

The Pennsylvania State University

The Graduate School

College of Medicine

**CHARACTERIZING BOVINE LEUKEMIA VIRUS INDUCED  
IMMUNOSUPPRESSION IN ADULT NEW ZEALAND WHITE RABBITS  
(*ORYCTOLAGUS CUNICULUS*)**

A Thesis in

Laboratory Animal Medicine

by

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## Abstract

A virus-induced immunosuppression model has yet to be fully described in the rabbit (*Oryctolagus cuniculus*). In this study we strive to characterize bovine leukemia virus (BLV) induced immunosuppression in the rabbit. BLV has been previously reported to induce persistent infection in rabbits resulting in an immunosuppression-like disease pathogenesis after a long (>12 months) incubation.<sup>1,2</sup> This model will be valuable to study concurrent viral infection in human immunodeficiency virus (HIV)/ AIDS patients. The purpose of this study was to evaluate parameters and establish criteria for bovine leukemia virus (BLV)-induced immunosuppression in rabbits. Proviral DNA (pBLV913) or virus producing fetal lamb kidney cells (BLV-FLK) were used to initiate infection in rabbits over the course of 6 months. Peripheral blood lymphocytes were collected biweekly to quantitate helper T, cytotoxic T, and B lymphocyte populations using anti-CD4, anti-CD8, and anti-MHCII antibodies respectively. Serum samples were collected for detecting anti-BLV antibody generation by ELISA. Our results showed that, within the confines of the short observation period, BLV did not significantly alter the peripheral blood lymphocyte populations. BLV-FLK inoculation resulted in significant lower body weights in rabbits when compared with those in the control rabbits. All of the BLV-FLK cell-inoculated but none of the pBLV913-inoculated rabbits produced antibodies against BLV Gp51. Longer duration of study is needed to fully understand the pathogenesis of BLV in rabbits.

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**Disclosures**

The authors have no conflicts of interest.

## Chapter 1

### Introduction

Human papillomavirus (HPV)-associated cancers are increasing in immunosuppressed patients, such as those concurrently infected with HIV, despite highly active antiretroviral therapy.<sup>3</sup> Rabbit cells transgenically expressing human CD4 and CCR5 have been reported to be highly permissive for HIV-1 infection.<sup>4</sup> No laboratory animal model is available to study HPV directly due to the restricted species tropism of the virus.<sup>5</sup> The New Zealand White rabbit is a well-established animal model for studying papillomavirus (PV) due to its susceptibility to the related virus, cottontail rabbit papillomavirus (CRPV). CRPV together with rabbit oral papillomavirus (ROPV) are surrogates for HPV infections and have played a pivotal role in the successful development of vaccines used clinically. We propose to establish a virus induced immunosuppression model to study coincidental PV infections in domestic rabbits.

To date, no rabbit virus is reported to infect rabbits and to induce an immunodeficiency syndrome. HIV infection of rabbits has failed to produce clinical symptoms of disease.<sup>6 7</sup> Bovine immunodeficiency virus (BIV), a lentivirus, can induce persistent infection in rabbits, however no clinical symptoms are observed in these animals over the course of two years.<sup>8</sup> Bovine leukemia virus (BLV), a delta retrovirus related to human T-cell leukemia viruses 1 and 2 (HTLV-1 and -2), infection leads to a reproducible immunodeficiency syndrome in 33-66% of BLV infected rabbits.<sup>2,9</sup>

BLV induces immunosuppression in both cattle and sheep prior to causing persistent lymphocytosis and lymphoma. The exact mechanisms of immunosuppression have not yet been elucidated, however they are multifactorial and include host genomics as well as the interaction of the oncogenes tax and its effects on p53. Cows with persistent lymphocytosis have altered mitogen response and altered lymphocyte populations and subpopulations. It has also been shown that these animals have decreased interleukin-2.<sup>10-14</sup>

BLV causes persistent lymphocytosis and B cell lymphoma in cattle and B cell leukemia in experimentally-infected sheep.<sup>15</sup> Transmission in cattle is blood borne requiring cell to cell transmission. Such transmission results from injecting cattle with contaminated needles, or by a hematophagous vector such as a mosquito. Feeding calves unpasteurized colostrum and milk may also cause infection. The disease in cattle has a high prevalence across the United States. In 1997 APHIS conducted a study through NAHMS which estimated 89% of US dairy operations had positive cattle, and 74.8% of those herds had at least a 25% seropositive prevalence using the agar gel immunodiffusion test (AGID). In 2007, APHIS conducted bulk tank milk ELISAs for antibodies to BLV and found a prevalence of 83.9%. APHIS reported that the incidence of developing lymphoma in infected cattle was 1-5%.<sup>16</sup>

Previous studies strongly support the idea that a model for immunodeficiency could be established by BLV infection in domestic rabbits. BLV has been shown to cause increased incidence of weight loss and secondary infections in rabbits inoculated with either the BLV plasmid p913, fetal lamb kidney cell suspension (BLV-FLK), or infected cattle lymphocytes.<sup>1,2,9,17</sup> This study examined differences in disease pathogenesis using plasmid DNA or BLV producing fetal lamb kidney cell inoculated rabbits. Measuring both virological and immunological parameters will aid in establishing a standard for clinical relevant immunosuppression by BLV infection.

This study will set the groundwork for studying co-infections in the BLV induced immunosuppressed rabbit model. The data gained from this study will be valuable to study other rabbit susceptible human pathogens in immunosuppressed rabbits as well.

## **Hypotheses**

1. Both BLV-FLK and plasmid inoculation routes will induce some level of immunosuppression

2. Immunosuppression using whole virus in a fetal lamb kidney (BLV-FLK) suspension will lead to greater increases in lymphocyte numbers, presumably nonfunctional lymphocytes, than immunosuppression with recombinant plasmid (pBLV913), thereby making the whole cell inoculation the more efficient method of immunosuppressing adult rabbits.

### **Specific Aims**

1. To quantitatively assess populations of leukocytes, especially lymphocytes and their subtypes, following inoculation with BLV either through plasmid (pBLV913) or BLV producing fetal lamb kidney (BLV-FLK) cells.

2. To determine the most effective way of virally immunosuppressing rabbits; either through inoculation with a recombinant plasmid or through inoculation with BLV-FLK cells.

## Chapter 2 Experimental Design

### Animals and Experimental Treatments

18 specific pathogen free (SPF) New Zealand White rabbits (NZW), (9 male, 9 female) [Crlc:KBL(NZW)], of Canadian origin weighing approx 1.9-2.3kg (approximately 8-11 weeks of age) were received from Charles River Laboratories and randomly assigned into groups by sex. Study groups consisted of three rabbits for negative control (2 females, 1 male), hereafter referred to as rabbits NC1-3. Three rabbits were used for positive control by administration of cyclosporine, (2 males, 1 female), referred to as rabbits PC1-3. Six rabbits were inoculated with BLV-FLK cells (3 males, 3 females), referred to as rabbits F1-6. Six rabbits were inoculated with pBLV913 (3 males, 3 females), referred to as P1-6. Animals were from a colony that previously serologically tested negative for at least 18 months for the following pathogens: reovirus, lymphocytic choriomeningitis virus (LCMV), parainfluenza types 1&2, and rotavirus by multiplexed fluorometric immunoassay (MFIA); and rabbit hemorrhagic disease virus (RHDV) by ELISA. Additionally, rabbits from the room historically tested negative for *Bordetella bronchiseptica*, *Pasteurella multocida*, *Pasteurella* spp., *Salmonella* spp., and *Pseudomonas aeruginosa* by nasal aspirate and bronchial wash, cilia-associated respiratory bacillus (CARB) by MFIA and PCR, and *Helicobacter* spp by PCR. *Treponema* (serology), *Lawsonia* sp. (PCR), and Tyzzer's disease (*Clostridium piliforme*) (PCR) were also excluded pathogens in this rabbit room. *Cheyletiella parasitovorax*, *Leporacarus (Listrophorus) gibbus*, *Psoroptes cuniculi*, *Notoedres cati*, trombiculid mites, ticks, lice and fleas, *Passalurus ambiguus* and other helminths were absent. *Eimeria stiedae*, other *Eimeria* spp., all Trichomonads (Trichomonadaceae), *Giardia* spp., *Hexamastix* spp., *Entamoeba* spp., *Enteromonas* spp., *Encephalitozoon cuniculi* were all absent from the rabbit colony.

All animals were housed in accordance to standards set by the Association for Assessment and Accreditation of Laboratory Animal Care and followed the Institute for Laboratory Animal Research's (ILAR) "Guide for the Care and Use of Laboratory Animals,

7<sup>th</sup> ed. (1996).” This experimental protocol was approved by the Penn State Hershey Medical Center’s Institutional Animal Care and Use Committee (IACUC). Animals were handled in order from negative control groups, positive control group (cyclosporine), and then the inoculated groups, first the BLV-FLK group and then the plasmid group. All animals were single housed and weighed weekly. Rabbits were anesthetized once every two weeks with ketamine (40mg/kg) and xylazine (5mg/kg) intramuscularly (IM) for phlebotomy using the central ear artery and BD Vacutainer® blood collection system. Total blood collected per time point did not exceed 10% of the total blood volume every two weeks, or 1% of the animal’s body weight per week. Complete blood counts were performed every two weeks to monitor lymphocyte counts and morphology as well as to check on the general health status of the animals. All rabbits were provided with autoclaved water and fed a commercially available irradiated rabbit feed (Teklad 2031 Global High Fiber Rabbit Diet) *ad libitum* and supplemented at least weekly with irradiated certified timothy hay cubes (Bio-Serv product#F1011) or sterile timothy hay mini-bales (Bio-Serv product#S1013). All rabbits received a rotating schedule of sanitized enrichment devices.

#### BLV-FLK production and inoculation

BLV-FLK cells were kindly donated by Dr. Katherine Boris-Lawrie from the Ohio State University. Persistently infected BLV fetal lamb kidney (FLK) fibroblast cells, hereafter known as BLV-FLK cells, were grown in Dulbeccos enriched medium. Medium consisted of 500ml Dulbecco’s modified Eagle’s medium (DMEM), 50ml heat-inactivated fetal bovine serum (Atlanta Biologicals) for a 10% solution, 5ml non-essential amino acids (Gibco 100×), 5ml sodium pyruvate (Gibco, 100×), 5ml HEPES buffer, 8ml NaHCO<sub>3</sub>, 5ml penicillin/streptomycin, and 5ml L-glutamine.

Virus was isolated for future experiments from both the supernatant of the BLV-FLK cell culture flask and also from sonicated BLV-FLK cells themselves according to a previously described protocol.<sup>18</sup> In brief, the cells were lifted from the plate with trypsin +

EDTA, washed three times in 1× PBS and subjected to sonication three times for 10 seconds each.

The supernatant of the sonicated cells was collected after centrifugation. Virus was also harvested from the cell culture medium after 6 days of BLV-FLK culture. The collected medium that was free of dead cells or cell fragments was further concentrated with 10% PEG8000. After thoroughly mixing, the solution was placed on ice for 1-12 hours (avg. 2 hours). The virus was then precipitated by centrifugation at 9,000rpm, 2°C, for 10 minutes. The supernatant was discarded and the virus was removed from the PEG8000 through a polyacrylamide bead column. First, the pellet was resuspended in TNE buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.4). This solution was then passed over a P-10 Bio-Gel polyacrylamide bead column (Biorad), 100-200 mesh. The bead column was washed three times in 1.5 bed volumes of 1x PBS to ensure collection of all virus. A serial of fractions was collected in 1.5ml eppendorf tubes and analyzed for presence of virus by ELISA. The virus was titrated using BLV positive calf serum as the primary antibody. BLV was found to be most concentrated in the 3<sup>rd</sup>, 4<sup>th</sup>, and 5<sup>th</sup> fractions. Those fractions were subsequently saved at -70°C for further analysis.

The BLV-FLK cells for inoculation of rabbits were harvested by lifting cells with EDTA+ trypsin, washing 3× with 1× PBS followed by cell counting. Each rabbit received 1x 10<sup>7</sup>-10<sup>8</sup> of BLV –FLK cells suspended in 0.5ml 0.9%NaCl injected subcutaneously.

To confirm the presence of BLV in BLV-FLK cells, a region of the BLV genome was amplified by reverse transcriptase PCR. The PCR product was sequenced by the Pennsylvania State University College of Medicine Core Facility. The PCR product was to BLV-FLK viral found to have 100% max identity with 96% query coverage, (fig 1). Primers and conditions are described under the Methods section for PCR. Primers used were pairs CHR1510, 1511(outer pair), and CHR1512, 1513 (inner pair).

