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The Characterization of Monoclonal Antibodies Specific to HPV45 L1 Protein

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Abstract

Cervical cancer is one of the leading causes of death from cancer around the world, resulting in nearly 300,000 deaths each year (Torre et al., 2012). Infection with high risk types of Human Papillomavirus is the primary etiologic event associated with the development of cervical cancer (Wallboomers, 1999). Since 2006, HPV vaccines have dramatically decreased cases of cervical cancer in developed countries. However, because these vaccines are expensive and require cold chain storage, their use in developing countries, where 85% of deaths from cervical cancer occur, has been limited (Torre et al., 2012; Kane, 2006). Development of second generation HPV vaccines that are inexpensive and thermodynamically stable at high temperatures is necessary to expand access to HPV vaccines to regions where they are needed the most.

The research project described in this thesis details the characterization of monoclonal antibodies specific to the L1 capsid protein of Human Papillomavirus type 45. The monoclonal antibodies were developed at NeoClone, then tested in three laboratory assays: enzyme linked immunosorbent assays (ELISAs), Western blots and pseudovirus neutralization assays. Identifying monoclonal antibodies specific to HPV45 L1 in each of these assays is important for the design and production of second-generation HPV vaccines.

During characterization, it was determined that the monoclonal antibodies were reactive toward a cross contaminant instead of HPV45 L1. Although the antibodies are contaminated, this thesis serves as a proof of concept for the characterization of HPV45 L1 monoclonal antibodies and can be used as a roadmap in further characterization experiments.

1. Introduction

Cervical cancer is the fourth most common cancer in women worldwide, with over 528,000 cases recorded in 2012 (Ferlay et al., 2015). In the US, where cervical cancer is the eighth most common cancer among women, the five year survival rate is 68% (Howlader et al., 2013). In developing countries that lack widespread screening programs and prevention methods, the survival rate is much lower. Of the 266,000 deaths from cervical cancer in 2012, 85% occurred in developing countries (Torre et al., 2012). Because cervical cancer is most prevalent among young women, it is the largest cause of years of life lost due to cancer in the developing world (Agosti et al., 2007). This disparity is largely due to the lack of screening programs or accessible vaccines, two measures that reduce cases of cervical cancer by 80% (Schiller, 2012; Denny, 2006).

The persistence of HPV DNA is the primary etiologic event associated with cervical cancer and has been found to be necessary for the development of 99% of all cervical cancers. (Bosch, 2002; Wallboomers, 1999). HPV is a non-enveloped, double stranded DNA virus that infects the stratified squamous epithelium (Schiller, 2012). The virus has a T7, right handed icosahedral structure made of 72 pentameric subunits that each consist of five interconnected L1 proteins (Chen, 2000, Baker 1991). Out of the 100 HPV types that have been identified, 40 can infect the genital tract and 15 are considered to be ‘high risk’ because they are associated with progression to carcinoma of the cervix, vulva, vagina, penis, anus and oropharynx (Munoz, 2003). ‘Low risk’ HPV types are associated with sexually transmitted genital warts as well as hand and foot warts (Garland et al., 2009; Iftner et al., 2003).

The development of HPV vaccines for the prevention of cervical cancer began in 1990 after the discovery that L1, the major capsid protein, can spontaneously self-assemble into a

capsid when expressed in certain cell systems (Kimbauer et al., 1993). The virus like particles (VLPs) produced by the self-assembly of L1 proteins resemble native virions and generate a humoral immune response that is directed against conformation epitopes on the L1 capsid protein (Stanley et al., 2006). One of the earliest vaccines, Cervarix® made by GlaxoSmithKline Biologicals, is produced using a baculovirus vector and contained VLPs for HPV type 16 and 18 which account for 70% of high risk HPV infections in the US (Stanley et al., 2006). Another early vaccine, Gardasil® made by Merck, is produced using a *Saccharomyces pombe* vector and contains VLPs for HPV types 6, 11, 16 and 18 (Stanley et al., 2006). The most recently developed vaccine, Gardasil 9®, contains VLPs from HPV types 6, 11, 16, 18, 31, 33, 45, 52 and 58 which account for 90% of all HPV infections (Joura et al., 2015). While these vaccines are effective prophylactic measures, they are only accessible in developed countries where deaths from cervical cancer are already dramatically reduced from effective screening programs (Schiller, 2012). These vaccines cost over $4.00 per vaccine to produce and are sold at $120 per dose in the United States. In addition, the vaccines require cold chain storage. Both the cost and storage requirements severely limit the use of these vaccines in developing countries. A second generation HPV vaccine that is low cost, single dose and does not require cold-chain storage is necessary to make prophylactic HPV prevention more accessible around the world.

To reduce the cost of vaccine production, second generation vaccines that use individual pentameric subcapsid particles instead of VLPs are being researched. These vaccines can be produced in *Escherichia coli*, a less expensive expression system (Gersch, 2012). While the VLPs need a cell nucleus in order to self-assemble, L1 proteins will spontaneously form pentamers, the base subunits of the HPV capsid, in bacteria (Thones et al., 2008). Protein pentamers initiate a similar immune response to VLPs when combined with adjuvants such as

monophosphoryl lipid A adsorbed to aluminum hydroxide (Yuan, 2001; Wu, 2011; Thones et al., 2008). In order to remove the need for cold-chain storage, these pentameres can be lyophilized, a process where the L1 capsomeres are embedded in glassy matricies at extremely high pressures to produce a dry, thermally stable powder vaccine (Hassett et al., 2015).

