Characteristics of a Truncation of the B22 Protein of Ectromelia Virus

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Introduction:

Viruses pose a deadly threat. Some are fifty times smaller than a red blood cell! They lurk on surfaces humans touch every day, waiting for the opportune moment to infect the perfect host. Humans and other organisms must find a way to fight this constant threat of siege by the microscopic. Luckily, the immune system exists to fight even the viruses that do get in. The immune system is constantly adapting to fight these microscopic invaders, but unfortunately, the viruses adapt as well. The B22 protein is a protein that has evolved in the Ectromelia virus (a poxvirus) to evade components of the mouse immune system. That evasion is examined here.

Poxviruses

Poxviruses are some of the most deadly viruses on this planet. They are double-stranded, DNA viruses with a range severity. Hosts include humans, mice, cows, chickens, and other animals. The virus enters the cells of its host and replicates within the cytoplasm. Poxviruses are extremely efficient and contain all required materials for DNA replication. The genome of poxviruses does not contain any introns, and maximum space efficiency is achieved by transcription of both strands of DNA [1].

Genetics provides the basis for the way in which poxviruses interact with the human immune system. Poxviruses have a double-stranded DNA genome. They enter host cells by fusing their outer plasma membrane with that of the host in a pH-independent process. Once inside the cell, the virus uncoats and replicates within the cytoplasm. Genes in poxviruses are separated into early genes, intermediate genes, and late genes. The early genes are activated directly after virus entry and either encode transcription machinery or help to evade the host immune response. The induction of intermediate genes usually aids with DNA replication, while

late genes usually encode the viral protein shell so that completed viruses can then invade new host cells [2].

One of the most deadly examples of a poxvirus that infects humans is smallpox. Smallpox killed thousands in the sixteenth century in Europe, and thousands more in the new world. Smallpox was declared eradicated in the 1970s by the World Health Organization, and only two stores of it now exist: in Russia and in the United States. A model organism for the study of smallpox is mice. The mouse immune system is comparable to that of humans. Therefore, the mouse reaction to smallpox makes an excellent model to predict what might happen if a smallpox outbreak were ever to occur in a human population, or if an outbreak of a related poxvirus were to erupt. Ectromelia virus (ECTV) naturally infects mice, and it makes a useful model to study how smallpox might interact with the human immune system.

In addition to serving as a model disease for smallpox, ECTV is studied to better understand overall virus biology, and predict possible virus vectors. A virus vector is a virus that is harmless in humans, yet can be engineered to express proteins from more harmful viral pathogens. This allows the body to create antibodies against these more harmful viruses without the risk of pathogenesis. This is the principle behind vaccination. Studying the interaction of ECTV with the mouse immune system may reveal ways to weaken the virus, which would make it the ideal virus vector.

Viruses and the Immune System

When a foreign agent enters the body, it is immediately met by the innate immune response. The innate immune response is activated by soluble proteins that bind to foreign substances and recruit effector cells that engulf the invader. The accumulation of effector cells at the sight of infection causes the hallmark redness and inflammation that typically accompanies a

cut or scrape. The innate immune response is very general, is activated immediately upon infection, and lasts only a short time [3].

The longer lived immune response is the adaptive one. While the innate immune system is activated immediately, the adaptive immune system is activated after four to seven days of fighting infection unsuccessfully. The innate immune response is general, and the same effector cells are activated for all pathogens, whereas the adaptive immune system is highly specific. Adaptive immune cells are created based on an organism's genome and based on pathogens it has encountered in the past. These cells are tailor-made to fight one particular pathogen, and one immune cell may never see its pathogen for the extent of its life [4].

CD4 T cells are an important part of the adaptive immune system that are evaded by ECTV. Within the mouse immune system, CD4 T cells play a main role in host defense. CD4 T cells are activated by mouse immune cells known as dendritic cells. These cells obtain peptide fragments from a virus (in this case, ECTV) and present peptides of the virus on their surfaces. Proteins from the virus enter the cell through phagocytosis in a small vesicle. The acidification of this vesicle causes the peptide to be broken down into small fragments. These fragments then bind to a molecule known as class II major histocompatibility complex (MHC-II), which brings fragments of the peptide to the cell surface. The CD4 T cells in circulation then recognize peptides on dendritic cell surfaces and become activated. Once activated, CD4 T cells release cytokines that then attract other immune cells to kill virus-infected cells. CD4 T cells may attract one of several different cell-types, including neutrophils and monocytes. They also stimulate B cells to produce antibodies against this particular virus [5].