5'TAGTACTCACCTCCCTGATCCACGTTCTGGAGCAAGATCAGCAACGCTTGATCACAGCAATTA  
CCAGACCCACTATAATTTGCTTAATGTGGCCTCTGTGGTTGCCAGAACCGACGGGGGCTTGATTG  
GTTGTACATCCGGCTGGGTTTTCAAAGCCTATGTCCCACAATTAATGAGCCTTGCTGTTTCCTGCGC  
ATTCAAAATGACTCCATTATCCGCCTCGGTGATCTCCAGCCTCTCTCGCAAAGAGTCTCTACAGAC  
TGGCAGTGGCCCTGGAATGGGATCTGGGGCTCACTGCCTGGGTGCGAGAAACCATTCTATTCTGTT  
CTAAGCCTGTTCTATTAGCCCTTTTTTTGCTCTTCTGGCCCCCTGCCTGATAAAAATGCTTGACCT  
CTCGCCTTTTAAAGCTCCTCCGGCAGGCTCCCCACTTCCCTGAAATCTCCTTAACCCCTAAACCCG  
ATTCTGATTATCAGGCCTTGCTACCATCTGCACCAGAGATCTACTCTCACCTCTCCCCCGTCAAACC  
CGATTACATCAACCTCCGACCCTGCTGGGGGAAGTCGCCAA 3'

Fig1: PCR product of BLV-FLK cells. Nested PCR was performed and the product was sequenced and found to have 96% homology to BLV-FLK viral DNA reported in Genbank.

### Plasmid production and inoculation

The BLV plasmid, pBLV913, was generously donated by Dr. David Derse (NIH). The plasmid was then used to transform Escherichia coli bacteria (Invitrogen Max Efficiency Stb12 Competent cells cat#10268-019), purified using the QIAGEN maxiprep plasmid purification kit and the Origene PowerPrep express plasmid purification kit according to the manufacturer's protocols. The plasmid was then purified further with cesium chloride ultracentrifugation. The purified plasmid was digested with EcoR1 and the resulting restriction digest compared to that described by Derse et al.<sup>19</sup>

### Cyclosporine

Three rabbits (2 males, 1 female) were randomly selected to be administered cyclosporine A (Sandimmune, Novartis) using a previously described dosing regime.<sup>20</sup> In brief, the rabbits were injected SQ daily with 15mg/kg (days 1-6), then 20mg/kg (days 7-29) and then tapering to 15mg/kg (days 30-termination of study) twice weekly.

## **Blood Collection and Processing**

Peripheral blood collected in EDTA vacutainer tubes was processed using Lympholyte®-Mammal (CedarLane®) to separate the peripheral blood lymphocytes (PBLs). Lympholyte solution isolates lymphocytes and monocytes. PBLs were washed twice with 1× PBS with 2% fetal bovine serum (FBS) and seeded in a U-bottom 96 well flask and labeled with anti-rabbit CD4 (Spring Valley Labs, Inc., 1:50 dilution in 1× PBS with 2% FBS) anti-rabbit CD8 (Spring Valley Labs, Inc., 1:50 dilution in 1× PBS with 2% FBS), and anti-MCHII (clone 2C4, ATCC). Because there are no commercially available anti-B cell antibodies validated for use in rabbits, MHCII was used as a surrogate to measure B lymphocyte, basophil, and monocyte populations. Cells were incubated on ice for 45 minutes. Cells were then washed twice with 1× PBS with 2% FBS) and treated with phycoerythrin (PE) conjugated anti-mouse IgG (diluted 1:50 in 1×PBS with 2% FBS), allowed to incubate on ice in the dark for 45 minutes and then washed twice with 1×PBS with 2% FBS. Cells were then collected and suspended in 2% paraformaldehyde and then counted using 1-color flow cytometry (FACS Calibur). The population of CD4:CD8 ratios and MHCII % were then calculated.

## ELISA

Serum ELISA using the VMRD test kit for BLV coated with GP51 molecule (part#284) was also tested. Positive control used was VMRD monoclonal mouse anti-GP51 antibody (catalog# BLV1 (BLV) Mab(gp51-G)) diluted 1:100 in 5% dry milk made in 1×PBS. Negative control used was rabbit serum from an un-manipulated rabbit housed in a different room. All serum samples were diluted 1:25 with the test kit serum-diluting buffer. Samples were tested in duplicates or triplicates and allowed to incubate for 1 hour at room temperature. Wells were then washed three times with 250ul of 1% wash solution (from the test kit). Primary antibody was added to wells: AP conjugated goat anti-mouse IgG F(ab')<sub>2</sub> (catalog no#31324, Pierce) was used for the positive control wells; AP conjugated goat anti-rabbit IgG&M (catalog #1410, Southern Biotech) was used for rabbit serum samples and negative control well. For development, 4-nitrophenyl phosphate disodium salt hexahydrate 5mg substrate/tablet (pNPP lot#109k-8206 Sigma Chemical Company) in 5ml of alkaline phosphatase buffer pH9.5 was used. Wells were allowed to develop for 20 minutes and read on the plate reader at 405nm using Dynex Revelation Quicklink software and a Thermo Labsystems Opsys MR plate reader. Results were interpreted as being positive when they were not statistically similar to the negative control value. Each plate was analyzed separately.

## RNA processing and RT-PCR

Total RNA was collected from peripheral blood lymphocytes using TRIzol (Invitrogen). A total of  $1 \times 10^6$  peripheral blood lymphocytes were suspended in 1ml TRIzol and then frozen at  $-70^\circ\text{C}$  until RNA extraction was performed. RNA extraction was performed according the manufacturer's protocol (Invitrogen). The pellet was then re-suspended in 20-40ul nanopure water and then incubated at  $55-60^\circ\text{C}$  for 10 minutes to ensure that it dissolved. RNA was then quantified using the Nanodrop and either used for RT-PCR or frozen at  $-70^\circ\text{C}$  for further processing.

Reverse transcriptase PCR (RT-PCR) (GeneAmp, Applied Biosystems) was performed using random hexamers with the following program:  $42^\circ\text{C}$  15 min,  $95^\circ\text{C}$  5 min,  $29^\circ\text{C}$  2 min (RT) followed by nesting PCR (*Taq* polymerase recombinant, Invitrogen) using outside primer pairs CHRI1510 and CHRI1511 followed by insider pairs CHRI1512 and CHRI1513 or CHRI1512 and CHRI1560 to yield DNA fragments of 854bp and 591 basepairs (bp) respectively. PCR program was as follows for both rounds of PCR:  $95^\circ\text{C}$  5min,  $95^\circ\text{C}$  30 sec,  $59^\circ\text{C}$  30 sec,  $72^\circ\text{C}$  30 sec (30 cycles),  $72^\circ\text{C}$  10 min,  $29^\circ\text{C}$  2 min.

Primers:

Pol (outer pair of nested) 854bp

5' GAACGACTCCAGGCCCTTCAA 3'...(KB2341) CHR1510

5' GTGGGACAGGGCTTGTCGAAG 3'...(KB3175) CHR1511

Pol (inner pair of nested) 591bp

5' GGAGGTTTGTGCATGACCTAC 3'...(KB560) CHR1512

5' CATTGGAGGTCTCCTAAGACC 3'...(KB561) CHR1513

Pol (second pair of inner nested) 300bp

5' GGACGATATCCTTATCGCTTCGCC 3' ...CHR1560

5' CATTGGAGGTCTCCTAAGACC 3' .....CHR1513

BLV env Primers 636bp

5' GTGACCTTAGGCCTAGCC 3'...(KB582) CHR1514

5' GTCGACTAAGGGCAGGGTCG 3'...(KB567) CHR1515

## **Virus Syncytium Assay**

Previous studies have used this cell line for measuring syncytium formation after adding BLV infected rabbit PBLs. Bat lung fibroblasts were purchased from ATCC (CCL-88) and cultured in Dulbecco's enriched medium (previously described). Once cells became confluent they were divided into a 12 well plate or a lysine-coated slide. After the cells became >50% confluent, 25ug/ml medium of diethylaminoethyl-dextran hydrochloride (DEAE-D, Sigma) was added to the wells, incubated for 30 minutes, and then removed. The monolayer was rinsed once and then rabbit PBLs ( $5 \times 10^5$  cells for 12 well plate,  $1 \times 10^4$  for slide well) in CTL medium were added to the wells. CTL medium consists of 500ml RPMI 1640 (Gibco), 55ml FBS-heat inactivated (Atlanta Biologicals), 5ml penicillin/streptomycin (100×), 5ml L-glutamine (100×), 5ml HEPES (100×), 5ml 2-mercaptoethanol (100× Sigma), 5ml sodium pyruvate (100×). These cells were incubated for 18 hours at 37°C and 5% CO<sub>2</sub> and then removed. The wells were washed in DMEM medium and incubated as previously described for an additional 4-5 days for observation of syncytium formation.<sup>21,22</sup>

Although the protocol is described, the results will not be incorporated in the thesis due to time constraints. Results and interpretations will be in the paper for publication.

## **Necropsy**

At the end of study, rabbits were sedated with 40mg/kg ketamine and 5mg/kg xylazine IM in the epaxial muscles, weighed and had blood collected. They were euthanized with an overdose of sodium pentobarbital and phenytoin sodium (Euthasol, Virbac) IV or intracardiac and then necropsied. Fecal samples and aerobic cultures of the nasal cavity were obtained. Sections of spleen, thymus, and mesenteric lymph node were frozen in liquid nitrogen. Other tissues were fixed in 10% neutral buffered formalin (NBF) for 48 hours and then transferred to 70% EtOH. Tissues were processed in an automated Tissue-Tek VIP processor and paraffin-embedded with a Tissue-Tek TEC embedding station. Sections were cut at 6  $\mu$ m for routine hematoxylin and eosin (H&E) staining. All tissues were examined by an ACVP diplomate pathologist. All images were obtained with an Olympus BX51 microscope and DP71 digital camera using MicroSuite Basic 2.6 imaging software.

## **Chapter 3 Results:**

### **Clinical signs of disease**

At the end of the study one rabbit per treatment group was selected for serology analysis to determine if the specific pathogen free disease status was retained throughout the experiment. Serology samples were sent to Charles River Laboratories for the Assessment Rabbit Serology Profile. Serology testing included *Encephalitozoon cuniculi*, CARbacillus, Treponema, *Clostridium piliforme*, parainfluenza virus types 1 and 2, reovirus, rotavirus, lymphocytic choriomeningitis virus, and *Toxoplasma gondii*. All rabbits in our study had negative fecal examination for parasites and ova and negative nasal conchae aerobic cultures for *Bordetella bronchiseptica* and *Pasteurella multocida* at necropsy. All serology results were negative.

Beginning around 6 months post inoculation several inoculated rabbits and the cyclosporine treated rabbits began showing signs of disease including reduced weight gain and decreased appetite. One rabbit had a slight head tilt (P5, plasmid group). This head tilt was not associated with gross lesions at necropsy. There were no lesions present in the brain or brainstem at necropsy which may have lead to a head tilt. Inner ears were not collected for histology.

### **Body Weight**

Body weight measurement was the most reliable marker of disease during the study (figure 2). Although all rabbits' body weights increased over time, the cyclosporine (CsA) treated rabbits and inoculated rabbits, both BLV-FLK and plasmids, gained weight at a slower rate than the uninoculated control group. Beginning at 2 months post-inoculation, the CsA treated rabbits had statistically significantly lower body weights compared to the controls. The BLV-FLK rabbits had body weights that were significantly lower than the controls beginning at 4.5 months post-inoculation. Both the CsA and the BLV-FLK rabbits

maintained their lower body weights for the duration of the study. Plasmid rabbits had significantly lower body weight than the negative control rabbits at one time point 5 months post-inoculation, however this was not found again during the study. Differences in weight between the BLV-FLK and plasmid groups were not significant.