The Garcea Lab at the University of Colorado at Boulder is currently working to produce a quadravalent, second generation HPV vaccine that protects against HPV types 16, 18, 31 and 45. This vaccine uses L1 capsomeres expressed in *E. coli* to reduce production costs. In addition, the HPV type 16 L1 capsomeres have been shown to be thermally stable with lyophilization without reduced immunogenicity (Hassett et al., 2015). In order to ensure consistency and quality control in vaccine production, reactive antibodies have been characterized for three of the HPV types incorporated into this vaccine, types 16, 18 and 31. This research project seeks to characterize monoclonal antibodies specific for HPV type 45. To create an antibody set unique for HPV 45 L1, the protein was expressed in *E. coli* cells, purified using column chromatography, then sent to a monoclonal facility (NeoClone, Madison, WI) where it was injected into mice. Cells producing antibodies to the protein were fused with hybridoma cells and subcloned, creating a wide range of monoclonal antibodies that were sent back to Boulder for testing in Western blots, enzyme-linked immunosorbent assays (ELISAs), and pseudovirus neutralization assays. The goal was to determine which antibodies should be used to identify HPV45 L1 in future vaccine development and experiments. However, due to a contamination in the production of the monoclonal antibodies of an unknown source, the antibodies were not reactive towards HPV 45 L1. This paper will provide an overview of the characterization of these monoclonal antibodies and serve as a template for future characterization protocols.

2. Materials and Methods

All research was conducted in the Garcea Lab at the University of Colorado at Boulder with the exception of section 2.2 which was completed by NeoClone.

2.1 HPV45 L1 Production

BLR DE3 *E. coli* were heat transformed with a plasmid containing kanamycin resistance gene and a gene encoding HPV45 L1 linked to a lac operon promoter. Transformed cells were then plated onto luria broth (LB) media plates with kanamycin incubated at 37ºC overnight to select for transformed colonies. Single colonies were inoculated into 3 ml of Terrific broth (TB) containing 50 µg/ml kanamycin diluted 1:1000 and grown for 6 hours at 30ºC with shaking. 200 µl of this culture was then inoculated into 50 ml of TB containing 50 µg/ml kanamycin and grown overnight at 30ºC with shaking. Two milliliters of the culture was inoculated into 6 baffled flasks each containing 500 ml TB with 50 µg/ml kanamycin for three liters total culture. The baffled flasks were incubated at 37ºC with shaking until the optical density at 600 nm reached 4.0. The flasks were then chilled to 25ºC in an ice water bath. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Research Products International, Mount Prospect, IL) was added to the culture to a final concentration of 0.2 mM to initiate HPV45 L1 expression. The induced culture was incubated at 25ºC with shaking until the optical density at 600 nm reached 8.0. The culture was pelleted by centrifugation at 5,800 x g for 15 minutes at 4ºC and stored at -20ºC overnight.

For purification, the frozen bacteria pellets were each resuspended in 50 ml homogenizing buffer (200 mM NaCl, 50 mM Tris, pH 8.0, 1mM EDTA, 1mM PMSF, 10%

glycerol, 0.01% Tween 80, 5 mM DTT, pH 8.1) with stirring at 4^{o}C. Once resuspended, the cells were lysed using a Panda homogenizer between 800-1000 bar. After one pass through the homogenizer, the lysate was cooled to 4^{o}C with stirring, then passed through the Panda homogenizer a second time. The lysate was centrifuged at 22,000 x g for 30 minutes at 4^{o}C which pelleted out insoluble bacterial proteins and cell debris (P1), leaving the L1 protein in the soluble fraction (S1). The S1 was run through a 300 ml Q Sepharose Fast Flow (QFF) (GE Healthcare, Piscataway, NJ) column equilibrated with running buffer (200 mM NaCl, 50 mM Tris, 10% glycerol, 5 mM DTT, pH 8.1). Ammonium sulfate was added to the flow through (30% saturation) and stirred for two hours at 4^{o}C in order to precipitate out the L1 protein. The solution was centrifuged at 13,000 x g for 30 minutes and the resulting pellet (AS-P) was stored at 4^{o}C overnight.

The pellet containing the L1 protein (AS-P) was resuspended in 10 mL Running Buffer (25mM NaCl, 50 mM Tris, 10% glycerol, 5 mM DTT, pH 8.5), passed through the Panda homogenizer at 500 bar, and centrifuged at 13,000 x g for 20 minutes. The conductivity and pH of the soluble fraction were adjusted to match the Running buffer using no-salt buffer (50 mM Tris pH 8.5, 5 mM DTT, 10% glycerol, pH 8.5). A 75 ml Q Sepharose High Performance column (QHP) (GE Healthcare, Piscataway, NJ) was equilibrated with Running buffer and the soluble fraction containing L1 was loaded onto the column. The L1 protein bound to the column and then was eluted over an increasing NaCl concentration gradient from 25 mM to 1 M NaCl Running buffer. The eluted fractions were analyzed using 10% Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) to determine which fractions contained the L1 protein (52 kD). L1 containing fractions were then pooled, quantified, flash frozen in liquid nitrogen and stored at -80^{o}C.