Ectromelia Virus and Immune Evasion

This is the normal course of defense during virus infection. However, research has

revealed that ECTV does not antagonize these normal pathways of host defense. There is no activation of CD4 T cells detected when they are infected with wild-type ECTV. ECTV that has been inactivated by ultraviolet light, however, does show activation of these cells, indicating that there is a genetic basis for the evasion of CD4 T cell activation by ECTV [data not published].

Hammarlund's research group investigated this phenomenon in Monkeypox virus (MPXV). This group found that MPXV does not activate CD4 or CD8 T cells during infection. They also found that the protein responsible for this phenomenon must play an active role in T cell inhibition. When MPXV was mixed with Vaccinia virus (VACV), which naturally does elicit a T cell response, even at a ratio of 1:10 MPXV to VACV no T cell response occurred. MPXV also inhibited CD4 and CD8 T cell activation by 80 percent when cells were experimentally stimulated in a nonspecific manner. When stimulated by CD3, cells do not need to present viral peptides in order to stimulate T cells. Yet, after infection with MPXV, even cells that were directly stimulated could not induce a T cell response. This indicates that MPXV plays an active role in suppressing the T cell response, rather than simply down-regulating one of the necessary stimuli to elicit a response. The group also found that an early gene may be responsible for this inhibition. After treating cells with cytosine arabinoside (AraC), a drug that inhibits intermediate and late gene expression, cells were infected with either VACV or MPXV. Researchers found that there was no measurable difference in T cell inhibition between cells that had been treated with AraC and cells that were grown in normal media, indicating that an early gene must be responsible for this inhibition [6]. This makes sense, as it is the early genes in poxviruses that usually inhibit host immune response.

The B22 Protein

In 2014, researchers from the same group found the family of proteins responsible for

inhibition of CD4 T cells by poxviruses. Researchers found that the gene product 197 of MPXV inhibits CD4 and CD8 T cells and can do so when acting alone in an optimized plasmid. They also found that the interaction between gene 197 and T cells does not disrupt the T cell plasma membrane, nor does it inhibit the ability of T cells to function on other cells in the future. CM9-specific T cells were stimulated with phorbol 12-myristate and 13-acetate (PMA), a substance that activates Protein Kinase C (PKC) and ionomycin (Iono), which is a calcium ion ionophore. Importantly, PMA/Iono stimulates T cells in an MHC-independent manner. Whereas MPXV197 inhibits T cell activation via peptide:MHC, it did not inhibit T cell activation by this PKC/Iono pathway, indicating that the interaction between T cells and MPXV197 does not inhibit the T cells from acting in the future, nor does it disrupt them structurally [7].

Researchers also established the importance of the B22 proteins as a family by comparing the protein product of gene 197 in MPXV, the gene product 219 in cowpox (CPXV219), and the B22 protein in Variola virus (VARVB22). These proteins all belong to the B22 protein family, and a homologue of these proteins is also found in ECTV. MPXV197 and VARVB22 were both studied in optimized plasmids, while CPXV219 was expressed in VACV, which normally stimulates both CD4 and CD8 T cells. When studied in isolation of the rest of their respective viruses, these proteins all caused inhibition of peptide-specific CD8 T cells from both humans and rhesus monkeys [8].

In vivo, mice that were infected with a knockout virus missing the vital gene 197 (MPXVΔ197) had significantly lower morbidity and mortality than mice infected with wild-type virus. Mice that were infected with MPXVΔ197 also had a shorter duration of fever and viral titers in the lungs were controlled much faster and fell more rapidly. There were also significantly higher amounts of both CD4 and CD8 T cells in the blood of animals infected with

MPXVΔ197 compared to wild-type virus fourteen days post-infection [7]. This is consistent with the idea that MPXV197 interferes with and inhibits both CD4 and CD8 T cell responses.

Further exploration of the CPXV219 has shown specific cleavage patterns and indicated a possible pathway that this protein follows during infection. Reynolds and Moss found that this protein is non-essential for virus replication in tissue culture. The N and C termini of this protein are loosely associated early in viral infection, although they are not tightly bound and present as two fragments during a Western Blot after being treated with SDS. Further experimentation revealed that the CPXV219 dissociates later in virus infection post-Golgi processing. The cleavage site for this protein was found to be approximately 600 amino acids from the N-terminus. Only the C-terminus then embeds in the plasma membrane of the host cell. This group also found that CPXV219 is heavily glycosylated, possibly adding to its immense size [8].

