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ENRICHMENT FOR VP1 ANTIGEN SPECIFIC B-CELLS

A Major Qualifying Project Report

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ABSTRACT

Murine polyomavirus, a double stranded DNA virus that causes multiple kinds of tumors, was one of the first viruses ever sequenced, and has proven to be an invaluable model in virology, cancer research, molecular biology and immunology. The purpose of this MQP was to use FACS staining to isolate viral antigen (VP1 major capsid protein)-specific B-lymphocytes using a double stain for VP1 and B220. A major portion of the project focused on designing a treatment to overcome the non-specific binding of antibodies to cell surface sialic acid residues. The enrichment of antigen-specific B cells succeeded, and these cells can be analyzed for gene expression patterns to further understand these virus-specific cells of the humoral immunity.

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ACKNOWLEDGEMENTS

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BACKGROUND

Introduction

Mouse Polyomavirus (PyV) belongs to the Papovavirus family. It was first isolated by S. Stewart and is still a widely studied virus for many reasons. First, this virus produces a large number of tumor types in its natural host, neonatally infected mice. An understanding of this tumor induction in mice will be helpful for cancer research in humans. Secondly, PyV-infected mice provide a good model to study immune responses to virus infection. PyV does not cause disease in adult mice with a normal immune system, but replicates to higher titer and causes pathology in immuno-compromised mice. This outcome of infection is similar to infections of humans with BK and JC viruses which are human polyomaviruses.

Viral Characteristics

The mouse polyoma virus possesses a double stranded, circular and supercoiled DNA of genome size of approximately 5.3 kb. The genome is non-enveloped and has an icosahedral capsid with a diameter of 40 – 55 nm. The nucleocapisds are isometric and they have 72 pentamers of capsomer proteins per nucleocapsid (Büchen-Osmond, 2004). Most of the virus is composed of structural proteins. Nucleic acid (DNA) makes up 12% of a complete virion.

Host Factors

Several polyomaviruses exist, but each is specific for only a certain species. The murine polyomavirus is only capable of replication in mice, not in any similar rodents (rats, chinchillas, hamsters, etc). But there are different polyomaviruses able to infect other animals. However the speed of murine PyV's life cycle and high rate of infectivity (*in vitro* and *in vivo*), in mice versus

other PyV is why they are used as model organisms in experimental research (Harbel, 1963). These other PyV can transform rat, hamster and human cells *in vitro*. Conversely, DNA can be injected or transfected making replication of viral DNA unnecessary.

The most frequent tumors induced by polyomavirus are mixed tumors of the salivary glands, respiratory tract mucous glands, subcutaneous sarcomas, mammary tumors and thymic epitheliomas (Row, 1960). Most of the *in vivo* experimental work is done by inoculating the newborn pups with the virus, so they can express the tumor induced by the virus. Then any changes in their health is closely followed and examined. The ability of the polyoma virus to induce tumors depends on the age of the host at the time of inoculation. Adult animals develop PyV-induced pathology (tumors) only if they are immunocompromised.

Genome Characteristics

Since polyomavirus was one of the first viruses to be sequenced and studied, its gene arrangement and regulation is well understood. Inside the virions, the DNA is supercoiled and compact. Murine polyomavirus' genome is composed of a 5.3 kb closed circular doubled stranded DNA. Despite its small size, the genome is known to encode 6 genes (Brinkman et al., 2004). This large amount of information packaging is accomplished by overlapping genes, and reading from both strands. The majority of viruses read only from one strand (+ strand) (University of Leicester, 2005).

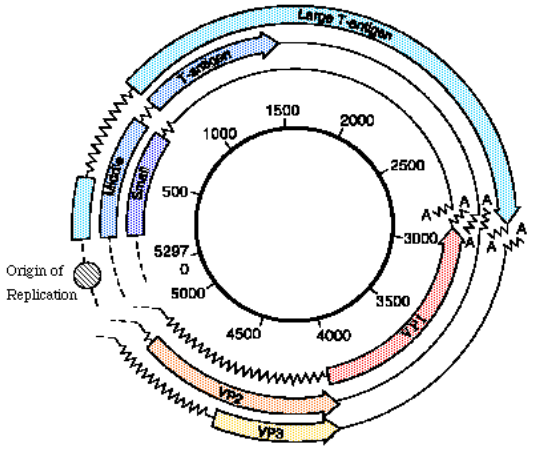

Figure 1. Polyomavirus Genome (University of Leicester, 2005).

The polyomavirus origin of replication (Fig.1 left middle) lies between non-coding areas responsible for transcriptional control. Three different T-antigens (large, middle, and small) are also encoded (on the opposite strand coding the structural proteins) (see table 1). PyV-infected mice with tumors express these T-antigen proteins in the tumor tissue. VP1 (major capsid protein) is encoded by a devoted open reading frame (no stop codons). This differs from VP3, which is contained within VP2 as an overlapping gene. VP1/2/3 encode viral structural proteins. Yet, if VP2 and VP3 are defective, viral capsids can still form from VP1 alone.

T-antigen:	**Large T**	**Small T**	**Middle T**
Size (amino acids):	785	195	421

Table 1. T-Antigen Lengths (University of Leicester, 2005).

VP1 is the only protein on the outside of the virion, and is responsible for attachment "to host cell membrane molecules containing terminal sialic acids" (Caruso et al., 2003). Almost

every murine cell contains surface sialic acids, which allows the virus to be efficient at binding. Four pockets on the VP1 pentamer are able to accommodate the sialic acids (see figure 2).

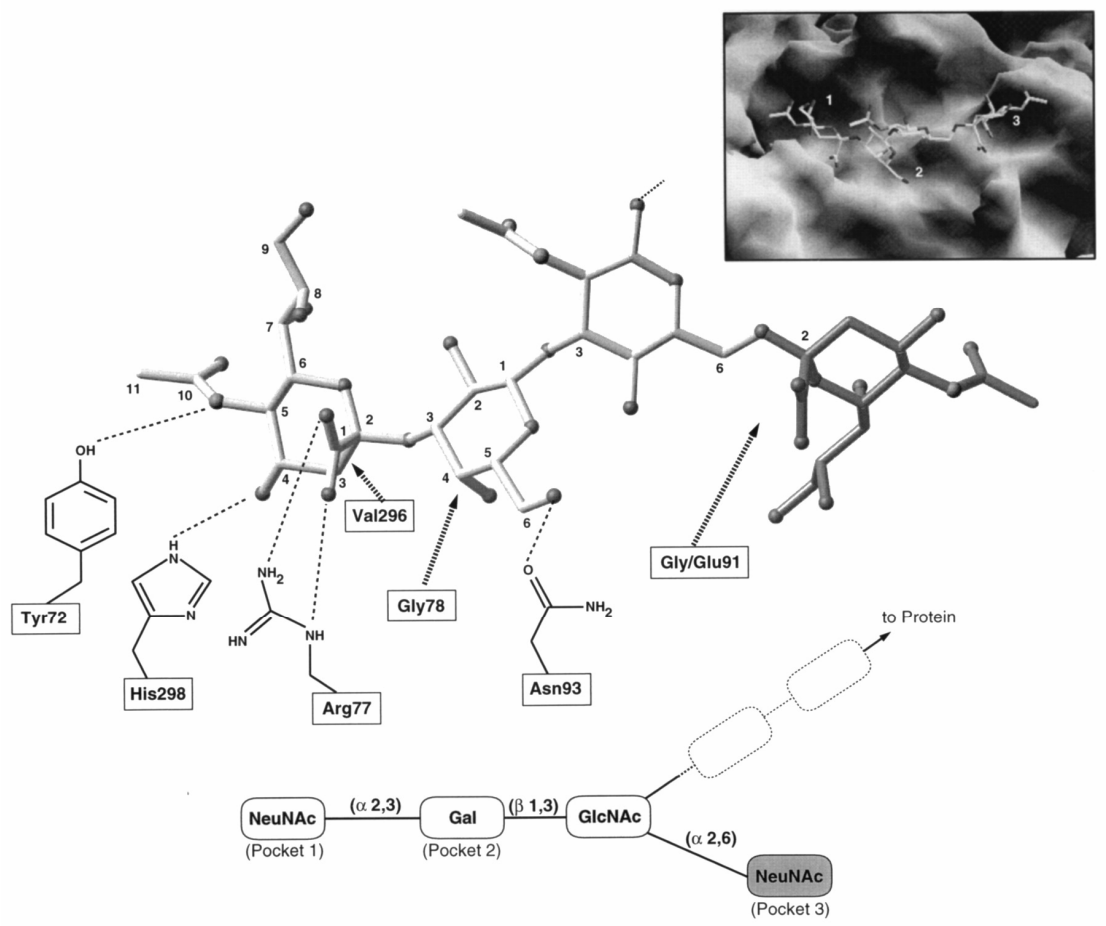

Figure 2. Interaction of VP1 with Sialic Acid Residues. Inset in the upper right quadrant shows VP1 bound to a sialic acid (α-2,6-linked sialic) residue. VP1 residues that interact via hydrophobic reactions with sialic acid receptor are shown as arrows, while hydrogen bonds with receptor are depicted as broken lines (Bauer et al., 1999).

Pocket 1 and 2 allow binding to the majority of sialic acids. Pocket 3 is able to bind alpha 2,6-linked sialic acids that would be part of a branched chain receptor. It is not known what pocket 4 is able to bind, but it may accommodate more complex oligosaccarides or proteins "in short O-linked glycans" (Stehle et al., 1994). A VP1 mutant will not be able attach correctly (see figure 3).

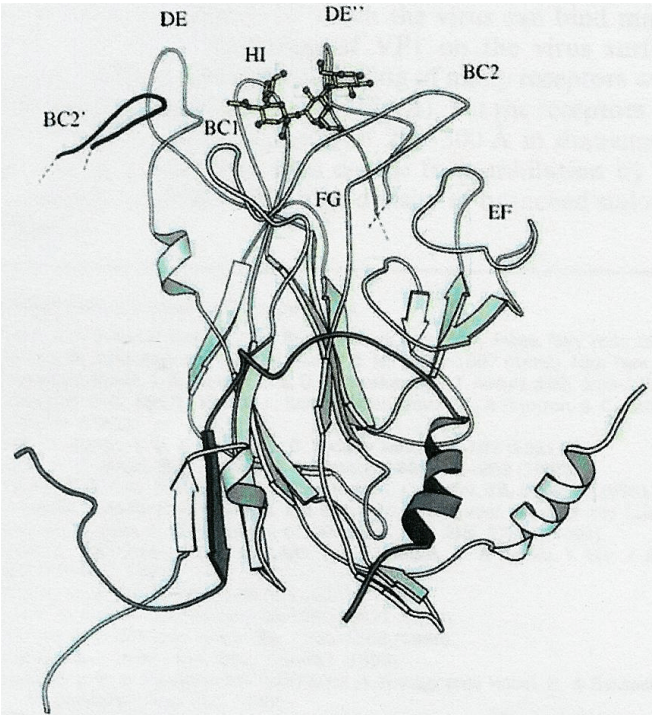

Figure 3. VP1 Monomer Bound to 3'Siayl lactose (receptor analogue) (Stehle et al., 1994).

