UNIVERSITY OF CALIFORNIA
SANTA CRUZ

Mechanistic and Structural Studies on Human Astrovirus Serotype 8

A thesis submitted in partial satisfaction of the requirements for the degree of

BACHELOR OF SCIENCE
In
MOLECULAR, CELL, AND DEVELOPMENTAL BIOLOGY

Thesis Submitted By:
Carolina Cuellar
April 2018
The Senior Thesis of Carolina Cuellar is approved by:

Rebecca M. DuBois on April 3, 2018

Acknowledgments

Thanks to the entire DuBois lab for their positivity, guidance, and friendliness throughout my time in the lab. Thank you so much to my PI, Dr. Rebecca DuBois, for all of her help, support, availability, and positive disposition to help and encourage me through my undergraduate career both personally and academically. You are such an inspiration.Your instruction and mentorship has been invaluable to my growth and confidence as a researcher. Special thank you to my graduate student mentor, Lena Meyer, for her guidance, instruction, and unwavering support throughout my entire time in the lab, you are a fantastic mentor and role model.

Thanks to the STEM Diversity Programs, especially the MARC program, for making it possible for me to work in my lab with their support. And special thanks to Yulianna Ortega, Xingci Situ, and Dr. Alan Zahler for keeping these invaluable programs running and always offering advice, support, and food when needed.

Thank you to Dr. Zia Isola, Jessica Arozqueta Basurto, Carmen Ma, Vanessa Venner, Yulia Zybina and Trevor Buck for being excellent editors and friends throughout the entire process of writing and editing my thesis.

Lastly, thank you to UCSC for my four years of education and the many opportunities I have had while being a student here.

Table of Contents

Mechanistic and Structural Studies on Human Astrovirus Serotype 8

Carolina Cuellar, Lena Meyer, Dr. Rebecca Dubois

Abstract

The human astrovirus is a primary cause of acute non-bacterial gastroenteritis in immunocompromised individuals, such as the elderly and infants. By the age of nine, an estimated 90% of children will have developed antibodies against the most common strain. Causing symptoms like vomiting, fever, diarrhea, and, more recently, meningitis, it is a relevant but poorly understood virus. The capsid of the virus contains globular proteins called spikes that are thought to be primarily responsible for attachment to the unknown host cell receptor. Although there are no vaccines or antiviral therapeutics to treat infection, current efforts in regards to viral treatment include identifying the unknown host cell receptor binding site and understanding the mechanism by which the spike infects human cells through identification and production of neutralizing monoclonal antibodies and studying their interaction with spike. Structural studies of HAstv spike and antibody binding are essential for identification of functional epitopes that are involved in receptor host cell attachment as well as possible therapeutic targets. The Human Astrovirus serotype 8 (HAstV8) spike was expressed and purified in order to perform mechanistic and structural studies with monoclonal antibody 2D9, which binds to a novel epitope not previously studied. Additionally, two engineered forms of the neutralizing antibody 2D9 were produced -- a humanized monoclonal antibody and a single chain variable fragment -- in order to increase the chance of crystallization. In conjunction to a solved structure of this complex, mechanistic studies done by a collaborator will allow for functional classification of epitopes and insights to viral biology. Future efforts will include binding studies of 2D9 with other strains in order to test for cross-reactivity for the purpose of vaccine design, and X-ray crystallography determination of the structure of 2D9-bound HAstV8 spike to understand binding epitopes and their functional role in neutralizing the virus. The goal of this project is to advance knowledge on the infective mechanism of a prevalent strain of the virus, HAstV8.

Introduction

Background and Significance

Overview of Viruses and Antibodies

Viruses are microscopic pathogens that infect a variety of organisms. In humans, symptoms are highly variable depending on the virus, but the mechanism of action is the same: overtake host cell machinery to multiply. Viruses are relatively simple infective agents that require a living host cell to survive and proliferate. A human's defense against viruses often comes in the form of an antibody, which are proteins produced by the adaptive immune system to protect against further infection.

Antibodies are found in a variety of isomers (IgG, IgD, IgA, IgM), which are distinguished by their type of heavy chain, with IgG isomers being the most abundantly found in humans (Misha Rahman, 2018). Although antibodies come in various isomers, for the purpose of this project, we solely refer to the IgG isomer. An IgG antibody contains various functional regions, including a heavy chain, a light chain, a fragment crystallizable region, a hinge region, a variable region, and twelve complementarity determining region (CDR) loops. While these regions serve various purposes, such as immunogenicity, the variable region is the antigenic site on the antibody that actively binds to pathogenic particles to fight infection. Although many antibodies are produced upon infection, only some will be neutralizing, or capable of completely stopping viral infection. These neutralizing antibodies are useful when it comes to identification of important infectious epitopes on viral proteins. Often the regions that are targeted by antibodies are necessary for infectivity or block important infective epitopes. This project hopes

to capitalize on this relationship in order to better understand the human astrovirus, specifically the capsid spike protein and its role in infection.

The Human Astrovirus and Its Significance

The human astrovirus (HAstV) is a cause of gastroenteritis in immunocompromised individuals, such as the elderly and infants. Eight distinct classical strains of the virus numbered 1-8 account for 8-9% of non-bacterial acute gastroenteritis in children worldwide. In addition to the virus' high variability, there are two other categories of non-classical strains that affect humans, HAstV-MLB and HAstV-VA/HMO, whose full pathogenic role is not yet fully understood (Bosch et. al., 2014). In typical strains, astrovirus causes symptoms like vomiting, fever, and diarrhea. Despite the fact that HAstV is a prevalent pathogen, with an estimated 90% of children having developed antibodies against the most common strain of the virus by the age of nine, it is still poorly understood (Elliott et. al., 2007). There are currently no vaccines or therapies, leaving infected individuals with no options in terms of treatment or prevention. This is due in part to the fact that the virus is highly variable but also largely attributed to the current gaps in knowledge in regards to the process of viral infection. The maturation of HAstV is dependent upon a series of proteolytic cleavages in the highly acidic environment of the stomach, particularly by trypsin, which allows infection of the gut epithelial cells. However, the particular host cell surface receptor by which the virus is taken into cells remains unknown (Carlos F. Arias, et al., 2017). This important insight into the viral life cycle is an important consideration when designing or creating antiviral therapeutics or vaccines.

Structurally, the virus resembles a five or six pointed star, due to its capsid protein creating a T=3 icosahedral symmetry throughout (**Figure 1)**. In addition to this shape, there are

globular spikes, formed by dimeric capsid spike proteins located on the surface, which are suspected to be required for host cell attachment to an unknown cell surface receptor. In addition, the spike protein elicits and binds HAstV-neutralizing antibodies. The goal of this project is to advance knowledge on the infective mechanism of the virus and develop possible therapies for infection by studying the structure and protein-antibody interactions of the capsid spike of a prevalent serotype of the virus, HAstV8.

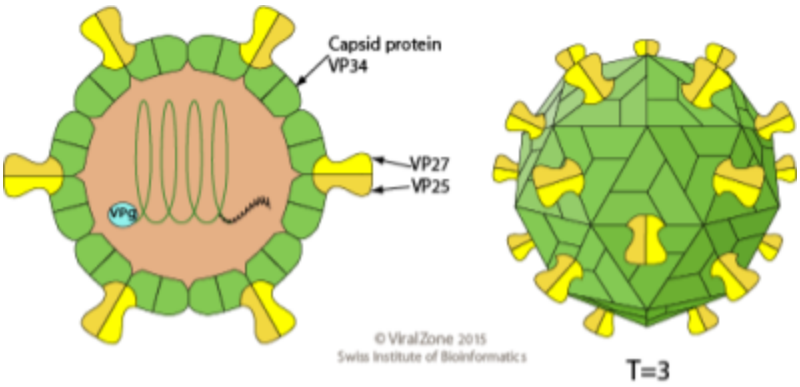

Figure 1. Illustration of Icosahedral structure of the Astrovirus due to capsid protein (green) and globular capsid spike located on surface (yellow). Adapted from Swiss Institute of Bioninformatics.