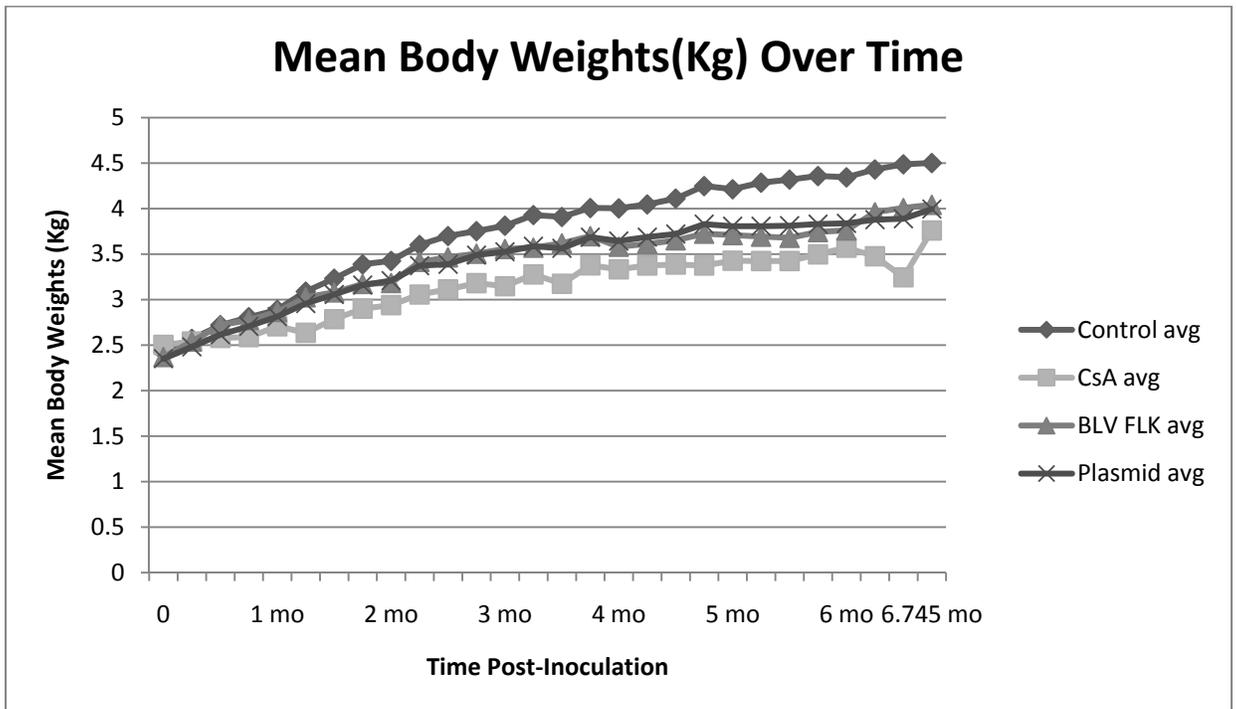


Fig 2. Mean body weights of rabbits over time post-inoculation. CsA group is significantly lighter than negative control rabbits beginning at 1.5 months post-inoculation until the end of the study. BLV-FLK group rabbits were significantly lighter than the negative control group for several time points between 5 and 6 months post-inoculation. Plasmid rabbits were lighter than the negative control rabbits at 5.5 months post-inoculation ( $p < 0.05$ , one-way ANOVA).

## **Lymphocyte counts**

Total lymphocytes counts, as well as other red and white blood cell parameters, were all within normal reference ranges for all rabbits for the duration of the study (data not shown for hematology). Reported reference range for rabbit lymphocytes is  $1600-10600 \times 10^3/\text{ul}$ .<sup>23</sup> Parameters measured included total white blood cell count, lymphocytes, heterophils, eosinophils, monocytes, platelets, total red blood cells, red and white cell morphology, hematocrit, and hemoglobin. No abnormal cells were seen on manual differentials. Each individual rabbit had a range of lymphocyte counts that did not alter significantly during the course of the study. Mean absolute lymphocytes counts for each test group over the course of the study are illustrated in figs 3.1 and 3.2. The cyclosporine treated group (positive control group) had significantly lower counts of lymphocytes on average than the plasmid group at 2 months post-inoculation. Figure 3.2 illustrates the initial sharp decline in lymphocyte numbers of the CsA group following initiation of treatment.

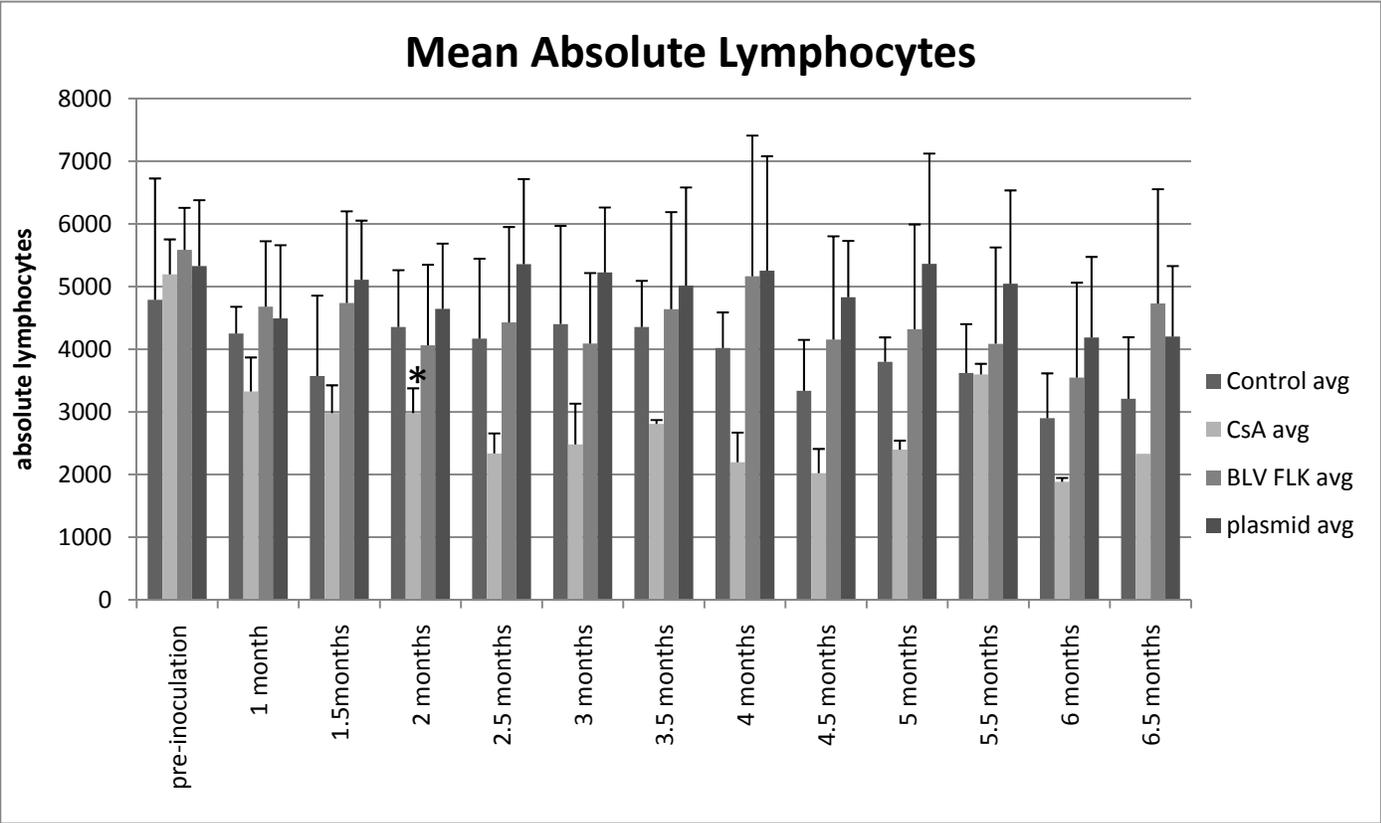


Fig 3.1: Total lymphocyte counts from rabbits that treated with CsA, BLV-FLK cells or pBLV913. \* CsA group was significantly lower than the plasmid group at 2 months post-inoculation (one-way ANOVA  $p < 0.05$ ).

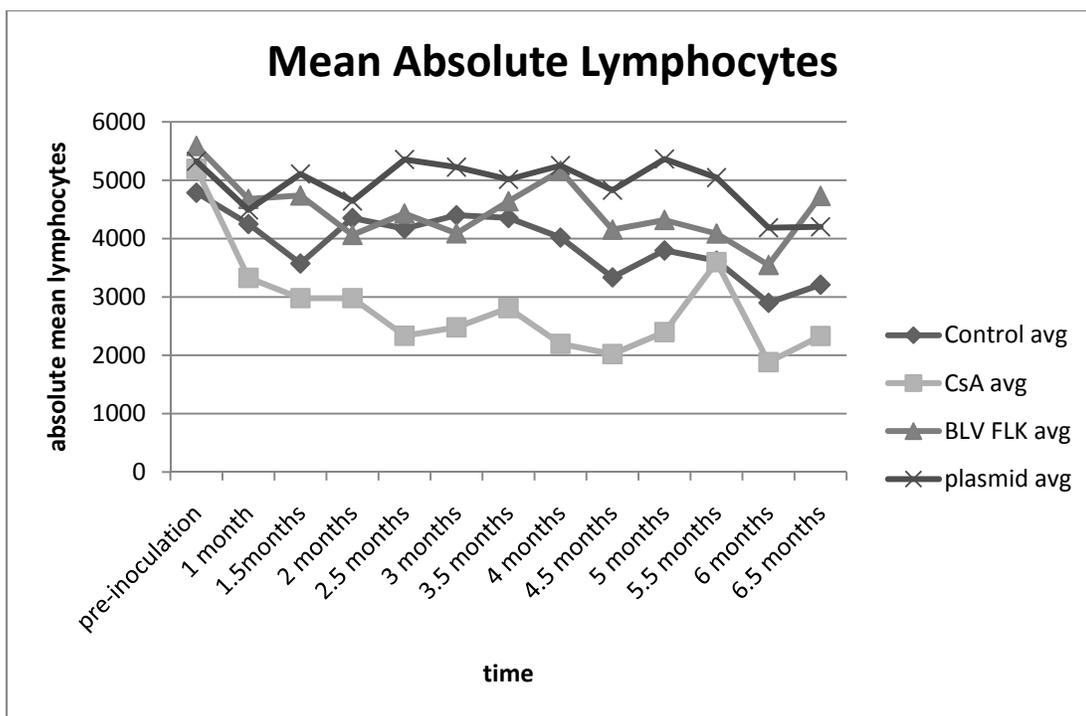


Fig 3.2: Average lymphocyte counts over time per test group illustrating the slight downward trend of the CsA group. CsA group was significantly lower than the plasmid group at 2 months post-inoculation (one-way ANOVA  $p < 0.05$ ).

### *Determination of infection*

### **RT-PCR**

RT-PCR was carried out as described in the Materials and Methods. Results varied and were overall inconclusive (table 1). The unreliable results may have been caused by low amounts of RNA extracted, contamination of samples with positive control RNA, or poor technique. All samples at time points 5.5 and 6 months post-inoculation were grossly

contaminated, with all samples including negative controls testing positive. Results of re-testing of those time points are in table 1 and are also inconclusive.

Pre-inoculation samples of RNA were not taken at the blood draw but will be available upon culture of original frozen lymphocytes. From this data it is concluded that RT-PCR was unreliable for proving infection status due to the high amount of variability and false positives most likely due to poor technique and lack of experience with this procedure.

RT-PCR	3 months	4 months	5 months	5.5 months	6 months	6.5 months	7 months
NC1	+	+	+	+	-	NT	NT
NC2	+	+	-	+	+	NT	NT
NC3	?	+	+	-	+	-	NT
PC1	?	-	-	-	-	NT	NT
PC2	-	n/a	n/a	n/a	n/a	n/a	n/a
PC3	-	+	+	+	-	+	NT
F1	+	+	+	-	-	NT	NT
F2	-	-	+	-	-	NT	NT
F3	-	-	+	+	+	-	+
F4	+	+	?	+	-	-	NT
F5	-	+	n/a	n/a	n/a	n/a	n/a
F6	-	+	+	-	+	+	+
P1	+	+	+	+	NT	-	-
P2	+	+	+	+	NT	-	NT
P3	+	-	+	+	NT	+	+
P4	-	+	+	+	NT	-	NT
P5	-	+	+	+	NT	NT	NT
P6	-	-	+	+	NT	-	NT

Table 1. RT-PCR results over time post-inoculation; NT: not-tested; n/a: no sample available; ?: inconclusive results.

Additional PCR testing of Rabbit NC1 for presence of viral RNA was performed due to positive results on ELISA. Rabbit NC1's final PBL collection sample as well as isolated splenic and lymph node lymphocytes were cultured in CTL medium. DNA and RNA samples were both extracted and analyzed for presence of viral sequences. Splenic lymphocytes were positive by nested RT-PCR. The PCR product from splenic cells was sequenced by the Penn

State Sequencing Core Facility. The sequence has 99% maximum identity to BLV-FLK pBLV913 plasmid (Genbank accession EF600696) (Fig 4).

```
5'ACCTTTAAGCCATTCCGGCACTCTCCCCGGACCGCCAGACCTTACCGCTATCCCTACACACCTT
CCACATATCATTTGCCTGGATCTCAAAGATGCCTTCTTCCAGATTCCAGTCGAAGACCGCTTCCGC
CCCTACTTTGCTTTTACCCTCCCTACCCCCGGGGGACTCCAACCTCATAGACGCTTTGCCTGGCGG
GTCCTACCTCAAGGCTTCATTAACAGCCCAGCTCTTTTCGAACGAGCACTACAGGAACCTCTTCGT
CAAGTATCAGCCGCCTTTTCCCAGTCTCTTCTGGTGTCTATATGGACGATATCCTTATCGCTTCGC
CTACAGAAGAACAACGGTCACAATGTTATCAAGCCCTGGCTGCCCGCCTCCGGGACCTAGGGTTT
CAGGTGGCATCCGAAAAGACTCGCCAGACGCCTTCGCCCCGTCCCCTTCTTGGGACAAATGGTCCAT
GAGCAGATTGCCACCTACCAGTCCCTACCTACCTTGCAGATCTCATCCCCAATTTCTCTTCACCAAT
TACAGGCGGTCTTAGGGACCTCCAATGAACCGCTGTATGGTGAAGAGAAATGGGGATGAGATCGC
AGGTAAGTGGGAGGAAGAGGAATTGCTCATGGACATTTGTCCAGAAGGGACGGGAAGGTCGGCG
AGTCTTTGGAGCACTGAAACCTAGGTCCGGAGCGGGAGCCGGGCTTGATACTTGTGACGTTGTCTT
CTGTAGGGAGGATAGGATTGCCTATGGAACCAAAGAACGGGAAAAGCGGTGAATTTGACAAAGT
CCGAATGCCTTCAAAAAAAAAAGGGTTTTATGAACCTGAGTAGACGCCGGCAAGCTTAAGTTGGATC
CCGGGGAGGGAGGGTAAAAAATAGGGGGAACAGTTTATGAAATGAAAAGGCTTTATAATCGGGA
TATATGTGCACGGGTGAAGACACGCCACTTGGCCCCGGGGGAACGCGAACGCTCTTGAAAATTTG
TCTGA 3'
```

Figure 4: PCR products isolated from rabbit NC1's splenic lymphocytes. The sequence has 99% max identity to BLV-FLK plasmid pBLV913.