Several loops in the VP1 monomer bind the sialic acid. "Residues 78 to 89 of the BC2 loop of the clockwise neighbor and residues 137 to 159 DE loop" of VP1 are crucial in allowing binding to sialic acids" (Stehle et al., 1994). A mutation in either VP1 Asn 93, Arg77 or His298 will destroy attachment ability and infectivity (see figure 4).

VP2 and VP3 are myristylated, and are thought to initiate endocytosis. The particles are guided to the nucleus by cytoskeleton-endocytic valcuole interactions (Leicester, 2005). Viral particles then enter the cell nucleus through nuclear pores, after which uncoating takes place (due to change in environment, it occurs spontaneously). If the virus contains VP2/VP3 mutations, the virus cannot uncoat and undergo replication (since VP2 and VP3 are involved in the uncoating).

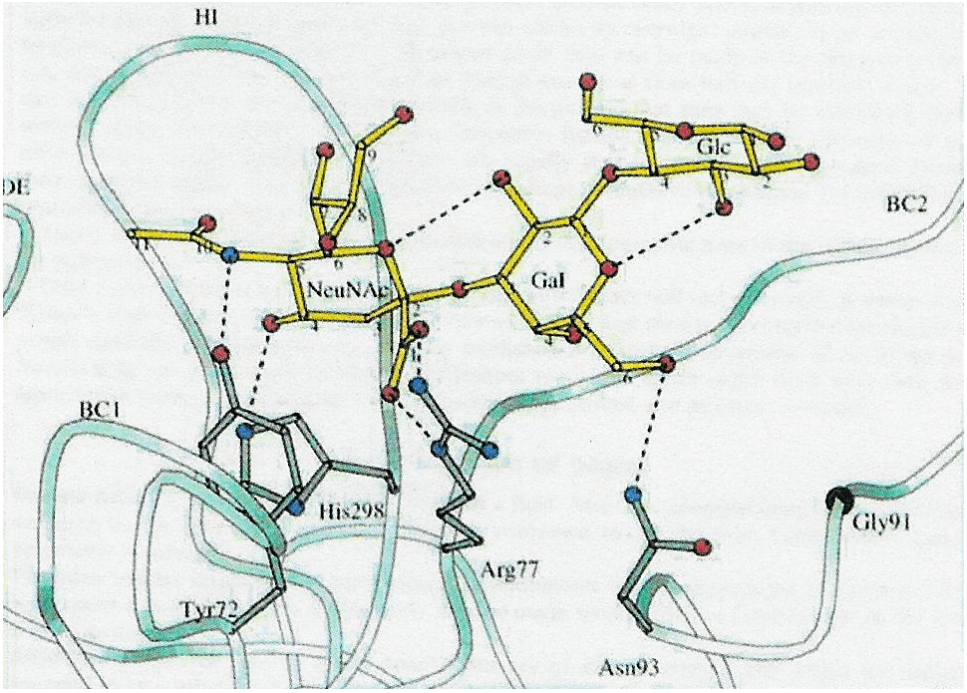

Figure 4. Binding of VP1 to 3-Sialyl Lactose (Stehle et al., 1994).

Once inside the nucleus, viral DNA associates with histones H2A, H2B, H3 and H4 of the host cell. This complex is called a "viral mini-chromosome" (Baek, 2005). The viral genes are expressed in a temporally coordinated fashion. Early genes are expressed first (before DNA replication) and proceed until late infection. The early proteins are non-structural (regulatory), and they initiate replication. As soon as viral DNA replication begins, a switch occurs to the late genes. These late genes are responsible for structural proteins (VP1, VP2, and VP3). Lastly, there is a regulatory region, composed of enhancers, origin of replication and transcription promoters. The entire polyoma life cycle is shown in Figure 5.

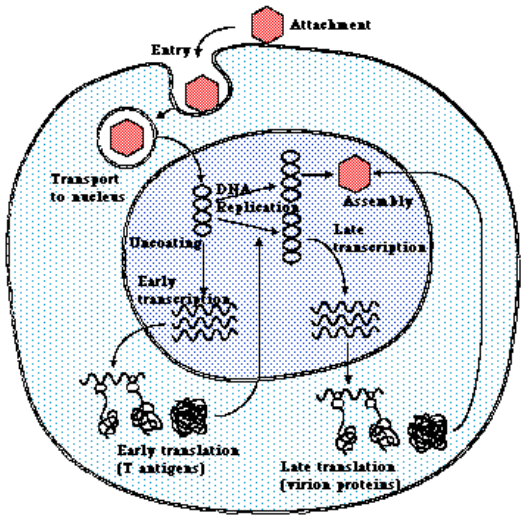

Figure 5. Polyomavirus Life Cycle (University of Leicester, 2005).

Transcription of the viral mini-chromosome to produce early mRNAs is completed by RNA polymerase present in the host cell. Around the origin of replication there are cis-acting regulatory signals, which guide transcription and T-antigens (trans-regulatory proteins). The T-antigens in turn direct further transcription and translation. Utilizing alternative splicing, 3 different early mRNA/T-antigens are produced (large, middle and small). The large-T antigen autoregulates transcription in the early portion of the promoter by binding it (see figure 6) (University of Leicester, 2005).

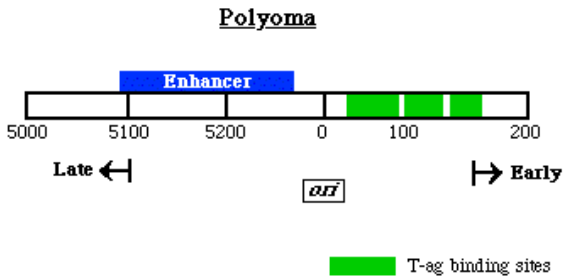

Figure 6. Polyomavirus Late and Early Regions (University of Leicester, 2005).

Also contained within the promoter are enhancer components, allowing activity (T-antigen transcription) in recently infected cells. Even though small T-antigen is not needed for viral replication, it enhances transcription of the late promoter (by binding to cellular proteins). When the large T-antigen concentration rises, it binds the origin of replication in the viral genome. DNA replication begins, and the late promoter becomes active. This causes early gene transcription to halt and switch to the infection's late phase. The T antigens enhance replication of viral DNA replication (binds the enhancer), and also transactivate late gene promoters. "After the onset of DNA replication, the balance between early and late gene expression is quickly reversed in favor of late products; by late in infection, late messages exceed the early ones by a factor of 20-80" (Liu and Carmichael, 1993).

The mechanism of early-late switch is not completely understood, but it is believed the large TAg (T-antigen) binds to a region opposite the viral origin of replication, and initiates viral replication. Experimental data has suggested that the "large TAg also transactivates the late promoter and represses transcriptional activity of early genes" (Liu and Carmichael, 1993). After replication, copies of DNA with the late promoter are then available for binding the transactivator. Cells that do not have viral DNA replication will not undergo a switch to late gene products. If a mutant virus has a deletion in the ori, TAg binding sequences, or frame-shift

mutations in the early region, the late gene product transition does not occur. Middle T-antigen may also be involved by altering host signal transduction pathways, which then modifies transcription factors (Liu and Carmichael, 1993).

DNA replication occurs due to a rise in the expression of Large T-antigen. It will bind to "DNA polymerase alpha & s/s DNA binding protein, both involved in DNA replication p53 & p105" (University of Leicester, 2005). Mouse polyomavirus relies completely on host cell machinery for its replication and assembly (since it has a limited genome size, it can not encode much). Viral genome replication occurs as T-antigen and DNA polymerase alpha interact directly. Tumor suppressor proteins bind to T-antigen, causing them to be inactivated. Cells previously stuck in G1 then are able to enter S phase (synthesis), again furthering DNA replication. T-antigen produces an environment conducive to viral replication, but also allows cells to replicate when they normally would not. By doing this, the virus transforms some cells into tumorgenic ones.

The frequency at which transformation occurs is a relatively low 1 per 10^5 cells in various cultured somatic mouse cells. The middle T-antigen also associates with "phosphatase 2A (PP2A), Src family tyrosine kinases, phosphatidylinositol 3-kinase (PI-3K) and an adaptor protein, Shc. They are necessary for cellular transformation" (University of Leicester, 2005). Interestingly, a single point mutation in position 250 (tyrosine to serine) prevents binding to Shc. Such mutant infected mice have different tumor morphology (University of Leicester, 2005).

Viral Assembly and Release

Polyomavirus proteins are first synthesized in the cytoplasm (on ribsomes), but need to get to the nucleus for virion assembly. These VP1, VP2 and VP3 structural proteins contain

nuclear localization signals which direct their aggregation in the nucleus. Chaperon proteins (hsc70 family) recognize these signals, bind the capsid proteins and escort them to the nucleus (Garcea et al., 2003). The capsid proteins self assemble in the nucleus (this has also been demonstrated *in vitro*) (Schmidt et al., 2000). If insect cells are infected with baculovirus expressing VP1, VP1 capsosomers are able to self assemble (Garcea et al., 1995). During polyomavirus infection host cell histones aid in packing the viral mini-chromosome in the newly formed capsids (by binding the capsids, and the assembly occurs spontaneously). A few of the newly formed virus particles are transported to the cell surface via cytoplasmic vacuoles (see figure 7 for structure). Most of the viral particles are released when the cell lyses. The virus interferes with normal cell growth by inhibiting apoptotic signals, which would normally lead to cell death (typical of most cancerous cells). Such interference is suspected to effect cellular metabolism and membrane synthesis, causing cell membrane weakening (the exact mechanism has not been determined). A weakened membran allows virons to escape easily during the release phase. The entire MPyV infection and replication (resulting in lysis) only takes 24-48 hours *in vivo*. Such an infection is also dependant on multiplicity of infection and which cell type is infected (University of Leicester, 2005).

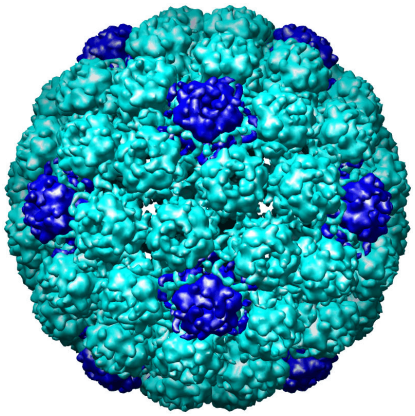

Figure 7. Complete Mouse Polyomavirus Virion (University of California, 2004).
The Outer shell contains 360 copies of VP1 (in pentamers).

Overall, despite being one of the simplest and smallest viruses, polyoma continues to prove its usefulness as a viral model. As recombinant DNA technology continues to advance, more and more details are elucidated from murine polyomavirus. Just like human polyomavirus (BK and JC), it produces an infection that is persistent and often silent in immuno-competent hosts. Thus, information gleaned from murine polyomavirus is relevant to human immunology as well.