Collaborators of this project in the Carlos Arias lab at the Universidad Nacional Autónoma de México immunized mice with various serotypes of the capsid spike in order to produce IgG antibodies that neutralize viral infection. Once isolated, these monoclonal antibodies were used to generate escape mutants. Escape mutants are mutated variants of pathogens which result from these pathogens altering their genotype and/or phenotype in order to avoid attacks by the host immune system. Escape mutants of HAstV spike, in which an antibody no longer neutralizes the virus, are obtained by adding enough antibody to cause selective stress in order to observe where mutations occur in the spike to evade neutralization. Studies of escape mutants provide possible locations of neutralizing epitopes as well as regions of potential infectious importance that can be further studied for mechanistic function and targeted for vaccines and therapeutics.

Epitopes targeted by neutralizing antibodies are indicative of areas of functional importance. In the case of HAstV spike, this importance may lie in viral attachment to the host cell receptor or internalization of the viral particle. Additionally, identification of these epitopes and their function is vital to the guidance of vaccine and antiviral therapeutic design. An antibody against HAstV8 spike has been isolated. This escape mutant of HAstV8 spike contains a mutation in residues 598-599 (**Figure 2)**, an area that differs from the location of antibody binding seen in the crystal structure done by a previous lab member, Walter Bogdanoff (Bogdanoff et. al., 2016). The mouse monoclonal antibody (mAb), 2D9, is believed to target this novel region and thus is a subject of study in this project. Localizing the neutralization of HAstv8 spike by 2D9 is key to identifying a possible functional neutralizing epitope. Through the implementation of protein engineering and X-ray crystallography, the intention is to better understand how this antibody binds and neutralizes HAstV8 spike, and why this novel region is targeted.

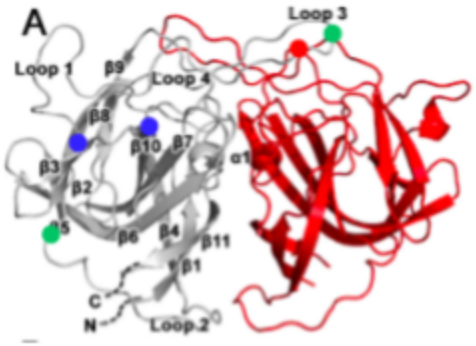

Figure 2. Mutation β10, denoted by a blue dot, can be seen away from loop one and is under investigation in this project.

Experimental Design

The experimental objective of this project is to crystallize the HAstV8 spike-2D9 complex using X-ray crystallography. This crystallized structure is valuable for insight into the role of the capsid spike in infectivity and as a target for neutralization. The HAstV8-specific monoclonal antibody 2D9 was engineered into two forms: a humanized monoclonal antibody (mAb) and a single chain variable fragment (scFv) (**Figure 3**). Humanization of mAb involves

insertion of the antigen binding site, known as the variable regions, into a "human backbone," or the constant region of an antibody found in humans. The engineering of an scFv involves solely expressing the aforementioned variable regions and connecting the heavy variable region and the light variable region by a flexible sequence of amino acids known as a linker. Due to the unpredictability of the crystallization of the complex, having multiple engineered forms of the

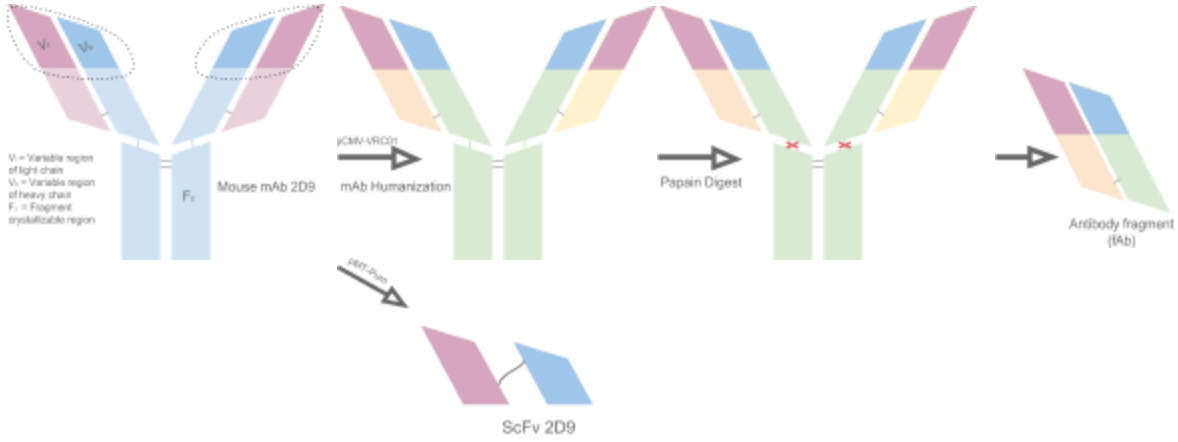

Figure 3. Schematic illustrates the identity of engineered forms of the monoclonal antibody in reference to their original form.

antibody allow for a higher chance of crystallization of these complexes. Additionally, the humanization of the mAb allows for binding studies to be done in human cells because this form will avoid the immunogenic response a mouse antibody would normally elicit. Western Blot and ELISA will be used to ensure specificity in binding as well as to ensure the adequate expression and purity of protein to be used in setting up crystal trials for X-ray crystallography. Additional cleavage of extraneous features by papain (to cleave the Fc fragment from the Fab fragment) and thrombin (to cleave the Strep and His purification tags from the scFv and spike protein, respectively) should allow for clearer binding visibility of the variable region on the antibody to the spike when crystals are imaged. By visualizing this HAstV8 spike-2D9 complex, there is

potential to find variable and conserved regions of this protein, as well as indications of sites of infectivity.

If crystallization of this complex is successful, it will provide new insights into this viral protein. By identifying possible sites of conservation as well as variability, there will be a greater grasp of the functional areas of the capsid spike as well as areas of cross-reactivity for use in vaccine design. Since neutralizing immunogen binding has not been studied on this particular region of the capsid spike, investigating and understanding this region will provide a more comprehensive view on the infectivity of the virus as a whole and offer new regions to explore in the realm of possible therapeutics and drug targets for all serotypes of the human astrovirus.

Materials and Methods

Note: Cloning of these constructs was done by previous lab members or graduate student mentor, Lena Meyer.

Table 1: Expression constructs

Gene	Plasmid	Relevant plasmid features	Expression cell type
Human Astrovirus Serotype 8 Capsid spike (HAstV8 spike)	pET52b	Ampicillin resistance, inducible expression via lac Z operon, Histidine tag	*E. coli*: BL21 (DE3)
Humanized Monoclonal Antibody 2D9 (mAb 2D9)	pCMV_VRC01	Secretion signal, Strep tag	Mammalian: Human Embryonic Kidney Cells 293
Single Chain Variable Fragment 2D9 (scFv 2D9)	pMT_Puro_Bip	Secretion signal, puromycin selection gene, inducible expression, Strep tag	Insect: Schneider 2

Production of Human Astrovirus Serotype 8 (HAstV8) Capsid Spike

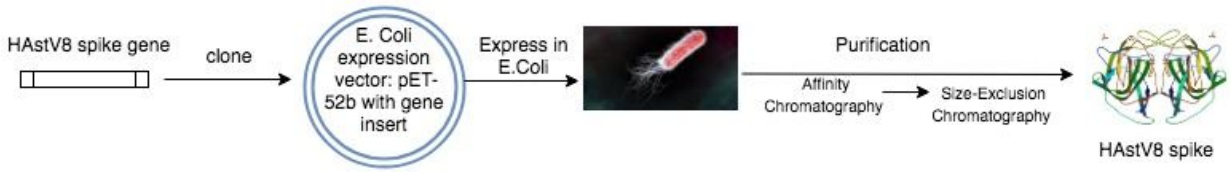

Figure 4. General method of expression of HAstV8 spike.

