONAL REGULATORS IN DAIRY CATTLE NATURALLY INFECTED WITH BLV

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**Abstract**

Bovine leukemia virus (BLV) is estimated to infect over 83% of dairy herds and over 40% of all dairy cows in the United States. While BLV only causes leukemia in a small proportion of animals, research indicates that BLV+ cattle exhibit reduced milk production and longevity that is distinct from lymphoma development. It is hypothesized that BLV negatively affects production by interfering with normal cattle immunity and increasing the risk of secondary infections. In particular, BLV+ cows demonstrate reduced circulating levels of both antigen-specific and total IgM. To investigate possible mechanisms by which BLV impairs IgM production, total plasma IgM and the expression of IGJ, BLIMP1, BCL6 and PAX5 were measured in BLV+ and BLV- cows. In addition, BLV proviral load and the relative expression of BLV TAX and 5 BLV microRNAs were characterized and correlated to the expression of selected endogenous genes. BLV+ cows exhibited lower total plasma IgM and lower expression of IGJ, BLIMP1 and BCL6. While BLV TAX and BLV miRNAs failed to correlate with IGJ expression, both BLV TAX and BLV miRNAs exhibited negative associations with BLIMP1 and BCL6. The results suggest a possible transcriptional pathway by which BLV interferes with IgM production in naturally infected cattle.

## Introduction

Bovine leukemia virus (BLV) is a δ-retrovirus that is the causative agent of enzootic bovine leukosis (EBL) (Kettmann et al., 1976). While the clinical stage of EBL is typically characterized by lymphosarcoma development, only a small percentage of infected cattle will progress to clinical disease (Kabeya et al., 2001). As a result, BLV infection is rarely tested for on commercial dairy operations. However, it is estimated that at least 83% of US dairy herds are BLV-infected. The within-herd infection rate is often between 25% to nearly 50%, and approximately 40% of all dairy cows in the US are infected (Bartlett et al., 2014).

While lymphosarcoma development is rare, approximately 30% of infected cattle will develop persistent lymphocytosis (PL), a benign, polyclonal expansion of the B cell compartment (Kabeya et al., 2001) that is associated with an increased proviral load (PVL) (Ohno et al., 2015). In addition, a growing body of research strongly suggests that BLV negatively interferes with immune function in infected cattle (Frie and Coussens, 2015). One striking phenotype is that BLV-infected cattle exhibit abnormal levels of serum IgM. BLV+ cattle exhibit lower total IgM (Meiron et al., 1985; Trainin et al., 1976); lower antigen-specific IgM (Frie et al., 2016; Frie et al., 2017; Puentes et al., 2016); and lower transcript levels encoding the immunoglobulin light chain in $IgM^+$ B cells (Teutsch and Lewin, 1996). What is puzzling is a potential mechanism by which BLV interferes with immune function. Although BLV is primarily transmitted within a host by cell-to-cell contact during early infection (Aida et al., 2013), chronic viral transmission within a host is characterized by proviral replication via the division of infected cells. However, multiple studies suggest that BLV is mostly transcriptionally inactive. Indeed, detecting BLV proteins is nearly impossible *ex vivo* (Gillet et al., 2007), and

evidence suggests that BLV protein expression is associated with rapid clearance of infected cells (Florins et al., 2012).

Recently, researchers discovered that the BLV genome encodes 5 pre-microRNA (miRNA) hairpins that produce 10 mature miRNAs in a genomic region previously thought to be inactive (Kincaid et al., 2012; Rosewick et al., 2013). BLV miRNAs are atypical in several ways: virally-encoded miRNAs are unusual in RNA viruses due to the potential for damaging genomic RNA (Kincaid et al., 2012) and BLV miRNAs are transcribed by RNA polymerase III (RNAPIII), as opposed to the typical miRNA biosynthesis that utilizes RNA polymerase II (RNAPII) (Burke et al., 2014; Kincaid et al., 2012; Rosewick et al., 2013; Van Driessche et al., 2016). Despite these unusual features, BLV miRNAs have been detected at physiologically relevant levels in both ovine B cell tumors (Rosewick et al., 2013) and in experimentally infected bovine calves (Gillet et al., 2016).

BLV miRNAs also provide a possible mechanism by which BLV interferes with host immunity because evidence suggests that transcription of BLV miRNAs by RNAPIII is independent of the transcription of BLV protein-coding genes by RNAPII (Rosewick et al., 2013). Research investigating the role of BLV miRNAs in BLV pathogenesis identified multiple potential genes that could be targeted by BLV miRNAs, including IGJ (Gillet et al., 2016). IGJ encodes the J chain, which is essential for the assembly and secretion of pentameric IgM and dimeric IgA, as well as for the transport of polymeric IgM and IgA across epithelial barriers via the polymeric immunoglobulin receptor (Hurley and Theil, 2011). In addition, IGJ expression has been found to be downregulated in peripheral blood isolated from BLV+ cows (Brym and Kaminski, 2016).

IGJ transcription occurs in terminally differentiating B cells that will become plasma cells, which secrete high levels of antibodies (Cho and Kang, 2005). B cell terminal differentiation is tightly regulated by a complex program of transcription factors. PAX5 is considered the master regulator of the B cell lineage and is expressed consistently from pro-B cells to mature B cells and its expression only declines after terminal differentiation (Nutt et al., 2011). BCL6 is a negative transcriptional regulator that specifically targets genes associated with DNA damage sensing, DNA damage response and DNA damage checkpoints. This is essential because BCL6 is primarily active in B cells during immunoglobulin light chain rearrangement and in germinal center reactions, during which somatic hypermutation and affinity maturation occur (Swaminathan et al., 2014). Indeed, mice without BCL6 expression fail to develop germinal centers and produce low-affinity IgG (Toyama et al., 2002). Of note, BCL6 upregulation is also associated with the development of B-cell lymphomas in humans (Basso and Dalla-Favera, 2010). Finally, BLIMP1 is considered the master regulator of plasma cell differentiation and will induce IGJ transcription (Nutt et al., 2011). While PAX5 and BCL6 repress BLIMP1 expression, BLIMP1, once expressed, will in turn repress PAX5 and BCL6 expression (Nutt et al., 2011).

In this study, we investigated whether BLV+ cows exhibited lower total IgM, as well as lower expression of genes associated with B cell function and differentiation, specifically IGJ, PAX5, BLIMP1, and BCL6. In addition, we investigated whether BLV PVL, BLV TAX expression or BLV miRNA expression correlated to measured phenotypes in BLV+ cows. We observed that BLV+ cows exhibited lower total plasma IgM, as well as lower IGJ, BLIMP1, and BCL6 expression. In addition, we were able to detect both BLV TAX expression and BLV miRNA expression. Finally, both BLV TAX and BLV miRNA expression correlated with

reduced B cell function or gene expression. These data suggest that lower plasma IgM in BLV+ cows could be related to viral interference in the transcriptional regulation that controls plasma cell differentiation.

## Methods

### Animals

30 adult, lactating Holstein cows housed at a commercial dairy farm in mid-Michigan were enrolled in this study. BLV+ cattle were initially selected on the basis of a positive milk ELISA diagnostic test (NorthStar Cooperative, Inc) and were subsequently screened based on their total leukocyte counts. BLV+ status was confirmed using a serum ELISA diagnostic test (NorthStar Cooperative, Inc). BLV+ cows were then matched with BLV- control cows (BLV status as determined by prior milk ELISA diagnostic test and confirmed using a serum ELISA diagnostic test) based upon age, lactation number, days in milk, reproduction status, and days until parturition (Table 4.1). All animal use protocols were reviewed and approved by Michigan State University Institutional Animal Use and Care Committee (AUF# 04/16-061-00).

### Whole blood, plasma, and B cell isolation

Whole blood and plasma samples were collected as previously described (Frie et al., 2016). Briefly, whole blood for BLV PVL quantification and total leukocyte counts was collected by coccygeal venipuncture into Vacutainer tubes containing the anticoagulant EDTA (Becton Dickinson). Freshly isolated whole blood was used to determine total leukocyte counts. Aliquots of whole blood were stored at -20°C for PVL quantification.

Whole blood was also collected by coccygeal venipuncture into Vacutainer tubes containing the anticoagulant ACD (Becton Dickinson) for plasma and peripheral blood leukocyte (PBL) isolation. Briefly, plasma aliquots with 0.1% sodium azide were stored at -20°C prior to ELISA analysis. PBLs were isolated using red blood cell lysis. Briefly, Vacutainer tubes were centrifuged at 2200rpm for 20min at room temperature and buffy coats were transferred to 50mL conical tubes. Red blood cell lysis solution (154mM ammonium chloride, 10mM potassium

**Table 4.1. Cow enrollment characteristics.** Data represent the mean and (range) on the day of sample collection.

| | BLV+ cows ($n$ = 15) | BLV- cows ($n$ = 15) |
|---|---|---|
| Age | 4 years 8 months (3 years 3 months – 8 years 1 month) | 4 years 8 months (2 years 11 months – 8 years 1 month) |
| Lactation number | 2.7 (1 – 6) | 2.8 (1 – 6) |
| Days in milk | 284 (216 – 431) | 291 (228 – 425) |
| Reproduction status | All pregnant | All pregnant |
| Days until parturition | 121 (88 – 155) | 118 (73 – 177) |
| Total leukocyte count (cells/μL) | $1.49 \times 10^4$ ($8.48 \times 10^3$ – $2.69 \times 10^4$) | $8.63 \times 10^3$ ($5.30 \times 10^3$ – $1.78 \times 10^4$) |

bicarbonate and 97μM tetrasodium EDTA in deionized water) was added at a ratio of 2:1 to the buffy coats and samples were inverted for 5min. PBLs were then centrifuged at 1500rpm for 5min at 4°C and washed with 25mL of 1X phosphate buffered saline (PBS). PBLs were counted with a hemocytometer.

$6x10^7$ PBLs were labeled for 30min at 4°C with 2.5 μg primary anti-bovine IgG2b antibody targeting surface IgM (SIgM) (clone PIG45A2; Washington State University) in 800μL staining buffer (PBS with 2% heat-inactivated horse serum, 10% ACD, and 0.09% sodium azide). Cells were washed with 3mL wash buffer (PBS with 10% ACD and 0.09% sodium azide) and centrifuged at 150xg for 5min at 4°C. Cells were then labeled for 30min at 4°C with 1 μg secondary anti-mouse IgG2b antibody conjugated to AlexaFluor 488 (Life Technologies). Cells were washed with 3mL wash buffer and centrifuged at 150xg for 5min at 4°C. Cells were suspended in 2mL 1X Hank's balanced salt solution (HBSS) and stored at 4°C. Live cells were first selected based on forward and side scatter gating and then were positively sorted based on fluorescence using an Influx Cell Sorter (Becton Dickinson). $1.5 \times 10^6$ $SIgM^+$ lymphocytes were collected for RNA extraction and qRT-PCR analysis. Data from B cell sorting were also used to calculate the mean relative percent of $SIgM^+$ B cells.