When evaluating the possible role of this protein during infection *in vivo*, researchers infected both BALB/c mice and CAST/Eij mice with both a knockout and a revertant virus. There was no significant difference in virulence in either colony of mice. There are several possible explanations for this result. One is that there is some redundancy in the cowpox genome, meaning that another gene compensates for the lack of 219. The other possible explanation is that gene 219 is nonfunctional in mice since the natural host of cowpox virus is not mice [8].

This same group further explored the B22 protein in ECTV, which they termed “C15.” BALB/c mice were infected with a virus containing a stop codon at the C15 gene (C15Stop virus) showed significantly higher survivorship than those infected with a revertant virus engineered to express the wild-type ECTV genome (C15Rev virus). Pathogenicity and symptoms among infected mice were too variable to measure simply because there was so much

inconsistency in time to death, so some mice received lower scores for pathogenicity, but succumbed to infection sooner. However, researchers did find that mice infected with the C15Stop virus had significantly more foot pad swelling than animals infected with C15Rev. This indicates that the immune system was more active in these mice. This supports the hypothesis that B22 inhibits the mouse immune system, specifically CD4 T cells, during ECTV infection. Without that protein, the immune system was better able to react to infection [9].

This paper also shows that the C15Stop virus had less dissemination than the C15Rev virus, indicating another possible function of the B22 protein as a facilitator of the spread of ECTV from one cell to another. The popliteal and inguinal lymph nodes in mice infected with the C15Stop virus showed significantly decreased viral titers compared to the lymph nodes of mice infected with the C15Rev virus. Tissue in these lymph nodes also showed less necrosis, as determined by immunohistochemical staining. Additionally, the spleen and liver showed significantly lower viral titers for mice infected with the C15Stop virus by day eight than they did for mice infected with the C15Rev virus. This is especially significant since viral titer in the liver indicates the lethality of ECTV infection. The spread of the virus in mice infected with the C15Stop virus seemed to be under much greater control than in mice infected with the C15Rev virus [9].

To explore the possible functions of T cells in this dynamic, researchers inhibited the action of either or both CD4 and CD8 T cells in mice prior to viral infection with either C15Stop or C15Rev. If C15 plays a role in facilitating virus spread and has no impact on T cells whatsoever, then theoretically the results seen in wild-type mice should match those seen in mice depleted of T cells. Researchers, however, found that actually mice that were depleted of CD4 or CD8 T cells or both could not fight off viral infection, and there was a very low rate of survival.

There was also no significant difference between the survival rates for mice infected with C15Rev or C15Stop, indicating that there is in fact some sort of interaction between C15 and T cells [9].

The Hersperger research group at Albright College created a knockout of ECTV containing Green Fluorescent Protein (GFP) in place of the B22 homologue (gene-product 174) of ECTV. A knockout virus missing gene 174 was used to infect mouse L929 cells expressing MHC-II. Infection with the knockout virus (ECTVΔB22) did result in activation of CD4T cell hybridomas, whereas infection with the wild-type ECTV did not [data not published]. This indicates that the B22 protein of ECTV does act to inhibit CD4 T cells. Unlike CPXV219, this protein seems to act alone.

A B22 Truncation

The goal of this project is to elucidate the portion of the B22 protein responsible for CD4 T cell inhibition. A truncation of the protein was created by deleting 500 amino acids from the C terminus. The C terminus is found outside the cell during virus infection. If the portion of the protein responsible for T cell inhibition is contained within the truncated region, T cell assays will reveal no T cell inhibition during transfection with the plasmid containing the truncated protein.

The first step of this project is to determine where the C-terminus of the truncated B22 protein is during virus infection. The C-terminus of the wild-type protein presents outside the cell during infection, and this is where the interaction between CD4 T cells and the host cell take place. Therefore, it would be plausible that if the C-terminus of the truncated B22 protein also presents outside the plasma membrane of the host cell, the region that inhibits CD4 T cells has probably not been disrupted.

CPXV219 undergoes cleavage during infection, which may or may not be true for ECTV protein B22. CPXV219 is cleaved 600 amino acids from the N-terminus, meaning that the entire transmembrane region and the region that inhibits T cells must be contained within the rest of the protein. Therefore, this project begins by cleaving the B22 ECTV protein 500 amino acids from the C-terminus. If this does not disrupt the portion of the protein that inhibits CD4 T cells, then another truncation can be attempted at a future date.