Expression of HAstV8 Spike

Gene construct pET52b_HAstV8_spike was expressed in *E. coli* strain BL21(DE3) by starting a 60 mL seed culture the previous day. The next morning, 10 mL seed culture was added to each 2800 mL Pyrex flask containing 1 L LB Media and 1 mL 1000X ampicillin. Flasks were

then left in incubator at 37˚C with 250 rpm until ocular density reached 0.6-0.8 cells/mL. Expression was induced with 1 mL 1000X Isopropyl β-D-1-thiogalactopyranoside. Once induced, the culture was left overnight at 18˚C with 250 rpm in incubator. Verification of protein expression via sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) gel and protein purification proceeded on the following day.

SDS-PAGE on HAstV8 Spike Expression

1X MES sodium dodecyl sulfate (MES-SDS) buffer was made by adding 25 mL NuPage 20X MES-SDS buffer to 475 mL of water. The buffer was added to an electrophoresis box containing a pre-prepared 4-12% polyacrylamide gel (PAGE) loaded with 25 uL pre- and post-induction samples that had been previously boiled for approximately fifteen minutes with 5X SDS. The gel was run for 40 minutes at 200V, then washed and stained with coomassie stain. The gel was imaged with an ImageQuant LAS4000 imager.

Purification of Human Astrovirus Serotype 8 spike

Table 2: Buffers prepared for the HAstV8 spike purification.

Buffer A (Wash Buffer)	20 mM Tris pH8, 500 mM NaCl, 20 mM Imidazole, 1 mM Dithiothreitol
Lysis Buffer	20 mM Tris pH8, 500 mM NaCl, 20 mM Imidazole, 1 mM Dithiothreitol, 1 mM protease inhibitor cocktail, 5 uL benzonase
Buffer B (Elution Buffer)	20 mM Tris pH8, 500 mM NaCl, 500 mM Imidazole, 1 mM Dithiothreitol

Dialysis Buffer	10 mM Tris pH 8, 150 mM NaCl, 1 mM Dithiothreitol
Sizing Buffer	10 mM Tris pH 8, 150 mM NaCl

HAstV8 spike was purified by affinity chromatography through the use of TALON metal affinity resin followed by size exclusion chromatography through the use of the ÄKTA chromatography system. Cell culture was pelleted in an J20 rotor at 8,000 rcf for thirty five minutes, after which the pellet was resuspended in Lysis Buffer and then sonicated to break open cells. Once the lysate was pelleted by centrifugation (F250 rotor at 14000 rcf for 35 minutes), the supernatant was 0.22 μm filtered and added to 6 mL of TALON resin and rotated at 4 °C for one hour. Lysate was then removed and TALON resin was washed (centrifuged at 3 rcf for 3 minutes then resuspended) 7-10 times with cold Buffer A. Finally, sample was eluted with 11 mL of cold Buffer B. The elution was dialyzed into Dialysis Buffer at 4 °C overnight, then purified by sizing on the ÄKTA instrument using the S75 16.600 column and Sizing Buffer. Elutions were collected in a fraction collector. Because of the amount of protein compared to column capacity, two separate injections were done.

SDS-PAGE gel on HAstV8 Spike Purification

The samples used for the gel were fractions of the elutions from sizing chromatography. Preparation of the PAGE gel, MES-SDS buffer, and samples was the same as the previous SDS-PAGE done on the expression.

Production of Humanized mAb 2D9 via Transient Transfection

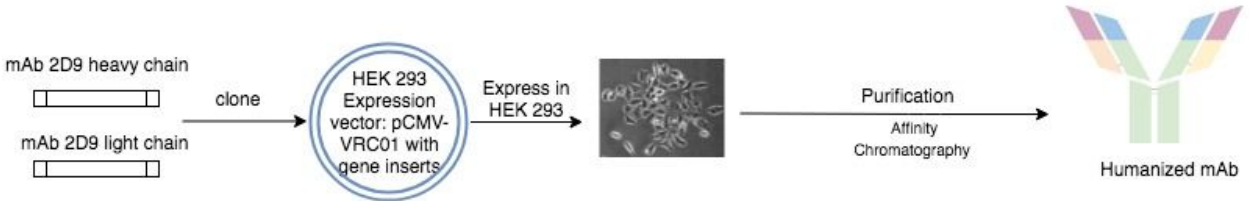

Figure 5. General method of expression of humanized mAb 2D9.

Transient Transfection of HEK 293 Cells with Humanized mAb 2D9

Ten million Human Embryonic Cells (HEK293) in 10 mL of HEK 293 cell media (Thermofisher - FreeStyle 293 media with 10 mL 100X pen/strep) were plated in a treated 75cm^2 flask. Then 2 µg of pCMV_VRC01_2D9_heavy chain DNA and pCMV_VRC01_2D9_light chain DNA were combined in a single eppendorf tube and 32 µL of Enhancer were added. After a five minute incubation at room temperature, a mixture of 740 µL Buffer EC and 60 µL Effectene were added to the DNA and Enhancer mix. This transfection complex was then added dropwise onto the top of the HEK-293 cells. The flask was rocked back and forth to ensure an even distribution and incubated at 37°C and 5% CO2 with humidity for 7 days.

Purification of Test Expression of Humanized mAb 2D9

Table 3: Buffers prepared for humanized mAb 2D9 purification.

Buffer PBS	100 mM phosphate buffer pH 7.2, 150 mM NaCl
Protein A Elution Buffer: EB	100 mM Glycine-HCl pH 2.8
Neutralization Buffer: NB	1.9 M Tris pH 8

The 10 mL of HEK 293 cells that were transiently transfected with humanized mAb 2D9 were transferred to a 15 mL conical tube and centrifuged at 3000g for 5 minutes. The supernatant was then 0.22 µm filtered. The filtered supernatant was then added to 100 µL of protein A agarose beads that were pre-washed with PBS. The mixture was incubated at room temperature for 30 minutes with rotation. After incubation, the mixture was centrifuged at 1000g for 1 minute, and all but 500 µL of media were pipetted out. The beads were resuspended in the 500 µL of remaining media. The bead/media mixture was transferred to a column sitting on a 2 mL eppendorf tube and centrifuged at 1000g for 1 minute. The beads were then washed three times by adding 500 µL PBS and centrifuging at 1000g for 1 minute. The column was placed into a new 2 mL eppendorf tube containing 20 µL of NB. 100 uL of EB were added to the column and then spun at 1000g for 1 minute three times. The elution was concentrated with a Pierce centrifugal concentrator (10kD MWCO) by centrifuging at 14,000g until the final volume was ~30 µL . This elution was then used in an ELISA.

Production of scFv 2D9 via Transient and Stable Transfection

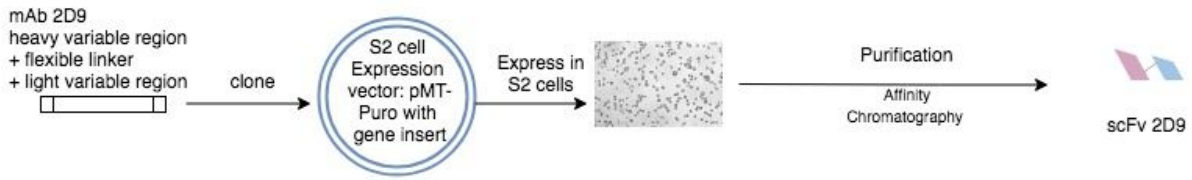

Figure 6. General method of expression of scFv 2D9.