2.2 Monoclonal Antibody Production

HPV 45 L1 protein purified in section 2.1 was sent to NeoClone for monoclonal antibody production. Over the course of three months NeoClone conducted three fusions. The antibodies produced by each fusion were screened with ELISAs by NeoClone then sent to Boulder for confirmation of positive clones. 42 total antibody-producing hybridoma cell lines were selected through this process. Antibodies from these lines were purified using protein affinity chromatography and aliquots were shipped back to Boulder for analysis using ELISA, Western blots and pseudovirus neutralization assays.

2.3 Enzyme-Linked ImmunoSorbent Assays (ELISA)

Serial dilutions of HPV45 L1 in 1x Phosphate-Buffered Saline (PBS) were added to a Polysorp 96 well plate (Nunc, Naperville, IL) with a final dilution of 0.25 µg, 0.13 µg and 0.06 µg of protein per well, each concentration was run in duplicate. A fourth well had no protein and served as a negative control. Each well had a final volume of 50 µl. The plate was covered with parafilm and left at 4^{o}C overnight to allow the protein to bind to the wells.

After binding overnight, the plates were washed three times with 50 µl per well Wash Buffer (1X PBS, 0.05% Tween20) and 100 µl per well of Block Buffer (5% non-fat dry milk in wash buffer) was added. The plates were incubated for one hour at 37^{o}C, then the Block Buffer was removed and 50 µl/well of each primary monoclonal antibody diluted 1:1000 in Wash Buffer was added. Positive controls were primary anti L1 antibodies and anti V5 monoclonal antibodies against L1 protein and conformationally correct HPV16 respectively. Negative controls were HPV45 L1 and a murine polyomavirus VP1 (MPyV VP1) structural protein against the anti V5 antibody. The plates were incubated at 37^{o}C for one hour, then washed three

times with 50 µl/well Wash Buffer. Fifty µl/well of anti-mouse horseradish peroxidase (HRP) conjugated IgG secondary antibodies diluted 1:5000 in Wash Buffer was added to all wells except where the anti L1 antibody was used. Fifty µl/well of anti-rabbit horseradish peroxidase conjugated IgG secondary antibodies diluted 1:5000 in Wash Buffer was added to wells where the L1 antibody was used. The plates were incubated at 37^{o}C for one hour. After incubation, the plates were washed three times with 50 µl/well Wash Buffer and 50 µl/well of TURBO 3,3' ,5'5-Tetramethylbenzidine (TMB) substrate (Thermo Scientific, Waltham, MA) was added. After five minutes the wells turned sufficiently blue and 50 µl/well 1M sulfuric acid was added to stop the reaction. Absorbance values were read using an ELx808 absorbance reader (Bio Tek, Winooski, VT) at a wavelength of 450 nm.

2.4 Western Blots

HPV45 L1, HPV16 L1 and MPyV VP1 at a concentration of 0.1625 µg/lane was run on a 10% SDS-PAGE (150 V, 500 mA, 75 minutes). HPV16 L1 and MPyV VP1, BLR cell lysate (BLR) and uninduced cell lysate (BLR cells + HPV45 L1 plasmid) were run to test for cross-reactivity. The gel was washed for ten minutes in Transfer Buffer (250 mM Tris, 2 M glycine), then transferred to a nitrocellulose membrane using a Hoefer IE70 Semi-dry Transfer Unit (Amersham Biosciences, Madison , WI) set at 15 V, 500 mA for 45 minutes. The membrane was blocked overnight in Block Buffer at 4^{o}C or for one hour at room temperature (TBST; 5% milk in Tris-buffered Saline, Tween20).

After blocking, the membrane was washed three times for 5 minutes each with Tris-buffered Saline, Tween20 (TBST). A primary antibody solution consisting of one HPV45 L1 monoclonal antibody diluted 1:1000 in TBST was added to the membrane and rocked at room

temperature for one hour. The membrane was then washed three times for 5 minutes each with TBST, and a secondary antibody solution consisting of goat-α-mouse-alkaline phosphatase antibody diluted 1:5000 in TBST was added. After rocking for an hour at room temperature, the membrane was washed three times for five minutes each with TBST and 20ml of developer consisting of BCIP (5-bromo-4-chloro-3'-inolyphosphate-p-toluidine) and NBT (nitro-blue tetrazolium chloride) in a 1X alkaline phosphatase (AP) developing solution (0.4 M Tris, 1 M NaCl, 0.2 M $MgCl_2$) was added and the blot was left to develop for 5 minutes. The membrane was rinsed in water, and allowed to air dry.