PROJECT PURPOSE

As discussed in the Background, Murine polyomavirus (MPyV) uses alpha 2-3 sialic acid residues present on the surface of many types of mammalian cells as receptors. But, the virus also engages antigen-specific B cell receptors on the surface of B-cells, eliciting an immune response. The purpose of this project was to isolate B-cells containing B-cell receptors (surface antibody), specific for the virus. If such cells can be isolated, they can be used to analyze changes in gene expression profiles that occur when B lymphocytes respond to viral antigens. Fluorescence activated cell sorting (FACS) was chosen as the isolation technique, using a double immunostain for VP1 (the main polyomavirus capsid protein) and B220 (CD45R) (a surface marker specific for B-cells). A substantial portion of the project focused on alleviating high levels of non-specific binding of VP1 to sialic acid residues present on the surface of B-cells.

MATERIALS AND METHODS

General Lab Reagents	
Source / Catalog Id (if applicable)	Description
GIBCO, cat # 11875-093	0.1 micron filtered RPMI w/ L-glutamine
Vector, cat # SA-5004,	Horse Radish Peroxidase Streptavidin, 1 mg/mL
1.75 µL 2-merecaptethanol, 50 mL FCS, 5mL L-glutamine (lab aliquoted)	Reagents added to RPMI
Fetal Calf Serum (lab aliquoted)	Added to several reagents
Ammonium Chloride (lab aliquoted)	For red blood cell lycing
Polyomavirus stock (lab aliquoted)	Used in several experiments
GFP-VP1 lab stock	Provided by Dr. Donald Tipper, at UMMS

Table 2. General Lab Reagents

FACS, Cell Sort and ELISPOT Reagents	
Source / Catalog Id (if applicable)	Description
FACS Buffer	FACS Buffer (1x PBS, 2mL 0.5 mM EDTA, 5mL FCS)
BD Pharmigen, cat # 553142, lot 41921	Fc Block:, Purified anti-mouse CD15/CD132, 0.5 mg/mL
BD Pharmigen, cat #553092, lot 41617	B220-APC: APC anti-mouse CD45R/B220 (RA 3-6B2), 0.1 mg/mL
BD Pharmigen, cat # 5527655, lot # 45976	CD19-APC: APC-Cy7 labeled anti-mouse CD19 (1D3), 0.1 mg/mL
UNLB, cat# 1010-01, lot F5004-T435	Anti-Ig: Goat-anti mouse Ig (H+L), Human, Absorbing, 1 mg/mL
Zymed, cat # 61-0140, lot 404874312	Biotin-Rabit-Anti-Mouse IgG1, 1 mg/mL (detecting antibody)
Vector, cat # BA-2020, lot Q0810.	Biotin-Anti-Mouse-IgM, mµ chain specific, affinity purified, made in goat, 1 mg/mL (detecting antibody)
Sigma, cat # N2876-100 UN, lot# 083K8630	Neuraminidase – from clostridium perfringes, (lyophilized powder, resuspened at 50 U/mL in PBS for stock solution)
Sigma, cat # D158550-1L, lot # 09708PC	N,N-Dimethylformamide 99%
Sigma, cat # A-6926, lot # 123K8204	3-Amino-9-Ethylcarbazole 20mg

Table 3. FACS, Cell Sort and ELISPOT Reagents

ELISA Specific Lab Reagents	
Source / Catalog Id (if applicable)	Description
VP1 (lab aliquoted)	Used for coating (1mg /mL)
Irrelevant Rat B-cell Hybridoma	M5/114.152 (IgG2b) hybridoma
VP1 specific Rat B-cell Hybridoma (IgG2b)	provided by Aaron Lukacher's lab at Emory University
Whey Dilution Buffer (lab aliquoted)	For dilution of antibodies/samples
Jackson Immuno Research Laboratories Inch, code 112-095-068, Lot # 55638.	1.5 mg/mL, of FITC-conjugated, Affinipure, Goat, anti-rat IgG + IgM (H+L)
BD Pharmigen, cat # 553653, lot # 0000080777.	PE anti-mouse CD4 (L3T4) (H129.19) 0.2 mg/mL
Zymed, lot # 10602944.	Biotin-Mouse-Anti-Rat kappa It chain, 1 mg/mL
Vector, cat # BA-4000, lot # E0727	Biotinylated, Anti-Rat (H+L) Affinity purified, Made in Rabbit, 1.5 mg/mL

Table 4. ELISA Specific Lab Reagents

General Lab ELISA Assay

ELISAs were performed to quantitate the levels of VP1-specific antibodies (VP1 coating), or to determine general serum levels of IgG2a, IgG2b, and IgM . VP1 coating of a 96-

well microtiter dish was performed by adding 3.5 µl of a 1 mg/ml stock of VP1 protein in 20 mM Tris pH 7.2, 1 mM EDTA, 5% glycerol, 1.0 M NaCl (provided by Bob Garcea) to 10 ml of Carbonate Buffer. 100 µl was added per well, except for control wells, then the plate was incubated in a humid box (Tupperware box lined with a wet paper towel) overnight, or at room temperate for 2 hrs minimum. The blocking solution was discarded, and replaced with 150 µl per well of Blocking Buffer (5% non-fat dry milk dissolved in Whey Dilution Buffer: 4% Whey, 0.5% Tween-20, 1x-PBS). The incubation was continued for at least 2 hrs. The blocking buffer was discarded, and the wells were washed once for 1 min with 150 µl per well with 2x Wash Buffer (0.2% Triton X-100 in 1x PBS). The wash was removed and immediately replaced (so the well does not go dry) with 100 µl of sample diluted in Whey Dilution Buffer, usually using a 1:500 sample dilution. Controls included no serum, no HRP, and non-viral serum. Plates were incubated at room temperature for at least 2.5 hrs. The sample serum was discarded, and the plate was washed twice 1 min each with 2x Wash Buffer, never allowing the well to dry.

When detecting IgG and IgM, 100 µl per well of isotype-specific biotin-labeled antibody (1:1000 dilution, 10 µL/10 mL Whey Dilution Buffer) was added, and incubated for 1 hour. The wells were washed twice in 2x Wash Buffer, then 100 µL/well streptavidin-HRP (1:500 dilution, 20 µL/ 10 mL Whey Dilution Buffer) was added, and the plate left at room temperature for at least 1 hour. When detecting IgA, IgG1, IgG2a, IgG2b, and IgG3, 100 µL/well of isotype-specific-HRP (Southern Biotech) (1:1000 dilution) was added (except to the HRP control), then the plates left at room temperature for at least 1 hour. The HRP soultion was discarded, and the wells were washed 2-3 times with 2x Wash Buffer.

The color forming reaction required the preparation of fresh TMB (Tetramethyl benzidine, Sigma) by dissolving 1 mg TMB tablet in 5 ml of 0.1 M phosphate/citrate buffer and

5 mL of water. 2 µL of 30% H_2O_2 was added just prior to use, and mixed thoroughly. 100 µL of this TMB substrate was added per well, and the plate was incubated at room temperature for 2-5 minutes. The positive wells were blue. The reaction was stopped by adding 25 µL/well of 2N H_2SO_4, then the OD was measured at 450 nm.

ELISA – General Antibody

The general lab ELISA protocol (above) was modified to determine the total Ab titer in hybridoma supernatant. A coating of Anti-rat IgG + IgM (Jackson Immuno Research Laboratories, cat# 112-095-068) was made by adding 3.5 µL per 10 µL Whey Dilution Buffer. All steps were performed as described earlier. Samples used were, supernatant from irrelevant and VP1-specific hybridoma. As standard practice, a positive control (day 14 polyomavirus infected mouse sera) and negative control (uninfected mouse sera) samples were also plated out. Each was compared at an initial dilution of 1:500 (diluted in Whey Dilution Buffer), and a 1/3 dilution series performed on the ELISA plate. A stock antibody was also used to calculate hybridoma antibody concentrations. The stock antibody used was PE anti-mouse CD4 (BD Pharmigen, cat # 553653) diluted identically to the samples. All subsequent steps were performed normally as described above. A Zymed, Biotin-Mouse-Anti-Rat kappa light chain (1 mg/mL) antibody was applied as the capture antibody. Every other step was done as in the general protocol.

ELISA – Hybridoma VP1 - Specific

A two plate ELISA was also performed to quantitate the amount of VP1 specific Abs in VP1 Specific and Irrelevant hybridoma supernatants. A standard VP1 coat was applied to two

plates. In exception to coat, plate 1 was carried out identically to the general antibody ELISA. Plate 2 differed in the use of a separate capture antibody. The capture antibody used was an Anti-Rat (H+L) (Vector, cat# BA-4000) antibody.

VP1-FITC labeling

For the initial VP1 staining, a labeling of FITC was done with a Pierce, EZ-label FITC Protein labeling kit. The VP1 sample labeled was at a concentration of 1 mg /mL. The labs stock was not in the correct buffer. BupH ™ Borate Buffer (pH 8.5) buffer was required for use in labeling. Thus, dialysis was performed using 50 µL of VP1 to get VP1 into the BupH buffer. The complete protocol for the dialysis is found on Page 3 of the labeling kit's instructions for "Removal of Excess Fluorescent Dye – For sample volumes of 100 µL or less. 100 uL of VP1 was dialyzed and split into two tubes (stained, unstained). To accomplish the labeling, FITC dye prepared by mixing 100 µL Dimethylformaide (DMF) to a pre-weighed FITC tube (both included in kit). It was determined that 1.1 uL of prepared FITC was needed to label 50 µL of VP1. 1.1 µL of FITC was added to stained tube, and allowed to incubate at room temperature for 1 hour. PBS was then mixed up (also included in kit), by adding a PBS packet to 500 mL of water. PBS is needed for second dialysis to remove excess dye. Another dialysis reaction was performed (1 hour), but instead used PBS in place of the BupH buffer. The VP1-FITC was then ready, and stored in a light block tube @ 4°C until future immuno-staining.

General FACS Protocol

The lab's basic FACS protocol was modified. Spleens were isolated from mice. Then, each spleen was ground in FACS Buffer using microscope slides (frosted ends). The cell

suspension was filtered through an RBC filter (neoprene mesh). The cell suspension was spun down (1330 rpm @ 4°C for 5 min), and the supernatant was discarded. The cells were resuspended in 5 mL (per spleen) of 155 mM NH_4Cl (kept on ice) and incubated for 5 minutes at room temperature to lyse red blood cells. Then the cells were washed twice with PBS and resuspended in PBS (1x) at 10^7 cell/ml cell density.

Generally, 200 µL or 250 µL of cell suspension was added per well (depending on the experiment): a control well for each fluorescent antibody used, and unstained control well, and any sample wells (infected, uninfected, enzyme treated, negative controls, etc.)
The plate was spun down, then Fc Block was added to each well, 100 µL / well (1:100 dilution, 20 µL Fc block / 2 mL FACS). Cells were then incubated in the dark (wrapped in tinfoil) for 30 minutes at 4°C. The plate was spun down, and the cells were washed by adding 200 µL/ well of FACS buffer, then they were spun down again. The washing process was repeated 2 times. Staining then proceeded by adding 100 µL/well staining antibody cocktail at 1:100 dilution (diluted in FACS buffer) to sample wells (I always made a little extra cocktail). A typical cocktail example for 7 Samples: added 8 µL of each antibody to 800 µL of FACS buffer, then added 100 µL / well. For control wells, 100 µL FACS buffer and 1 µL staining antibody was added, for unstained control no antibody was added. The plate was incubated in the dark (wrapped in tinfoil) for 30 minutes @ 4°C. After incubation the plate was spun down and washed 2 additional times (w/FACS buffer). Finally, the cells were fixed by adding 100 µL / well Cytofix™ (paraformaldehyde) and incubated at room temperature for 2 minutes. 100 µL per well FACS buffer was then added, samples were usually run the next day on LSR II.