Methods:

Creation of the Plasmid

A plasmid containing the B22 protein of ECTV was used to engineer a plasmid containing a truncation of this protein, missing 500 amino acids from the C-terminal end. DNA primers were designed to be complementary to the start of the B22 protein and primers were designed to be complementary to the desired endpoint of the truncation, 1500 nucleotides from the end of the B22 protein. These primers were used in a Polymerase Chain Reaction (PCR) to replicate the desired region from the plasmid containing B22. A restriction digest was then used to reveal sticky ends on a commercially available plasmid backbone. The backbone also contained a Myc tag complementary to the C-terminus of the new protein. The backbone was then sealed to the truncation using a ligation reaction and grown in *Escherichia coli*. The sequence of the truncation was confirmed by commercial sequencing techniques.

Confirmation of Plasmid by DNA Analysis

A restriction digest was used to separate the truncated B22 protein from its plasmid backbone. These two pieces of DNA were then run in a 0.75 percent agarose gel for 90 minutes.

The plasmid containing the full-length B22 protein also underwent a restriction digest and was run next to the truncated protein as a control.

Western Blot

A Western blot was used to confirm the suspected lengths of the full-length B22 protein and the truncation. 293T cells were transfected with either the full-length B22 plasmid or with the 500 amino acid truncation. Transfections were done using Lipofectamine 3000 for a duration of 24 hours. Cells that had not undergone transfection were included as a control. They were then isolated using a kit with a standard protocol. The amount of protein in the samples was then quantified using a Qubit system with standard protocol. The isolated protein was prepared for the Western blot by adding SDS sample buffer in a one to one ratio and boiling for four minutes at 100 degrees Celsius. The samples were then run in a 4 to 20 percent gradient pre-cast SDS-PAGE gel at 200 volts for one hour with 100 micrograms of sample per well. The gel was run in 1x Tris-Glycine SDS buffer. It was then rinsed in a solution containing three percent Trizma base and fourteen percent glycine in methanol, hereafter referred to as Western blot transfer buffer. The blot was transferred overnight at twenty volts. The blot was incubated for one hour in a one microgram per microliter concentration of Mouse anti-Myc primary antibody. It was incubated in secondary antibody for one hour. The secondary antibody used was Goat anti-mouse. The gel was then developed using a developing solution for one hour and visualized.

Immunofluorescence Microscopy

293T cells were seeded on a chamber slide and transfected with either the full-length B22 protein or the 500 amino acid truncation. Transfections were done using Lipofectamine 3000 for

a duration of 24 to 48 hours. Untransfected cells were used as a control. Cells were fixed to the slide using 5% formalin. For intracellular staining, plasma membranes were then disrupted using acetone at negative 20 degrees Celsius for ten minutes. For extracellular staining, this step was skipped. Cells were then incubated in a mixture containing one percent bovine serum albumin and two percent fetal bovine serum in phosphate-buffered saline, hereafter referred to as blocking buffer. Cells were then incubated in Mouse anti-Myc antibody for thirty minutes followed by Goat anti-mouse antibody for twenty minutes. This antibody is light-sensitive, and this incubation was done in the dark. The slide was mounted using mounting media containing DAPI double stranded DNA stain and visualized using a Zeiss Axiostar plus epifluorescence microscope. Images were captured with an Optronics camera system.

Results:

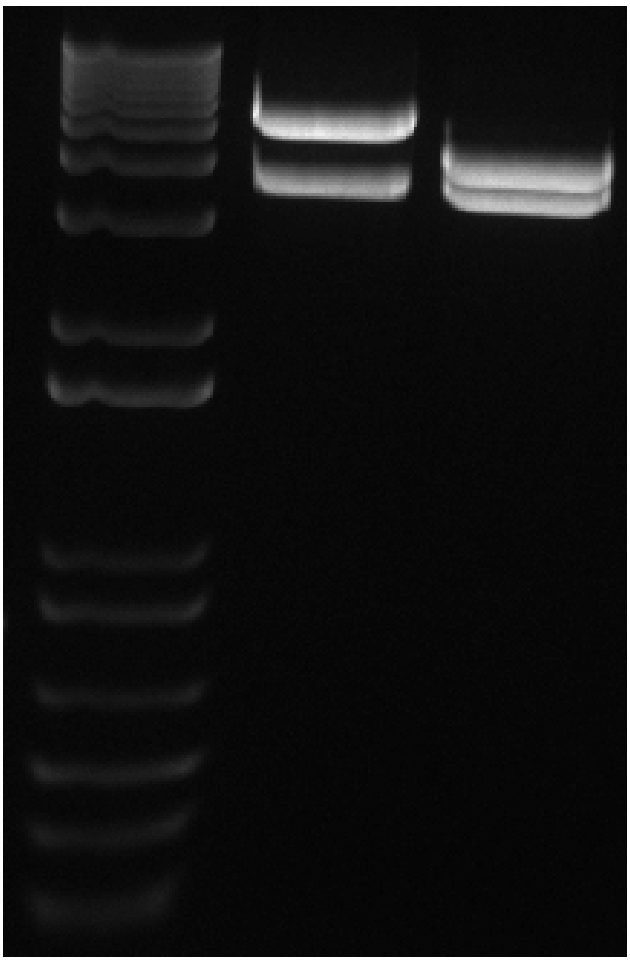
<u>The truncation plasmid contained a gene that was 500 amino acids shorter than the wild-type B22 gene</u>

A restriction digest of the plasmids containing the wild-type B22 gene and the 500 amino acid truncation revealed that the backbone of both plasmids were roughly the same size (Figure 1). The gene of interest encoding the full length protein was also 1500 nucleotides longer than the gene encoding the truncation (Figure 1). This corresponds to a difference of 500 amino acids in the final truncated protein. The difference in size of the truncated protein was also confirmed by Western Blot. A Western Blot of 293T cells transfected with the plasmid containing the full-length B22 protein was visualized next to 293T cells transfected with the plasmid containing the truncation. The full-length protein isolated from these cells clearly appeared to be 500 amino acids longer than the truncation (Figure 2). These samples were also run next to untransfected cells for comparison and no background cellular proteins appeared in the area of interest (Figure 2).

The 500 amino acid truncation still has an extracellular C-terminus

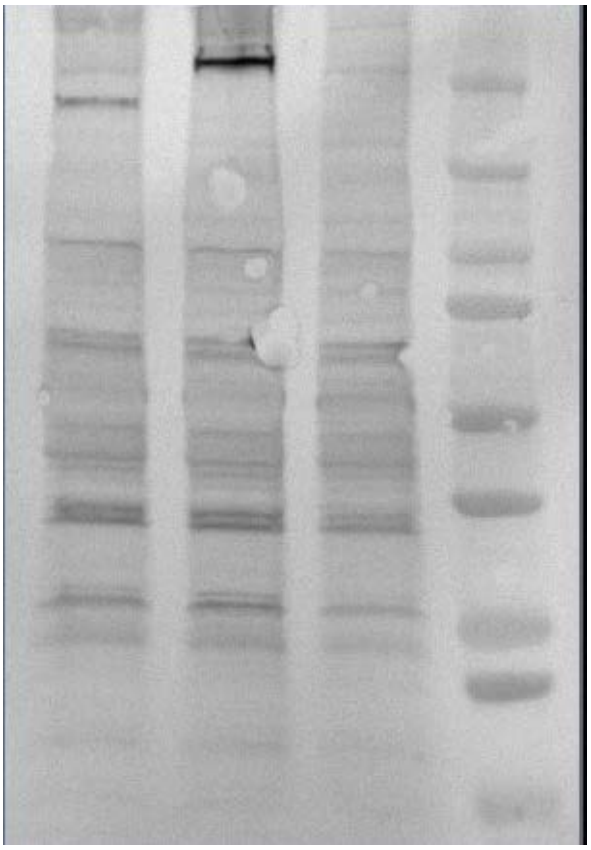
Intracellular and extracellular staining was conducted upon 293T cells that had been grown in a chamberslide and transfected with either the full-length B22 protein or the 500 amino acid truncation. The antibody used in the staining was specific for the Myc tag, which had been engineered into each plasmid to be expressed on the C-terminal end of the finished protein. Previous work had shown that the C-terminus is extracellular in the wild-type B22 protein. Intracellular staining confirmed that the protein was present in the cells and that the Myc tag was able to be stained effectively from both proteins (Figure 3 A and B). Extracellular staining revealed that the C-terminus was extracellular in both the full-length B22 protein and in the 500 amino acid truncation (Figure 3 D and E). An extracellular stain of untransfected cells eliminated the possibility of a false result from background antibody binding (Figure 3 C).