2.5 Pseudovirus Neutralization Assays

293T augmented cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; supplemented with 10% Fetal bovine serum (FBS), 1% Antimicrobial-Antimycotic, 1% MEM, 1% Glutamax) with routine passaging at 80-90% confluency. After two passages, the cells were washed with PBS, trypsinized and counted. They were then centrifuged at 2000 x g for 5 minutes and resuspended in DMEM minus phenol red (DMEM-PR) with a final concentration of $3x10^5$ cells/ml. One hundred µl/well of this suspension was added to the internal wells of a Greiner CellStar 96-well cell culture plate. One hundred fifty µl/well DMEM was added to the exterior wells to prevent evaporation from interior wells. The plate was covered and incubated for 2 hours at 37^{o}C. In a round-bottom 96 well plate, six serial dilutions (1:80, 1:320, 1:1280, 1:5120, 1:20480, 1:81920) of HPV45 L1 monoclonal antibodies in supplemented DMEM-PR were added to a final volume of 50 µl/well for the lowest dilution and 40 µl/well for all other dilutions.

In a new round-bottom 96 well-plate, HPV45 pseudovirus (containing a Secreted Embryonic Alkaline Phosphatase (SEAP) reporter plasmid) at a dilution of 1:250 in DMEM-PR

was added to a final volume of 120 µl/well using siliconized pipet tips. Thirty µl/well from the monoclonal antibody dilution plate was added to the pseudovirus plate using siliconized tips. Anti Bovine Papillomavirus (BPV) antibody diluted 1:50 in DMEM-PR was used as a negative neutralization control. 1 mg/ml heparin was used as a positive neutralization control. The background control consisted of wells containing only 293TT cells in DMEM-PR. The pseudovirus/antibody mix was left for one hour on ice, then 100 µl/well of the pseudovirus/antibody mix was added to the plated 293TT cells and the plate was incubated at 37ºC for 72 hours.

After incubating for 72 hours, 50 µl of cell media per well was removed and placed in a new round-bottom 96 well plate and centrifuged for 5 minutes at 1000 x g. In a Costar white 96-well optiplate, 45 µl/well of 1x Dilution Buffer (Great Escape Chemiluminescence Detection Kit 2.0) and 15 µl/well of clarified supernatant was added. The optiplate was sealed and wrapped in coverfoil, then incubated at 65ºC for 30 minutes. After incubation, the optiplate was chilled for 3 minutes on ice, then brought to room temperature. Sixty µl/well chemiluminescence substrate (4-methylumbelliferyl phosphate, MUP) from the detection kit was added and allowed to incubate for one hour at room temperature. After the hour, the optiplate was read for chemiluminescence values in a Synergy 2 Multi-mode plate reader (BioTek, Winooski, VT).

3. Results

The results reported below were obtained in the Garcea Lab at the University of Colorado, Boulder. Experiments characterizing HPV45 L1 monoclonals were conducted from October, 2015 through February, 2016. Because the HPV45 L1 monoclonal antibodies made by NeoClone were found to cross react with a contaminant after testing with ELISA and Western blots, the monoclonal antibodies were not tested for reactivity in a pseudovirus neutralization assay. Results for the pseudovirus neutralization assay were obtained using sera from mice injected with purified HPV45 L1 capsomeres as a proof of concept.

3.1 ELISAs

All 42 monoclonal antibodies were tested for reactivity in ELISAs (Fig. 1). Reactivity was assessed using absorbance values for the wells containing each monoclonal with higher absorbance values reflecting higher reactivity of the monoclonal antibody to the HPV45 L1 protein. Positive controls were anti L1 antibodies which bound to HPV45 L1 and anti V5 antibodies which bound to HPV16 L1. Negative controls were anti V5 antibodies which did not recognize HPV45 L1 or MPyV VP1. Absorbance values ranged from 0 relative absorbance units (RAU) to 2.744 RAU. 14 of the monoclonal antibodies were reactive in the ELISA with absorbances above 0.5 RAU. 4 antibodies were strongly reactive with an absorbance above 2 RAU.

3.2 Western Blots

All 42 of the HPV45 L1 monoclonal antibodies were tested for reactivity in Westerns (Fig. 2). Visible banding on the Western blots was used as an indication that the monoclonal

antibodies identified and bound to HPV45 L1 on the blot. This banding was the result of a reaction between the developer and the alkaline phosphatase (AP) enzyme on the secondary antibody. In this reaction, the alkaline phosphatase enzyme hydrolyzes BCIP in the developer to a form that is oxidized by NBT, creating a dark blue diformazan precipitate visible on the membrane. If the monoclonal antibody is reactive to proteins on the membrane, then the antibody binds to those proteins. The secondary anti mouse-AP antibodies are then able to bind to the primary monoclonal antibody, providing AP for the developer reagents to react with. If the monoclonal antibody is not reactive to proteins present on the blot, then there is no initial binding of primary antibody and no subsequent binding of the secondary antibody leading to no visible banding on the blot.