FACS Protocol w/ Neuraminidase Treatment and GFP Stain

When performing a plate with neuraminidase treatment, the cells had to resuspended between 1-3 million cells/ mL or the treatment would not work. The protocol was the same as the general one, but the treatment step was placed prior to the Fc Block step. Neuraminidase (Sigma, type V, from clostridium perfringes) was used at a concentration of 1 unit /mL (dissolved in TC-PBS 1X). Diluted neuraminidase was then added 200 µL/ well and the plate was allowed to incubate at 37°C for 1 hr. The plate was washed 2-3 times (spun down, washed) with 200 µL/well with FACS Buffer. Then the plate enters the normal Fc block stage.

For a GFP-VP1 stain, only 0.1 µL GFP-VP1 for 100 µL well was needed (0.1 µL/ 100 µL PBS). This step was done after the Fc Block step. A GFP-VP1 stain was optimally done to separate this reagent from any other staining antibodies, and it was diluted in PBS instead of FACS buffer. After incubating for 30 minutes at 4°C, the plate was spun down (1330 rpm @ 4°C for 5 min). The plate was flicked, and its wells were washed by adding 200 µL/ well of FACS buffer. Generally 2-3 washes were done after the GFP stain. Any additional staining then proceeded after the GFP stain.

FACS Protocol/ Cell Sort

Five spleens from C57BL/6J spleens at day 7 of infection with 2 x 10^6 pfu of polyomavirus were isolated for staining. The cells were prepared as described in the FACS protocol section. The staining itself was then done using 500 million cells at 10 million cells/mL (50 mL total volume). A plate was prepared concurrently with control wells. 200 µL of suspension was added per well, and spun down. A control well for each fluorescent antibody used (APC-B220 and GFP-VP1) was done. Both the 50 mL conical and plate were spun down

(1330 rpm @ 4°C for 5 min) and the supernatant was discarded. The Fc block was then added to each well, 100 µL / well (1:100 dilution, i.e. 20 µL Fc block / 2mL FACS). The Fc Block was also added to a 50mL conical tube (500 µL Fc block / 50 mL FACS). The plate and 50 mL conical were incubated in dark (wrapped in tinfoil) for 15 minutes @ 4°C.

Both samples were then spun down, and washed once (100 µL/well of FACS buffer, and 50 mL of FACS buffer in tube). Staining antibodies were added (100 µL/well) to control wells: 1 µL APC-B220 / 100 µL FACS Buffer , and 0.4 µL GFP / 100 µL FACS Buffer. For the 50 mL conical tube (main stain), 90 µL of APC-B220 was added, and 50 µL of GFP-VP1 was added in 50 mL of FACS buffer (90 µL APC-B220 + 50 µL GFP-VP1/50 mL FACS buffer). Stains were incubated in the dark (wrapped in tinfoil) for 30 minutes @ 4°C.

The conical tube was resupended at 30 million cells /mL (~13 mL). The sample at 30 million cells/mL was placed into snap cap tubes. Control wells were pipetted from the plate to bullet tubes. Collection media was made (50% FCS – 20 mL, 50% RPMI -20 mL) for the collection of cells after sorting. Cells were sorted in the UMMS sort facility (~4 hours) into two populations. One population sorted out was APC-B220+ lymphocytes, and the other were APC+ / GFP-VP1+. During the time the cells were being sorted an ELISPOT plate was coated with VP1 (1 mg/mL), anti-Ig (1mg/mL) or no coat control. The coat was made by adding 3.5 µL of VP1 or anti-Ig to 10 mL carbonate buffer. VP1/Anti-Ig/No Coat were used to coat 2 columns each. The ELISPOT plate was allowed to incubate for 4 hours at 37°C. After coating, the plate was blocked by adding RPMI + 10% FCS and incubated for 30 minutes at 37°C. After cell sort was finished cells were given 30 minutes to discharge (they are fragile).

The APC+ and APC/VP1 double-positive (+/+) sorted samples were spun down and resupended at maximal cell density to be still be able to coat 6 wells (266 µL each, i.e at least

1600 µL of each suspension was needed). APC positive cells were resuspended at 5 million cells/mL, and double positive cells were resuspended at 500,000 cells /mL (suspension media used was 10% FCS and RPMI). Media + 10% FCS was added (200 µL/well) to all wells except the top row. In the top row, 266 µL of either APC + (first 6 wells) or +/+ (last 6 wells) was added of the cell suspension. A ¼ dilution was then performed by removing 66 µL of cells from the top row and continuing down the plate. The plate was allowed to incubate at 37°C for 4 hours. During the incubation time, detecting reagent was made. 11 mL ELISPOT wash (1X PBS, 0.1% Tween 20) was added to 220 µL FCS, 11 µL anti-IgG biotin, and 11 µL anti-IgM – Biotin.

After incubation, the plate was flicked, and washed 3 times with 150 µL/well ELISPOT wash buffer. Detecting reagent was added to all wells (100 µL/well) and the plate was allowed to incubate overnight at 4°C in a humid box (to prevent drying out). The plate was washed 3 times with 150 µL/well ELISPOT wash buffer. Streptavidin-HRP solution was made by adding 220 µL FCS, and 11 µL SA-HRP to 11 mL ELISPOT Wash Buffer. The SA-HRP was added (100 µL/well, and the plate incubated at room temperature for 45 minutes. The plate was then washed 3 times with ELISPOT Wash Buffer (150 µL/well). Developing reagent was made by placing a 20 mg AEC tablet (Sigma) in 2mL of DMF (N,N-Dimethylformamide). The solution was vortexed, and 1 mL was added to 30 mL of 0.1 M Sodium Acetate. Developing Solution was then filtered through a 0.2 micron (or 0.4 micron) filter. Immediately before developing, 15 µL of H_2O_2 was added to the solution and then pipetted 100 µL/well on plate. After 5 minutes of plate developing, brown spots appear. The plate was then washed vigorously with water and given time to dry.

* Alternative Neuraminidase Method: before the Fc Block, cells were resuspended at 1 million cells / mL in PBS, then treated with 40 Units of neuraminidase per 400 million cells for 1

hr @ 37°C. Cells were washed 3 times in FACS buffer, and then resuspended at 10 million/cells mL for staining.

RESULTS

The original goal of this project was to sort out antigen-specific (VP1) B-cells using FACS staining. Mice were infected i.p. with PyV, then splenocytes were taken during the peak of infection (day 7). B-cells specific to VP1 are very low percentage, thus taking them from a pool of infected mice increases the chances of selecting for such cells. A double stain for B220 (CD45R) (a B-cell-specific marker) and VP1 viral capsid should produce a small percentage of double positive cells that are both B-cells (B220+) and antigen specific (VP1). The problem was that B-cells (and most other cells) contain sialic acid residues which also bind VP1, producing non-antigen specific staining.

Why search for such cells in the first place, particularly primary cultures? The argument can be made that B-cells in culture are not accurate representations of an intact immune system response. Methods similar to this project have been used, but never with primary splenocytes. As these double positive cells are such a low percentage of naturally occurring B-cells, it made the project a bit more challenging. But once enrichment for such B-cells succeeds they can be studied on the genomic level. What exactly makes those cells so special during, before, and after viral infection can be learned.

The first method used to reduce non-specific binding was to use neuraminidase to cleave the sialic acid residues present on the surface of the cells prior to staining with VP1. The strategy is shown in figure 8 below. With non-specific binding of VP1 to sialic acid minimized (right panel), more VP1 should be free to engage viral-specific antibody on the B-cell surface.

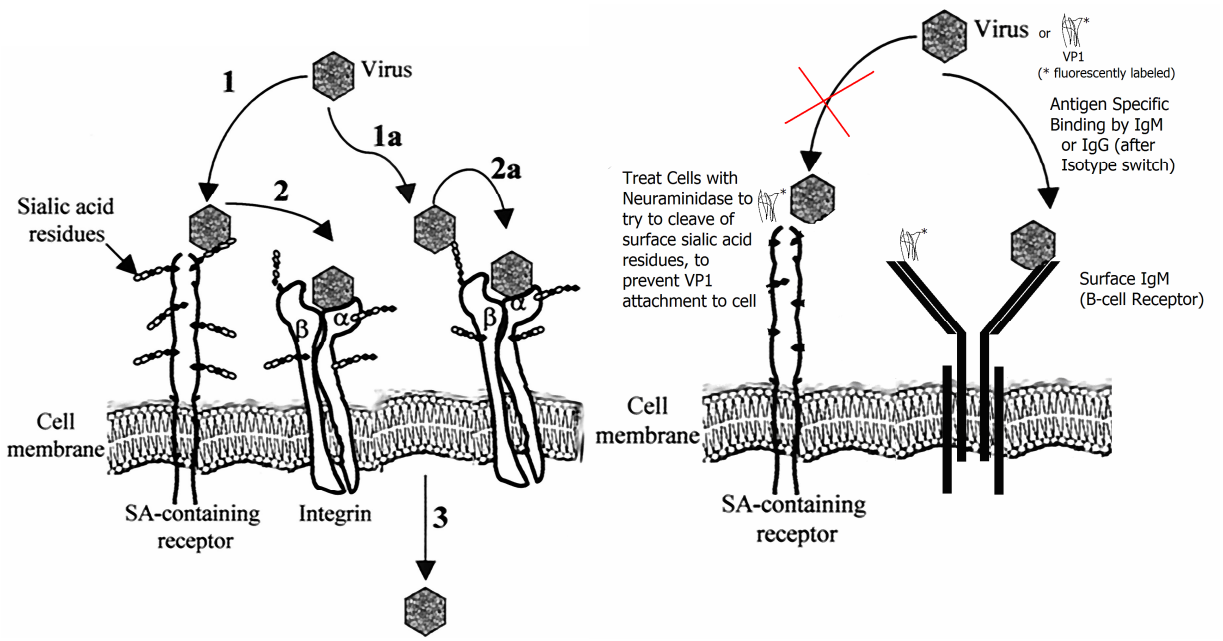

Figure 8. Theoretical Neuraminidase Treatment. VP1 major capsid protein (that binds cells) is able to bind terminal alpha 2,3 sialic acid residues (left panel), thus neuraminidase needs to be used to remove sialic acid (right panel), leaving surface IgG as the primary means for binding the VP1 stain. Modified from Caruso et al., 2003.