**Total leukocyte count quantification**

The total leukocyte count (TLC) was determined using a Z1 Coulter Particle Counter (Beckman Coulter) according to instrument specifications. Briefly, 40μL of whole blood was diluted in 20mL 1X PBS and 6 drops of zap oglobin II (Beckman Coulter) to lyse red blood cells. Samples sat at room temperature between 2min and 30min before running on the particle counter. Each sample was run in triplicate and the average TLC was recorded as cells/μL.

**Total IgM quantification**

Total plasma IgM was quantified using the bovine IgM ELISA quantitation set (Bethyl Laboratories) following the recommended protocol. Briefly, flat-bottomed ELISA plates (Thermo Fisher Scientific) were incubated with 100µL coating antibody diluted in coating buffer for 1h at room temperature. Wells were washed 5X with 200µL wash buffer and then 200µL wash buffer was incubated for 30min at room temperature to block the plate. Wells were washed 5X with 200µL wash buffer and 100µL plasma diluted 1:10,000 in wash buffer was incubated for 1h at room temperature. Wells were washed 5X with 200µL wash buffer and 100µL anti-IgM-HRP diluted 1:100,000 in wash buffer was added to all wells and incubated for 1h at room temperature. Wells were washed 5X with 200µL wash buffer and 100µl TMB substrate (Thermo Fisher Scientific) was added to all wells and incubated for 15min at room temperature in the dark. 100µL of stop solution was added to all wells. The optical density was measured at 450nm using a SpectraMax M5 microplate reader and total IgM was quantified using a standard curve according to kit protocol. All samples were run in duplicate and plates included blank controls.

**BLV proviral load quantification**

DNA was extracted from whole blood using the DNeasy blood and tissue kit (Qiagen) using a modified kit protocol (Frie et al., 2017). DNA was quantified using a Nanodrop-1000 and purity was assessed using A260/280 ratios. qPCR was performed using 60ng DNA and Power SYBR Master Mix (Applied Biosystems) in 50µL reaction volumes run in triplicate on a 7500 Fast Real-Time PCR system (Applied Biosystems).

Primers targeting the BLV provirus (BLV; Table 2) (Nishimori et al., 2016) or β-actin (ACTB; Table 4.2) were used to relatively quantify the amount of BLV provirus in BLV+ cows. BLV was normalized to ACTB and the abundance of provirus was relatively quantified using

**Table 4.2. Primer sequences for qPCR and qRT-PCR.** All primer pairs were used at a final concentration of 300nM per reaction except for PAX5, in which both primers were used at a final concentration of 100nM per reaction.

| Gene target | Forward primer (5' – 3') | Reverse primer (5' – 3') |
|---|---|---|
| BLV (gDNA) | ACT TTC AGA CCC CCT TGA CTG ACA | AAA CCT CTG CCC TGG TGA TTA AGG |
| ACTB (gDNA) | TCC CTG GAG AAG AGC TAC GA | GGC AGA CTT AGC CTC CAG TG |
| IGJ (cDNA) | TGA CCC CGG ATT CCT GCT AT | GAT AAG CAG TTG TGC AGC CAG |
| PRDM1 (cDNA) | AAA CGT GTG GGT ACG ACC TT | CTT CAG TCC CCT CTG CCA AC |
| PAX5 (cDNA) | AAA ATT ACC CGA CTC CTC GGA C | GTG GCC GTC CAT TCA CAA AAA |
| BCL6 (cDNA) | AGG AAA CCT CTC ATT TTA GAG TGC | CGG CGA GGC CAT TTT TCT TC |
| TAX (cDNA) | GGA GCT ACA CCA TTC ACC CC | TCA GAG CCC TTG GGT GTT TC |
| PPIA (cDNA) | GCA AGC ACG TGG TAC TTT GG | TTG CTG GTC TTG CCA TTC CT |
| HPRT (cDNA) | TGC ACT ACG AGC CTA AAG AC | TCC AGT CAA TAG TGG TGT GGT |
| ACTB (cDNA) | TGG AAC GGT GAA GGT GAC AG | CAA TCA AGT CCT CGG CCA CA |

$2^{-\Delta Ct}$ (Schmittgen and Livak, 2008). BLV primers amplified targets at significantly higher expression in BLV+ cows (Figure 4.1A).

**Cellular gene expression quantification**

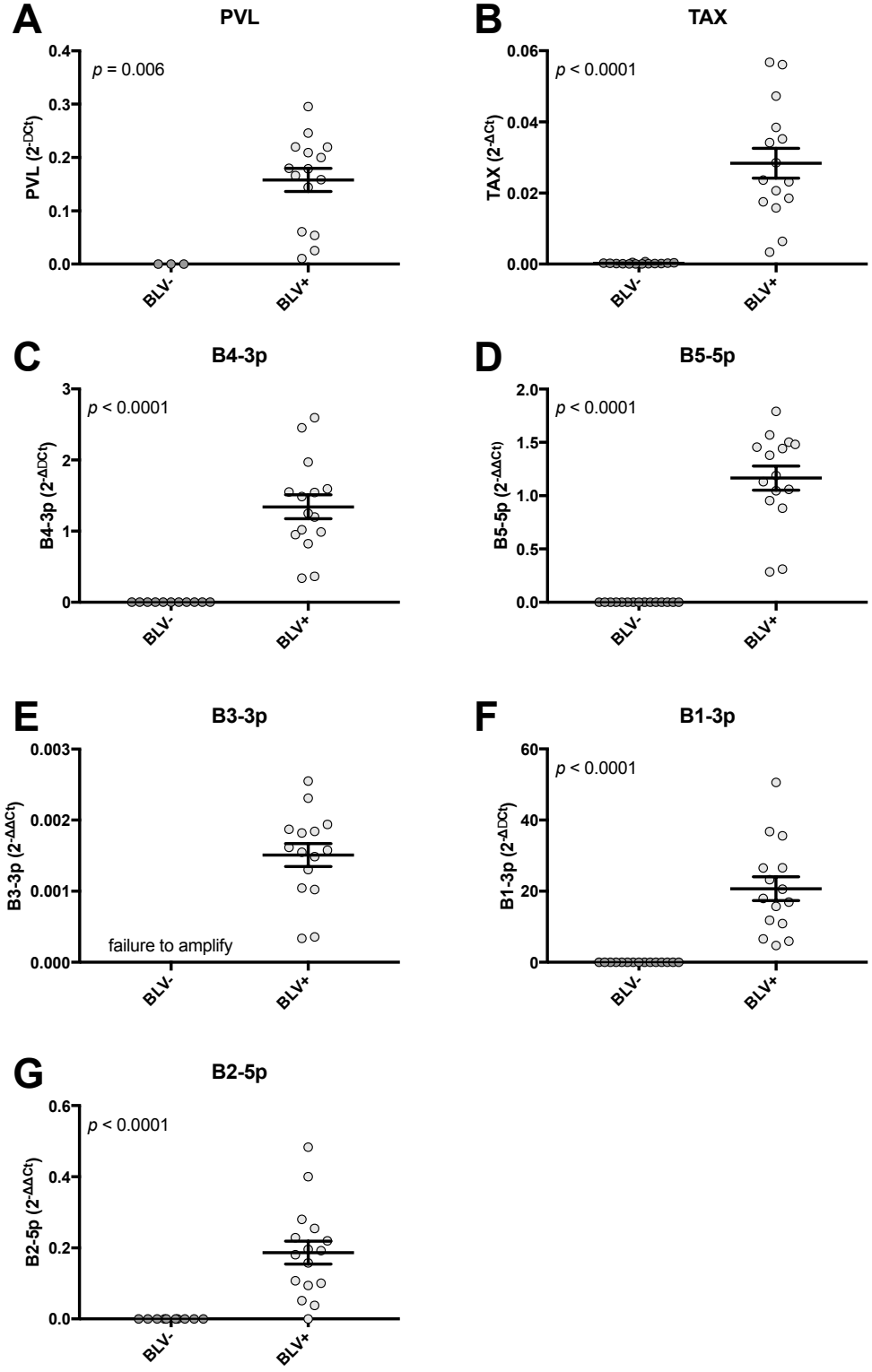
Total RNA was extracted from $1.5 \times 10^6$ $SIgM^+$ B cells using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions using the on-column DNase digestion protocol. Extracted RNA was quantified using a NanoDrop-1000 and purity was assessed using A260/280 ratios. 71.4ng RNA was reverse transcribed using the high capacity cDNA reverse transcription kit (Applied Biosystems). qRT-PCR was performed using 3µL of cDNA diluted 1:10 and Power SYBR Green PCR Master Mix (Applied Biosystems) in 50µL reaction volumes run in triplicate on a 7500 Real-Time PCR system (Applied Biosystems).

IGJ, PAX5, BLIMP1, BCL6, TAX, PPIA (Roussey et al., 2016), HPRT, and ACTB were assayed for each cow (Table 4.2). NormFinder software (Andersen et al., 2004) was used to determine the most stably expressed internal control genes (PPIA, HPRT and β-actin). Subsequently, PPIA alone was used to relatively quantify IGJ, PAX5, BLIMP1, BCL6 and TAX expression. Expression in both BLV+ and BLV- cows was assessed using $2^{-\Delta Ct}$ (Schmittgen and Livak, 2008). TAX primers amplified targets at significantly higher expression in BLV+ cows (Figure 4.1B).

**BLV microRNA expression quantification**

10ng of extracted RNA from $SIgM^+$ B cells was reversed transcribed using the high capacitiy cDNA reverse transcription kit (Applied Biosystems). BLV miRNA B4-3p, B5-5p, B3-3p, B1-3p, and B2-5p expression was measured using stem-loop reverse transcription primers (Thermo Fisher Scientific) and Taqman microRNA assays (Thermo Fisher Scientific). U6 was used as an endogenous control and *C. elegans* miR-39 (Cel-39) (Norogen

**Figure 4.1. Relative expression of BLV targets in BLV+ and BLV- cows.** A) PVL. BLV+ *n* = 15, BLV- *n* = 3. B) TAX. BLV+ *n* = 15, BLV- *n* = 15. C) B4-3p. BLV+ *n* = 15, BLV- *n* = 15. D) B5-5p. BLV+ *n* = 15, BLV- *n* = 15. E) B3-3p. BLV+ *n* = 15, BLV- *n* = 15. F) B1-3p. BLV+ *n* = 15, BLV- *n* = 15. G) B2-5p. BLV+ *n* = 15, BLV- *n* = 15. Bars indicate the mean ± SEM.



Inc.) was added to the RT reaction as a spike-in control. Samples were assayed in triplicate on a 7500 Real-Time PCR system (Applied Biosystems). BLV miRNA expression was relatively quantified using $2^{-\Delta\Delta Ct}$ (Schmittgen and Livak, 2008). BLV miRNA and U6 expression were normalized to Cel-39 and then BLV miRNA expression was normalized to U6 expression (Sourvinou et al., 2013). BLV miRNA primers amplified targets at significantly higher expression in BLV+ cows (Figure 4.1C-D, F-G) or failed to amplify targets in BLV- cows (Figure 4.1E).

**Statistical analysis**

Outliers were detected using Grubbs test and removed from analysis. Significance between BLV+ and BLV- cows was assessed using a Students unpaired t- test. Correlation between parameters in BLV+ cows was assessed by calculating the Pearson correlation coefficients. Data were analyzed using Prism 7.0b (GraphPad Software) and significance was determined as $p < 0.05$.