Prior to initiation of the Western blots, the purified HPV45 L1 was run on a coomassie gel to determine the size of the L1 monomer (Fig. 3A). In the gel, as with previous studies on HPV L1, the protein was visualized in a band at ~55 kDa. In the first 14 Western blots, however, there were no bands visualized at 55 kDa. Instead, the monoclonal antibodies reacted with proteins at ~100 kDa and <55 kDa, neither of which had visible bands on the coomassie. To determine if the monoclonals were contaminated with proteins from the BLR *E. coli* cells used to express the HPV45 L1 protein, the six monoclonal antibodies that were found to be reactive in the initial Western blots and all subsequent monoclonal antibodies were tested for reactivity in Western blots that also contained BLR cell lysate (BLR) and uninduced cell lysate (BLR cells + HPV45 L1 plasmid) (BLR+plasmid) (Fig. 3B). In addition, positive controls of anti L1 antibody and the Gardasil sera antibody (reactive toward HPV45 L1 and HPV16 L1) were tested for reactivity against HPV45 L1, MPyV VP1, HPV16 L1, BLR and 45un. As expected, the anti L1 reacted with the L1 monomer at ~55 kDa in the HPV45 L1 lane, the HPV16 L1 lane, the BLR

lysate and the BLR+plasmid (Fig 3B). The more specific Gardasil antibody reacted with the L1 protein in both the HPV45 lane and the HPV16 lane (Fig 3c).

3.3 Pseudovirus Neutralization Assay

Because the monoclonal antibodies were found to be cross reactive to a contaminant in the Western blots, none of the antibodies from NeoClone were tested in the pseudovirus neutralization assay. Instead, the assay was performed as a proof of concept using sera from mice injected with purified HPV45 L1 capsomeres with alum. Sera was taken from the mice at day 21 and day 35 day after initial injection of a low dose (LD) or a high dose (HD) of HPV45 L1 capsomeres. Reactivity for the sera was determined by the difference between the anti BPV value and each antibody value, divided by the difference between the anti BPV value and the heparin value. Percent neutralization, 100 x the fractional neutralization value, was determined and plotted for the six serial dilutions of the antibodies (Figure 4).

The HD sera obtained after 35 days had the highest percent neutralization. The HD sera obtained after 21 days and the LD sera obtained after 35 days had similar percent neutralizations that were lower than the HD, day 35 sera. The lowest percent neutralization was seen in the LD sera obtained after 21 days.

4. Discussion

The results from the Western Blots provide strong evidence that these monoclonal antibodies were produced to a contaminant in the purified HPV45 L1 protein. The source of this contaminant is unknown; it could have occurred during protein purification or production of the monoclonal hybridomas by NeoClone. Regardless of the source, the contamination dramatically limited the analysis and use of these 42 antibodies. Although none of the antibodies are candidates for use in further ELISAs, Western blots or Pseudovirus neutralization assays, the data from this research has provided a proof of concept for the characterization of monoclonal antibodies for HPV45 L1.

ELISA testing was used to determine which monoclonals were reactive to conformationally intact HPV45 L1 bound to a plate. In the ELISAs, four monoclonal antibodies (1H6D7, 1H6B9, 1H7C10 and 1H7D12) were classified as strongly reactive with absorbance's greater than 2 RAU. Six monoclonal antibodies had an absorbance between 1 RAU and 2 RAU and were determined to be moderately reactive. Five antibodies had an absorbance between 0.5 RAU and 1 RAU and were classified as weakly reactive.

Western blotting was used to determine which antibodies were reactive to denatured HPV45 L1 protein. A coomassie gel run prior to initiation of the Western blots showed that the HPV45 L1 protein is ~55 kDa. No other bands appeared in this coomassie gel, indicating that the initial purified HPV45 L1 protein sent to NeoClone for antibody production was uncontaminated. None of the 40 monoclonal antibodies tested reacted with the HPV45 L1 protein at ~55 kDa. Three monoclonal antibodies (7B1H8, 7B1E8 and 2B8B11) reacted with an unknown protein at ~100 kDa. A band at ~100 kDa was also present in the BLR lysate and BLR+plasmid for 2 of the monoclonals. In addition, 4 of the monoclonal antibodies (4E6H11,

4E6G12, 5H5C11 and 5H5D8) reacted with an unknown protein at <55 kDa. A band at <55 kDa was also present in the BLR lysate and BLR+plasmid in 17 of the Western blots. Interestingly, the ~100 kDa and the <55 kDa bands were not visible in the coomassie gel. The lack of reactivity with denatured HPV45 L1 protein indicates that the antibodies were reacting to cross contaminants instead of HPV45 L1 protein. Because the Western results revealed monoclonal antibody reactivity with contaminants, we can extrapolate that the monoclonal antibodies found to be reactive in the ELISAs were also reacting to contaminants, not to HPV45 L1.

An interesting finding worth noting is that the monoclonals found to be strongly or moderately reactive in the ELISAs were not strongly reactive in the Western blots. Only two antibodies (4E8B12 and 7B1H8) that were weakly reactive in the ELISA also showed reactivity in the Westerns. This difference is due to the structural integrity of the proteins in the assays. In ELISA, the protein's natural conformation is preserve whereas the proteins used in Western blots are denatured by SDS in order to migrate through the acrylamide gel effectively. These differences in the structure of the proteins cause different epitopes to be exposed that are not normally recognized by the same antibody. This difference shows the necessity for a characterization project; the antibodies that are useful in ELISAs might not be useful in Western blots.