## **ELISA:**

All rabbit pre-inoculation serum samples tested negative for GP51 by ELISA (Fig 5.1). Starting one month post-inoculation, 2 out of 6 of the BLV-FLK cell inoculated rabbits tested positive (Fig 5.2). By 6 months, all six BLV-FLK cell inoculated rabbits were positive by ELISA (Figs 5.2-5.6). No plasmid inoculated rabbits tested positive at any time during the length of the study. Additionally, at 6 months post-inoculation one negative control rabbit (NC1) tested positive on ELISA, however this sample was repeated and found to be negative.

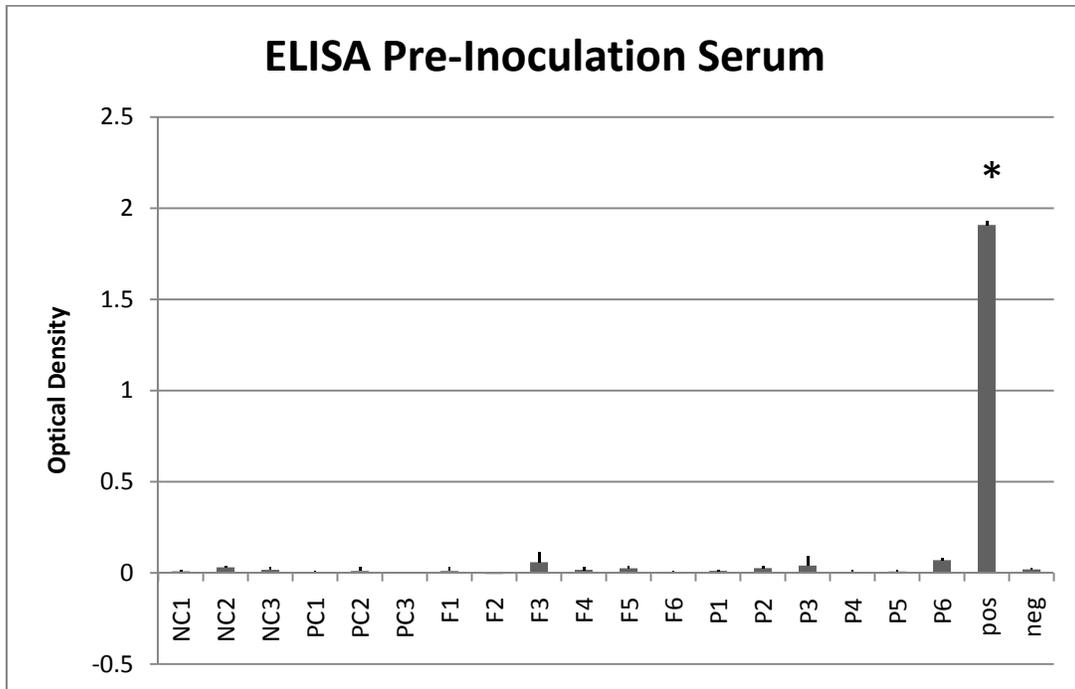


Fig 5.1: Pre-inoculation serum ELISA optical density. All test samples are negative.

\*Positive control is positive (student's t-test  $p < 0.05$ ).

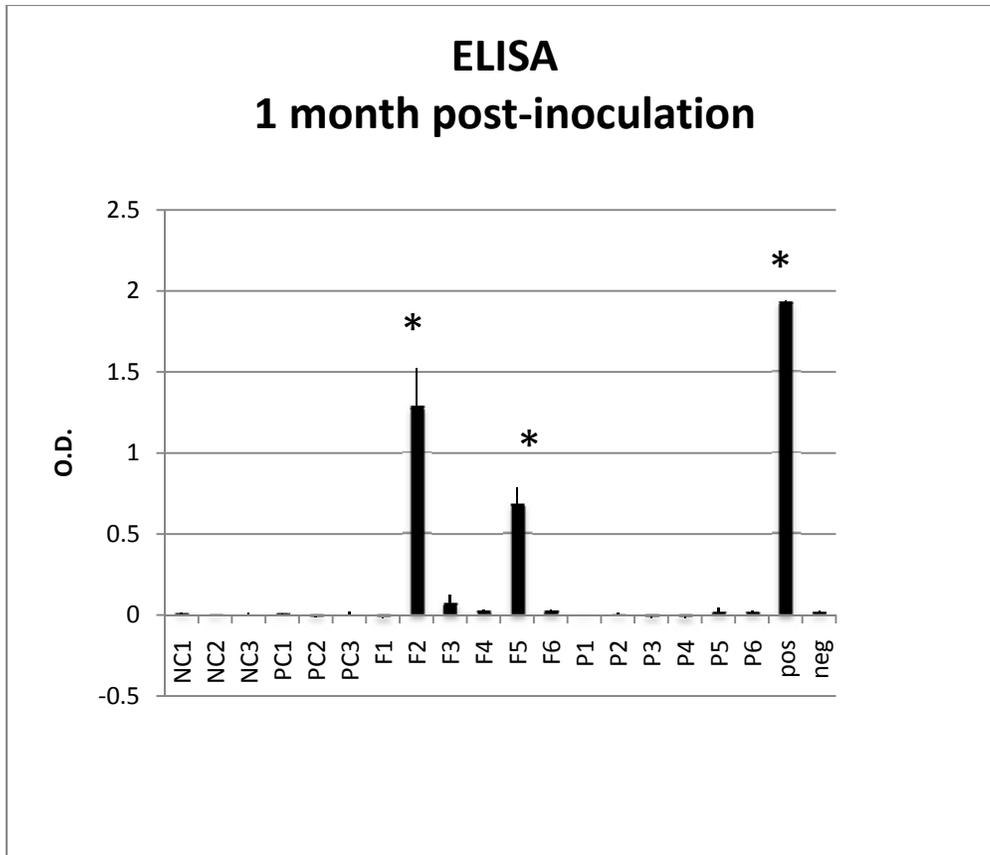


Fig 5.2: ELISA results 1 month post-inoculation (student's t-test  $P < 0.006$ )

\*FLK-BLV inoculated rabbits F2 and F5 test positive.

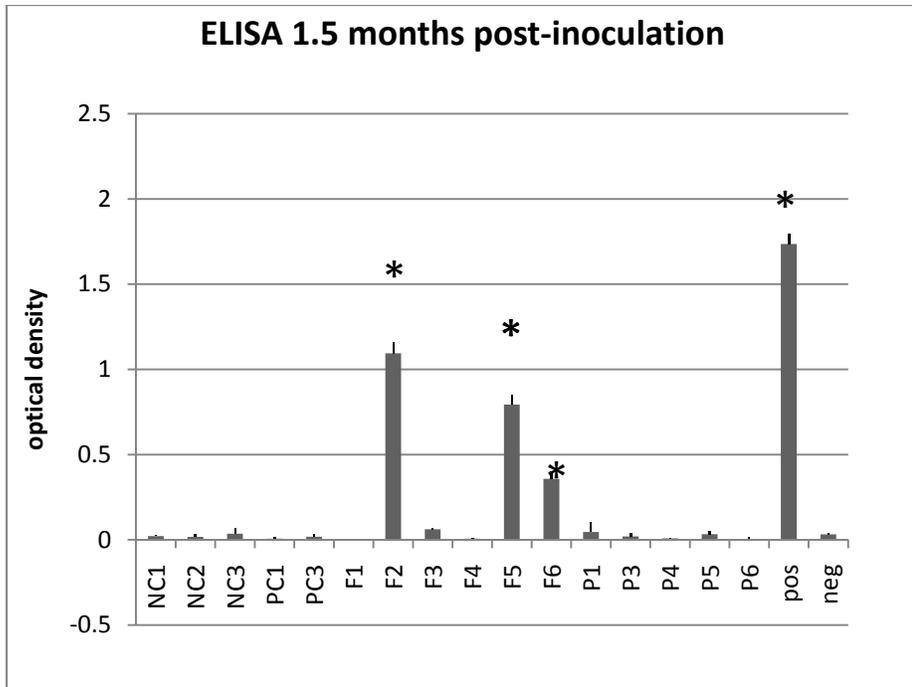


Fig 5.3: 1.5 months post-inoculation serum ELISA (student's t-test  $P < 0.05$ )

\*BLV-FLK inoculated rabbits F2, F5, and F6 test positive.

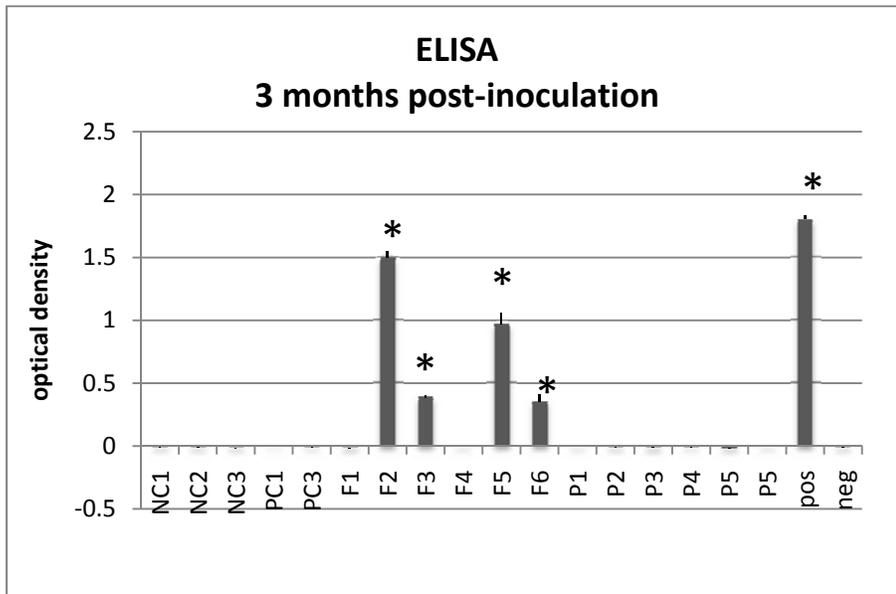


Fig 5.4: 3 months post-inoculation ELISA. \*BLV-FLK rabbits F2, F3, F5, and F6 are positive (student's t-test  $p < 0.05$ )

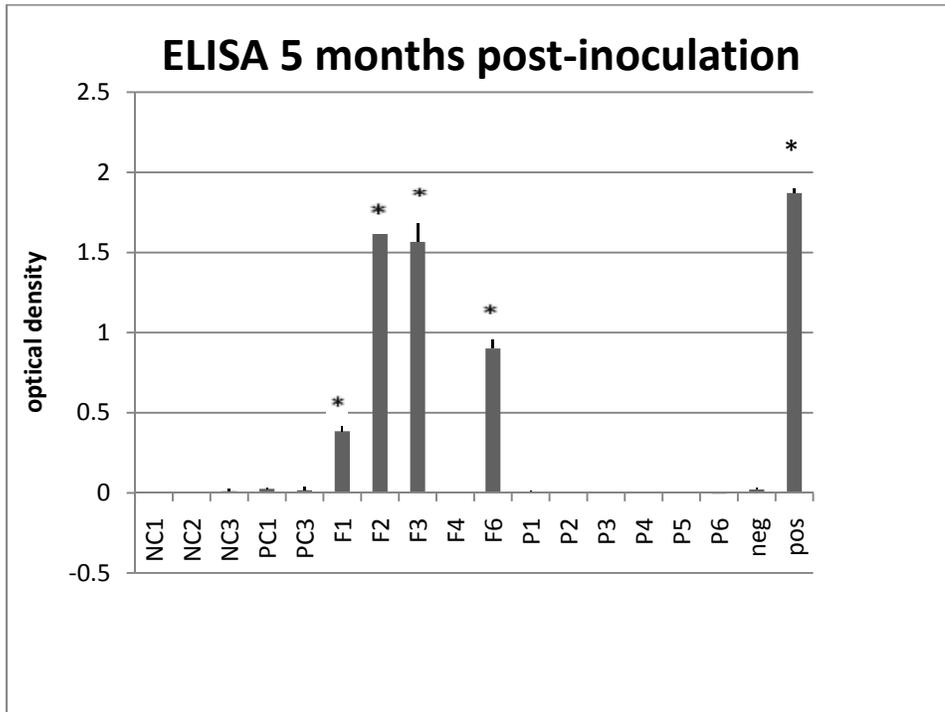


Fig 5.5 5 months post-inoculation. \*BLV- FLK rabbits F1, F2, F3, and F6 test positive. Positive control is positive (student's t-test  $p < 0.05$ ; F5 died and was not-tested)