## Results

### BLV+ cows demonstrate reduced antigen-specific and total plasma IgM

Previous research had found reduced antigen-specific IgM in BLV+ cows (Figure 4.2A-C) (Frie et al., 2016; Frie et al., 2017). If lower observed IgM levels were the result of reduced IGJ expression in BLV+ cows, then the total plasma IgM concentration should also be reduced in those BLV+ cattle. Total IgM concentrations were measured in plasma collected on day 0 from cattle enrolled in previous time course studies (Frie et al., 2016; Frie et al., 2017) and BLV+ cows did exhibit a significant reduction in total plasma IgM ($p = 0.0007$) (Figure 4.2D).

### Higher levels of BLV TAX and miRNA expression are associated with increasing PVL

It was critical to establish how different measurements of viral activity correlated with each other in order to interpret any significant associations that were detected between viral expression and host phenotypes. PVL was significantly positively correlated to B5-5p (Figure 4.3C), B3-3p (Figure 4.3D), and B1-3p (Figure 4.3E). PVL also exhibited a trending positive correlation with both TAX (Figure 4.3A) and B4-3p (Figure 4.3B).

In contrast, TAX expression only exhibited a trending positive correlation with the expression of one miRNA, B3-3p (Figure 4.4C). When comparing BLV miRNA expression between individual miRNAs, most measured miRNAs significantly positively correlated with each other. However, B2-5p expression was only positively associated with B5-5p (Figure 4.5E).

Taken together, these data suggest that expression of both BLV TAX and BLV miRNA is higher in animals with increasing PVL. However, BLV TAX and BLV miRNA expression levels do not appear to be related to each other. In addition, four BLV miRNAs (B4-3p, B5-5p, B3-3p, and B1-3p) show closely correlated expression levels, while one BLV miRNA (B2-5p), does not.

**Figure 4.2. IgM in plasma from cattle enrolled in previous studies.** All plasma samples were collected on day 0 prior to vaccination or antigen exposure. A) Anti-*L. hardjo* IgM. BLV+ *n* = 20, BLV- *n* = 20. b) Anti-*L. pomona* IgM. BLV+ *n* = 18, BLV- *n* = 18. C) Anti-KLH IgM. BLV+ *n* = 8, BLV- *n* = 8. D) Total plasma IgM. BLV+ *n* = 26, BLV- *n* = 24. Bars indicate the mean ± SEM.

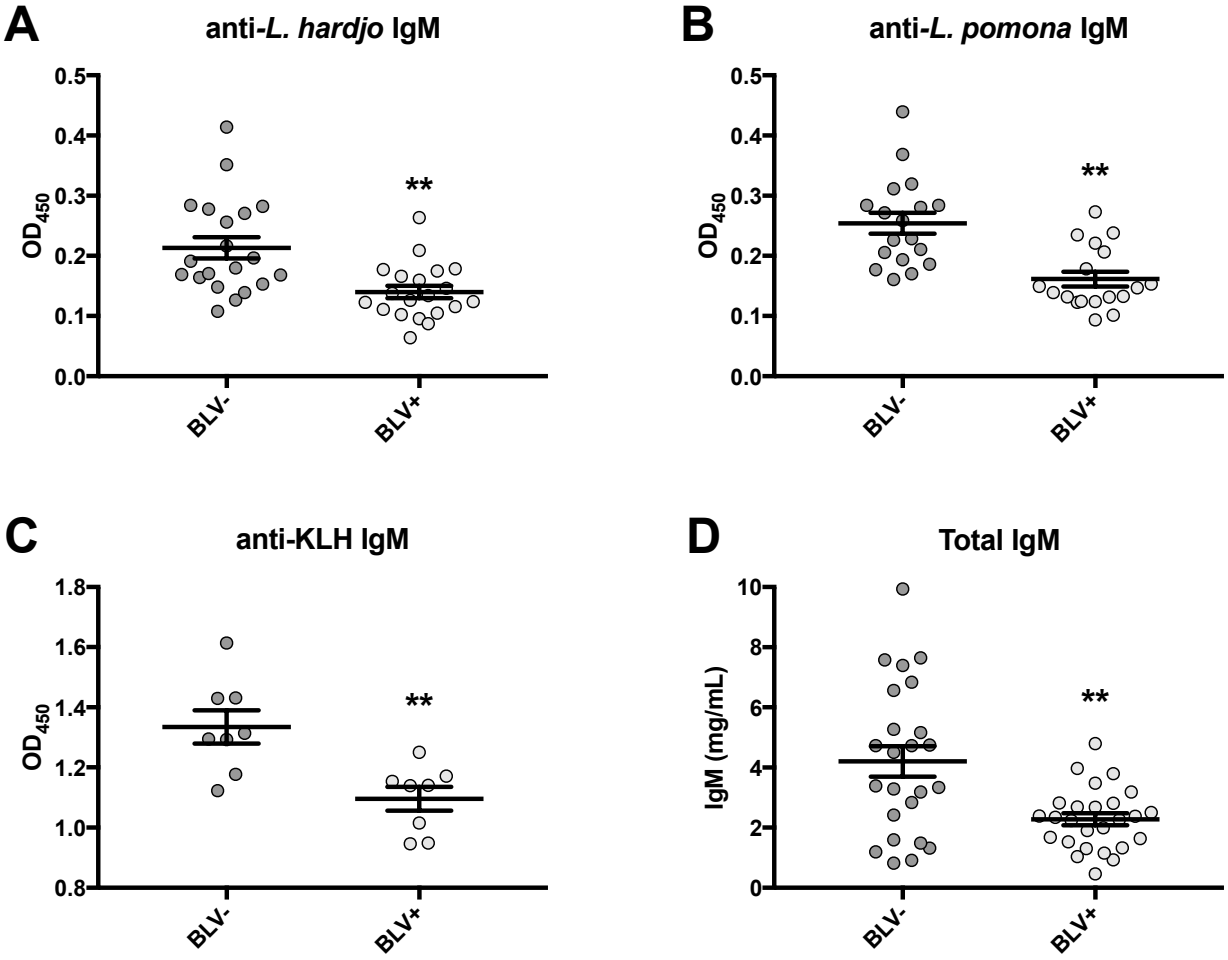


**Figure 4.3. PVL correlations with BLV TAX or BLV miRNAs.** PVL in BLV+ cows correlated with A) TAX, B) B4-3p, C) B5-5p, D) B3-3p, E) B1-3p, F) B2-5p. *n* = 15.

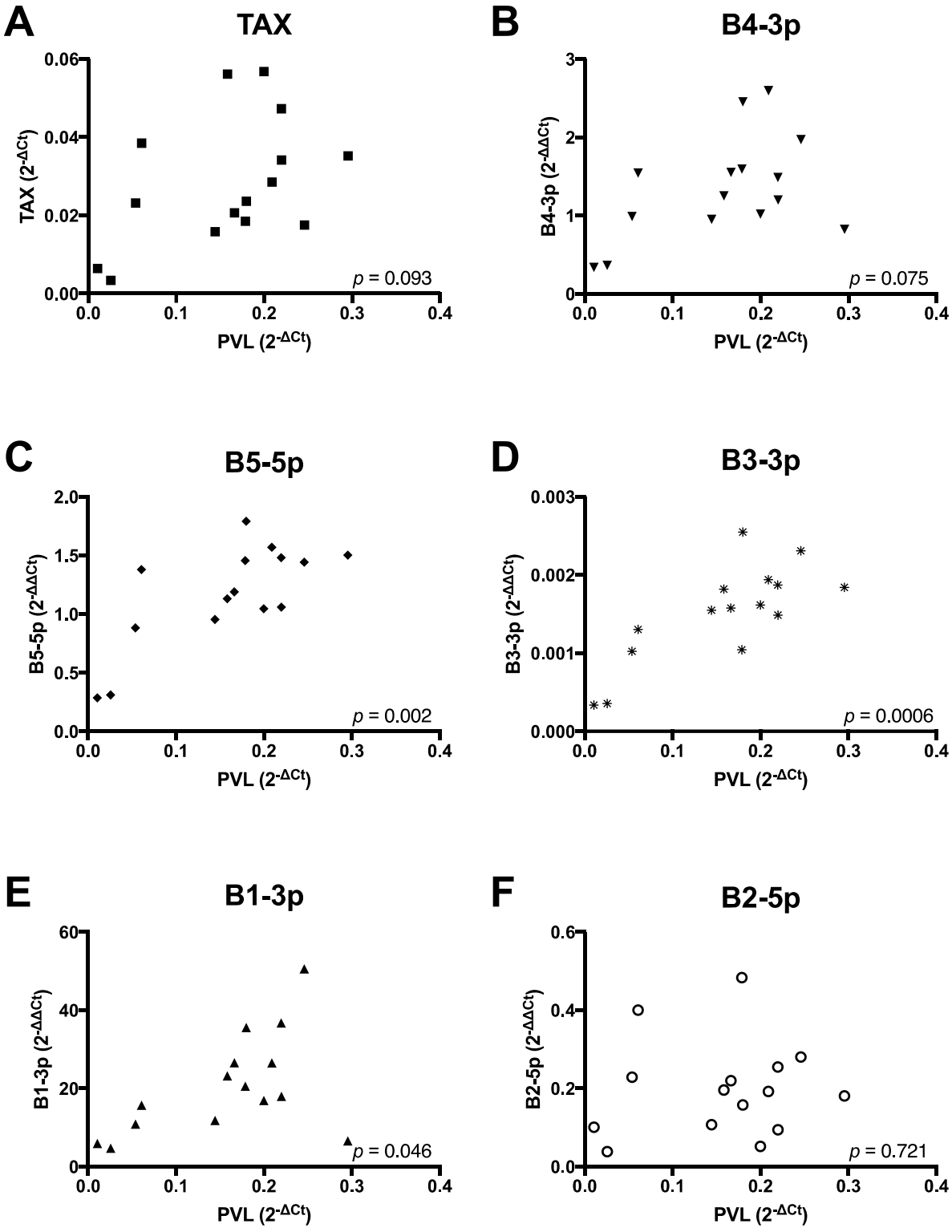


**Figure 4.4. TAX correlations with BLV miRNAs.** TAX in BLV+ cows correlated with A) B4-3p, B) B5-5p, C) B3-3p, D) B1-3p, E) B2-5p. *n* = 15.