Transfection of S2 Cells with scFv 2D9

The day prior to transfection, five million Schneider 2 (S2) cells were added to 5 mL of serum-containing Schneider's Drosophila media in an untreated 25 cm^2 flask. The following day, 2 µg of spin-filtered pMT_Puro_Bip_scFv_2D9 were combined in a single eppendorf tube and

16 µL of Enhancer were added. After a five minute incubation at room temperature, 20 µL of Effectene Transfection Reagent were added and mixed by pipetting. This mixture was incubated for 15 minutes at room temperature. The cells were washed by transferring cells to a 15 mL conical tube and centrifuging for 10 minutes at 500g. Afterwards, the cells were resuspended in 4 mL of new medium and placed back into the flask. 1 mL of new media was added to the mixture of Effectene Transfection Reagent, DNA, and Enhancer. This mixture was then added dropwise to the cells while rocking back and forth to ensure an even distribution and the cells were left at 28˚C overnight. The following day, the cells were spun down by centrifuging at 500 g for 10 minutes and resuspended in 2 mL of fresh medium and 1 mL of conditioned media (0.22 µm filtered media in which cells had been growing). Then cells were added back to the flask. The following steps differed depending on stable or transient transfection.

For the Transient Transfection

Copper chloride was added to the flask to a final concentration of 500 µM to induce expression. Purification was done five days later.

For the Stable Transfection

25 µL of puromycin were added to the flask for selection, Every 4-5 days for 4 weeks, the cells were spun down and resuspended in fresh media along with the re-addition of puromycin. Cell aliquots were frozen down in order to thaw for future cultures. Once ready to express, copper chloride was added to a final concentration of 500 µM and the purification was done five days later.

Purification of scFv 2D9

Table 4: Buffers prepared for scFv 2D9 purification

Strep-Tactin Binding Buffer	50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA
Strep-Tactin Elution Buffer	25 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM Desthiobiotin

The induced S2 cells were spun down on the centrifuge (400 g for 10 minutes for 4 mL or 6000 g for 10 minutes for 4 L) and the supernatant was 0.22 μm filtered. 1M Tris pH 8.0 was added until the pH was approximately 8.0 and then the supernatant was centrifuged at 6000 g for ten minutes. The supernatant was once again 0.22 μm filtered and added to a Strep-Tactin column (pre-washed with Strep-Tactin Binding Buffer) and let flow through by gravity. The column was then washed with 5x Strep Binding buffer and then eluted with 5x Strep Tactin Elution Buffer. The elutions were collected in a fraction collector. The elutions were then pooled and concentrated and used in a Western Blot, ELISA, and in X-ray crystallography trials.

Anti-Strep Western Blot on scFv 2D9

Table 5: Buffers prepared for anti-Strep Western Blot.

Towbin Transfer Buffer	25mM Tris, 192mM Glycine, 20% Methanol, 0.035% SDS
PBS Buffer	4 mM KH2PO4, pH 7.4, 16 mM Na_2HPO_4, 115 mM NaCl
Blocking Solution	PBS buffer with 3 % BSA and 0.5 % v/v Tween 20

Primary Antibody Solution: HRP-conjugated Strep-Tactin	5 ml PBS buffer with 0.1 % v/v Tween 20, 5 ul HRP-conjugated-Strep-Tactin (1000X)
Wash Buffer	PBS buffer with 0.1 % v/v Tween 20

After running SDS-PAGE as with HAstV8 spike, the gel was placed in Towbin Transfer Buffer and left on the rocker for 15 minutes. Additionally, two filter papers and a blot membrane activated with Methanol were also placed in Towbin Transfer Buffer for 15 minutes. The gel-membrane stack was then assembled by stacking in the following order a filter paper, gel, membrane, and filter paper, and placed into a stainless steel cathode. The assembled stack was dried thoroughly with paper towels until it was barely damp. Then stainless steel cathode was run at 15V for 15 minutes. The Western Blot was performed in accordance to Millipore Sigma SNAP i.d. Protocol. The membrane was placed protein side down in a pre-wet membrane holder and the bubbles were rolled out. The membrane holder was placed in SNAP i.d. holder frame so the protein side faced up and the frame was closed. 30 mL of Blocking Solution was added and then the vacuum was turned on. After the frame was empty, 5 mL of Primary Antibody Solution was added and left to incubate for 10 minutes at room temperature. Following the incubation, the vacuum was turned on and the frame was allowed to dry. Afterwards, the frame was washed four times with 30 mL of Wash Buffer while the vacuum was turned on. Once the frame was empty, the membrane was removed and 1 mL of each bottle of Detection Agent (luminol and stabilized peroxide solution) was placed on the membrane and incubated at room temperature for 3

minutes. The membrane was imaged on the chemiluminescence setting on the ImageQuant LAS4000 imager.

Enzyme-Linked Immunosorbent Assay (ELISA) on scFv 2D9 and HAstV spike 1/8

The day before the ELISA, scFv 2D9 was diluted to 5 µg/mL in 10 mL of buffer in which protein is contained. The 10 mL of diluted protein was vortexed and then 150 µL was placed in each well of 96 well plate. The following day, the plate was washed in Dr. Phillip Berman's lab by graduate student mentor Lena Meyer. 150 µL of 5% BSA (w/v) in PBS was added to each well and incubated for 1 hour. 150 µL of primary antibody, HAstV8 spike, that was diluted to 1µg/mL with 5% BSA (w/v) in PBS was added to the first well and 1:3 dilutions were carried out on subsequent wells. This was incubated for 1 hour at room temperature. The secondary antibody was diluted to 5 µg/mL in 5% BSA (w/v) in PBS and 150 µL were added to each well and left to incubate for 1 hour at room temperature. One phosphate-citrate buffer (PCB) tablet was added to 100 mL ddH_2O and dissolved, then two tablets of Ortho-Phenylenediamine (OPD) and 12.5 µL Hydrogen Peroxide (HP) were added. 100 µL of this mixture was added to each well and let react for 12 minutes. 100 µL of Sulfuric Acid was added to each well to stop the reaction and then the plate was imaged by Lena in Phillip Berman's lab imager by reading the absorbance at 490 nm.

Crystal Trials of scFv 2D9 bound to HAstV8 spike

A 96-well plate was set up with drops of protein in each well as well as drops of solution from the Microlytic-MCSG1 crystal screen. Each drop contain either a 1:1 or a 2:1 µL ratio of protein to solution. This 96-well plate was set up using the Aria machine, and then incubated at 22 ˚C. The plate was checked several days later for crystal formation with a microscope.

Results and Discussion

HAstV8 Spike Expression and Purification

Our initially planned method of expression and purification of HAstV8 spike was successful. We found that the protein expressed well and was very pure with only initial TALON purification, with a yield of approximately 54 milligrams of protein. We decided to proceed with size exclusion chromatography to ensure absolute purity for crystallography and obtained a final yield of approximately 47 milligrams of protein. Yields and general purity can be qualitatively observed in the gel samples (**Figure 7)** in which HAstV8 spike is 29.150 kDa in weight.