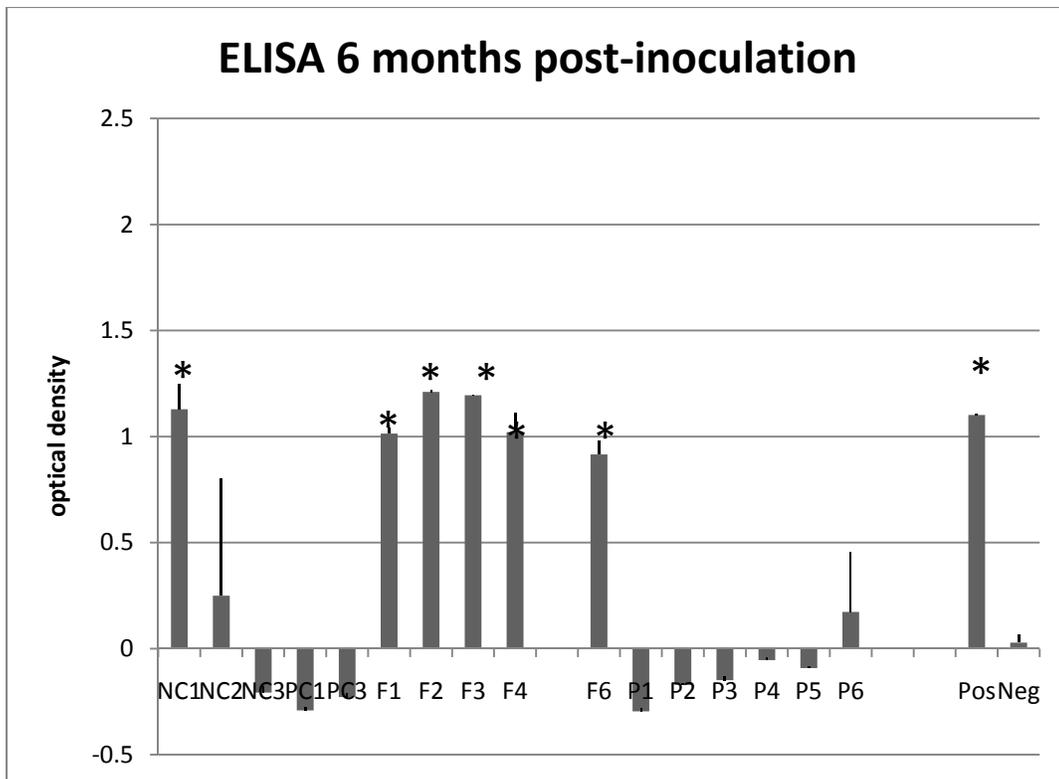


Fig 5.6. Last samples from each rabbit. 6-7 months post-inoculation

\* NC1 (negative control), BLV-FLK #s F1, F2, F3, F4, and F6 are all positive. F5 had died and was not tested. Sample NC1 was repeated and found to be negative (student's t-test  $p < 0.05$ ).

## **CD4:CD8 lymphocyte ratios**

The CD4:CD8 ratios did not stay within the reference range (2.8+/-0.8) for the duration of the study when measured using fresh PBLs (fig 6), however there were no significant differences between the negative control group and either BLV test group at any time point.<sup>24</sup> There are several time points in which the standard deviation is very great within test groups, such as the pre-test time point. This is thought to be due to human error or an uneven aliquotting of cells, the presence of erythrocytes in the sample, or inadvertently gating non-lymphocytes during flow-cytometric analysis. The ratios at different time points varied; however, there were no significant differences between the BLV experimental groups (either plasmid inoculated or BLV-FLK cell inoculated) and the negative controls. At two time points the CsA group was statistically higher than the negative control group and both BLV test groups (one-way ANOVA). This data is in agreement with previously published data on HTLV-1 infection in rabbits.<sup>25</sup> Cows with persistent lymphocytosis as a result of BLV have been found to have decreased CD4 and CD8 lymphocytes as when compared to control animals.<sup>11</sup>

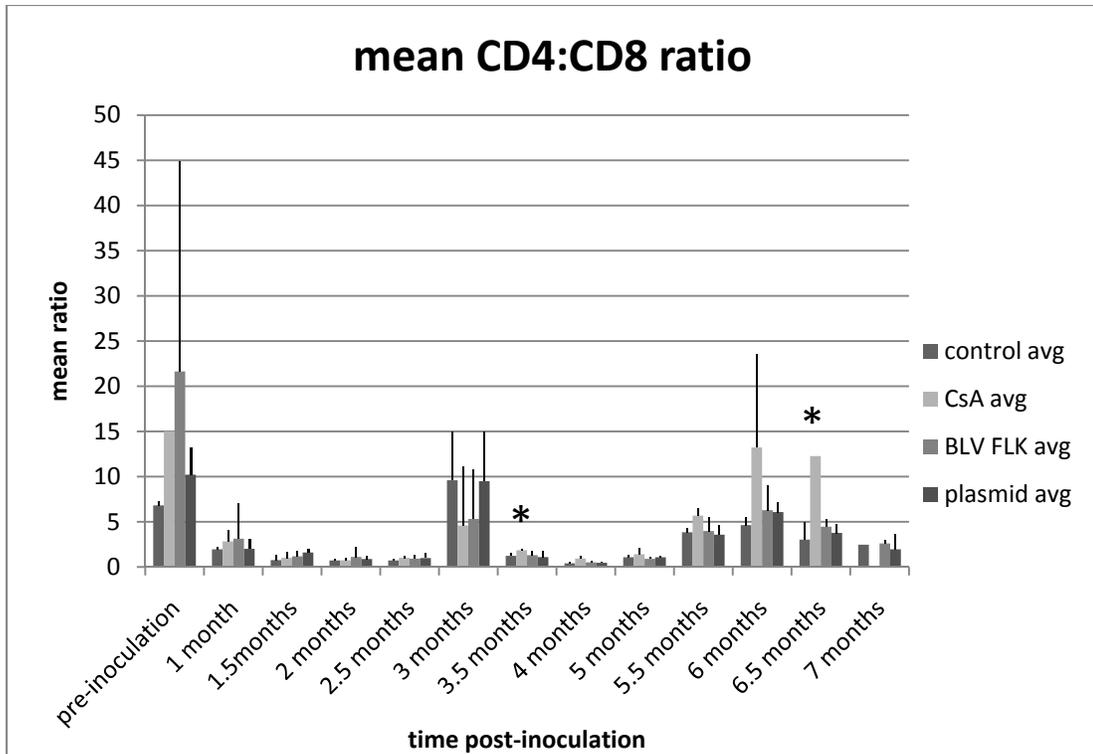


Fig 6: CD4:CD8 ratio of treatment groups over time. There were no significant differences between groups. \*CsA (positive control) was significantly greater than the other three test groups 4 months and 6.5 months post-inoculation. ( $p < 0.05$ , one-way ANOVA)

### MHCII:

The percentage of the leukocyte population that stained positively for MHC class II did not change significantly during the course of the study for most samples and time points (fig 7). MHCII stains both B lymphocytes, monocytes, and basophils. The CsA treated group had two time points in which the relative MHCII+ population was significantly lower. The final time point showed that the plasmid group had significantly higher MHCII expression than the negative control (one-way ANOVA). The number of animals present in the plasmid group at the last sampling was reduced to only two rabbits and there were no CsA rabbits still alive at that time point.

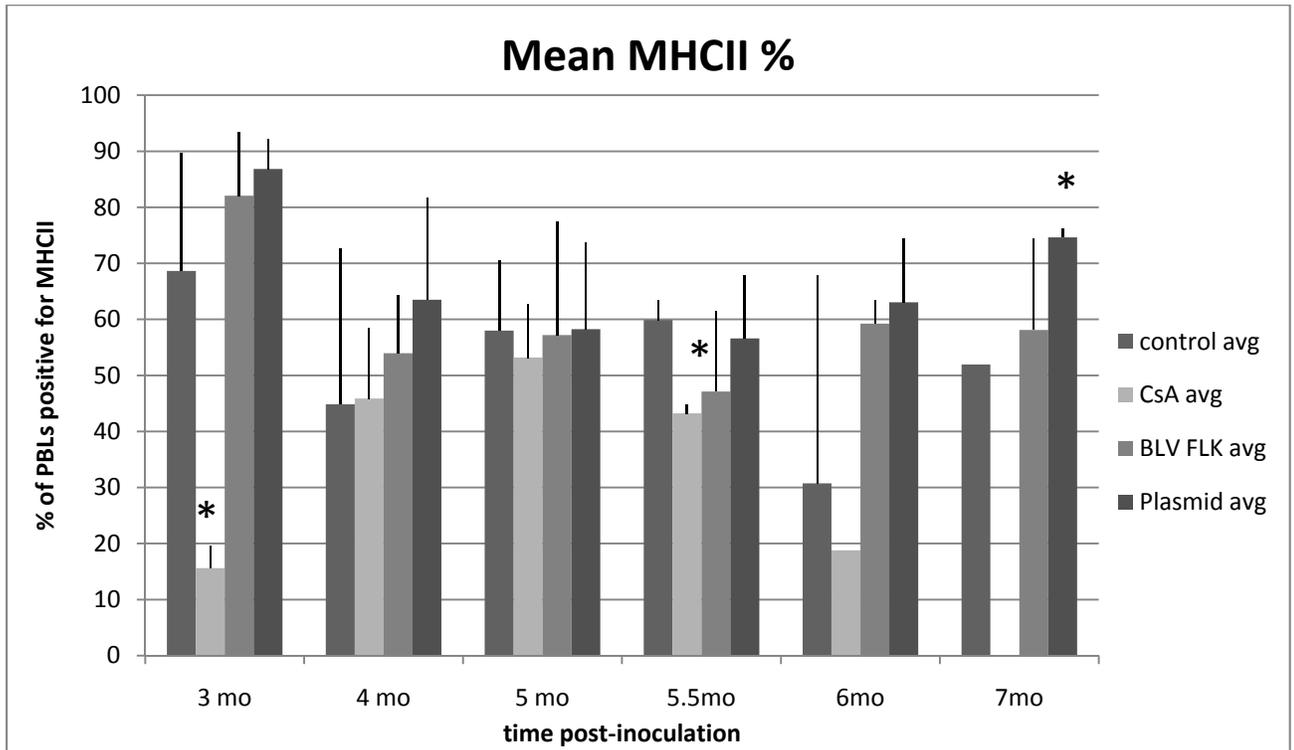


Fig 7: Mean %MHCII + PBLs per test group over time post-inoculation. CsA group was statistically lower than the negative control group at 3 months and 5.5 months post-inoculation. The plasmid group was statistically higher than the negative control group at 7 months post-inoculation (One way ANOVA,  $p < 0.05$ ).

## Pathology- Necropsy results

A total of 9 diagnostic necropsies were performed. All rabbits on study, including those not necropsied, tested negative for fecal parasites and ova and tested negative for growth of *Bordetella bronchiseptica* and *Pasteurella multocida* by aerobic culture of the nasal conchae at necropsy or at time of euthanasia. Two rabbits were necropsied before the end of the study: one, NC2, was euthanized due to a fractured leg, and another rabbit, F5, died under anesthesia prior to routine phlebotomy. Overall there were no significant changes in the erythroid to myeloid ratios of bone marrow or the percentages of cells in the bone marrow. The spleens, mesenteric lymph nodes, and thymuses of all rabbits were similar with no findings consistent with leukemia or lymphoma development and there were no findings associated with immunosuppression other than occasional non-specific enteritis. One cyclosporine treated rabbit (PC2) had hypocellular lymphoid tissue consistent with immunosuppression from cyclosporine administration. This rabbit also had bronchopneumonia which could be due to an opportunistic infection from resulting immunosuppression. Rabbits F3 and F5 (BLV-FLK inoculated) had splenic marginal zone hyperplasia (Figs 9.1, 9.2). The marginal zone of the spleen contains B lymphocytes. Splenic marginal zone hyperplasia is a rare but nonspecific finding and has been associated with the development of B-cell lymphoma.<sup>26</sup>

### Necropsy Summary

Rabbit	Age/sex	Cause of death	Final diagnosis (es)
NC2	10 mo/F	Euthanized- end of study	Liver, hepatitis, lymphohistiocytic, multifocal and peri-portal, subacute to chronic, mild to moderate
PC2	3 mo/M	Euthanized- fractured hind leg	1. Tibia, left, simple spiral fracture of the mid-proximal diaphysis with proximal-caudal displacement and severe hemorrhage.