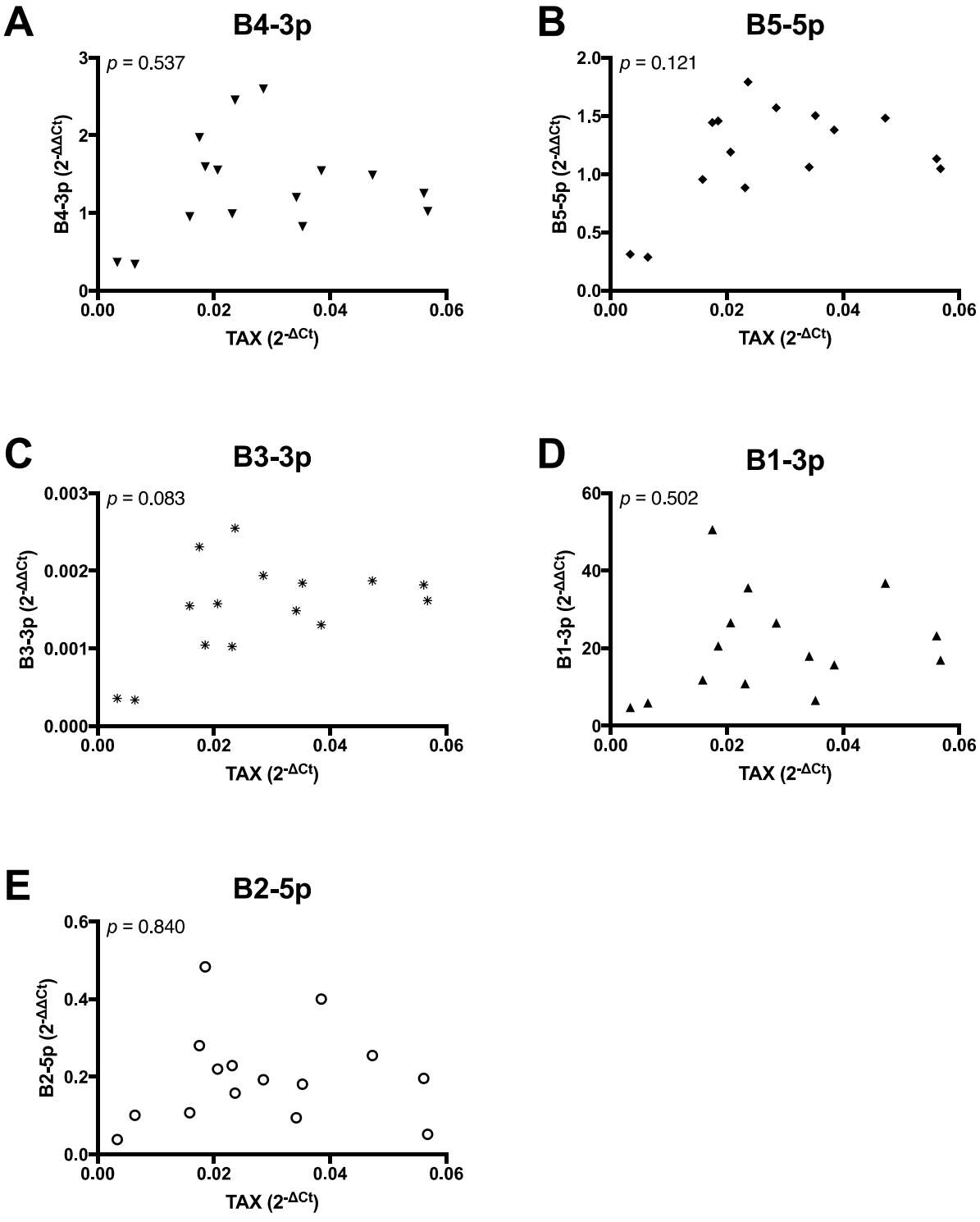
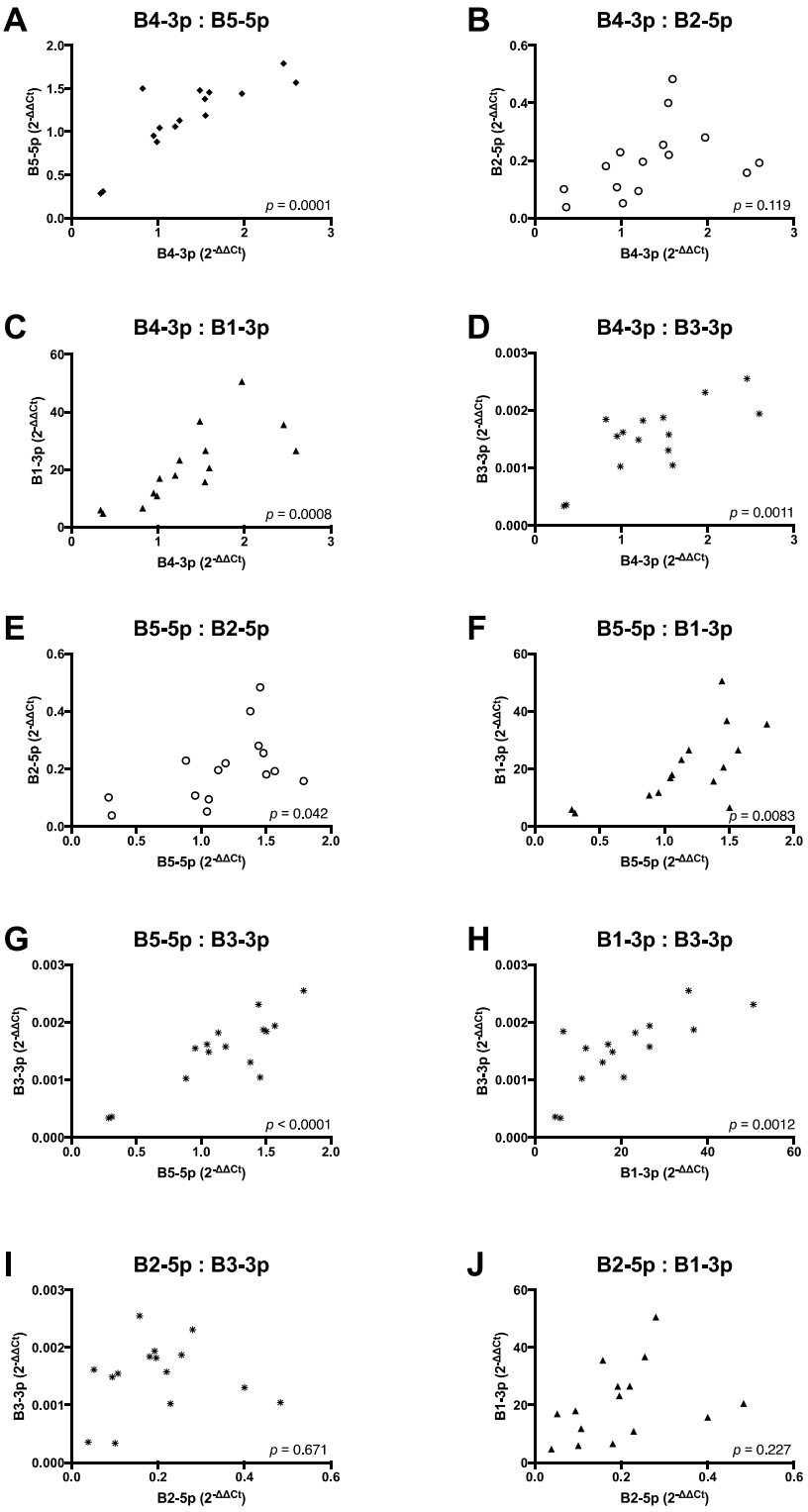


**Figure 4.5. BLV miRNA correlations.** A) B4-3p : B5-5p. B) B4-3p : B2-5p. C) B4-3p : B1-3p D) B4-3p : B3-3p. E) B5-5p : B2-5p. F) B5-5p : B1-3p. G) B5-5p : B3-3p. H) B1-3p : B3-3p. I) B2-5p : B3-3p. J) B2-5p : B1-3p. $n$ = 15.



**B cells from BLV+ cows demonstrate reduced IGJ expression**

BLV+ cows in this study were found to exhibit a significant increase in the prevalence of $SIgM^+$ B cells in peripheral blood ($p < 0.0001$) (Figure 4.6A), as expected. Similarly to the results obtained from a larger sample size, there was a near-significant reduction in total plasma IgM ($p = 0.053$) (Figure 4.6B). When the relative expression of IGJ in $SIgM^+$ B cells from BLV+ and BLV- cows was compared, BLV+ cows demonstrated a significant 2-fold reduction ($p = 0.008$) (Figure 4.6C).

We next wanted to determine whether BLV PVL or BLV activity correlated to the observed phenotypic abnormalities in BLV+ cows. PVL (Figure 4.7A), B5-5p (Figure 4.7D), B3-3p (Figure 4.7E), and B1-3p (Figure 4.7F) expression were all positively correlated to the percentage of $SIgM^+$ B cells. However, only a trending positive association was observed between TAX and the percentage of B cells (Figure 4.7B).

In contrast to the observed associations between viral expression and the percentage of B cells, only TAX expression exhibited a negative association with total plasma IgM (Figure 4.8B). Finally, when the relationship between viral expression and IGJ expression was examined, no associations were detected, despite the overall reduction in IGJ expression in BLV+ cows (Figure 4.9).

**B cells from BLV+ cows exhibit decreased BLIMP1 and BCL6 expression**

Although BLV+ cows were found to express less IGJ in $SIgM^+$ B cells, lower IGJ expression did not correlate to any measurements of viral expression. To investigate how BLV expression might indirectly affect IGJ expression *in vivo*, transcription factors regulating B cell differentiation were subsequently analyzed. While BLV+ cows demonstrated no difference in

**Figure 4.6. Percent B cell prevalence, total plasma IgM and IGJ expression in BLV+ and BLV- cows.** A) Percent B cell prevalence. BLV+ *n* = 15, BLV- *n* = 15. B) Total plasma IgM. BLV+ *n* = 15, BLV- *n* = 15. C) IGJ expression. BLV+ *n* = 14, BLV- *n* = 15. Bars indicate the mean ± SEM.

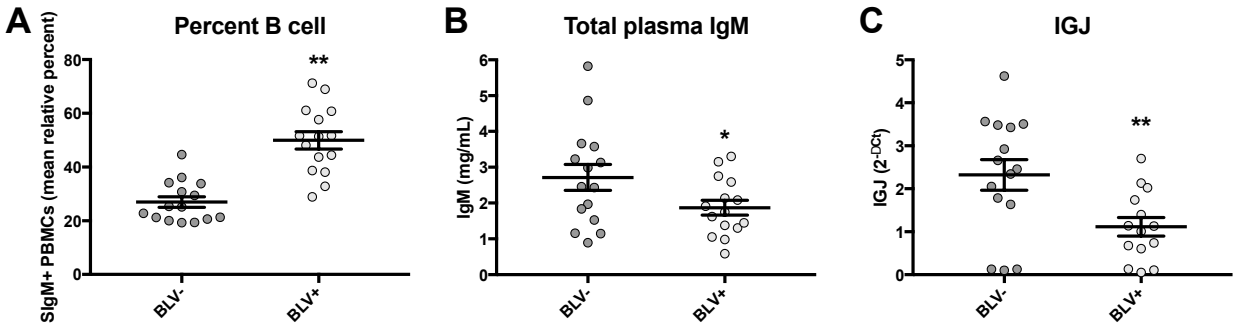


**Figure 4.7. Percent B cell prevalence and BLV expression in BLV+ cows.** The percent B cell prevalence in BLV+ cows correlated with A) PVL, B) TAX, C) B4-3p, D) B5-5p, E) B3-3p, F) B1-3p, G) B2-5p. $n = 15$.