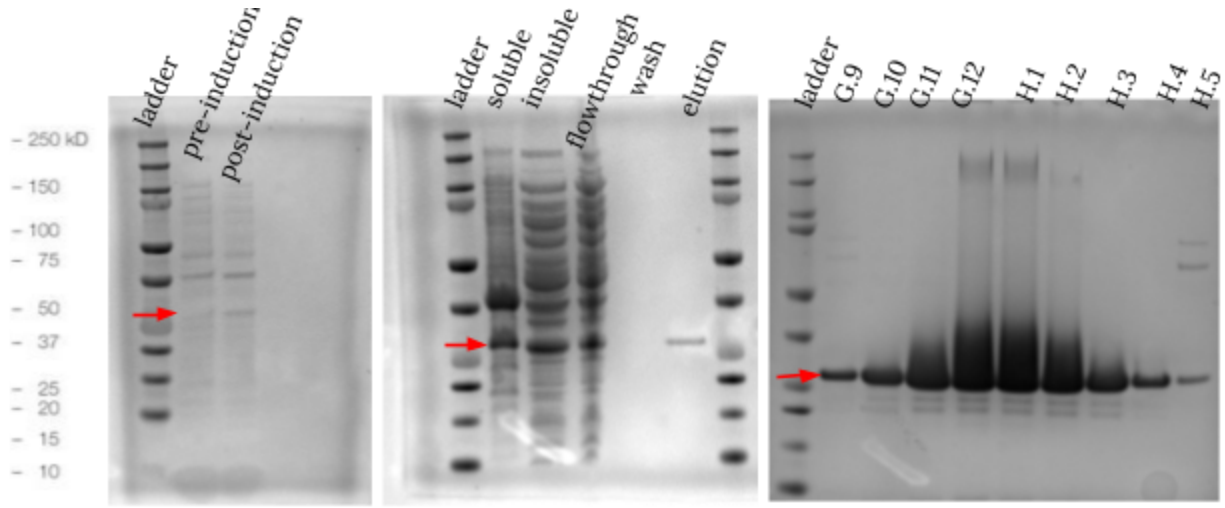

Figure 7. Left shows pre and post induction samples taken before and after the addition of IPTG to induce expression, the appearance of the band at approximately 30 kD is indicative that HAstV8 began expressing once induced. Middle shows fractions taken throughout purification of HAstV8 spike in the following order: Soluble, Insoluble, Flow-through, Wash, Elution. Right shows samples from fraction collector in which our protein of interest should have eluted.

The chromatograms (**Figure 8**) obtained by size exclusion chromatography show the first and second injection of TALON-purified HAstV8 spike in reference to size standards. The UV 1 280 (blue) curve shows the fraction of the total volume of injection at which the protein elutes. Size exclusion chromatography works by eluting larger proteins sooner, therefore the sooner the peak, the larger the protein. Observation of the largest peak in early fractions is indication that

the protein is eluting at the proper size of ~60 kDa (its native dimeric form). The smaller peak before the peak of interest is most likely aggregate protein.

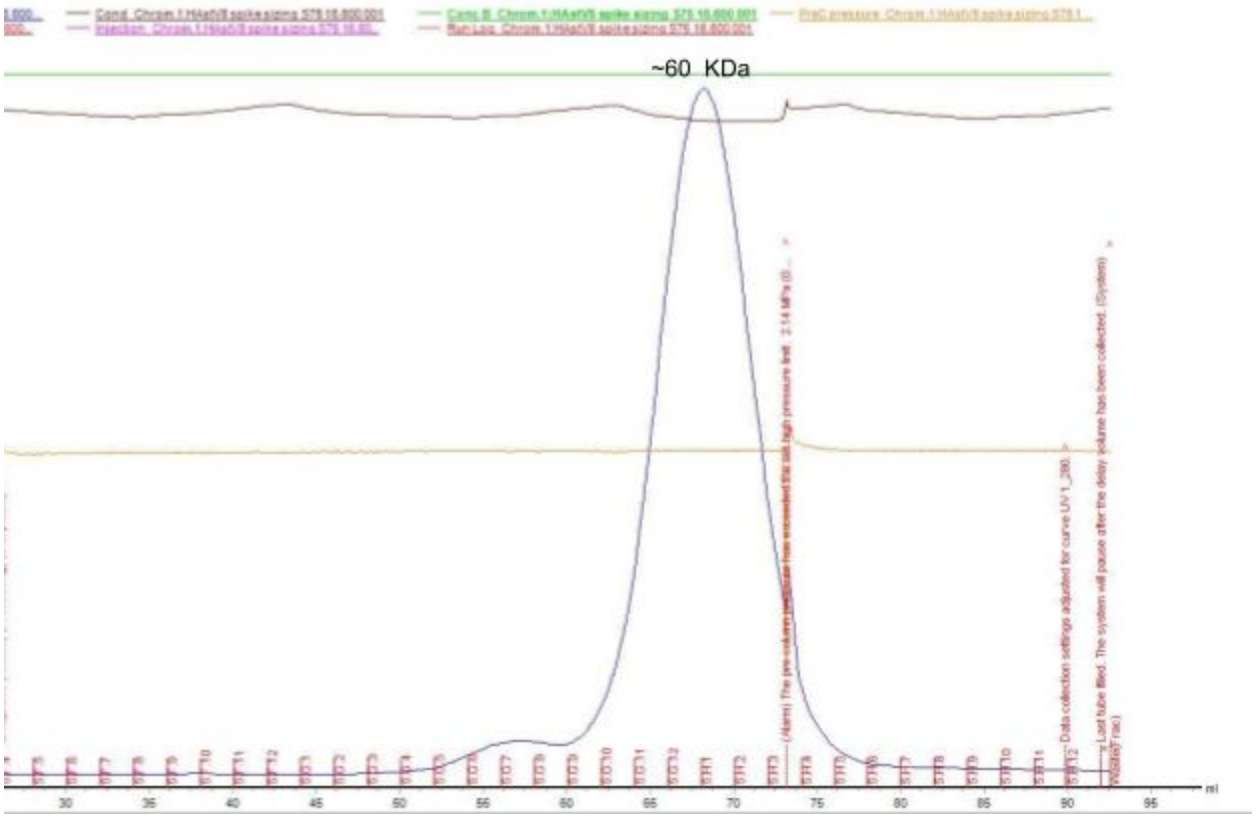

Figure 8. Size Exclusion Chromatograms from HPLC machine. Large peak is HAstV8 spike. Molecular weight standards are found on **Figure 11**.

Verification of Binding of HAstV8 Spike to humanized mAb 2D9

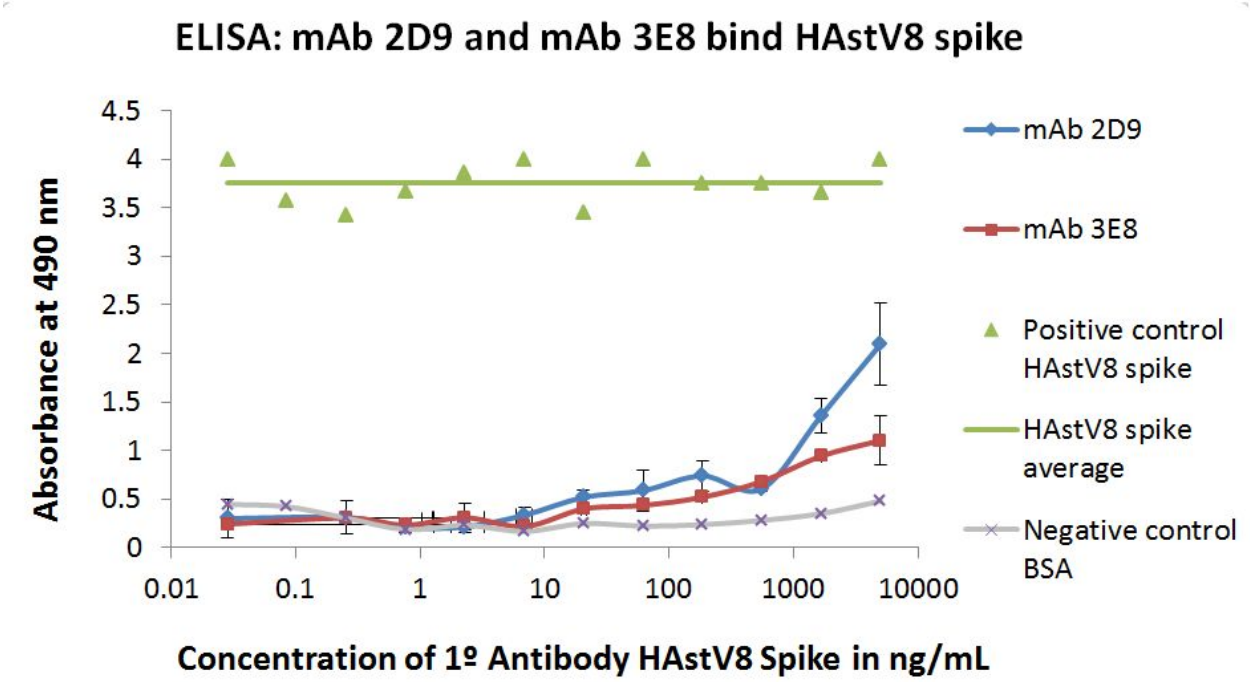

Figure 9. ELISA done with humanized mAb 2D9 and HAstV serotype 8 spike to test for confirmation of humanized spike/mAb binding (HAstV8/2D9).