Figure 1: Restriction Digest of Full-Length B22 Plasmid and 500 Amino Acid Truncation



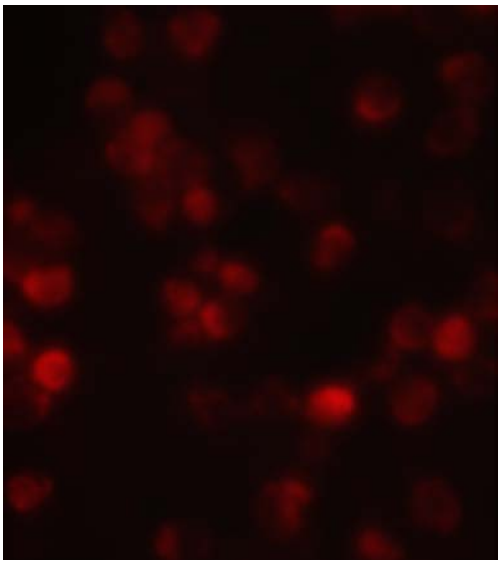
A restriction digest was carried out on the plasmid containing full-length B22 protein (right) and on the one containing the 500 amino acid truncation (left). The plasmid backbone was the same length in both plasmids (bottom band). The gene of interest (top band) was approximately 1500 nucleotides shorter in the truncation than in the full-length gene.

Figure 2: Western Blot Showing the Size Difference Between Full-Length B22 and the 500 Amino Acid Truncation

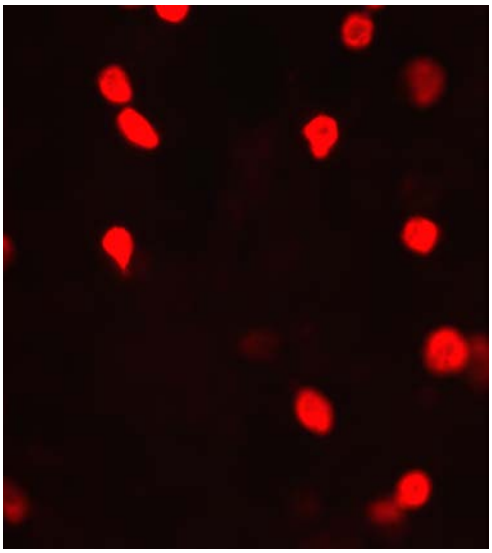


A Western Blot shows the size difference in complete proteins. The topmost band shows that the 500 amino acid truncation (left lane) is indeed 500 amino acids shorter than the full-length B22 protein (second lane). The mock-infected cells show no such similar bands (third lane). The ladder also confirms that the size difference between the full-length B22 protein and the truncation is in fact 500 amino acids (right lane).

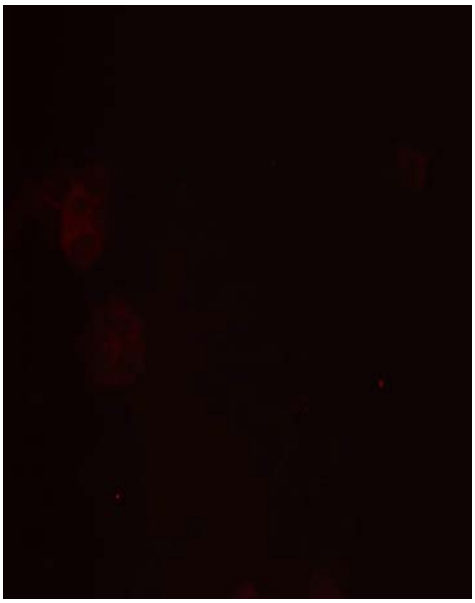
Figure 3: Immunofluorescence Staining of Cells Transfected with Full-Length B22 or the 500 Amino Acid Truncation



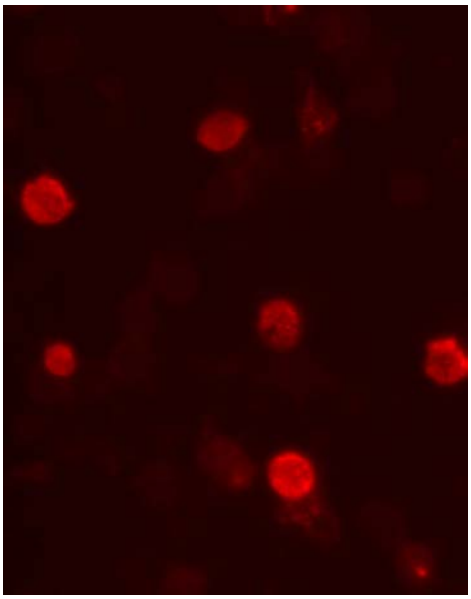
A.