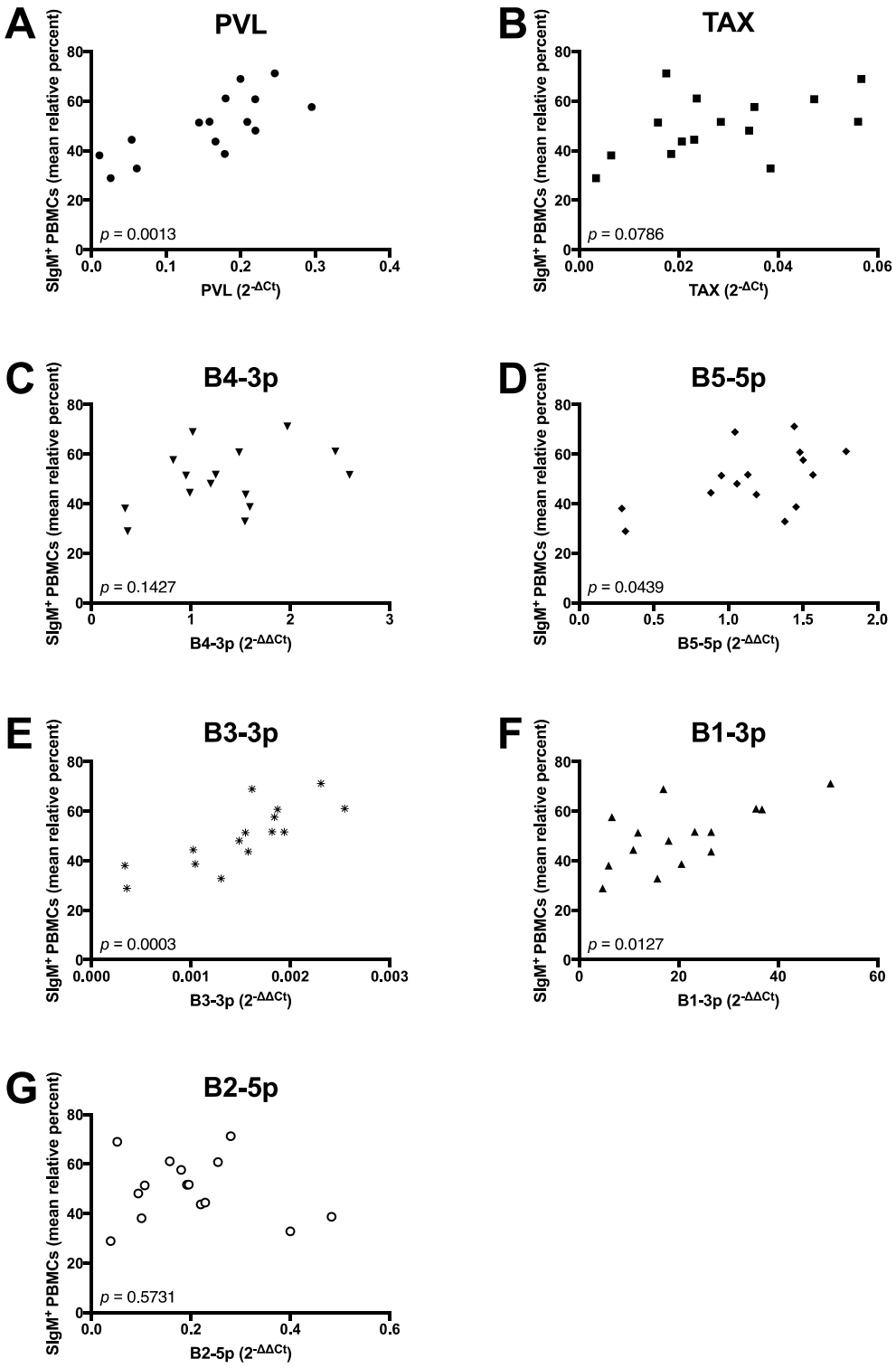


**Figure 4.8. Total plasma IgM and BLV expression in BLV+ cows.** The concentration of total plasma IgM in BLV+ cows correlated with A) PVL, B) TAX, C) B4-3p, D) B5-5p, E) B3-3p, F) B1-3p, G) B2-5p. $n = 15$.

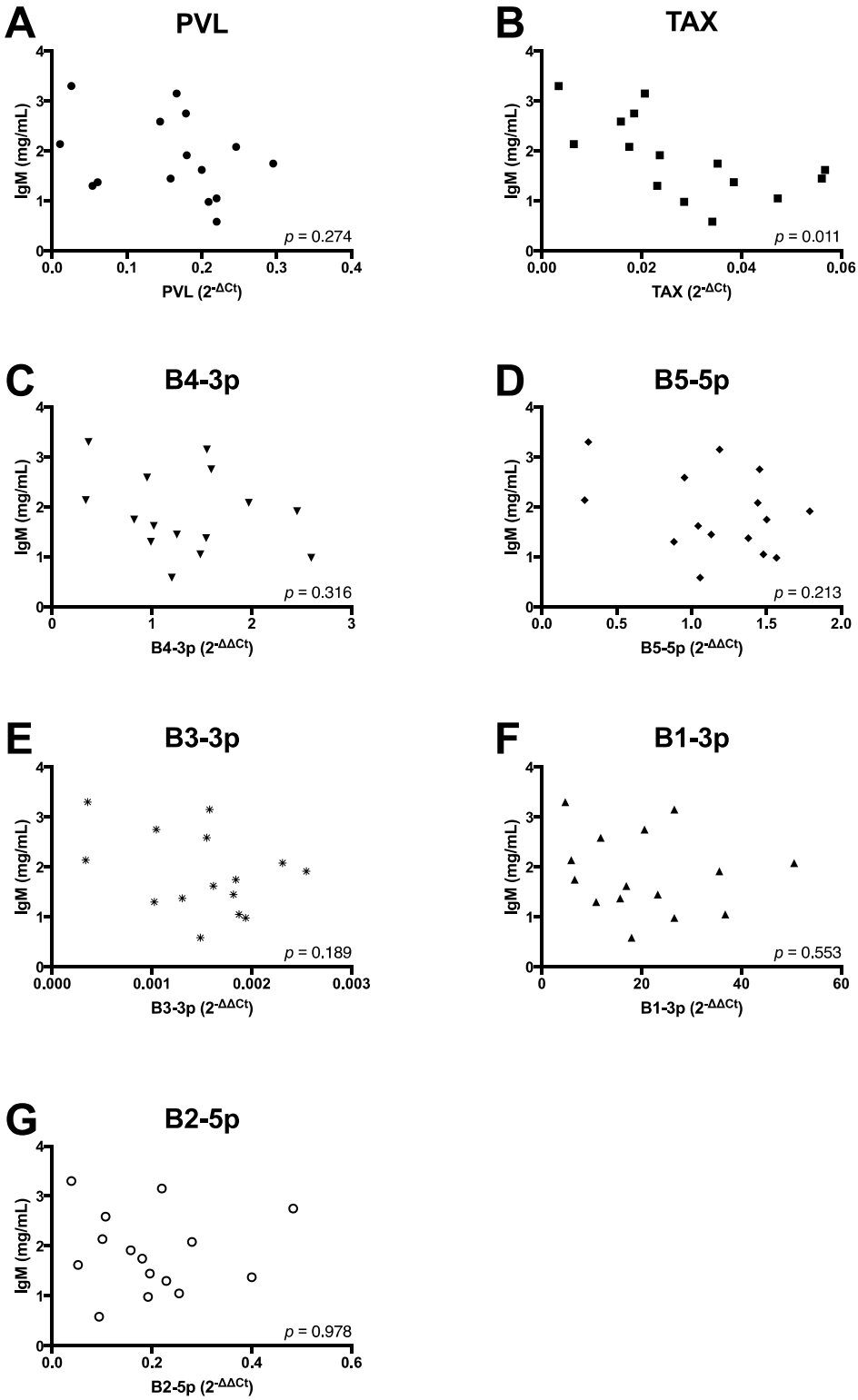
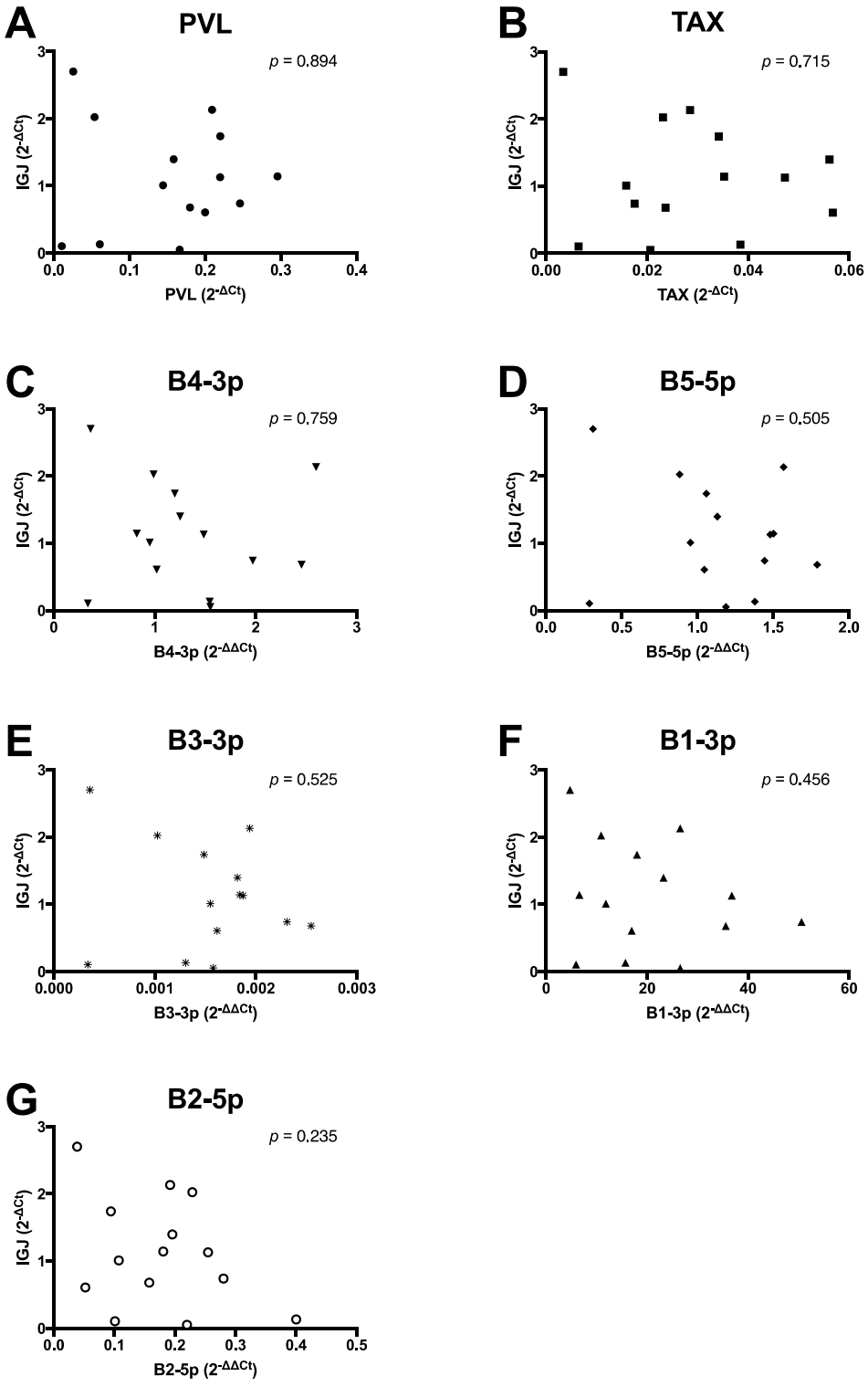


**Figure 4.9. IGJ expression and BLV expression in BLV+ cows.** The expression of IGJ in BLV+ cows correlated with A) PVL, B) TAX, C) B4-3p, D) B5-5p, E) B3-3p, F) B1-3p, G) B2-5p. $n = 14$.