Additionally, HAstV8 spike was tested for binding to mAb 2D9 to ensure that antibody and antigen binding remained consistent (**Figure 9**). This was done by an enzyme linked immuno-absorbancy assay (ELISA). The binding curve clearly demonstrated that 2D9 and spike 8 still bound, indicating that both proteins were properly expressed. The proper next steps will include re-expressing on a larger scale to perform a Western Blot to confirm size as well as reaffirm that it was properly expressed. In addition to a Western Blot, further ELISAs with other serotypes will be helpful in potential identification of conservation of neutralization amongst other serotypes of HAstV spike. Furthermore, crystallization of the antibody/spike 8 complex will allow identification of the binding epitope of mAb 2D9.

Expression and purification of scFv 2D9

The scFv 2D9 was expressed, purified, and eluted at the correct size and an anti-Strep Western Blot indicated the expressed protein is in fact scFv 2D9 (**Figure 10**) at ~ 30 kDa. Due to the success of test expressions of stably and transiently transfected scFV 2D9, a large 4 liter expression was done and sufficient quantity was obtained for formation of the HAstV8 spike/scFv 2D9 complex.

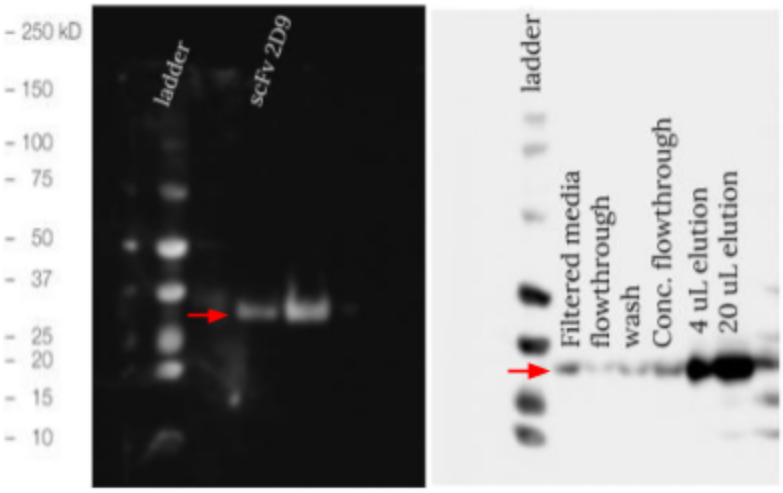

Figure 10. Left shows stable transfection of S2 cells expressing 2D9 Right shows Western Blot demonstrating purification samples of transient S2 cells expressing scFv 2D9. scFv 2D9 is ~32 kDa.

Verification of Binding of HAstV8/HAstV1 Spike to scFv 2D9

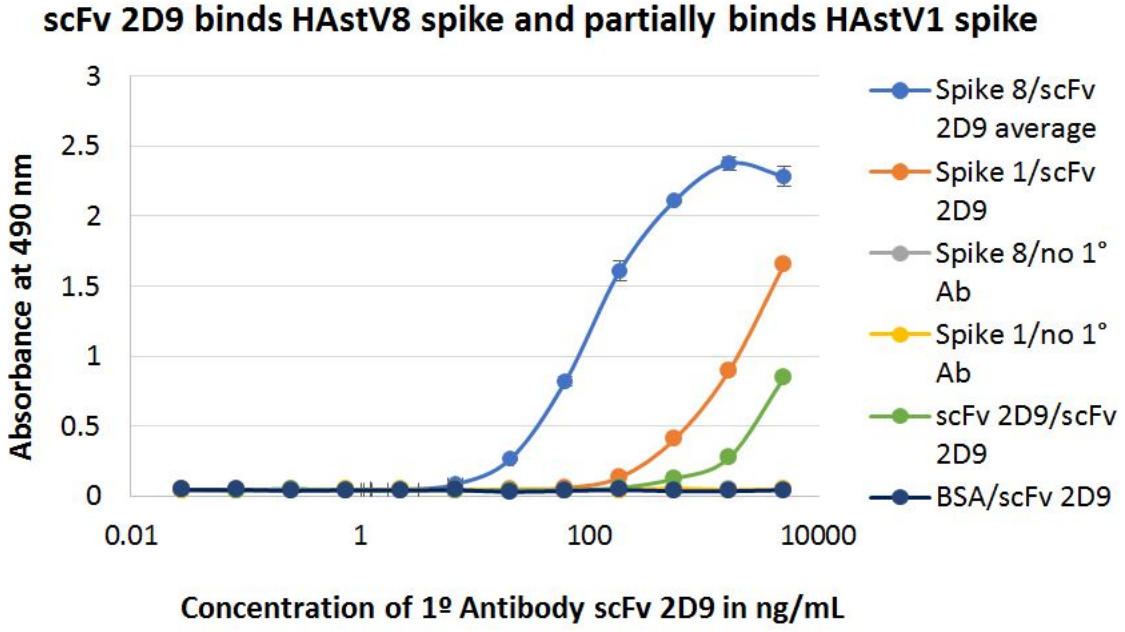

Figure 11. ELISA done with scFV 2D9 and HAstV serotypes 1 and 8 spikes to test for confirmation of spike/scFv binding (HAstV8/2D9) as well as potential for cross reactivity with another strain (HAstV1/2D9).

In addition to the qualitative determination of the proper expression of scFv 2D9 by Western blot, an ELISA was done on HAstV spike serotype 1 and 8 with HAstV8-specific scFv 2D9 for quantitative verification of correct binding and possible cross-reactivity with HAstV1 spike (**Figure 11**). Binding can be seen between HAstv8 spike and scFv 2D9 (light blue), as expected, but partial reactivity of scFv 2D9 with HAstV1 spike (orange) was also observed. This may indicate that there is partial conservation between this epitope in HAstV1 and HAstV8 spike. Furthermore, this result implicates that there may be some region of function in the area where 2D9 binds. It is not known whether the area where 2D9 binds is the same in both HAstV1 and HAstV8 spike, but crystallography can be done to further explore these interactions. Nonetheless, with evidence of binding fidelity to our protein of interest and successful production of the protein, we were able to proceed with the formation of the HAstV8 spike/scFv 2D9 complex.