The pseudovirus neutralization assay is another useful test in monoclonal antibody characterization because it evaluates how effectively antibodies recognize a conformationally accurate HPV45 L1 viral capsid in solution. In this assay, monoclonal antibodies are mixed with pseudoviruses containing SEAP plasmids and incubated with 293T augmented cells for 72 hours. During this incubation, any pseudoviruses that are not neutralized by the antibodies are able to infect the cells. The secreted alkaline phosphatase (SEAP) plasmids includes an SV40 origin

sequence so its expression is initiated by SV40 Large T Antigen in the 293T cells. The secretion of SEAP from the cells into the media is measured using a chemiluminescent reporter system. Antibodies with a strong affinity for the pseudovirus will neutralize and prevent pseudovirus entry, therefore decreasing the SEAP expressed from the cells. Titrating each monoclonal shows the overall efficacy of each antibody: antibodies capable of high neutralization at low concentrations are more effective at recognizing fully formed virus capsids, making them ideal antibodies for use in vaccine development. Although the pseudovirus neutralization assay performed in this study did not test any monoclonal antibodies, its success proved the importance of this assay in future characterization studies of monoclonal antibodies.

5. Conclusion

The HPV vaccines that are currently on the market are not accessible in many parts of the world where they are needed the most. The high cost of producing vaccines in eukaryotic cell systems and the need for cold chain transport severely limit the use of current vaccines in developing countries. Second generation vaccines produced with lyophilized HPV L1 capsomeres are less expensive and thermodynamically stable without loss of efficacy. In order to control the quality of these vaccines and produce them on a large scale, monoclonal antibodies specific to each HPV serotype in the vaccine are necessary. Out of the four HPV serotypes in the second generation HPV vaccine that the Garcea lab is developing, only HPV45 lacked unique monoclonal antibodies. The focus of this project was to characterize monoclonal antibodies developed by NeoClone toward purified HPV45 L1protein in order to determine antibodies that could be used in ELISAs, Western blots and pseudovirus neutralization assays, three assays that are crucial to the vaccine development and production process.

Although the monoclonal antibodies were made to a cross contaminant instead of HPV45 L1, this project serves as a proof of concept for the characterization of HPV45 L1 monoclonal antibodies. The characterization of these antibodies in future projects will allow for better quality control and faster production of a second generation tetravalent HPV vaccine that is low cost and thermodynamically stable.

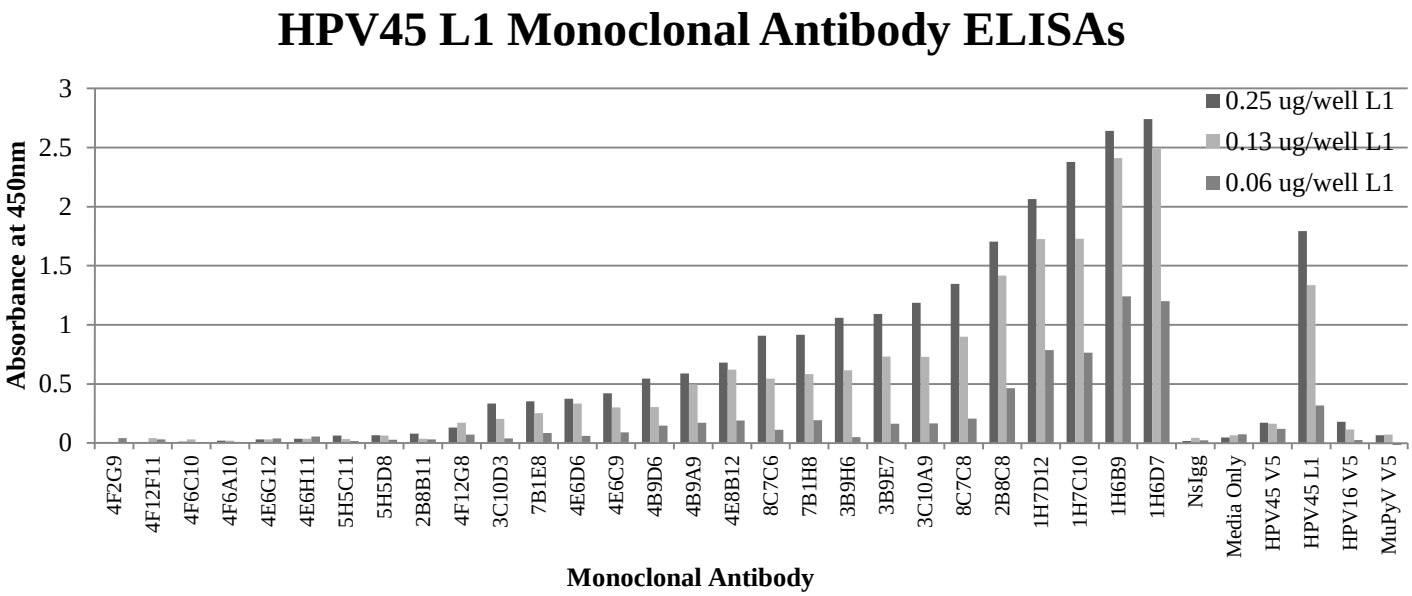

Fig. 1. **HPV45 L1 monoclonal antibody ELISA absorbance data**. Each monoclonal antibody was run with three dilutions of HPV45 L1 protein: 0.25 ug/well, 0.13 ug/well, 0.06 ug/well. The plate reader was set at 450nm wavelength end point absorbance.