PAX5 expression (Figure 4.10A), they did show a significant 5-fold reduction in BLIMP1 expression ($p < 0.0001$) (Figure 4.10B) and a significant 7-fold reduction in BCL6 expression ($p = 0.002$) (Figure 4.10C).

When viral expression was compared to BLIMP1 expression in BLV+ cows, the expression of PVL (Figure 4.11A), B4-3p (Figure 4.11C), B5-5p (Figure 4.11D), B3-3p (Figure 4.11E), and B1-3p (Figure 4.11F) were all negatively associated with BLIMP1 expression. TAX expression also demonstrated a trending negative association with BLIMP1 expression (Figure 4.11B).

Unlike the associations observed between viral expression and BLIMP1 expression, BCL6 expression was only negatively associated with B4-3p (Figure 4.12C), although a trending negative association was also observed with B2-5p expression (Figure 4.12G). Interestingly, a trending positive association was also observed between TAX and BCL6 (Figure 4.12B).

**Figure 4.10. PAX5, BLIMP1, and BCL6 expression in BLV+ and BLV- cows.** A) PAX5 expression. BLV+ *n* = 15, BLV- *n* = 15. B) BLIMP1 expression. BLV+ *n* = 15, BLV- *n* = 14. C) BCL6 expression. BLV+ *n* = 15, BLV- *n* = 15. Bars indicate the mean ± SEM.

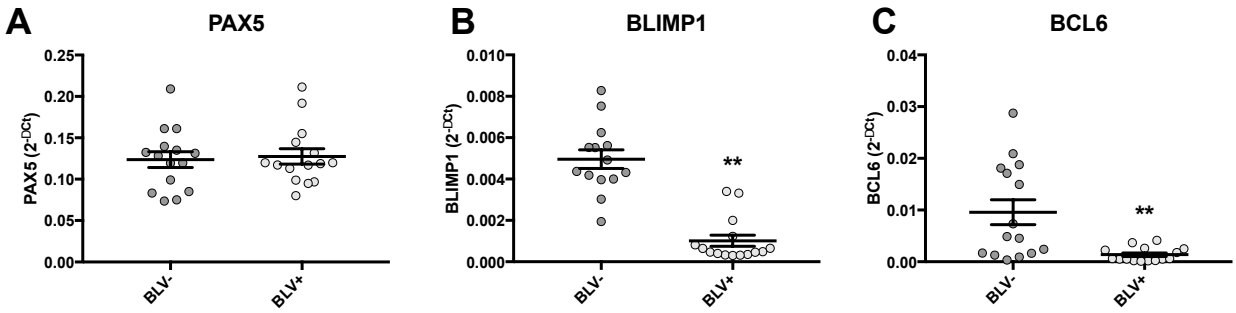


**Figure 4.11. BLIMP1 expression and BLV expression in BLV+ cows.** The expression of BLIMP1 in BLV+ cows correlated with A) PVL, B) TAX, C) B4-3p, D) B5-5p, E) B3-3p, F) B1-3p, G) B2-5p. $n = 15$.

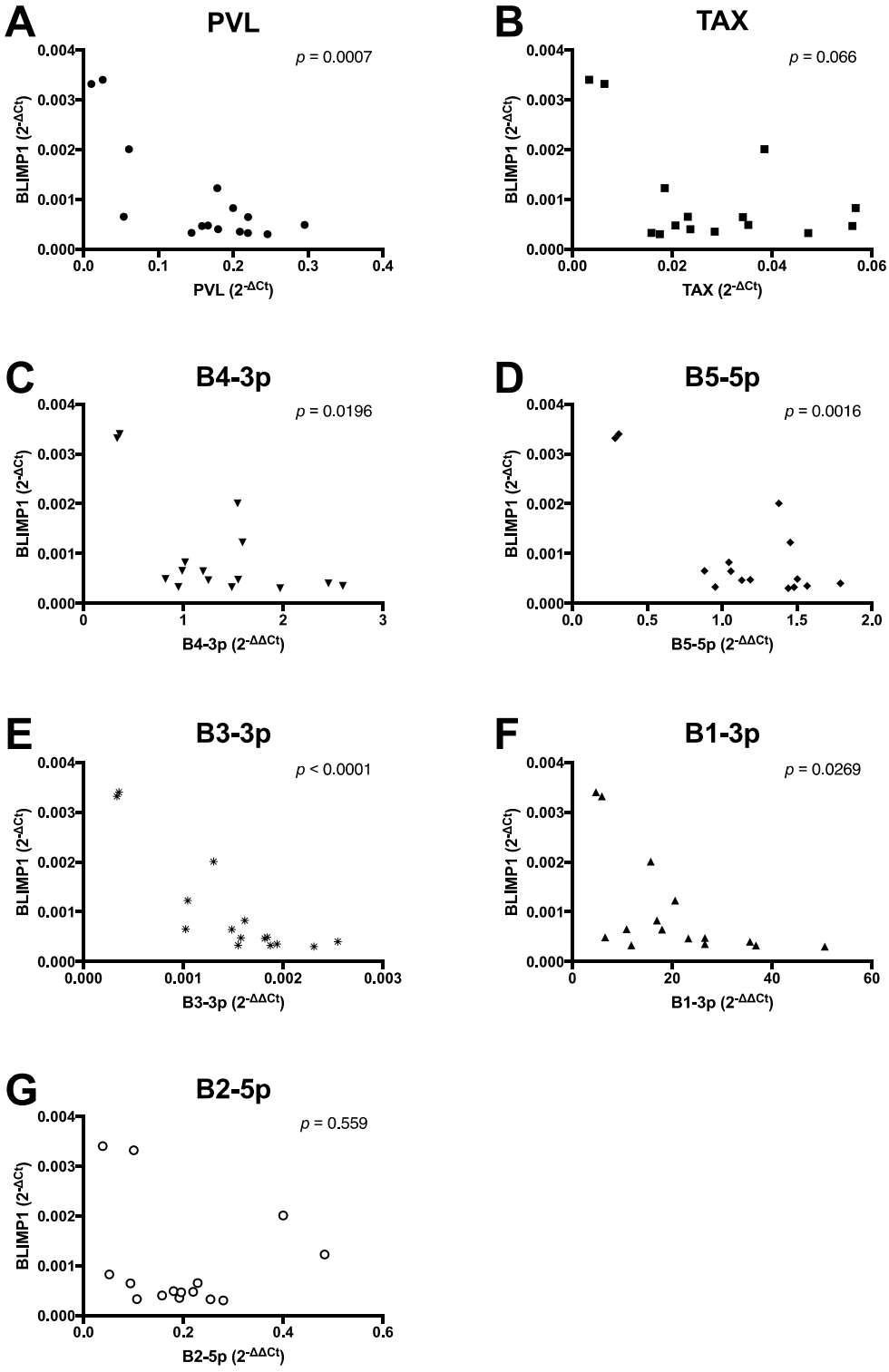
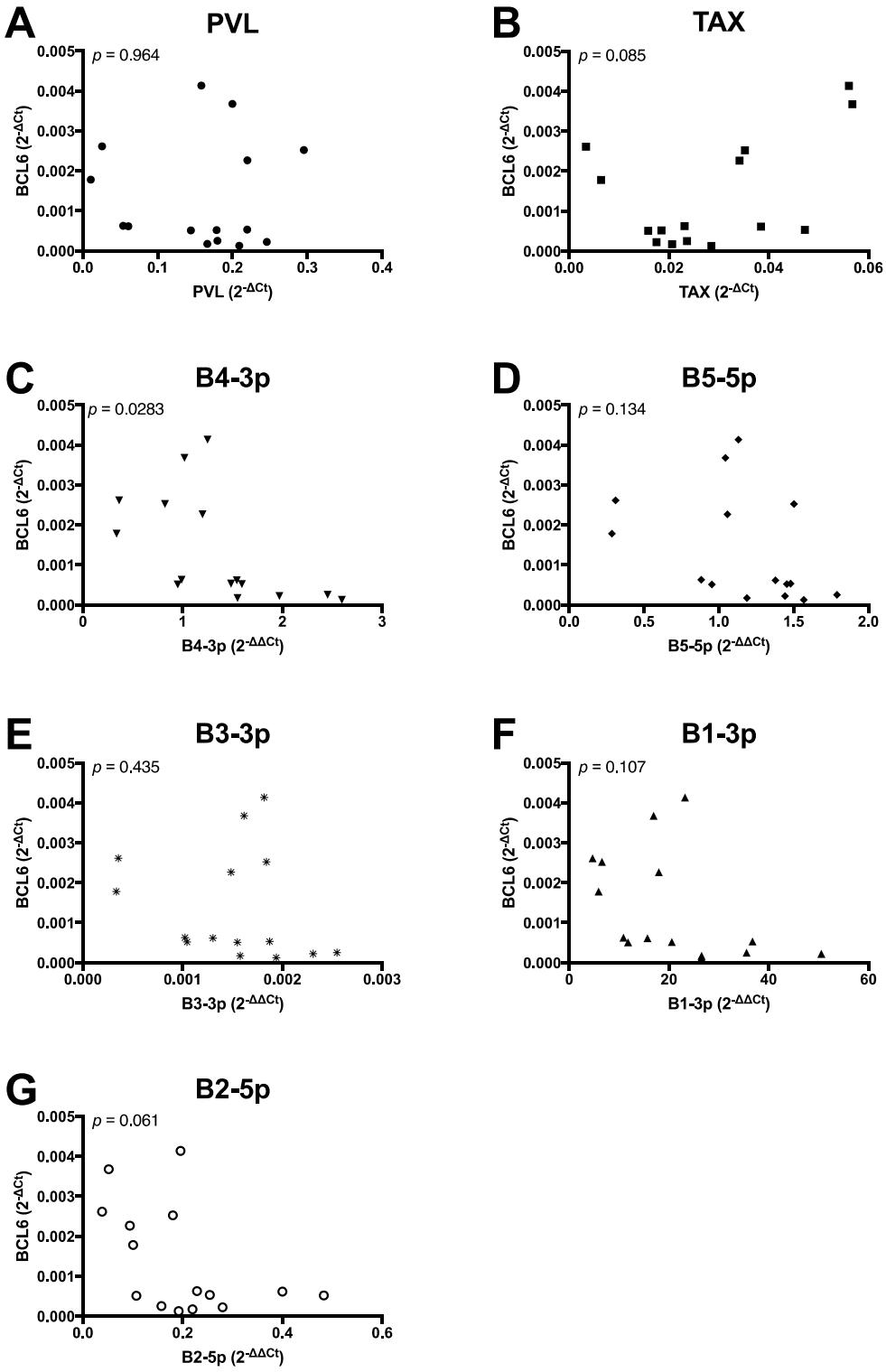


**Figure 4.12. BCL6 expression and BLV expression in BLV+ cows.** The expression of BCL6 in BLV+ cows correlated with A) PVL, B) TAX, C) B4-3p, D) B5-5p, E) B3-3p, F) B1-3p, G) B2-5p. $n = 15$.