Formation of HAstV8 spike/scFv 2D9 complex

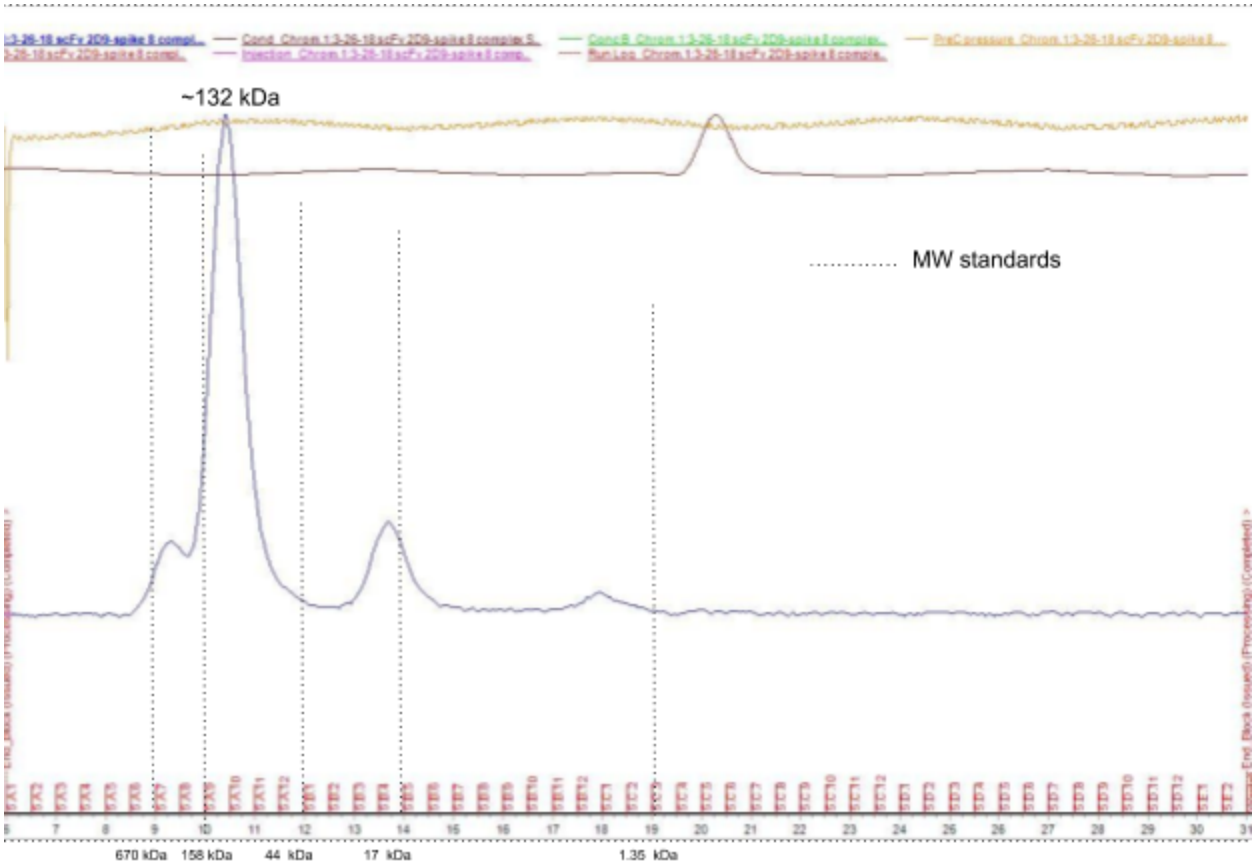

Figure 12. Chromatogram showing elution of HAstV8 spike/scFv 2D9 complex at correct size.

Following a 4 liter expression and purification of scFv 2D9, we had enough quantity of both proteins to proceed with the formation of the complex for crystal trials. The formation of the HAstV8 spike/scFv 2D9 complex was done by Lena Meyer. In order to create the complex, both proteins were incubated together with a ~3.5 molar excess of scFv 2D9 to ensure binding with dimerized HAstV8 spike. Additionally, thrombin was added to to cleave the Histidine and Strep tags that were added to purify HAstV8 spike and scFv 2D9, respectively, so as to not impede potential crystal formation. After incubation, the complex was run through the size-exclusion column and the chromatogram obtained (**Figure 12**) shows the elution of the

complex at the correct size, 132 kDa. HAstV is a dimer, therefore the molecular weight reflects two HAstV spikes and two scFvs bound. The additional peak following our peak of interest is the excess scFv 2D9 that was added during incubation. An SDS PAGE (**Figure 13**) run on fraction elutions from the chromatogram, specifically of the large peak and the small peak show that the unbound proteins run at the correct size and that the small peak is scFv 2D9.

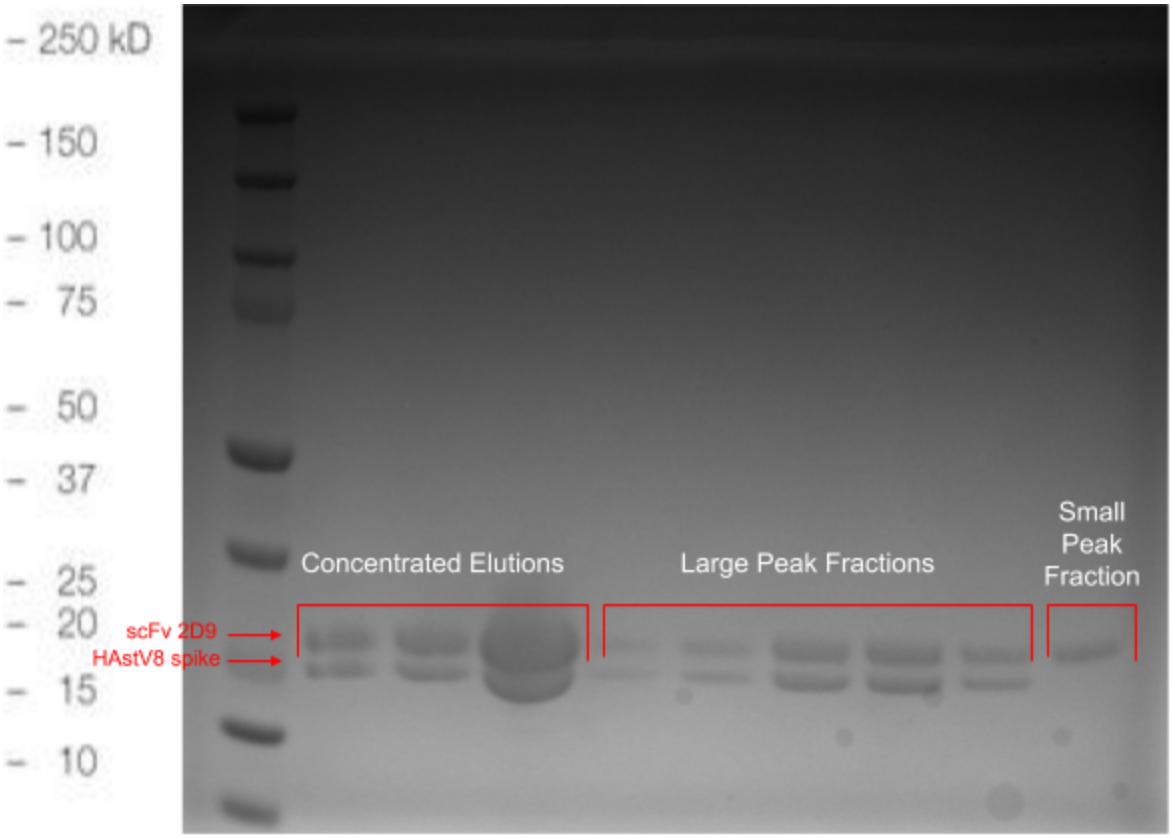

Figure 13. SDS PAGE run on fractions eluted from size exclusion chromatography purification corresponding to important peaks.