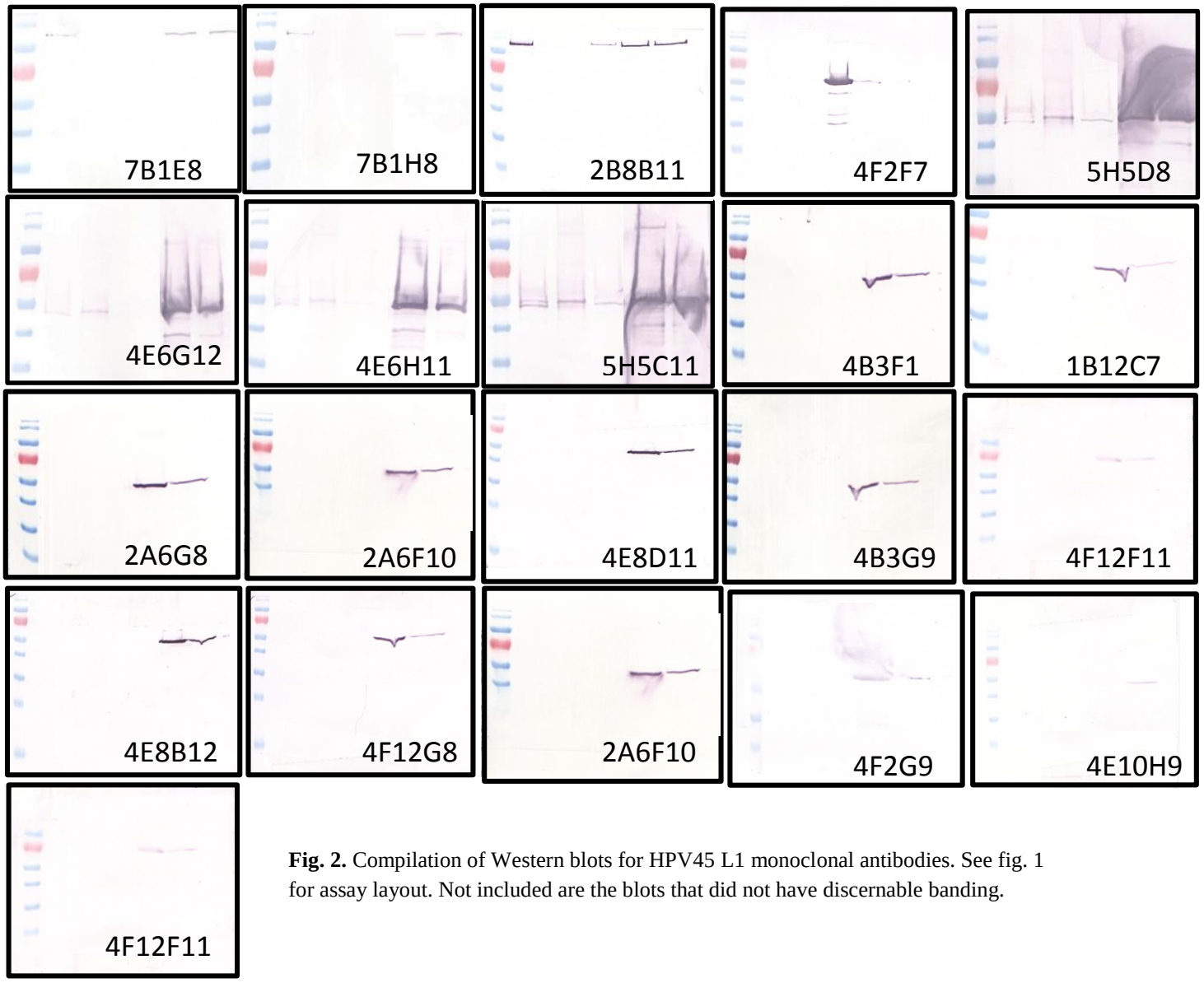

Fig. 2. Compilation of Western blots for HPV45 L1 monoclonal antibodies. See fig. 1 for assay layout. Not included are the blots that did not have discernable banding.