			<p>2. Lung, pneumonia, granulomatous, multifocal, subacute, mild to moderate (Fig 6).</p> <p>3. Haired skin, dermatitis, ulcerative, focal, severe, acute, with suppurative furunculosis and folliculitis.</p> <p>4. Liver, coagulative necrosis, focally extensive, acute, moderate (infarct), with heterophilic inflammation</p>
F3	10 mo/M	Euthanized- End of study	<p>1. Cecum and jejunum, enterotyphlitis, plasmacytic, multifocal, subacute, mild</p> <p>2. Spleen, marginal zone hyperplasia, multifocal, mild</p>
F4	10 mo/F	Euthanized- End of study	No significant gross or histological findings were seen.
F5	5 mo/F	Found dead after administration of anesthesia	<p>1. Lung, interstitial pneumonia, lymphohistiocytic, multifocal, subacute, mild</p> <p>2. Liver, cholangitis, lymphohistiocytic, multifocal, subacute, mild</p> <p>3. Spleen, marginal zone hyperplasia, multifocal, mild</p>
F6	10 mo/F	Euthanized- End of study	No significant gross or histologic lesions were present.
P1	10 mo/M	Euthanized- End of study	No significant gross or histologic lesions were present.
P3	10 mo/M	Euthanized- End of study	No significant gross or histologic findings.
P6	10 mo/F	Euthanized- End of study	1. Liver, hepatitis, periportal and random, eosinophilic and lymphohistiocytic, multifocal, subacute, mild-moderate, with

			biliary hyperplasia 2. Jejunum and cecum, enterotyphlitis, plasmacytic and eosinophilic, multifocal, subacute, mild
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Rabbit NC2 was euthanized at the end of the study. She was apparently healthy for the duration of the study. There was mild to moderate periportal hepatitis which showed no organisms on special stains. Differential causes of hepatitis in a rabbit include *Eimeria stiedae*, *Enterocytozoon bieneusi*, and *Cryptosporidium parvum*. Charles River tests for *C. parvum* and *Eimeria stiedae*. The SPF rabbits are tested free of these pathogen, however there is no testing done for *E. bieneusi*. It is possible that she became contaminated with a pathogen upon housing at central. Other differentials include hepatitis E virus.<sup>27</sup> A Steiner-Chapman stain of the liver is negative for organisms, including *Helicobacter spp.*

Rabbit PC2 was euthanized due to an acute fracture of the tibia. Incidental findings of pneumonia and hypocellular lymphoid tissues are consistent with experimental immunosuppression brought on by chronic dosing with cyclosporine A (fig 8). An etiologic cause of interstitial pneumonia was not found despite the use of special stains including GMS, Wright-Giemsa, and Ziehl-Neelsen acid-fast, as well as a negative nasal conchal aerobic culture. This does not exclude the possibility of organisms present that were not visualized by the stains. Differential diagnoses include *Pneumocystis oryctolagi*, *Bordetella bronchiseptica*, and *Pasteurella multocida*. The dermal lesion was the result of injection with ketamine/xylazine and was being treated prior to euthanasia. Infarction in the liver could be related to extension of the pneumonia, but can also be observed as an incidental finding in laboratory animals.

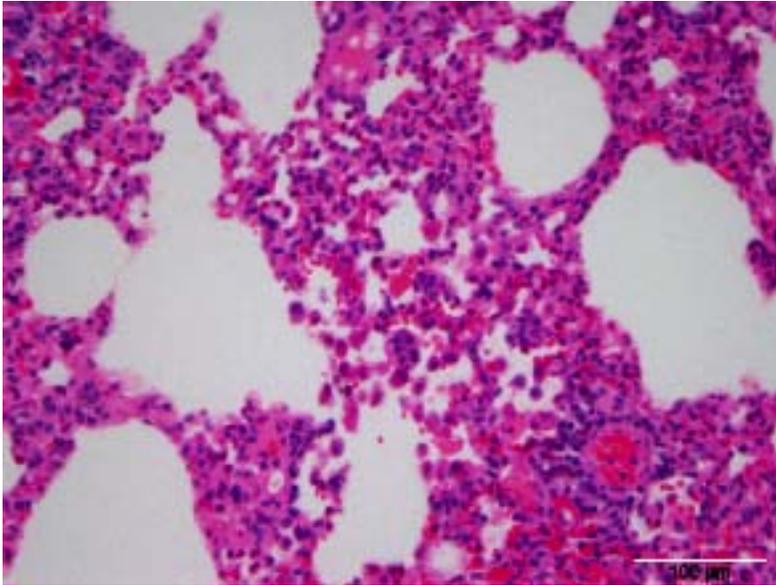


Fig 8: Rabbit NC2, lung, H&E: Granulomatous pneumonia with epithelioid macrophages and giant cells

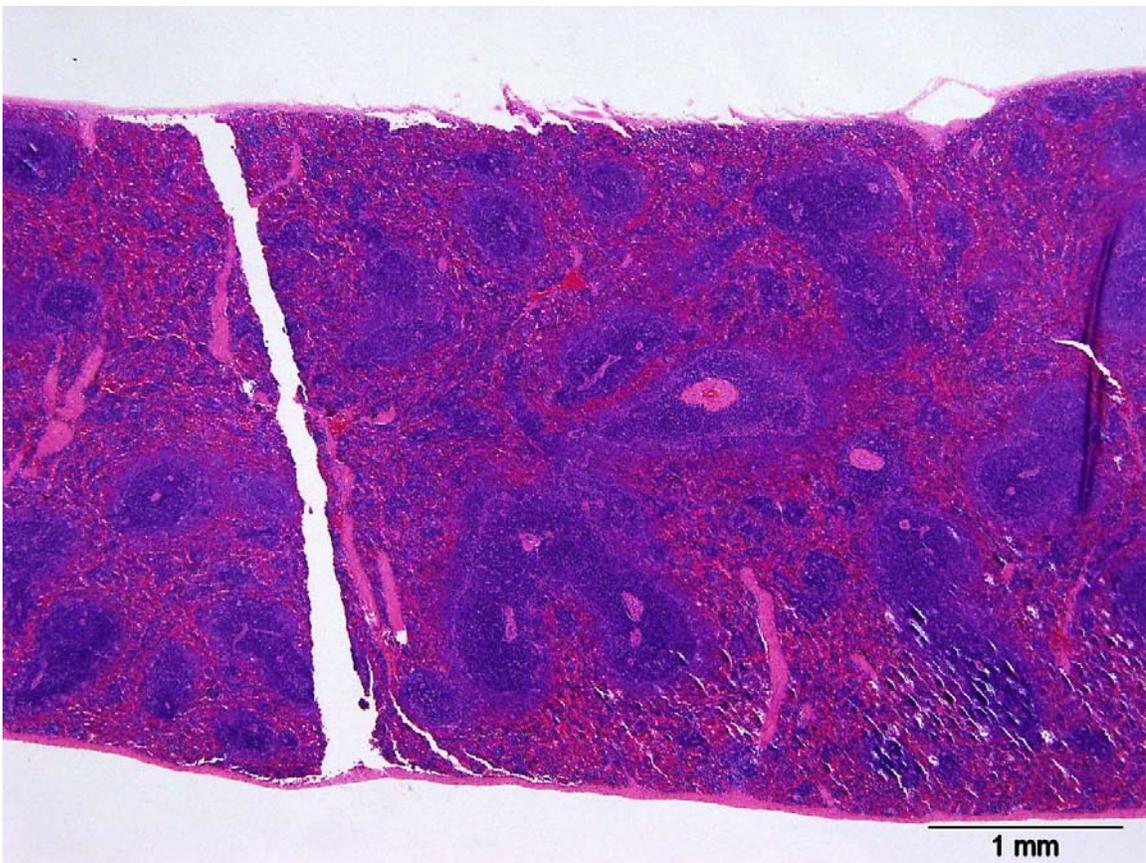


Fig 9.1: Rabbit F3. Mild multifocal splenic marginal zone hyperplasia (H&E).

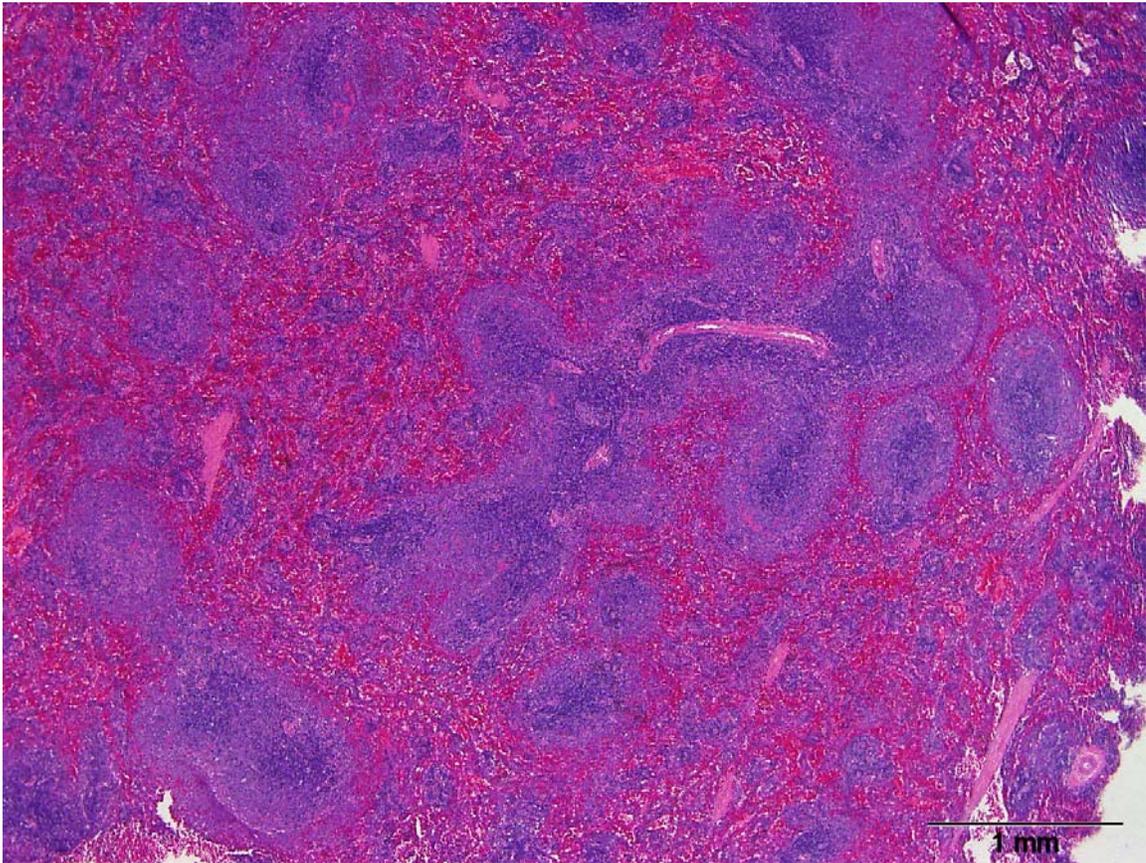


Fig 9.2: Rabbit F5. Mild multifocal splenic marginal zone hyperplasia (H&E).

Rabbit F3 had moderate plasmacytic enterotyphlitis. No organisms were seen histologically. Causes of plasmacytic enterocolitis include *Lawsonia intracellularis* (proliferative enteropathy), *Cryptosporidium parvum*, *Eimeria spp.*, rotavirus, and coronavirus. Contamination with a pathogen could have occurred while being housed at CAQ. Additionally, there was mild splenic marginal zone hyperplasia (fig 9.1).

Rabbit F5 was found dead shortly following injection with 25mg/kg ketamine and 2.5mg/kg Xylazine IM. The mild interstitial pneumonia is most likely due to subclinical infection with *Pneumocystis oryctolagi* or *Bordetella bronchiseptica*. Marginal zone

hyperplasia of the spleen is of questionable significance (Fig9.2). It has been shown that marginal zone lymphoma, a B-cell lymphoma, arises from previous findings consistent with marginal zone hyperplasia.<sup>26</sup>

Rabbit P6 had mild to moderate periportal hepatitis which showed no organisms on special stains. Differential causes of hepatitis in a rabbit include *Eimeria stiedae*, *Enterocytozoon bieneusi*, and *Cryptosporidium parvum*. Charles River tests for *C. parvum* and *Eimeria stiedae*. The SPF rabbits are tested free of these pathogen, however there is no testing done for *E. bieneusi*. It is possible that she became contaminated with a pathogen upon housing at central. No organisms were seen. Other differentials include hepatitis E virus.<sup>27</sup> Multifocal plasmacytic and eosinophilic enterotyphlitis was present histologically. No organisms were observed. Causes of plasmacytic enterocolitis include *Lawsonia intracellularis* (proliferative enteropathy), *Cryptosporidium parvum*, *Eimeria spp.*, rotavirus, and coronavirus.<sup>28 29</sup> Contamination to a pathogen could have occurred while on study.