**Discussion**

In this study, we investigated the impact of BLV infection on the concentration of total IgM in naturally infected dairy cows, as well as potential viral factors driving reduced IgM. IgM production was investigated because 1) our research had previously found reduced antigen-specific IgM in BLV+ cows after vaccination (Frie et al., 2016) and after primary antigenic exposure (Frie et al., 2017), and 2) because IgM is functionally important for protection against infectious agents. Natural IgM is produced in the absence of antigenic stimulation and recognizes pathogen-associated molecular patterns, including carbohydrates, phospholipids, and nucleic acids (Panda and Ding, 2015) and is an important component of innate immunity. In addition, IgM is also important during the initial adaptive immune response through its early production and high avidity. In cattle, IgM is especially effective at activating complement against extracellular bacteria and has been found to delay or prevent the onset of experimentally induced *E. coli* septicemia in neonatal calves (Logan and Penhale, 1971a, b).

BLV has two distinct known mechanisms that could ultimately affect IgM production: 1) the transcription and production of viral proteins, including Tax (Aida et al., 2013), and 2) the transcription of virally-encoded miRNAs (Gillet et al., 2016). Tax is the transactivator of BLV transcription and a candidate as a driver of BLV-induced oncogenesis (Aida et al., 2013). However, a puzzling aspect of BLV pathogenesis in cattle without lymphoma is the difficulty in detecting viral transcripts or proteins *in vivo* (Lagarias and Radke, 1989; Tajima and Aida, 2005). Evidence suggests that BLV persistence favors transcriptionally inactive provirus (Gillet et al., 2013), which is likely the result of the rapid clearance of infected cells expressing BLV (Florins et al., 2012). In recent years, BLV miRNAs have emerged as a possible source of observed immune abnormalities in BLV+ cows. BLV miRNAs have been observed *in vivo* in

both experimentally infected sheep (Rosewick et al., 2013) and calves (Gillet et al., 2016). In fact, comparing the transcriptomes of cells infected with WT BLV to cells infected with a mutant BLV missing the entire miRNA-encoding region demonstrated that WT-infected cells specifically exhibited downregulated IGJ expression (Gillet et al., 2016). As a result, we investigated whether BLV miRNA expression correlated to decreased expression of genes important for antibody production, which would suggest possible direct targets of BLV miRNA activity and suppression.

Although BLV+ cows exhibited a significant two-fold reduction in IGJ expression in comparison to BLV- cows, IGJ expression was not correlated to any measure of viral activity, suggesting that IGJ is not a direct target of BLV miRNAs. When the expression of transcription factors PAX5, BLIMP1 and BCL6 were examined, both BLIMP1 and BCL6 were found to be significantly downregulated in BLV+ cows. BLIMP1 expression was negatively correlated with the expression of four out of five tested BLV miRNAs, suggesting that BLIMP1 may be the direct target of one or more BLV miRNAs. In addition, BLIMP1 also exhibited a trending negative association with TAX expression, although it is unclear how TAX would interfere with BLIMP1 expression. In fact, it is possible that elevated BLIMP1 expression could suppress TAX expression by interfering with BLV transcription driven by endogenous factors. Bovine IRF1 and IRF2 (Kiermer et al., 1998) and Spi-B (Dekoninck et al., 2003) transcription factors can bind to the BLV promoter and induce transcription. BLIMP1 can both antagonistically bind to IRF1 and IRF2 target sequences (Doody et al., 2010) and directly repress the expression of Spi-B (Shaffer et al., 2002).

In contrast to the results observed with BLIMP1, only one BLV miRNA exhibited a significant negative correlation with BCL6 expression, although a second BLV miRNA did

exhibit a trending negative correlation. What was most intriguing was a trending positive association between TAX and BCL6. This was the only measurement where TAX and BLV miRNAs demonstrated opposite associations with the target gene. In addition, elevated BCL6 expression is associated with B cell lymphoma development in mice (Nutt et al., 2011) and Tax is associated with the accumulation of DNA damage and can induce transformation *in vitro* (Aida et al., 2013). The data suggest that BCL6 may be directly repressed by one or more BLV miRNAs, while TAX might actually promote BCL6 expression.

Overall, this study has illustrated that expression of both BLV miRNAs and BLV TAX can be detected in $SIgM^+$ B cells isolated from the periphery of naturally infected dairy cows. Moreover, the negative association between BLV miRNAs or BLV TAX and BLIMP1 or BCL6 suggests that BLV activity might suppress genes that are essential for either plasma cell differentiation or germinal center formation. Importantly, our results do not suggest that BLV interferes with IgM production specifically, but more likely interferes with overall antibody production. Although we have not previously observed a consistent decrease in plasma IgG (Frie et al., 2016; Frie et al., 2017), it is possible that IgM levels are more obviously affected because of the presence of natural IgM, which is enhanced by BLIMP1 (Savage and Baumgarth, 2015), or that T cell signaling during class-switching lessens the impact of BLV infection on B cell functions. Interestingly, reduced BCL6 is associated with impaired germinal center reactions and affinity maturation, even while maintaining a normal concentration of IgG (Toyama et al., 2002), but it remains to be seen if class-switched antibodies in BLV+ cows have reduced affinities. What is clear is that B cells from BLV+ cows exhibit reduced expression of transcriptional regulators of antibody production and reduced levels of plasma IgM, supporting a possible mechanism through which BLV infection interferes with host immune function.

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# CHAPTER 5: SUMMARY AND CONCLUSIONS

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**Specific Aims**

This dissertation aimed to investigate if bovine leukemia virus infection negatively impacted the immune system of infected dairy cattle. The following specific aims were designed to specifically address our research question.

**Specific Aim 1:** To investigate whether BLV infection interfered with healthy immune reactivity *in vivo* in naturally infected dairy cattle.

**Subaim 1.1:** To assess B and T cell responses to routine vaccination in adult, lactating Holstein cows infected with BLV.

**Subaim 1.2:** To characterize B and T cell responses to a primary and secondary antigenic exposure in adult, lactating Holstein cows infected with BLV.

**Specific aim 2:** To investigate a potential mechanism by which BLV infection interfered with IgM production in naturally infected adult, lactating Holstein cows.

**Overall Conclusions and Limitations**

To investigate aim 1, we initially investigated how BLV+ cows responded to routine vaccination. Vaccination is an essential component of disease management strategy for dairy producers and an impaired response to vaccination would be highly relevant in a production setting. To assess the response to vaccination in BLV+ cows, markers of B and T cell immunity were measured over 28 days following a routine boost vaccination with Bovi-Shield GOLD® FP® 5 L5 HB, which is a multivalent vaccine that is protective against several different viruses and five species of *Leptospira*. Immune responses from BLV+ cows were then compared to responses from BLV- cows. BLV+ cows consistently demonstrated lower levels of antigen-specific IgM against both viral and bacterial antigens. BLV+ cows might also produce less IgG2 against viral antigens, but this effect of BLV infection appears to require a high level of antigen exposure for detection. We only observed lower IgG2 production against BHV1 in two cohorts of cattle that had received 12 Bovi-Shield boosts on average before the start of our study. In contrast, BLV+ cows did not show any deficiency in antigen-specific IgG1 production, nor did they exhibit a decrease in anti-viral neutralizing antibody titers. When we used *in vitro* stimulation of primary PBMCs to investigate B and T cell activation, B cells from BLV+ cows appeared to be more reactive against BHV1 stimulation when measuring $CD25^+$ B cells. However, B cells from BLV+ cows also appeared to be less responsive to *in vitro* stimulation when measuring the relative surface expression of MHCII. $CD4^+$ and $CD8^+$ T cells from BLV+ and BLV- cows reacted similarly to antigen-specific and mitogenic stimulation *in vitro*. However, when we measured IFNγ production from γδ T cells, γδ T cells from BLV+ cows actually appeared more reactive and had a higher population of $IFN\gamma^+$ T cells than those from BLV- cows. Overall, we observed a reduced antibody response to vaccination in BLV+ cows,

but the *in vitro* data suggested that the immune function in BLV+ cows is not necessarily suppressed, but likely dysregulated (Frie et al., 2016).

Although investigating vaccine responses in BLV+ cattle was highly relevant to dairy producers, a major limitation of the study design was our inability to control for how many vaccine boosts received by BLV+ cows occurred before or after the onset of BLV infection. To address this issue, we exposed BLV+ and BLV- cattle to KLH, a novel antigen the cattle had not previously been exposed to, and recorded both primary and secondary B and T cell responses over 28 days. As observed after vaccination, BLV+ cows exhibited lower antigen-specific IgM following primary exposure to KLH, while IgG1 and IgG2 levels were not affected in BLV+ cows. When we characterized circulating B and T cell populations, we found that BLV+ cows specifically exhibited an increase in $CD5^{dim+}$ B cells, but also demonstrated a large reduction in the $CD45R0^{+}$ B cell population, as well as a reduction in MHCII surface expression. Similarly, BLV+ cows had a significant decrease in circulating $CD45R0^{+}$ γδ T cells, although $CD45R0^{+}$ $CD4^{+}$ and $CD8^{+}$ T cell populations were unaffected. Surprisingly, we also observed a significant increase in BLV proviral load (PVL) after both primary and secondary exposure to KLH.

Finally, we repeated *in vitro* PBMC cultures to investigate B and T cell responses to KLH and mitogenic stimulation, although we observed consistent reactions to *in vitro* stimulation regardless of the sampling date. Unlike the results observed after vaccination, B cells from BLV+ cows showed a smaller $CD25^{+}$ population after antigen-specific and mitogenic stimulation, suggesting reduced reactivity. Although we hypothesized that KLH stimulation *in vitro* might induce BLV protein production, we did not observe any more $BLV^{+}$ B cells in KLH-stimulated cultures over unstimulated cultures. However, we did observe that BLV protein expression was primarily found in activated $CD25^{+}$ B cells. We also observed that $BLV^{+}$ B cells

from BLV+ cows exhibited a significant increase in MHCII surface expression and responded normally to mitogenic stimulation. In contrast, $BLV^-$ B cells from BLV+ cows actually failed to increase CD25 surface expression. When we investigated IFNγ or IL4 production by T cell subsets *in vitro*, we observed a larger proportion of $IFN\gamma^+$ γδ T cells from BLV+ cows. However, we observed a larger proportion of $IL4^+$ $CD4^+$ and $CD8^+$ T cells from BLV+ cows across all culture conditions. Interestingly, while the relative production per cell of IFNγ or IL4 from $CD4^+$ and γδ T cells was similar between BLV+ and BLV- cows, this was not the case for cytokine production by $CD8^+$ T cells. While $CD8^+$ T cells from BLV+ cows showed a slight increase in average IFNγ production, they actually demonstrated a large decrease in average IL4 production in BLV+ cows, despite BLV+ cows having a larger population of IL4-producing $CD8^+$ T cells (Frie et al., 2017).