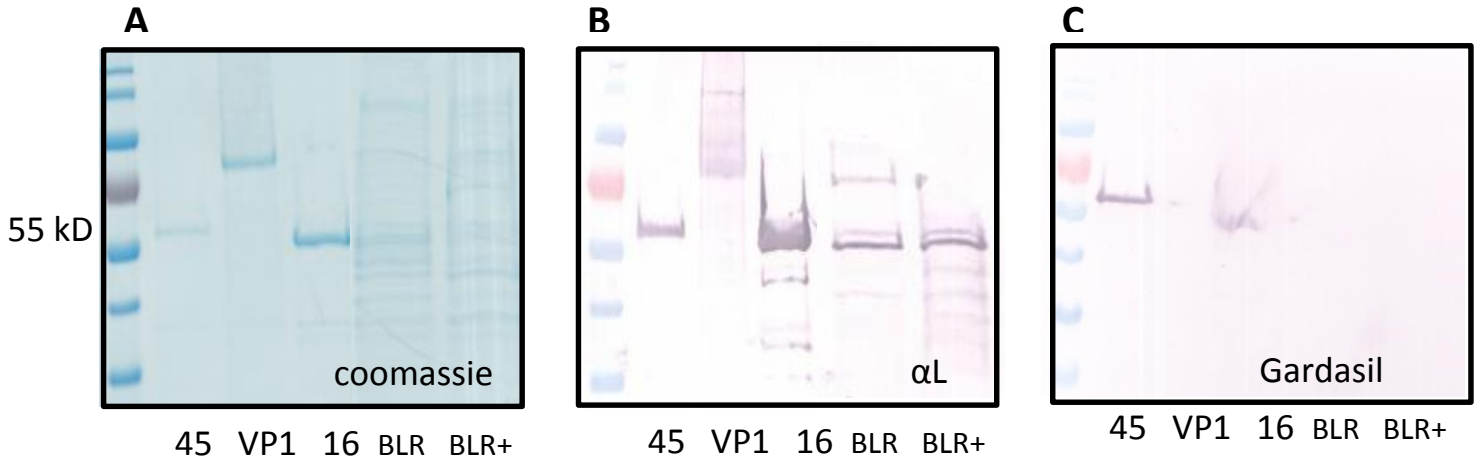

Fig. 3. A. Coomassie gel with the five proteins evaluated in the Western blots. **B**. Layout of HPV45 monoclonal antibody Western blot using anti L1 as the primary antibody. The 55 kD band in lane one depicts the size of the HPV45 L1 protein. **C.** Western blot using the Gardasil antibody which identifies HPV45 L1 and HPV16 L1.

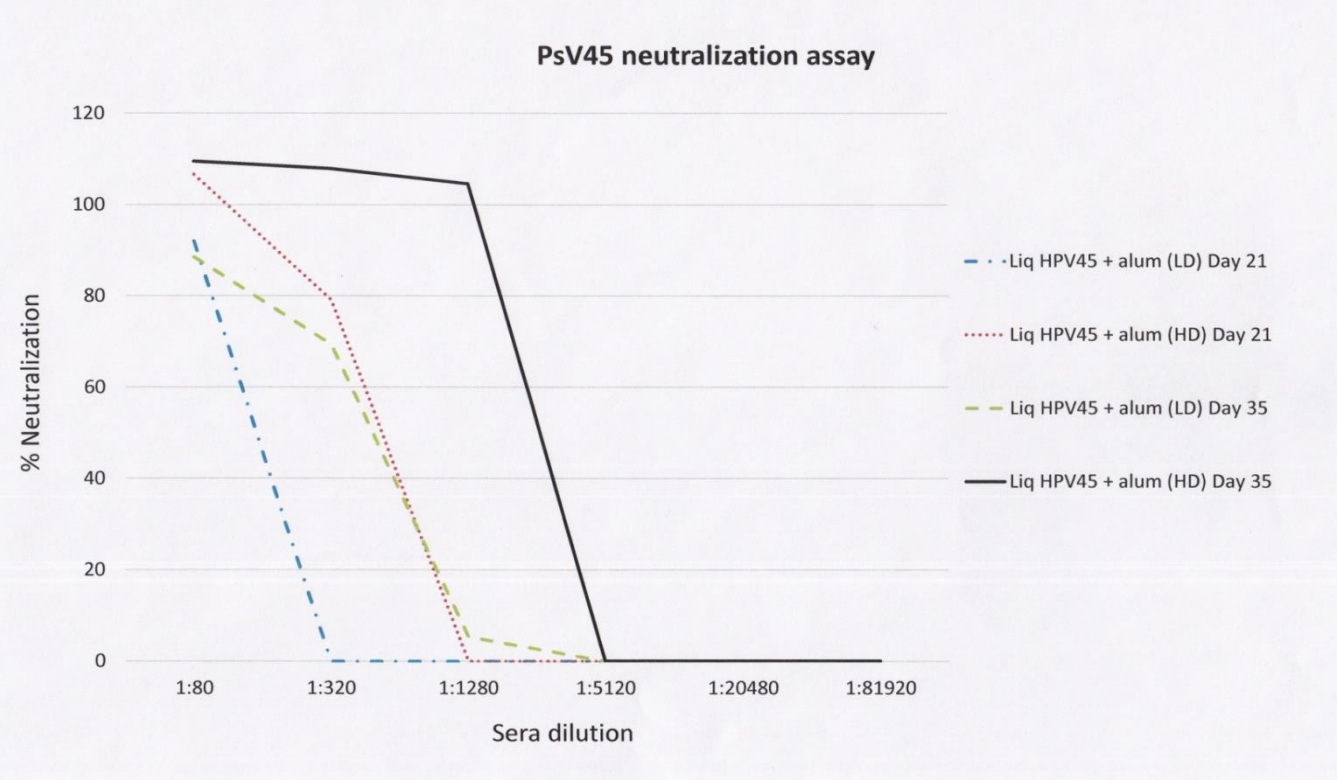

Fig. 4. Proof of concept pseudovirus neutralization assay data. Four samples of sera obtained from mice injected with liquid HPV45 L1 protein were run over six serial dilutions. Percent neutralization values were generated by comparing luminescence values of the antibody wells to a positively neutralizing heparin control.

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