Crystal Trials of HAstV8 spike/scFv 2D9 complex

Crystal trials were set up using the Microlytic-MCSG1 Formulations that contain 96 different solutions optimized for crystal formation. The method of crystal formation we used was vapor diffusion, in which a solution is added to the protein sample in a closed environment. Water is drawn out of the protein-containing droplet as the environment reaches equilibrium. In this process of dehydration, the protein droplet becomes slowly more concentrated and should provoke crystal formation. To maximize the probability of crystallization, 1:1 and 2:1 ratios of HAstV8 spike/scFv 2D9 complex to each solution were used and then the 96-well plate was left at 22 ˚C for several days. Seven wells -- A6, A8, B8, C1, C10, E6, E11 -- contained crystals hits (**Figure 14**) under the following conditions:

A6- 0.2 M Ammonium Sulfate 0.1 M Bis-Tris: HCl, pH 5.5 25 % (w/v) PEG 3350

A8- 0.1 M Tris: HCl, pH 7 20 % (w/v) PEG 2000 MME

B8- 0.17 M Sodium Acetate 0.085 M Tris: HCl, pH 8.5 25.5 % (w/v) PEG 4000, 15 % (v/v) glycerol

C1- 0.1 M Ammonium Acetate 0.1 M Bis-Tris: HCl, pH 5.5 17 % (w/v) PEG 10000

C10- 1 M Lithium Chloride 30 % (w/v) PEG 6000, 0.1 M Sodium Acetate

E6- 0.2 M Potassium Sulfate 20 % (w/v) PEG 3350

E11- 1 M NaH_2PO_4/K_2HPO_4, pH 8.2

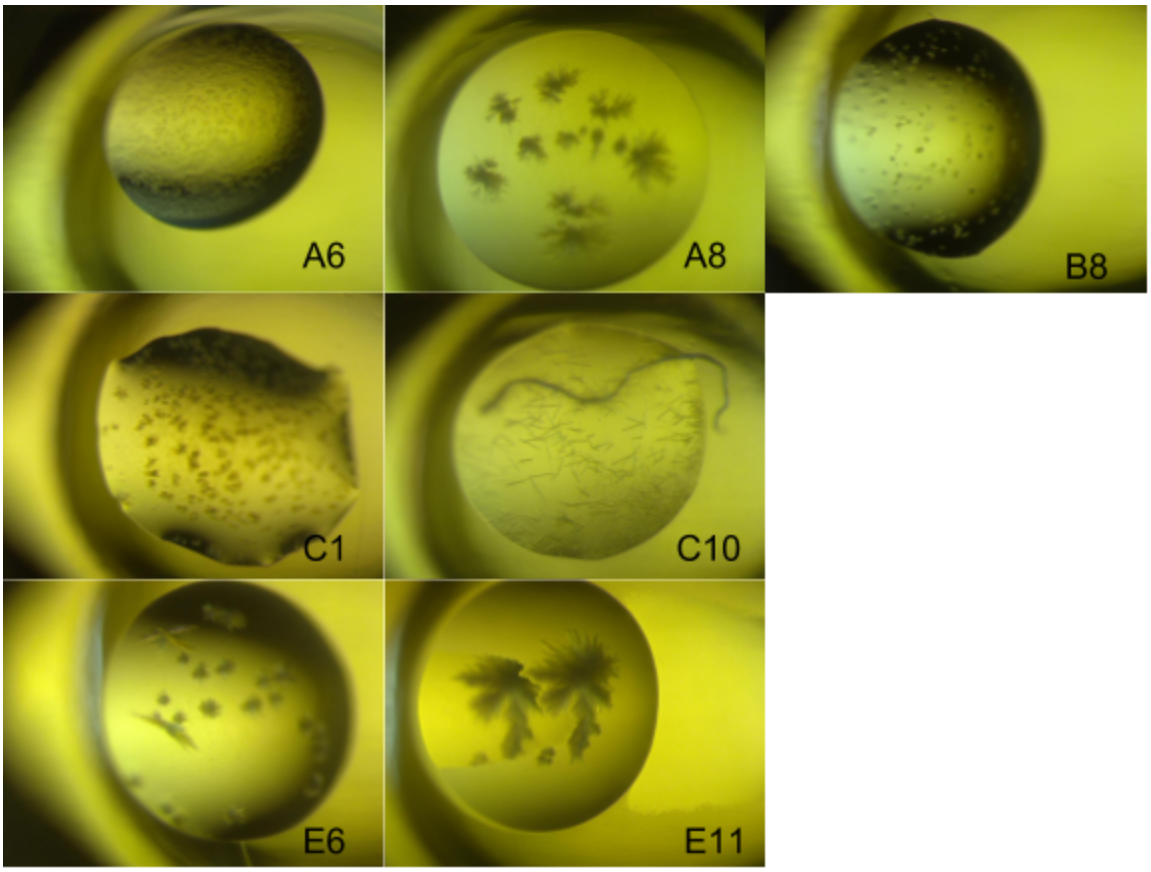

Figure 14. Crystal formations of the HAstv8 spike/scFv 2D9 complex in various wells under aforementioned conditions.

Although crystals were obtained, many were in clusters, which is not desirable for X-ray diffraction. Therefore, optimization of crystal formation is necessary to obtain quality resolution diffraction data. Nonetheless, formation of any crystal is promising.

Conclusion

While the Human Astrovirus is a common and widespread pathogen, methods of treatment and prevention for it are not. The most important step in developing vaccines and treatments against this disease lie in identification of functional epitopes as well as discovering their mechanistic importance. Through this project's exploration of a HAstV8 spike neutralizing antibody, 2D9, we are moving towards identifying an epitope targeted for neutralization through the employment of protein engineering and X-ray crystallography. The expression of engineered forms of the antibody alongside its corresponding spike have allowed us to obtain crystals of the bound complex for optimization, that we will ultimately use in collecting diffraction data. We aim to solve the protein structure in order to identify this neutralizable epitope. Additional studies done by a collaborator will lead to the determination of the functional importance of this epitope and whether this particular region is responsible for prevention of receptor host cell attachment or internalization of the viral particle. Both the identification and functional classification of epitopes on HAstV spike are imperative for expanding our knowledge on its viral biology as well as guiding vaccination design.

References

- Walter A. Bogdanoff, Edmundo I. Pereza, Tomás López, Carlos F. Arias, and Rebecca M. DuBois. Structural basis for the escape of human astrovirus from antibody neutralization: broad implications for rational vaccine design J. Virol. JVI.01546-17; Accepted manuscript posted online 25 October 2017
- Bogdanoff WA, Campos J, Perez EI, Yin L, Alexander DL, DuBois RM. 2017. Structure of a Human Astrovirus Capsid-Antibody Complex and Mechanistic Insights into Virus Neutralization. J Virol 91
- Bosch, Albert, Rosa M. Pintó, and Susana Guix. "Human Astroviruses." Clinical Microbiology Reviews 27.4 (2014): 1048–1074. PMC. Web. 2 Apr. 2018.
- Elliott EJ. Acute gastroenteritis in children. BMJ : British Medical Journal. 2007;334(7583):35-40. doi:10.1136/bmj.39036.406169.80.
- Cordey S, Vu D, Schibler M, et al. Astrovirus MLB2, a New Gastroenteric Virus Associated with Meningitis and Disseminated Infection. Emerging Infectious Diseases. 2016;22(5):846-853. doi:10.3201/eid2205.151807.
- Rahman, Misha. "Immunoglobulins | Classes and Subclasses." Bio-Rad, www.bio-rad-antibodies.com/immunoglobulins-classes-subclasses.html.
- Méndez E, Muñoz-Yañez C, Sánchez-San Martín C, et al. Characterization of Human Astrovirus Cell Entry. Sandri-Goldin RM, ed. Journal of Virology. 2014;88(5):2452-2460. doi:10.1128/JVI.02908-13.
- "Astroviridae." Viralzone, viralzone.expasy.org/27?outline=all_by_species.

- Samuel Cordey, Diem-Lan Vu, Marie-Celine Zanella, Lara Turin, Aline Mamin Laurent Kaiser. Novel and classical human astroviruses in stool and cerebrospinal fluid: comprehensive screening in a tertiary care hospital, Switzerland. Emerging Microbes & Infections (2017) 6 ;September 2017
- Kelly A. Dryden, Mariana Tihova, Norbert Nowotny, Suzanne M. Matsui, Ernesto Mendez, Mark Yeager. Immature and Mature Human Astrovirus: Structure, Conformational Changes, and Similarities to Hepatitis E Virus,Journal of Molecular Biology, Volume 422, Issue 5,2012, Pages 650-658, ISSN 0022-2836, https://doi.org/10.1016/j.jmb.2012.06.029.