## Chapter 4 Discussion

A rabbit model of viral-induced immunosuppression was proposed to resemble human papillomavirus infection under the condition of immunosuppression induced by retroviral infection such as human immunodeficiency virus. The rabbit /papillomavirus model has been a surrogate model for HPV infection in human and played a pivotal role in the successful development of vaccines for clinical use. In order to study the confounding effects of HIV-1 induced immunosuppression on patients with HPV, co-infection of rabbits with CRPV and bovine leukemia virus (BLV) has been proposed. Bovine leukemia virus has been previously shown to infect rabbits and result in an immunosuppressed state over the course of an extended incubation time, up to 24 months. This is similar to HIV-1 induced AIDS in humans. This project's aim was to evaluate the effects of BLV infection on the immune system, specifically the CD4 and CD8 lymphocytes as well as MHCII expressing cells (B lymphocytes, monocytes, and basophils), and develop parameters to measure BLV induced immunosuppression in domestic rabbits.

Several retroviruses such as HIV-1 and even BIV-1 have been tested in domestic rabbits for immunosuppression effect and failed to produce clinical signs of disease over time.<sup>7,8,30</sup> Rabbits inoculated with these viruses produce antibodies and it is possible to recover viral RNA from peripheral blood lymphocytes. Because rabbits do not show clinical disease, these viral agents are not ideal for studying immunosuppression. Additionally, there are significant biosafety concerns for working with HIV infected rabbits.

Because there is no naturally occurring immunosuppressive viral disease of rabbits, alternatives were sought to re-create this disease state. Rabbits infected with bovine leukemia virus were shown to develop an AIDS like syndrome followed by secondary opportunistic infections several months post-infection. No rabbits have developed lymphocytosis, leukemia, or lymphoma. Previous studies have not reported peripheral lymphocytes subtype ratios. At this point it is still unclear as to which lymphocyte population BLV infects in rabbits. This will be an important area of future study in this model.

The results of this experiment were consistent with previous experiments in the length of time until infection was detected. Other than decreased weight gain there were no obvious clinical signs of disease in the inoculated rabbit experimental groups. Due to the strict biosafety precautions and usage of personal protective equipment, this study may have a decreased incidence of secondary opportunistic infections. Furthermore, rabbits used in this study were purpose-bred and free of many rabbit pathogens commonly seen in conventionally housed colonies. The most common clinical sign of disease in previous studies was respiratory infection. All rabbits on this study tested negative for growth of *Bordetella bronchiseptica* and *Pasteurella multocida* by aerobic culture of the nasal conchae at necropsy and were presumed negative upon delivery.

The most significant finding in this study was the unexplained reduced weight gain shown in the BLV-FLK inoculated group of rabbits compared to that of the normal and plasmid inoculated groups. Decreased appetite or fecal production was not noted for any of the BLV-FLK inoculated rabbits, however daily food consumption and fecal output was not measured. All three cyclosporine treated rabbits and one plasmid- inoculated rabbit (P5) were frequently reported as having decreased appetites, slower weight gain, and decreased grooming for the majority of the study. It is unclear if the virus progression consumes energy in itself or if it causes a state of nausea or decreased appetite by another mechanism. There were no specific pathological findings on necropsy to suggest any reason for decreased appetite. Future studies may employ the use of metabolic cages for rabbits, or at least daily weights on food and volumes of water to quantitatively determine if there is decreased intake or increased expenditure.

The role of the injection of FLK cells (without virus) was not examined. In retrospect, a second control group using injection of non-BLV producing FLK cells should have been used. It is possible that the presence of the FLK cells contributed to decreased weight gain through unknown mechanisms, possibly causing a systemic inflammatory foreign body response or causing immune system activation.

The ratio of CD4:CD8 lymphocytes remained stable for the duration of the study when test groups are compared. Unlike HIV-1 infection, there was no major loss or destruction of CD4 T lymphocytes. Other research has also concluded that BLV does not alter the CD4:CD8 ratio in cattle.<sup>31</sup> However, these values have never been reported in the rabbit model. Infection of rabbits with a related virus, HTLV-1, also did not alter the CD4:CD8 lymphocyte ratio.<sup>25</sup> Destruction of at least 50% of CD4 lymphocytes is a hallmark of HIV-1 pathogenicity in humans and leads to the devastating immunosuppressed states of its hosts.<sup>32</sup> While this rabbit model does not mimic that depression of CD4 counts it may still be useful for studying immunosuppression in rabbits, however the exact mechanism remains to be elucidated.

The mechanism of immunosuppression in cattle has not been fully elucidated; however there is evidence that it is multifactorial taking into consideration host genetic profile as well as the ability of the BLV oncogene *tax* to disrupt tumor suppressor genes of infected host cells. Different haplotypes of the bovine MHC DRB3 alleles are associated with either resistance or susceptibility to persistent lymphocytosis from BLV. In cattle, infected lymphocytes accumulate mutations, especially of p53. Chromosomal alterations occur in tumor cells which may or may not be required for the progression to full malignancy. Peripheral blood B lymphocytes have reduced rates of apoptosis following infection with BLV, which contributes to a lymphocytosis. *Tax* is an oncogene causing cell proliferation as well as transformation of B lymphocytes leading to decreased ability to respond to antigenic stimulation.<sup>13</sup> Additionally, it has been shown that BLV infected cows with persistent lymphocytosis (PL) have decreased serological response to immunization against J5 Escherichia coli bacterin, and also that BLV PL cows have decreased circulating IL-2, IL-12, and interferon gamma, whereas BLV positive cows had increased amounts of IL-12 and interferon gamma.<sup>11,12</sup> Cows infected with BLV have significantly lower CD4 and CD8 counts.<sup>10,11,14</sup> It has also been shown that PL cows have decreased neutrophils than controls.<sup>11</sup>

MHCII expressing PBL populations remained relatively constant throughout the duration of the study. There were variations between time points but there were no

statistically significant changes between test groups. Onuma *et al.* reported that 30-40% of rabbit lymphocytes are B cells, similar to the results observed in this study.<sup>2</sup> While MHCII is not ideal for measuring B lymphocytes specifically, it was the only anti-rabbit antibody available at the start of the study. MHCII is also expressed on the surface of monocytes and basophils. Monocytes are also included in the peripheral blood lymphocyte population due to the inability of the Lympholyte® solution to separate these two groups. While every effort was made on flow cytometry to select lymphocytes only, there is some degree of overlap between these two cell populations. Ideally antiCD79 alpha would have been used as a more specific B cell marker. An anti-rabbit B cell marker (Antigenix) was found at the conclusion of the study and tested on cultured lymphocytes. Cultured lymphocytes did not react positively to the stain, however when tested on fresh lymphocytes the percentage of stained cells was similar to that of those that stained positive with anti-MHCII.

Lymphocyte responsiveness using the mitogen concanavalin-A to measure T cell function of harvested purified lymphocytes *in vitro* was undertaken but yielded inconsistent results. Additional testing is warranted, including testing the humoral immune system such as through the production of antibody following challenge with a novel antigen *in vivo*. The negative results obtained in this study are most likely an artifact of error than true results. In follow-up studies taken at later time points it was shown that BLV-FLK inoculated rabbits had valid statistically significant decreases in the response to the mitogen as compared to negative control and plasmid inoculated rabbits, however these rabbits were all co-infected with CRPV at that time. It is unknown whether the additional CRPV infection increased the pathogenicity of BLV from BLV-FLK cell inoculation, or if the longer duration of BLV infection has lead to decreased the decreased immune response.

Ten rabbits total had complete diagnostic necropsies performed. Three of these rabbits were not planned necropsies. One of these rabbits, PC3 (CsA treated), was necropsied after a following study with co-inoculation with CRPV. The results of that necropsy, other than benign fibroadenomatous mammary hyperplasia induced by CsA administration, were not significant.<sup>33</sup> Another CsA rabbit was necropsied 4 weeks into the study due to a

fractured femur. This rabbit showed signs consistent with immune suppression including granulomatous pneumonia. One BLV-FLK rabbit, P5, died under anesthesia and was subsequently necropsied. This rabbit had minimal marginal zone hyperplasia of the spleen. An additional BLV-FLK inoculated rabbit, F3, also had minimal marginal zone hyperplasia. No other rabbits necropsied had this finding. Marginal zone hyperplasia is a nonspecific finding and is rarely reportedly in rabbits. The marginal zone is composed of B lymphocytes. Whether this hyperplasia was in fact due to BLV stimulation is not known. Immunohistochemistry or *in-situ* hybridization on frozen tissue sections could be used to determine if BLV infection played a role in the development of marginal zone hyperplasia.

Bovine leukemia virus induces immunosuppression in rabbits after a prolonged incubation period. Although viral antigen was detected as early as 4 weeks post-inoculation, clinical signs of disease, consisting of decreased weight gain, were not detected until 4.5 months after inoculation with BLV-FLK cells. There were no hematologic parameters significantly affected by inoculation with BLV in either FLK or plasmid delivery. The ratios of CD4:CD8 lymphocytes were not affected. The level of MHCII expressing cells was also not affected; however the pre-test measurement of MHCII was not reliable due to the lymphocytes being cultured with IL-2 and supernatant from Con-A stimulated uninfected rabbit splenic cells.

There were differences found between the inoculation groups. Whole DNA inoculation using plasmid pBLV913 proved to be unreliable in the time frame examined. The plasmid inoculated rabbits did not show statistically significant changes in body weight from the negative control group until 6 months post inoculation. On the contrary, the BLV-FLK inoculated group showed statistically significant decrease in weight gain as compared to the negative control group starting at 4.5 months post-inoculation. Unlike the BLV-FLK group, the plasmid inoculated rabbits never sero-converted on ELISA. The BLV-FLK rabbits began to sero-convert starting one month post-inoculation. All of the BLV-FLK rabbits were testing positive by the conclusion of the study.

Future directions of classifying this model of immunosuppression include quantitative RT-PCR of longitudinal data sets as well as clarifying the presence of the virus in the peripheral blood lymphocytes since RT-PCR proved to be unreliable. The data gained from qPCR will clarify how quickly the virus began to replicate by either inoculation mode. An *in vitro* assay of infectivity, the syncytium formation assay, will address the question of how effective the different modes of inoculation are at inducing infectivity.

More specific evaluation of the humoral immune system through antibody production assays is also warranted. Evaluation of the humoral immune system can be accomplished by inoculating test rabbits with a novel antigen and measuring antibody production against that antigen. This test has been done with mouse serum protein <sup>2</sup>.

The use of BLV as a model of HIV-like immunosuppression in rabbits to study co-infection with CRPV will require further refinement. At this point, either infection with BLV alone or co-infection with HIV-1 and HTLV lead to decreased weight gain and an increase of opportunistic infections after long incubation times.<sup>30</sup> The main differences are that BLV does not alter the CD4:CD8 ratio as HIV does and also BLV does not affect lymphocyte counts.<sup>32</sup>

## **Chapter 5 Literature Review**

### **Bovine Leukemia Virus:**

Bovine leukemia virus is a type C lymphotropic retrovirus and is the etiological agent of enzootic bovine leukosis.<sup>9</sup> BLV causes B cell lymphocytosis and lymphoma in cattle. It can experimentally be infective to sheep<sup>15</sup>, goats, chimpanzees, and rabbits.<sup>2</sup> Bovine leukemia virus is a retrovirus related to HTLV-1 in humans, a virus that induces T cell leukemia. APHIS reported that the incidence of developing lymphoma in infected cattle was 1-5%.<sup>16</sup> BLV has been shown to cause increased incidence of weight loss and secondary infections in rabbits inoculated with the BLV plasmid pBLV913, infected fetal lamb kidney cell suspension, or infected cattle lymphocytes.

Transmission in cattle is blood borne requiring either an inoculation or spread of disease by a hematophagous vector such as a mosquito. The most common route of infection of cattle is repeated injections with a contaminated needle. Rectal palpation is also thought to be a mode of transmission. Feeding calves unpasteurized colostrum and milk may also lead to infection.

The disease in cattle has a high prevalence across the United States, in 1997 APHIS conducted a study through National Animal Health Monitoring System (NAHMS) which estimated 89% of US dairy operations had positive cattle, and 74.8% of those herds had at least a 25% seropositive prevalence using the agar gel immunodiffusion test (AGID). In 2007, APHIS conducted bulk tank milk ELISAs for antibodies to BLV and determined a herd prevalence of 83.9%.<sup>16</sup>

### **BLV genome:**

The genome is 8714 nucleotides long. Gag, pol, env, prt, tax, rex, RIII, and GIV comprise the eight open reading frames. Seven alternatively spliced RNAs have been found. On the extremities of the viral genome are long terminal repeat sequences (LTRs), similar to other retroviruses. These LTRs consist of U3, R, and U5, which repeat consecutively. There are transcriptional regulatory sequences within the U3 region and the 5' area contains

polyadenylation signals. The R region is where the viral mRNAs are initiated, starting at the first base; and the R 3' area contains the polyadenylation site.<sup>34,35</sup>

BLV is a lymphotropic retrovirus that possesses genes coding for “trans-activating” products, similar to HIV-1 and HTLV-2. All of these contain trans-regulatory protein that activates viral gene expression.<sup>9</sup> The tax and rex regions are essential for oncogenesis as they alter the host genome.