VP1-FITC Stain

Initially, VP1 was labeled using a FITC labeling kit. Sometimes the staining looked fairly good, but this was not reproducible. One would expect more VP1-specific B-cells in the spleen of PyV- infected mice, but the staining with VP1-FITC was inconsistent. Neuraminidase treatment was attempted using the VP1-FITC and B220 stains. Neuraminidase is used by several bacteria and viruses to degrade sialic acid residues. Influenza virus uses neuraminidase, as it cannot bind effectively with sialic acid present. Without neuraminidase to remove sialic acids, influenza is unable to enter host cells and infect them. This is interesting, because polyomavirus uses the same sialic residues as a receptor. If sialic acid residues are removed, polyomavirus is unable to bind and be endocytosed by the host cell. Thus, neuraminidase is being used to prevent viral binding of PyV, rather than allow its entry as in influenza (fighting fire with fire). After treatment it was hoped that the sialic acid receptors would be removed and only B-cell receptors

would continue to bind fluorescently labeled-VP1, making isolation of such cells easier. Figure 9 shows the best VP1-FITC stain achieved without neuraminidase. The top right quadrant (high staining for B220 + VP1) contains 0.74% of the cells in the infected sample versus only 0.56% in the uninfected (0.014% to 0.036%). B220 staining is also increased in the infected sample, as would be expected during the peak of any infection (42.2 % versus 57.4 %). During an infection's peak (day 7-8) viral titer is high and the amount of antigen-specific B-cells fighting it increases. Even so, the total percent of these B-cells is very small, much less than one percent of all B-cells. Therefore, many events (cells) much be sampled in order to see any difference in infected samples, or to get a significant amount of antigen-specific B-cells.

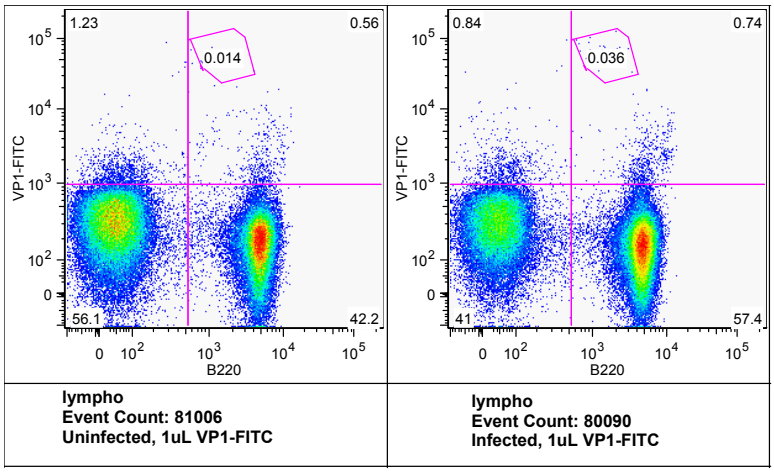

Figure 9. FACS Analysis of Uninfected and Infected Murine Lymphocyte Samples Stained with VP1-FITC. Uninfected splenocytes (left panel) and infected (right panel), both using 1:100 dilution of VP1-FITC.

VP1-staining had hints of success, but unfortunately a new batch VP1-FITC frequently had to be prepared, with each labeling reaction producing different staining results. It was concluded that trying a dilution of VP1-FITC may be more effective at reducing background (see figure 10).

One would hope that the B-cell receptors specific for VP1 would have a much higher affinity then a common sialic acid residue (present on most cells Therefore dilutions of FITC-VP1 were first performed to determine a point where most of the double positive cells were those desired antigen specific cells. Since, if there was an excess of FITC-VP1 it would bind not only to specific B-cell receptors, but then the extra VP1 would bind sialic acid residues.

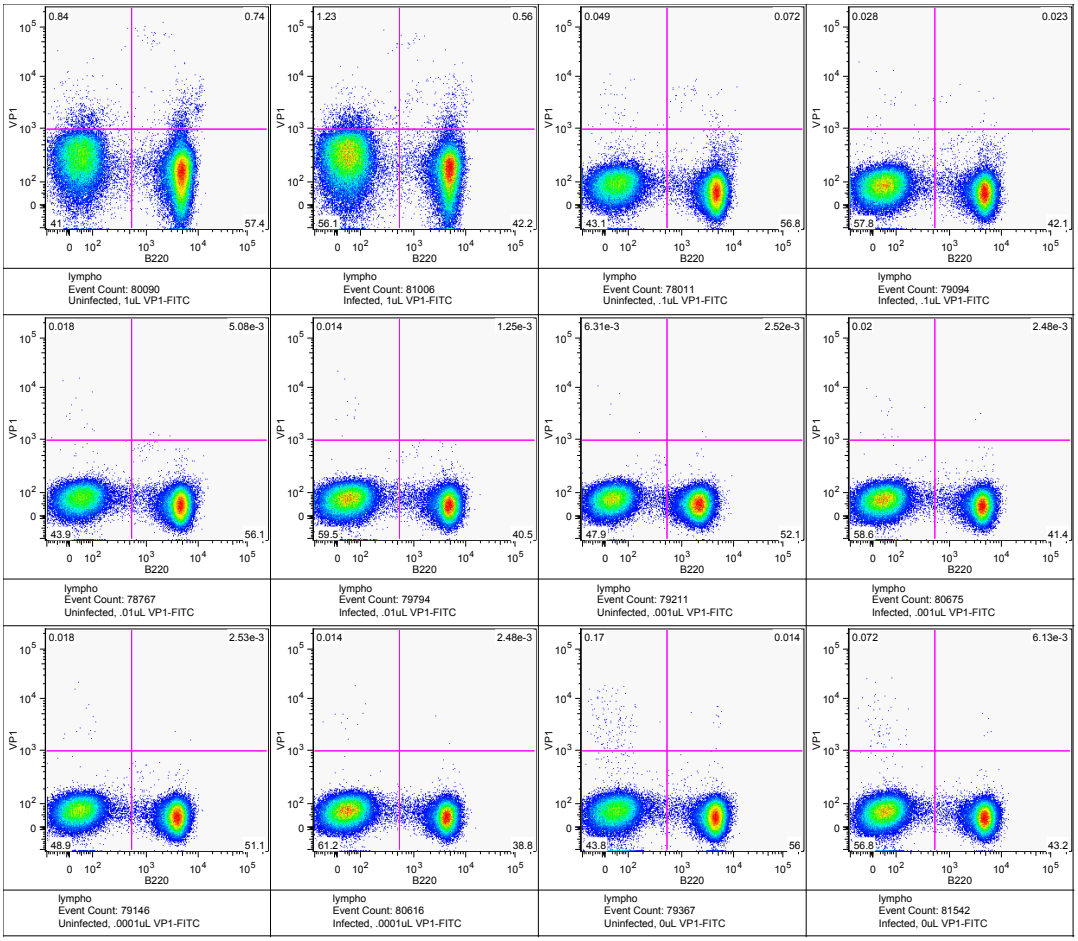

Figure 10. Effect of Diluting the VP1-FITC Lymphocyte Stain. Data shown in paired panels, uninfected (left) and infected (right). Highest amounts of VP1-FITC stain are shown in the upper left, and the most dilute in the lower right.

The background was significantly reduced using 0.1 µL stain instead of 1 µL, but the infected samples contained less double positives (upper right quadrant), not consistent with

infection. During an infection, one would suspect more of such cells. The VP1-FITC approach was tested several more times, with and without neuraminidase, but it was still not reproducible.

VP1-GFP Stain

In place of VP1-FITC it was decided to use a genetically engineered VP1-GFP made in yeast. A large amount of this reagent was obtained, alleviating the issue of having to constantly make new batches. It was also less likely that it would have any cross-reactive dye since the dialysis after the FITC labeling reaction never removed 100% of the un-reacted FITC, which may have contributed to irregular staining. Also the neuraminidase used was changed to Sigma brand, as the New England Biolabs was ineffective, even at extremely high concentrations. The dilution of neuraminidase, temperature of incubation, and what the enzyme was dissolved in were changed over many experiments. Some papers suggested 37˚C, others 4˚C, and no paper gave a real definition of how many units of neuraminidase should be used per given amount of cells. Such variables made it difficult to optimize the stain, however using the VP1-GFP did produce uniform staining throughout the assay optimization.

Several dilutions were tested of the VP1-GFP stain, and it was determined that 0.1 µL per 100 µL well produced the same results as 1 µL, so this 10-fold dilution was used for the rest of the project. Likely the GFP-VP1 is at higher concentration than FITC-VP1 (confirmed by ELISA). Unlike with VP1-FITC, the GFP-VP1 staining was virtually the same every time 0.1 µL/ 100 µL well was utilized. Some of the earliest data using this staining dilution is shown in Figure 11 below. In Figure 11, uninfected and infected lymphocytes were stained using VP1-GFP and CD19-APC (another B-cell marker often used in place of or concurrently with B220). Staining with CD19 provided identical data as B220 when both were used.

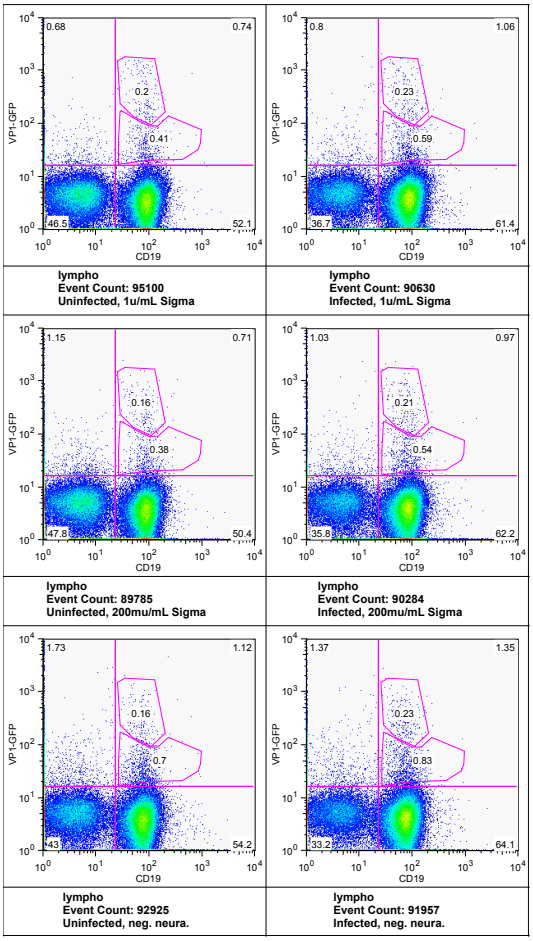

Figure 11. Early GFP Neuraminidase Lymphocytes Stain. Left column contains uninfected samples, and right contains infected. The top row is treated with 1 u/mL neuraminidase, middle with 200 mu/mL and the bottom is a negative control for treatment.

Also in this figure, 200 µL/ well of neuraminidase dissolved in PBS was used to treat infected and uninfected lymphocytes. Concentrations of 1 u/mL and 200 mu/mL and a negative (untreated row) were tested. In the upper left panel of each set (VP1-positive cells that are not B-cells) for uninfected samples (left column), 1.73% non-neuraminidase-treated (bottom row) was reduced to 0.68% using 1 u/mL neuraminidase (top row), and to 1.15% using 200 mu/mL (middle row). In virally exposed samples (right column), 1.37% was brought down to 0.8% using 1 u/mL, and to 1.03% using 200 mu/mL. These are non-B-cells, thus such dramatic cut

downs in VP1-positive cells are indicative of a working neuraminidase treatment. Reduction of non-B-cell receptor binding is also seen in the upper right quadrant (B-cells). The subpopulations (circled) in the upper right quadrant that are lower VP1 staining (middle), show a large reduction after neuraminidase treatment. Thus, the other super populations, likely contain the true double positives. Such data suggests that the 1 u/mL concentration was a bit more effective. A few other stains produced similar results.