Overall, our investigation into the *in vivo* function of the immune system in BLV+ cows demonstrated both reduced and abnormal immunity. While optimal immune function is often best described by balance, reduced antibody levels in BLV+ cows is a clear indication of reduced immune reactivity. In addition, the observed decrease in $CD45R0^+$ B cells and $CD45R0^+$ γδ T cells suggests a reduced effector or memory B (McHeyzer-Williams and McHeyzer-Williams, 2005) and γδ T (De Maria et al., 1993)cell compartment and, thus, an overall reduction in immune function through the development or maintenance of effector or memory populations. More puzzling is the observed differences in T cell activation *in vitro* and the paradigm of IFNγ-driven Th1 versus IL4-driven Th2 polarization (Brown et al., 1998). While γδ T cells from BLV+ cows had a higher population producing IFNγ after mitogenic stimulation, both $CD4^+$ and $CD8^+$ T cells from BLV+ cows had a higher population producing IL4 under all culture conditions. When comparing the balance of IFNγ to IL4, it is possible that classical αβ T cells

from BLV+ cows are more likely to polarize to a Th2 phenotype, which could have significant downstream effects when it comes to infection control. However, while T cells from BLV+ cows appeared to favor Th2 polarization, $CD8^+$ T cells from BLV+ cows actually produced less IL4 than IL4-producing T cells from BLV- cows, suggesting that BLV infection could specifically interfere with $CD8^+$ T cell cytokine signaling. With multiple data points demonstrating either reduced or abnormal immunity in both the B and T cell compartments in BLV+ cows, our research strongly suggests that BLV+ cows have system-wide immune abnormalities, which increases the likelihood that BLV+ cows are more susceptible to secondary infections.

Although we learned a great deal from our studies, there were several limitations to our investigations into the *in vivo* immune function of BLV+ cows. While we were able to track immune parameters and observe abnormalities in BLV+ cows, we were unable to directly test whether those abnormalities translated to an increased risk of contracting secondary infections or a worse clearance of secondary infections in BLV+ cows. Another shortcoming was our inability to work with immune cells isolated from tissues beyond the blood. While immune cells circulate as a part of their life cycle, their primary functions are often occurring in secondary lymphoid tissues. In particular, using immune cells from a draining lymph node would be illuminating when looking for antigen-specific immune responses *in vitro*. However, obtaining tissues from cattle for live cell isolation was beyond the scope of this dissertation. Finally, while we did track IgM and IgG plasma levels over time, we have not explored immunoglobulin levels in other tissues, particularly mucosal IgA and milk immunoglobulins. Considering the importance of mucosal barriers for host immunity in adult cattle and colostral immunoglobulins for passive immunity in juvenile calves, it is important to know whether antibody levels in these tissues are affected in BLV+ cows.

For aim 2, we specifically investigated whether lower IgM in BLV+ cows was the result of lower IGJ expression, and whether lower IGJ expression was correlated to higher BLV miRNA expression. Although we did not expect to detect transcripts encoding BLV proteins (Frie and Coussens, 2015), we included TAX expression as a measure of viral transcription to ensure that associations we observed were specific for BLV miRNA expression. We did observe an overall reduction in total plasma IgM and a reduction in IGJ expression in B cells from BLV+ cows, which suggested that BLV miRNAs targeting IGJ could explain reduced IgM concentrations in BLV+ cows. While we observed detectable levels for both BLV TAX and BLV miRNAs in B cells from BLV+ cows, only TAX negatively correlated with total plasma IgM and no measurements of BLV activity correlated to reduced IGJ expression.

We next investigated several transcription factors that are important for B cell activity to determine whether an upstream regulator could be a direct target of BLV activity. While PAX5, the master regulator determining the B cell lineage (Nutt et al., 2011), was not different between BLV+ and BLV- cows, both BLIMP1 and BCL6 expression were significantly depressed in B cells from BLV+ cows. In fact, BLIMP1, the primary transcriptional regulator of plasma cell differentiation (Nutt et al., 2011), negatively correlated with both TAX and BLV miRNA expression. BCL6, a transcription factor critically important for germinal center B cell maturation (Swaminathan et al., 2014), also negatively correlated with two BLV miRNAs and actually demonstrated a trending positive association with TAX. Despite four out of five BLV miRNAs positively correlated with each other, our data still suggests that both BLIMP1 and BCL6 could be direct targets of one or more BLV miRNAs. In addition, our data suggests that BLV TAX may play a more active role in pathogenesis than previously appreciated. In total, these results suggest that reduced IgM levels in BLV+ cows is due to BLV interference in

transcriptional regulators of plasma cell differentiation, as opposed to direct interference with IGJ expression.

While we considered it critical to investigate BLV miRNA expression in naturally infected adult cows, the evidence we gathered is only correlative. Considering the complex interplay between host immunity and viral pathogenesis, we cannot determine any causative relationship between BLV TAX or BLV miRNA expression and suppressed immune phenotypes in infected cattle. In addition, while we included one mature miRNA from each pre-miRNA hairpin encoded by BLV, which were also the five most highly expressed BLV miRNAs in sheep (Rosewick et al., 2013), we did not directly test the expression of all 10 mature miRNAs. Finally, although miRNAs function through the suppression of transcription of target gene transcripts, we did not test whether reduced IGJ, BLIMP1, or BCL6 expression corresponded to reduced protein expression, although lower IgM data would support that the lower transcript levels observed in BLV+ cows are biologically relevant.

**Future Experiments**

While this dissertation has clearly demonstrated reduced plasma IgM in BLV+ cows, it remains unknown whether BLV+ cows have abnormal levels of other immunoglobulin isotypes across different biological compartments. Assessing the plasma concentrations of IgG and IgA in BLV+ cows would provide additional evidence to determine whether BLV infection interferes with global antibody production, or if BLV infection more directly affects IgM production. In addition to plasma immunoglobulin levels, it would be beneficial to investigate immunoglobulin levels secreted into milk and onto mucosal surfaces. Reduced immunoglobulin levels in milk could be highly significant in terms of disease risk management for calf rearing in dairy herds. The passive transfer of maternal immunity to calves is extremely important to protect calves from contracting infections before their own immune systems mature, and while passive immunity is mostly conferred through colostrum (Hurley and Theil, 2011), depressed antibody levels in milk would be suggestive of reduced antibody levels in colostrum. It would be especially interesting to investigate IgM and IgA levels in milk because PIgR mediates the transport of pentameric IgM and dimeric IgA across epithelial barriers into milk (Hurley and Theil, 2011), and we have observed lower IGJ transcripts in B cells from BLV+ cows. Investigating immunoglobulin levels on mucosal surfaces would also complement our research because of the importance of dimeric IgA, in particular, for mucosal immunity (Hurley and Theil, 2011).

In addition to measuring the antibody levels in BLV+ cows, it would be highly informative to investigate the functionality of antibodies from BLV+ cows. We found that circulating B cells from BLV+ cows demonstrated reduced BCL6 expression and BCL6-deficient mice fail to develop germinal centers, which are essential for class-switching and

affinity maturation (Toyama et al., 2002). *In vivo*, these mice demonstrated an equivalent titer of IgG antibodies after immune stimulation when compared to WT mice. However, BCL6-deficient mice exhibited a reduction in IgG affinity, as well as a shorter-lived antigen-specific IgG response. It would be critical to examine IgG affinity because equal IgG titers between BLV+ and BLV- cows could mask impaired immunity in BLV+ cows and because BCL6 is regulated both transcriptionally and post-translationally (Nutt et al., 2011), thus we do not currently know if lower BCL6 expression has biological relevance for humoral immunity in BLV+ cows.

Another intriguing aspect of overall immunity in BLV+ cows is the possible skew towards Th2 immunity. Based on the *in vitro* production of IFNγ and IL4 by T cells from BLV+ and BLV- cows, it would be highly informative to investigate whether BLV+ cows favor Th2 polarization after immune stimulation. Favoring Th2 polarization could make BLV+ cattle more susceptible to secondary infections (for example, intracellular bacteria and viruses) that require Th1 or Th17 polarization for optimal immunity. Understanding whether BLV+ cows were more at risk for specific secondary infections would allow for herd management practices that could directly address specific risks in BLV+ cows. Considering the difficulty in performing experimental infections in cattle, two alternative options would include 1) *in vitro* stimulation of PBMCs from BLV+ and BLV- cows using known Th1 or Th2 antigens, or 2) epidemiological investigations for an association between BLV infection and a higher incidence of infectious diseases with characterized Th profiles. To investigate Th1 or Th2 polarization, a similar design could be used based upon our *in vitro* PBMC stimulation using KLH. However, PBMCs could be isolated from cattle exposed to the Startvac mastitis vaccine, which has been shown to induce IFNγ production in favor of IL4 production after *in vitro* stimulation using *S. aureus* or *E.* coli (Piepers et al., 2017). Interestingly, J5 vaccination for *E. coli* mastitis in BLV+ cows induced

lower antigen-specific IgG2 titers in BLV+ cows (Erskine et al., 2011), and IgG2 production is favored over IgG1 production after B cell stimulation by IFNγ (Estes and Brown, 2002). For epidemiological studies, it would be especially interesting to investigate whether BLV infection were a risk factor for cattle progressing to clinical Johne's disease, as Johne's disease progression has often been associated with a switch from Th1 to Th2 immunity (Coussens, 2004). The combination of both approaches would be ideal to account for both a physiological relevance at the herd level, as determined by epidemiological studies, in addition to specific mechanisms within cattle as shown by IFNγ or IL4 production in response to antigenic stimulation.

Finally, while this dissertation found associations between BLV expression and abnormal B cell phenotypes in BLV+ cows, a causal relationship between BLV activity and dysregulated transcription in BLV-infected calls has yet to be established. Based upon the associations we observed between BLV miRNA expression and BLIMP1 and BCL6 expression, we could test whether BLIMP1 and BCL6 are direct targets of BLV miRNAs using a luciferase reporter assay and BLV miRNA-expressing vectors. It is also possible that BLV miRNAs do not directly target BLIMP1 or BCL6 expression and the associations we observed were downstream effects from the suppression of a direct target of BLV miRNAs. To test that hypothesis, BLV miRNAs could be ectopically expressed in B cells isolated from BLV- cows and BLIMP1 and BCL6 expression would be assessed. In addition, antibody production in BLV-transduced or control B cells could be assessed to determine whether IgM production was impaired in B cells expressing BLV miRNAs. A complementary study design could be used to ectopically express TAX in B cells from BLV- cows to investigate whether TAX expression can increase BCL6 expression, as we observed a trending positive association between TAX expression and BCL6 expression.

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