### **BLV Virion:**

BLV is a C-type retrovirus, making it an oncovirus. It has C-type particles seen with electron microscopy; these are crescent-shaped formations on the cell membrane host cells that are associated with viral budding of the C-type retroviruses. The BLV virion is approximately 90-110nm in diameter with a core of 60-80nm<sup>36</sup>. Negative staining of the BLV virus using uranyl acetate shows a diameter of 120-150nm with projections, which is similar to other murine and feline C-type particles. The core is round, ovoid, or polygonal, and electron dense. The core is surrounded by an electron lucent area, which is enclosed by a double-layered membrane.<sup>13,36</sup>

Visualizing BLV budding has been documented as difficult by previous investigators. The budding BLV particles have identical morphological appearance as budding murine C-type particles, from the murine leukemia virus. Both viruses have crescent shaped budding, which then becomes a more ring-like core with a translucent center.<sup>36</sup> The BLV virus is apparently very fragile and difficult to image with electron microscopy due to spontaneous disruptions during the negative staining procedure. They often were deformed in ultrathin sections compared to typical murine C-type particles.<sup>36</sup>

### **BLV as a model of human and animal disease:**

Rabbits have successfully been infected with bovine foamy virus (previously known as bovine syncytial virus), bovine immunodeficiency virus (BIV), bovine leukemia virus (BLV), human T cell leukemia virus, and human immunodeficiency virus (HIV).<sup>1</sup> All of these viruses are retroviruses. It was found that rabbits infected with BIV had leukocyte counts that were within the reference range for the species (5200-12500/mm<sup>3</sup>) and had no evidence of clinical disease at the time of euthanasia (maximum 114 days post-inoculation).<sup>1</sup>

Although BLV has been shown to experimentally infect several species including goats, sheep, chimpanzees, cattle, and rabbits; its oncogenic capabilities seem to be limited to that of sheep, with less oncogenesis observed in goats and cattle.<sup>2</sup> Persistent anti-BLV antibody has been found in dogs and cats following experimental inoculation, but no other signs of disease were noted.<sup>9</sup> It has been reported that rabbits infected with BLV show a fluctuation in BLV antibody titers followed by a rapid drop of the titer to zero.<sup>2,37</sup> Onuma *et al.* found that following subcutaneous inoculation of rabbits 2 or 4 months old with either peripheral blood lymphocytes from cattle with persistent lymphocytosis at a concentration of 2-20 x10<sup>4</sup> cells, or inoculation with BLV-FLK cells 2-20 x 10<sup>4</sup> cells, the earliest detection of BLV antibody was at 13 days post inoculation. The longest latency was 46 days post inoculation, by 6 weeks all rabbits had a positive titre against BLV. Not all rabbits tested positive throughout their lifetime, by 15.5 months post inoculation 4 out of 6 rabbits converted to testing negative. Only one of the rabbits tested antibody positive at 17 months post-inoculation. Alternatively, it took two months for rabbits to test positive for BLV using the syncytium assay. However, not all rabbits every tested positive for syncytium assay even though they all tested positive by other methods.<sup>2</sup>

Immunofluorescence testing (IF) was performed following short term culture of PBLs with con-A at 5 months post-inoculation but only 7/10 rabbits tested positive and less than 1% of cells were antigen positive. There were similar results at 9 months post inoculation but only 6 of the original 7 positive rabbits tested positive at that time.<sup>2</sup> Wyatt *et al.* were able to demonstrate the BLV genome in rabbits infected for up to 18 months. Within 6 months of infection with BLV, 1/3 of rabbits had decreased T cell responsiveness to phytolectin stimulation while 2/3 showed signs of clinical disease: conjunctivitis, rhinitis, severe weight loss, and death.<sup>9</sup>

BLV infected rabbit PBLs ( $4 \times 10^6$  cells/rabbit) were injected SQ into 1 year old rabbits. The rabbits seroconverted to antibody positive 2 weeks later and produced antibodies for at least three month. However, when 5-day old bunnies were inoculated with the same amount of cells none of them seroconverted.<sup>2</sup>

No significant differences in erythrocyte and lymphocyte counts were found in rabbits infected with BLV versus the controls for 10 months post inoculation. Five rabbits had signs of upper respiratory disease 2.5 months after BLV inoculation, two of which had pulmonary abscesses at necropsy.<sup>2</sup> At necropsy, 6 out of the 9 infected rabbits had pulmonary lesions and 1 uninfected rabbit had pulmonary lesions. One rabbit that died of pneumonia had alveolar hemorrhage and pulmonary thrombi, which cultured *Bordetella bronchiseptica*.<sup>2</sup> At 17 months post-inoculation, Onuma *et al.* found that the percentage of T cells of inoculated rabbits was similar to those of uninfected rabbits (50-70%); whereas the number of Ia positive cells slightly decreased to 5-15% in four rabbits and 20-30% in the remaining 2 rabbits versus the 30-40% for uninfected rabbits.<sup>2</sup> Therefore, there was a slight decreased in Ia expressing cells 17 months post inoculation with BLV. Since rabbits infected with BLV develop an immunodeficiency syndrome, as characterized by an increase in the rate of infection and significantly lower antibody titers to mouse serum following SQ challenge and suppressed mitogen responses.<sup>2</sup>

### **HTLV in humans:**

Human T-cell leukemia virus type 1 is an oncogenic, retrovirus that is endemic in areas of Japan, West Africa, and the Caribbean. Like HIV, it targets CD4+ cells. The virus only causes leukemia in 3-5% of the infected individuals and only after a long incubation time of approximately 40-60 years. Clinically, individuals present with skin lesions, generalized lymphadenopathy, hepatosplenomegaly, peripheral blood lymphocytosis, and hypercalcemia. Once the disease manifests clinically it is rapidly progressive and often fatal within months to a year. HTLV-1 is associated with a demyelinating syndrome known as “tropical spastic paraparesis.” HTLV-1 is spread through sexual contact, blood products, or breast-feeding. There is no oncogene within the viral genome. Similar to BLV, HTLV1 contains a coding region in its genome known as *TAX*. The *TAX* gene is essential for viral

reproduction. The TAX protein also activated host cell gene transcription involved in the proliferation and differentiation of T cells. TAX also inhibits ATM-mediated cell-cycle checkpoints and interferes with DNA repair functions; both of which lead to the formation of neoplastic T cells. The neoplastic cells then are able to replicate in the absence of IL-2.<sup>38</sup>

### **HIV infection in humans**

AIDS patients have an increased risk for several tumors including non-Hodgkins B-cell lymphoma, cervical cancer in women, and *Kaposi's sarcoma*. Primary central nervous system lymphoma is 1000-fold more common in AIDS patients than in uninfected individuals. Epstein-Barr virus is a known polyclonal mitogen for B cells and is thought to play a role in leukemia/lymphoma development in AIDS patients. Epstein-Barr viral genome is found in 50% of systemic B-cell lymphomas and almost all lymphomas involving the central nervous system. Women with AIDS are ten times more likely to have cervical dysplasia associated with human papillomavirus due to their immunosuppressed state.<sup>38</sup> Kaposi's sarcoma is caused by the interaction between HIV and human herpesvirus 8 (HHV-8).

### **Detection of BLV and its effects:**

There are several different methods used to detect BLV infection including: 1. immunodiffusion test using glycoprotein antigen described.<sup>2</sup> 2. ELISA using sucrose gradient purified BLV as antigen fixed with 2% glutaraldehyde.<sup>2</sup> 3. Immunofluorescence of peripheral lymphocytes stained with anti-BLV p24 monoclonal antibody.<sup>2,39</sup> 4. Humoral immune response against mouse serum protein. 5. Blastogenesis test.<sup>2,21</sup>

### **HIV-1 infection in rabbits**

To date, HIV-1 infection in rabbits has produced infection characterized by persistence of RT-PCR products in the brain and proviral PCR products found mainly in the spleen. The PBLs were a poor source of virus.<sup>6</sup> Antibodies to gag and env proteins by western blotting were also detected but no overt signs of disease were seen. In these experiments, A3.01 cells (a human T-lymphocyte line) were infected *in vitro* with HIV-1, (strain LAV) and monitored for reverse transcriptase activity and evidence of syncytia

formation. At the time of peak infection the HIV-1-infected A3.01 cells were injected intravenously into rabbits. Control animals were injected with uninfected A3.01 cells. The dose of infected cells ranged from  $1 \times 10^6$  cells to  $5 \times 10^7$  cells. These data have shown that the strongest antibody responses are against gp41, p55, and p24.<sup>40</sup> Reina *et al.* induced HIV-1 infection in SPF New Zealand White rabbits housed in BSL3 conditions using cell free supernatant from H9 cells persistently infected with HIV1, through IP route following prior induction of aseptic peritonitis.<sup>7</sup>

Rabbits have also been co-infected with HTLV-1 and HIV-1, which resulted in weight loss, diarrhea, and evidence of neurologic dysfunction in all co-infected rabbits.<sup>30</sup>

### **RELIK: rabbit endogenous lentivirus type K**

An endogenous lentivirus type K of lagomorphs, specifically the European rabbit *Oryctolagus cuniculus*, was recently discovered. RELIK is thought to be one of the oldest viruses found, its earliest estimated date of origins is between 7-11 million years ago via analysis of segmentally duplicated insertions. There are no functional copies of the virus and it is referred to as a “genomic fossil.” The sequence has been pieced together from numerous broken copies of archived sequences. This is the first endogenous retrovirus of rabbits and the first lentivirus of rabbits. Comparison of sequences between RELIK and BLV do not have significant similarity, thereby diminishing the possibility of false positives in RT-PCR of inoculated rabbits being due to endogenous RELIK sequences.<sup>41,42</sup>

### **Cyclosporine A:**

Cyclosporine A (CsA) is an immunosuppressant commonly used in organ transplant recipients to ward off graft rejection. Cyclosporine A induces immunosuppression of both B and T lymphocytes by inhibiting the phosphatase activity of calcineurin by binding to cyclophilin. Calcineurin inhibition results in blockage of nuclear factor of activated T cells (NF-AT). This block is caused by the inhibition of translocation of the cytosolic component of NF-AT, which results in failure of activation of gene transcription. The subsequent lack

of NF-AT leads to decreases in interleukin (IL) 4, IL2, and CD40 ligand. IL2 and IL4 stimulate T cell differentiation.<sup>43</sup> CD40 ligand plays a role in stimulating immunoglobulin class switching as well as activating antigen presenting cells (APCs) (NCBI- CD40). The dosing regime for CsA followed that of one previously reported to induce consistent immunosuppression in rabbits.<sup>20</sup>

Cyclosporin administration in rabbits has been shown to cause decreased food intake, decreased weight gain, and decreased levels of CD4 and CD8 cells in the peripheral blood.<sup>20</sup> Although CsA decreases overall levels of CD4 and CD8 cells, it does not affect the CD4:CD8 ratio in rabbits treated with either 10mg/kg or 20mg/kg CsA two to three times per week.<sup>20,25</sup> Host immunity was suppressed in both inbred and outbred rabbits as measured by mitogen response test to concanavalin-A following treatment with CsA.<sup>20</sup> Administration of CsA leads to increased pathogenicity of both human T cell leukemia virus -1(HTVL-1) through enhanced virus expression, and also cottontail rabbit papillomavirus (CRPV) through delayed papilloma regression.<sup>20,25</sup> Since it has been proven that cyclosporine affects both B and T cell lymphocyte function, and alters the course of two other viral infections, it was chosen as the treatment for the positive control group of rabbits.

### **Lymphoid Organ Development:**

As rabbits mature the CD4/CD8 ratio decreases. In the peripheral blood of the adult rabbit (*Oryctolagus cuniculi*), percentages of lymphocyte subsets are as follows: CD4: 29.1 +/-4.6; CD8: 10.9 +/-3.3; CD4+CD8+: 1.9+/-0.7; CD79 $\alpha$  (B cells): 42.5+/-4.7; and the CD4/CD8 ratio: 2.8+/-0.8.<sup>24</sup> Lymphocyte reference values for the adult rabbit from a peripheral blood sample are 30-85%; or 1.6 – 10.6 x10<sup>3</sup>/ul.<sup>23</sup>

Rabbits have been shown to express MHCII on granulocytes in addition to resting B cells, and monocytes in the peripheral blood. MHCII is also expressed on resting T cells in horses, mink, pigs, ferrets, and dogs. However, this has not been reported in rabbits.<sup>44</sup>

Detection of rabbit B and T cell subsets has been determined by Onuma using monoclonal antibodies L11/135 (pan T -T cell), and 2C4 (against Ia antigen) and found that

50-70% of normal rabbit lymphocytes reacted with T cell marker, whereas 30-40% reacted with B cell marker.

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