Using the GFP-VP1, it became clear that the neuraminidase treatment works best under the following conditions: 0.2 units of neuraminidase in 1X PBS (1 Unit/mL per well), at low cell densities, and at 37°C. Even so, we questioned how well GFP-VP1 actually binds sialic acid residues and B-cell receptors. ELISA data confirmed GFP-VP1 is more concentrated than unlabeled VP1 or FITC-VP1 (each was used as a coat, and infected serum with antibodies was used as a sample in ELISAs). Yet, this did not confirm that B-cell receptors where actually responsible for the specific binding.

Therefore, two rat isotype-matched (IgG2b) B-cell hybridomas were obtained. One hybridoma was specific for VP1 of polyomavirus, and the other was an irrelevant (M5/114.152) rat hybridoma. In theory, the specific hybridoma should stain with a higher affinity for GFP-VP1 if it truly staining specifically. This experiment is detailed below in Figure 12.

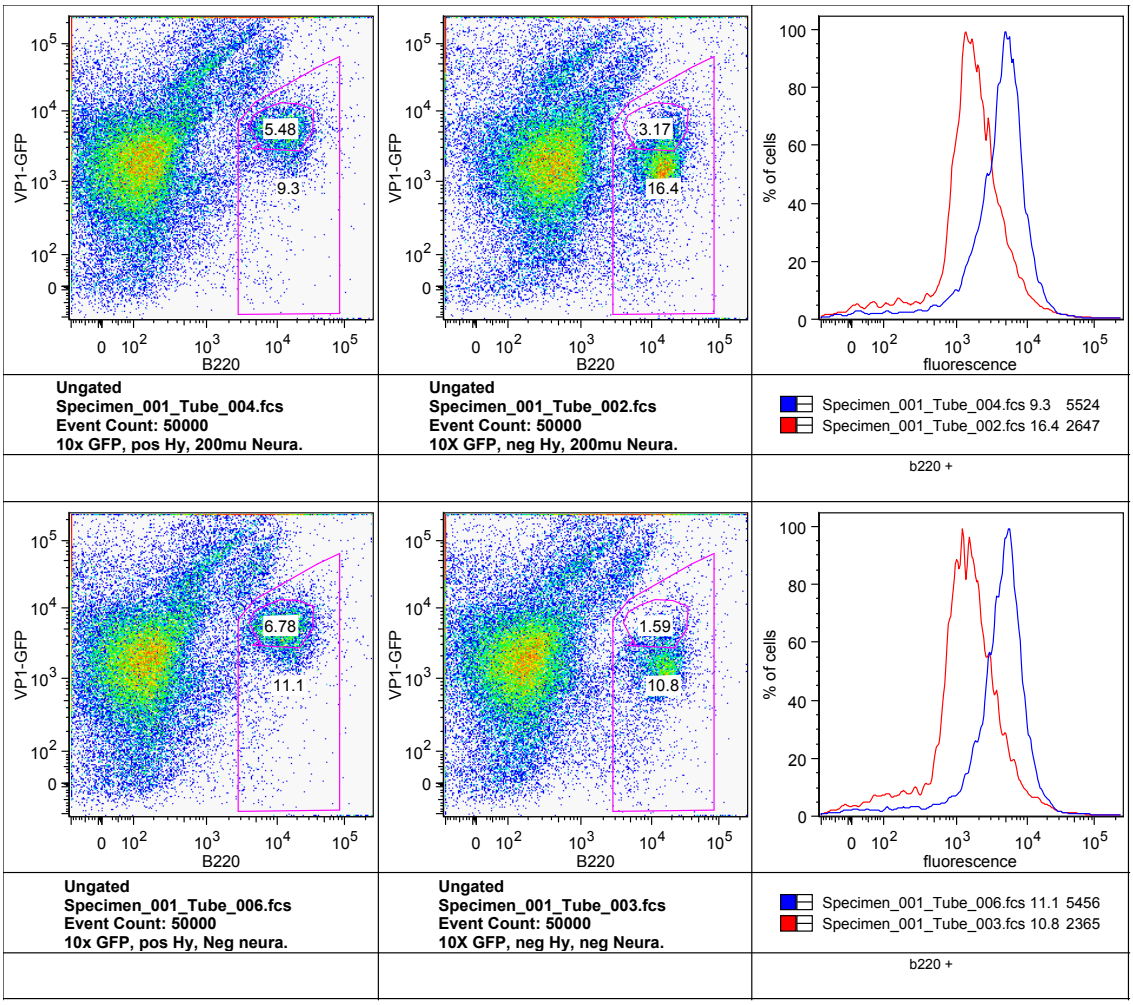

Figure 12. Hybridoma Comparison Stain. Positive Hybridoma is VP1 specific and Negative Hybridoma in non specific. Top row is neuraminidase treated, and bottom is untreated. The last column contains a comparison of the fluorescence of the large group (all B220+). The first number is the % of cells and the last number is the main fluorescence intensity. Key: 10X; (1:1000 fold dilution), neg Hy = negative hybridoma, pos Hy = VP1-specific hybridoma,. 200 mu Neura. = 200 mu of neuraminidase. Neg Neura = no neuraminidase.

The top row of Figure 12 contains the specific (positive) anti-VP1 hybridoma (left panel) and negative (non-specific) hybridoma (middle panel). Both hybridomas produce a IgG2b antibody, except the positive is specific for VP1 antigen. The large subpopulations represent the B220+ cells which are compared in fluorescence intensity in the 3rd column. Within that is a smaller group, gated around VP1-specific B-cells. Neuraminidase treated high affinity B-cells compose 5.48% (in the positive control) and drop to 3.17% in the negative. This is congruent with the fact anti-VP1 antibody producing cells should have a higher affinity than a non-specific

antibody producing cell. Without this information, one might suspect it was just a non-specific antibody (any IgG2b type) with the ability to bind VP1. It is also quite clear that both hybridomas were not pure clones, they contain a lot of non-hybridoma cells. Hybridomas unfortunately are not very stable, and often get outgrown by other cells. Regardless, this serves as a good positive control. In the non-treated bottom row, the specific hybridoma still has a higher fluorescence as expected. While the neuraminidase treatment reduced binding in the specific hybridoma, it appeared to increase in it a small amount in the negative. This increase in the irrelevant hybridoma might be due to the sialic acids not being completely washed off the cell after cleavage. Other stains have produced similar results. Either way, there is a clear increase in the fluorescence intensity for the hybridoma making anti-VP1 antibodies. This would support the hypothesis that the GFP-VP1 is staining the antigen-specific B-cells.

Test of Hybridoma Specificity

An ELISA was done to confirm that the VP-1 hybridoma produced antibodies specific for VP1 (Figure 13) (Y-axis). The question had been raised over whether VP1 was really binding sialic acids / B-cell receptors specifically. Thus, a general antibody ELISA was performed (X-axis) where the plate was coated with an antibody targeted to all rat/mouse IgM + IgG antibodies. Using such a coat should allow any antibody present in the hybridoma supernatant to bind and be detected. Supernatant from each hybridoma was collected to have its antibody concentration assayed. It was compared to a stock CD4 antibody of known concentration.

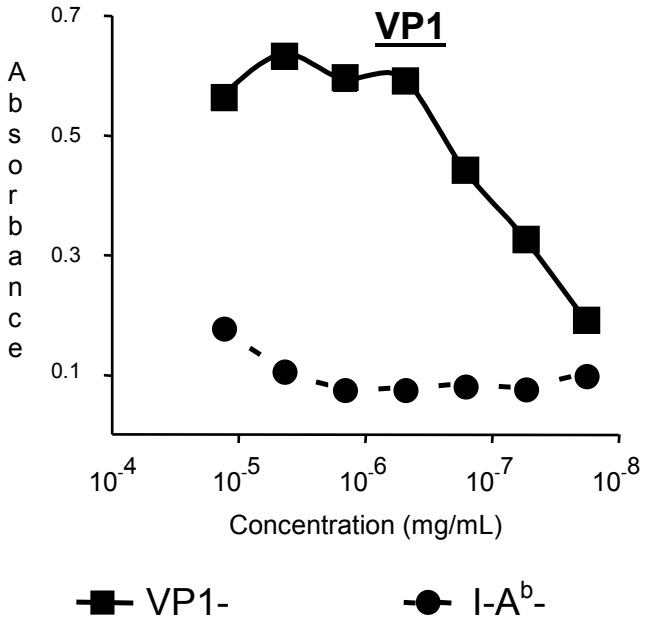

Figure 13. Hybridoma Comparison Using VP1-Specific ELISA. The y-axis denotes the absorbance values for hybridoma supernatant in a VP1-specific ELISA. A quantitative general antibody ELISA was also performed on both hybridoma supernatants and displayed on the x-axis.

Infected and uninfected mouse sera were also tested, and positive and negative controls. A dilution series was performed for each antibody-containing sample. As can be seen on the X-axis, both hybridoma supernatants did contain approximately equal antibody titer. As the isotypes are matched, each produces about the same antibody level, the only difference between the hybridomas was VP1 specificity.

To test the VP1-binding ability of the antibody in the hybridoma supernatants, another ELISA was completed using VP1 as a coat to only bind VP1-specific antibodies instead of all of them. Two contrasting plates were done using different capture antibodies. One plate used the mouse-anti-rat kappa light chain capture antibody, and another with rabbit anti-rat IgG antibody. The rabbit anti-rat appeared to produce a more consistent dilution curve, possibly as the reagent was much fresher. Even so, both yielded data that confirmed much higher absorbance levels for

VP1-specific hybridoma (Y-axis in figure 13). At higher antibody concentrations it is clear that the VP1-specific hybridoma reacts much brighter than the irrelevant hybridoma. As antibody concentrations decrease, so does the VP1-specific absorbance in the specific hybridoma. Conversely, the irrelevant hybridomas VP1-specific absorbance says at background levels regardless of concentration. Such data was completely congruent with the fact the specific hybridoma does indeed produce antibodies specific for VP1. It also confirmed that the GFP-VP1 truly binds specific antibodies for VP1, and it's not caused by GFP or any other cross-reactive cell property.

Cell Sorting

With all the data supporting the dual GFP-VP1 and B220 stain as valid, an attempt was made to select for the double positives in hope of seeing enrichment for primary VP1-specific B-cells isolated from mice spleens. To accomplish this, a pool was done of 5 infected mice, an optimized stain performed, and the double positive B-cells sorted. The cells were then tested in an ELISPOT assay (see Materials and Methods). Unlike an ELISA, an ELISPOT assay yields information about living cells (and their ability to secrete hormones) rather than a static dead cell component without an intact membrane. This sorting experiment is the climax of the entire project. The purpose was to isolate VP1-specific living primary B-cells for analysis. If the cell sort selecting for double positives was performed, and the ELISPOT confirmed an enrichment of VP1-specific B-cells, gene expression studies could be performed in the future. See figure 14 for a graphical representation of the process.

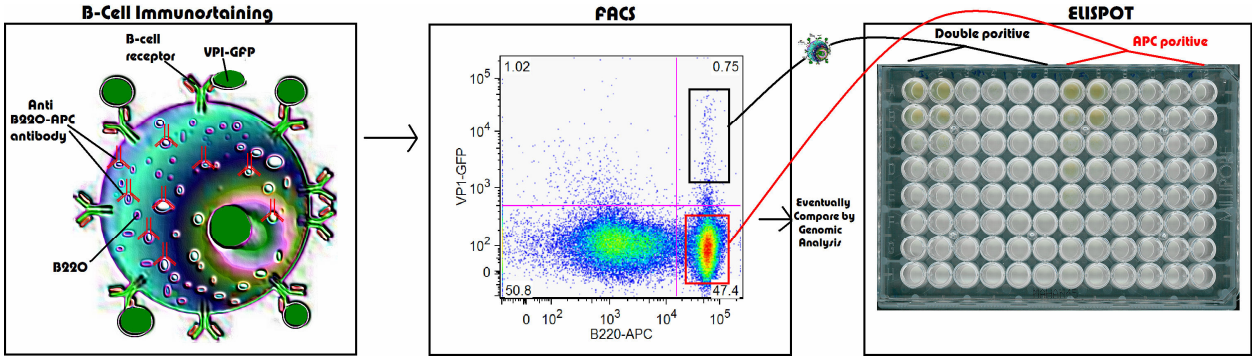

Figure 14. Process for Selecting Antigen (VP1)-Specific B-Cells. Cells are first stained with fluorescently labeled VP1 followed by B220 (b-cell marker). Double positives and B220 positives were sorted out and compared via ELISPOT. The goal is to use this method to compare the enriched population for differences on the genomic level.

A double stain was performed, and two populations were isolated by a FACS cell sort. No neuraminidase treatment was used since neuraminidase treatment often adversely affects cell viability (notable in some earlier staining). It was hoped the amount of GFP-VP1 staining would be high enough so that a majority of specific B-cell receptors would stain preferentially over lower affinity sialic acid residues. Two populations of B220-APC + (B-cells) and double-positive cells (B220-APC/VP1-GFP) were isolated. In theory the double positives should show a large enrichment for antigen specific cells. A VP1-specific ELISPOT was performed using both populations (Figure 15).

	VP1-GFP/B220-APC +/+ (500,000 /mL)						B220-APC + Only (5 Million /mL)					
	Ig	*Ig*	*VP1*	*VP1*	*NC*	*NS*	*Ig*	*Ig*	*VP1*	*VP1*	*NC*	*NC*
1:1	x	x	x	x	x	-	x	x	10	8	6	7
1:4	x	x	25	27	12	-	x	x				
1:16	x	x					x	x				
1:64	37	71					x	x				
1:256							32	8				
1:1024												

Figure 15. ELISPOT Data for APC-Positive B-Cells and Double-Positive VP1 B-cell Populations. Left column is the dilution of sample. Key: Ig = Ig coat on plate, VP1 = VP1 coat on plate, NC = no coat, NS = no sample, x = too many spots to count, - = no spots.

Approximately 10.33 $\cdot 10^6$ APC-positive cells and 6.574 $\cdot 10^5$ double-positive cells were isolated from approximately 400 million splenocytes via a FACS cell sort. APC-positive cells were resuspended at 5 million cells/mL, and double positives resuspended at 500,000 cells /mL, these cells were then tested in an ELISPOT for specific VP1 and general antibodies (Ig) (see Figure 15). It is evident that not only are the double-positives enriched for VP1-specific B220+ cells, but also for antibody secreting cells. Even with the +/+ cells at lower dilution (500,000/mL versus 5 million/mL), the plate had a tremendously increased amount of colonies (spots) for both VP1 and Ig. Additionally, the +/+ took a higher dilution just to be able to count the amount of VP1-specific colonies. Uncoated lanes were done as negative control, so that the actual amount of VP1-specific colonies could be determined. Double positives only had one such lane as there was only a limited amount available (even with a cells sort, only a limited amount can be obtained). With such data the percent of VP1 specific cells can be elucidated, along with the increase enrichment, as shown in Figure 16.

	VP1-GFP/B220-APC +/+	B220-APC + Only
Countable Concentration	**125,000 cells /mL**	**5 Million /mL**
µL / well	**200**	**200**
Initial # cells / well	**25,000 cells/ well**	**1 million cells / well**
Average # VP1 colonies	**26**	**9**
Background (No coat) colonies	**12**	**6.5**
Actual Vp1 colonies (ave. – NC)	**14**	**2.5**
Concentration of specific VP1 colonies	**14 colonies /25,000 cells = 5.6 x $\cdot 10^{-4}$ colonies / mL**	**2.5 colonies/1 million cells = 2.5 x $\cdot 10^{-6}$ colonies /mL**
Percentage of cells	**(14/25,000)(100) = 0.56 %**	**(2.5/1,000,000)(100) = .00025%**
% Enrichment	**(0.56)/(0.00025) = 224 times more or 22400%**	

Figure 16. Enrichment Calculations for the ELISPOT Data.

Calculations (see figure 16) show that there were 224 times greater (22400%) enrichment for VP1-specific cells in the double positive population compared to the starting population. This augmentation should be more than enough to see changes on the genomic level. The double

positives contained 0.56% VP1-specific B-cells, and only 0.00025% in the B220+ samples. Neither number is incredibly high, as such cells are quite rare *in vivo*. Despite this, the enrichment did work! The projects main aim was achieved. The only way to improve the purity even more would be to use neuraminidase.

Neuraminidase treatment optimization was tested (temperature, dilution, buffer, incubation time), using an increasing dilution of cells (10 million, 3 million, and 1 million cells /mL (see Figure 17).

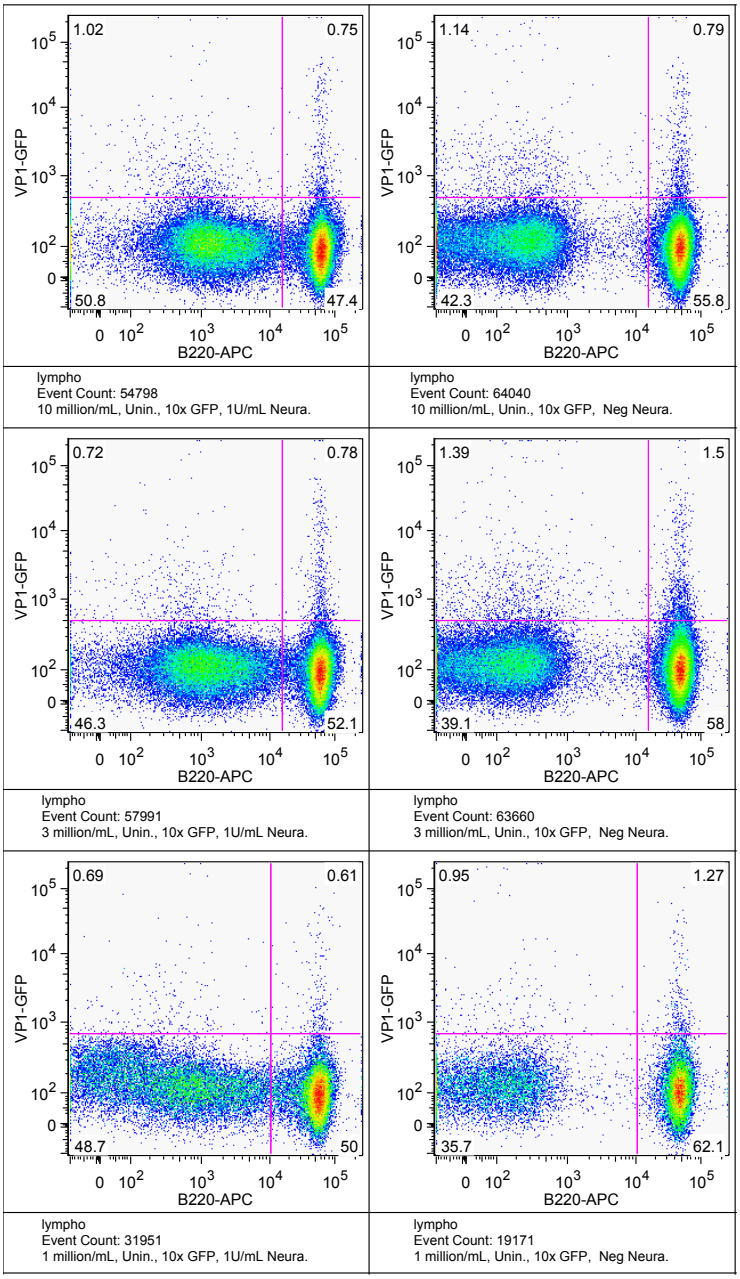

Figure 17. Neuraminidase Cell Dilution Stain. Only uninfected splenocytes were tested. A 1:1000 (10 fold dilution of normal) GFP stain was performed on different cell dilutions. The top row contains cell dilutions of 10 million/mL, middle 3 million/mL, and bottom 1 million/mL. Left columns are 1U/mL neuraminidase treated samples. While the right column contains untreated samples. Note the reduction in VP1 binding in the upper quadrants.

The percent of VP1-positive cells decreased dramatically in both B220 + and B220 negative upper quadrants in the lower cell dilutions. The upper left quadrant (non-B-cells staining for VP1) showed a large decrease in both the 1 and 3 /million cell dilutions. Most of the staining during the project was done at 10 million cells /mL to facilitate the FACS analysis. But the neuraminidase enzyme is fairly inefficient; therefore the lower cell dilutions work significantly better. Such dilutions produce more reduction of non-specific binding to sialic acid residues. Moreover, the ratio of enzyme to cell numbers would increase (which would settle less as well). Neuraminidase was probably overwhelmed with the amount of substrate (sialic acids) at higher dilutions. It's also possible, cell density was so high that cells clumped and just did not allow neuraminidase to even access the sialic acids.

When expanding this approach to infected and uninfected splenocytes (see Figure 18), the infected sample did contain more VP1/B220 double positives as expected. Additionally, the infected samples had a lot more B220+ B-cells in general (bottom left quadrant) in the infected samples. Neuraminidase treatment decreased the amount of B-cells in the infected samples, from 73.6% to 59.8%. Both upper right quadrants in infected and uninfected samples showed decreased VP1 binding. The difference is more drastic in the infected samples (0.86% to 0.67%).

Using this neuraminidase treatment, another FACS stain (Figure 18), cell sort, and ELISPOT (Figures 19 and 20) were performed. The experiment was a repeat of the previous experiment (pool of 5 infected mice), only the neuraminidase treatment was done.

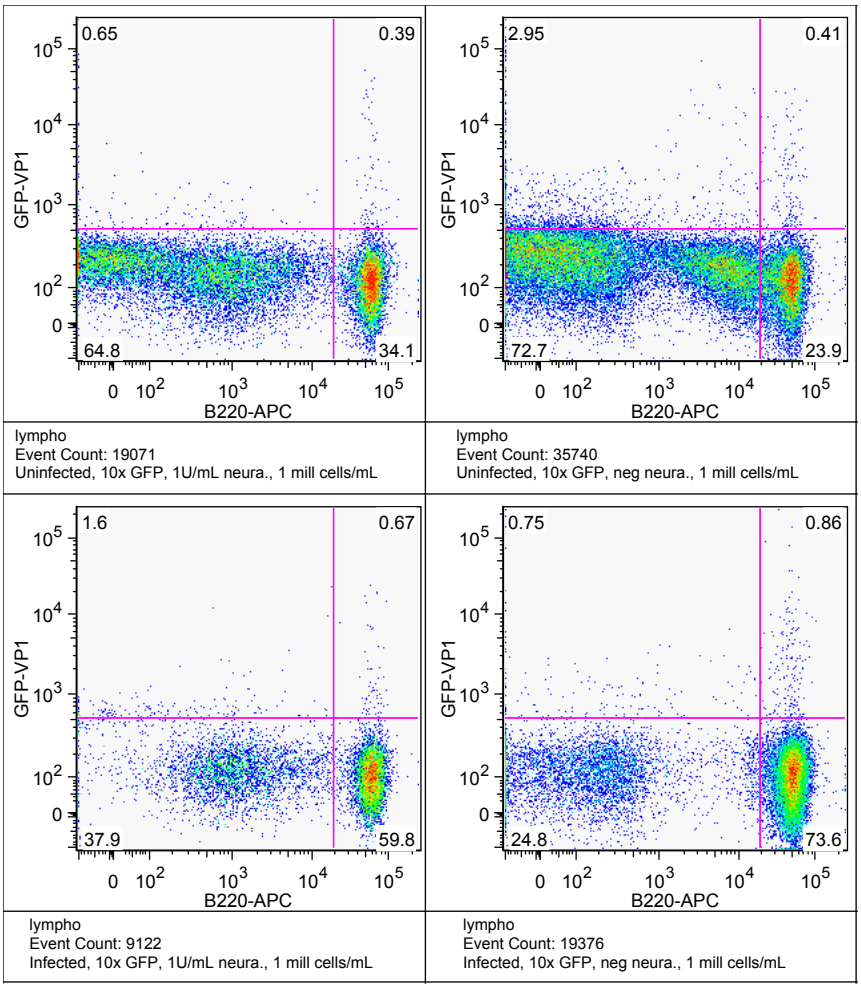

Figure 18. FACS Analysis of Infected and Uninfected Cells Using Neuraminidase at a Low Cell Density. Uninfected (top row) and infected (bottom row) splenocytes were used. A 1:1000 (10 fold dilution of normal) GFP stain was performed on different cell dilutions. All cells were stained at a concentration of 1 million cells/mL. Left column are 1U/mL neuraminidase treated samples. While the right column contains untreated samples, note the reduction in VP1 binding in the upper quadrants, especially in the infected samples.

For this neuraminidase treatment, the cells were resuspended at 1 million cells / mL, and 400 million cells were treated with 40 Units of neuraminidase for 1 hr @ 37°C. Unfortunately, this treatment appeared to be too harsh to the cells. Of 400 million cells, only about 40 million survived. Another plate was also done with unsorted infected and uninfected cells. This plate served as a control to make sure infected mice do have a larger amount of VP1–specific cells. The data for both plates are present in figures 19 and 20.

	VP1-GFP/B220-APC +/+ (43,000 /mL)						B220-APC + Only (1.875 Million /mL)					
	Ig	*Ig*	*VP1*	*VP1*	*NC*	*NC*	*Ig*	*Ig*	*VP1*	*VP1*	*NC*	*NC*
1:1	x	x	-	-	-	-	x	x	x	X	-	-
1:4	x	x	-	-	-	-	x	x	X	X	-	-
1:16	10	9	-	-	-	-	x	x	5	9	20	22
1:64							x	x	-	-		
1:256							7	9	-	-	3	4

Figure 19. ELISPOT Data for APC + and Double-Positive Populations with Neuraminidase Treatment. Left column is the dilution of sample. Key: Ig = Ig coat on plate, VP1 = VP1 coat on plate, NC = no coat, x = too many spots to count, - = no spots.

	Uninfected Unsorted (20 million / mL)						Infected Unsorted (20 million /mL)					
	Ig	*Ig*	*VP1*	*VP1*	*NC*	*NC*	*Ig*	*Ig*	*VP1*	*VP1*	*NC*	*NC*
1:1	x	x	x	x	x	-	x	x	X	x	x	x
1:4	x	x	x	x	x	-	x	x	X	x	x	x
1:16	x	x	x	x	x	-	x	x	23	18	11	8
1:64	x	x	8	12	2	6	x	x				
1:256	5	10	1	2	1	1	x	x				
1:1024							x	x				
1:4096							14	20	-	-	-	-

Figure 20. ELISPOT Data for Unsorted Infected and Uninfected Splenocytes. Left column is the dilution of sample. Key: Ig = Ig coat on plate, VP1 = VP1 coat on plate, NC = no coat, x = too many spots to count, - = no spots.

This ELISPOT confirmed the presence of more antibody producing VP1-specific cells in the infected samples versus the uninfected. Unfortunately the neuraminidase treated cells barely showed any spots/colonies positive for Ig or VP1. The plate did have some spots but they were infinitesimally small. Perhaps neuraminidase treatment may only be useful from an analytical perspective, not for sorting. Since the entire process works without the neuraminidase by limiting the amount of GFP-VP1, such treatment may be unnecessary (and also too expensive). As the enrichment (2240%) without treatment was quite good, it will be able to be useful in studying the antigen-specific B-cells.

DISCUSSION

The purpose of this project was to develop a FACS sorting protocol for isolating PyV major capsid protein VP1-specific B-cells from spleens of PyV- infected mice. These cells will be studied further for uncovering pathways of B cell responses to virus infection. To achieve this unusual enrichment, a double stain for VP1 (antigen specific) and B220+ (B-cell specific) proteins was developed. The only problem was that in addition to binding cell surface antibody, VP1 also binds cell surface sialic acids causing non-specific binding. Two methods were utilized to get around this. A dilution of VP1-fluorescently tagged stain, and neuraminidase treatment of the cells. A stain dilution should reduce staining to only higher affinity B-cells. Conversely, neuraminidase would digest any surface sialic acids that would act as non-specific receptors.

The research literature was not much help on this project. Other papers using neuraminidase poorly specified exactly how treatment was to done. Additionally, no studies were published on treatment of primary B-cells with neuraminidase. Primary cells are a lot more fragile than cell lines, thus numerous optimization experiments had to be done before optimizing a working stain and neuraminidase treatment. Factors such as temperature, cell density, time, incubation reagent were proven to be crucially important. The key was to achieve reproducible staining, and acceptable cell viability. Also, ELISAs and hybridoma stains were performed to confirm that the GFP-VP1 molecules were actually binding via VP1 to B-cell receptors (surface bound antibodies). Only a VP1-specific hybridoma antibody (at same concentration as an isotype matched hybridoma) was able to successfully bind the GFP-VP1 reagent at a limiting dilution.

During the course of the experiment both a VP1-FITC and VP1-GFP stain were utilized. As time progressed it became clear the VP1-FITC stain did not produce reliable data. Several

batches had to be labeled, and each was somewhat different in staining efficacy. Thus, yeast produced VP1-GFP was used an alternative. VP1-GFP had the benefit of being highly concentrated, allowing all the experiments to be done with the identical reagent. Data from the VP1-GFP stains also proved to be much more consistent. Neuraminidase was also used as a way to remove background sialic acid staining by removing them. Unfortunately, an effective treatment decreased cell viability and proliferation greatly. Treatment with neuraminidase removes sialic acid residues, which are present in high numbers on most cells. With these stripped, the cell has a hard time proliferating. The goal of the project was to select for viable antigen specific B-cells, which neuraminidase treatment did not prove effective.

In the end, a cell sort without neuraminidase yielded the best results, producing 224 times more VP1/B220 antigen-specific enrichment, versus a B220-positive B-cell population. When the same procedure was done with a neuraminidase treatment known to work at the small scale, results were poor, the ELISPOT plate showed only very small spots due to the harshness of the neuraminidase treatment on cell viability. Thus, the neuraminidase treatment might be too caustic to cells when used in conditions that effectively remove sialic acid. It is possible that one might be able to further improve the conditions, so that cells survive better and that treatment still removes background.

An alternative method would be to block the sialic acid residues instead of removing them. Instead of chewing up the cell surface to remove sialic acids, a sialic acid-binding protein could be used. Possibly the cells would be better of this way. If all the sialic acids could be blocked, then only specific-B-cell receptor binding to VP1 should remain. Another advantage would be that washing after treatment would not have to be so vigorous. With neuraminidase treatment, sialic acid residues might be cleaved, but they could still stick to the cell. Caruso et al (2003)

hypothesize that VP1 is only required for the initial cell-virus interaction. That if cleaved sialic acid is not completely washed off, they could bind VP1 and allow secondary attachment to the cell. *α4ß1* Integrin was shown to be a likely secondary receptor once VP1 is bound to an alpha 2,3 sialic acid (Caruso et al., 2003).

One such possible blocking agent may be Myelin-Associated Glycoprotein (MAG). MAG has a site in its N-terminus that is able to bind certain "sialyated glycans and gangliosides" (Vinson et. al, 2001). Vinson and colleagues were able to show that treatment with neuraminidase on neuron cells which bind GT1b and GD1a prevented their attachment. Such GTlb and GDla receptors, which contain alpha 2,3 sialic acids, stimulate growth which is blocked by stripping sialic acid residues. In another paper (Tsai et al., 2003) show the same GT1b and GD1a gangliosides serve as effective polyomavirus receptors. They prepare some yeast cell membrane extracts which contain various gangliosides (GT1b and GD1a), treat some of the extract with virus, and some with virus and neuraminidase. "Treatment of the ER proteoliposomes with α2,3-neuraminidase abolished binding activity" (Tsai et al., 2003). Those neuraminidase treated and containing virus showed no virus fractions bound to the yeast extract. Whereas virus had bound especially to GT1b and GD1a. They postulate that such gangliosides are likely viral receptors. Moreover, it was shown in the previously mentioned paper that MAG binds to both these gangliosides. Such treatment may be very useful in removing background in the future. Commercially prepared MAG is often linked to a human Ig domain that can be fluorescently tagged. This could be useful for staining active B-cell sialic acids that could act as potential viral receptors. Such data would be quite interesting for receptor and non-specific binding studies.

In another paper (Weitkamp et al., 2003), they attempt a similar method to select for B-cells that are specific for rotavirus. They use a double stain for CD19-PE and VLP-GFP (virus like particle linked to GFP). They sort out the double positives, culture them *in vitro*, put them into an ELISA to quantitate antigen-specific cells, and then use them for RT-PCR. From then on genes of interest can be cloned into a vector an analyzed. This is similar to the goal of this project, except that primary B-cells would be used, instead of cultured. Cultured B-cells are often not-representative of a truly viral activated B-cell. The double-positive population would then be compared to a B220-positive B-cell population. Since this project's enrichment was successful, one would hopefully be able to observe an increased amount of certain signals in the double positives. These could then be studied even further! Whether it is by neuraminidase, GFP-dilution, or MAG blocking methods, there is a bright green future for research of polyoma VP1-specific B-cells.

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