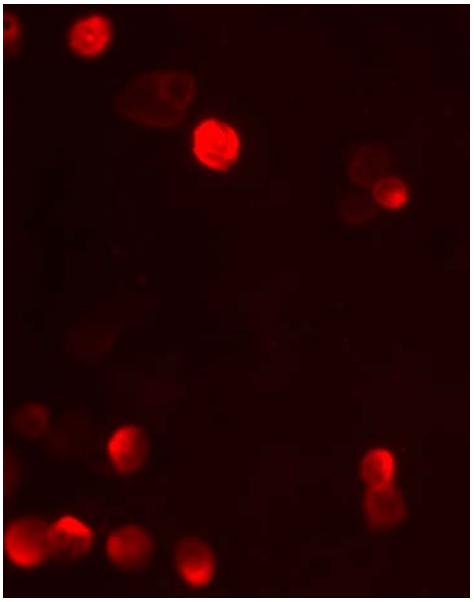
B.



C.



D.



E.

A. Intracellular staining of the full-length B22 protein in 293T cells after 24-hour transfection. B. Intracellular staining of the 500 amino acid truncation of the B22 protein in 293T cells after 24-hour transfection. C. Extracellular staining of untransfected 293T cells. D. Extracellular staining of the full-length B22 protein in 293T cells after 24-hour transfection. E. Extracellular staining of the 500 amino acid truncation of the B22 protein in 293T cells after 24-hour transfection.

Discussion:

The B22 protein plays an important role in evading the mouse immune system during ECTV infection. If isolated, this protein could have medicinal uses, or medications could be designed to render it ineffective in case of ECTV infection. Without this protein, the virus can be easily detected by the mouse immune system and can be eradicated without medication. Since ECTV is so similar to smallpox, a medication that renders the B22 protein ineffective could also work to combat smallpox in the case of an outbreak. Therefore, this protein is a very important candidate for further research.

B22 is the largest gene in the poxvirus genome. Therefore, if the protein is to be studied further, it would be useful to narrow its study to one particular site. In order to elucidate the region of B22 that inhibits CD4 T cells, a 500 amino acid truncation of the protein was created. The C-terminus of this truncation was then visualized in cells that had undergone a 24-hour transfection with a plasmid containing the truncation. Immunofluorescence microscopy showed that the Myc tag located at the C-terminus of the truncation was present outside the host cell during transfection.

The next step in this project would be to conduct CD4 T cell activity assays on cells transfected with the plasmid containing the B22 500 amino acid truncation. No activity by CD4 T cells would indicate that the region of B22 that inhibits these cells was not disrupted by the 500 amino acid truncation. A possible next step then would be to truncate a larger region of B22

until the region of B22 that inhibits T cells is disrupted. This would be evident if a T cell activity assay of cells transfected with a plasmid containing the new truncation revealed no T cell activity.

A key next step in this project is to engineer a plasmid that contains a tag other than the Myc tag. 293T cells are derived from humans, and they will naturally express the human Myc tag when it is made available. This leads to a prominent background band in any Western Blots that is the same length and intensity in untransfected cells, cells transfected with the full-length B22 protein, and in cells transfected with the 500 amino acid truncation. Therefore, a next step in this project is to engineer new plasmids expressing a different marker on the C-terminal end of the genes of interest to reduce background staining.

The region of the B22 protein that inhibits CD4 T cells could be very useful. Many autoimmune diseases result in overactivation of the immune system. This protein could be used to suppress the immune system in the context of these diseases. An ECTV with an altered B22 protein could also make an excellent candidate for a vaccine vector as it could infect human cells while carrying very little risk of pathogenicity. This protein is important for further study, and its large size makes it advantageous to find and utilize only the portion of the B22 protein that inhibits CD4 T cells. This project warrants further exploration.

Literature Cited

1. Acheson, NH. 2007. Fundamentals of molecular virology. Hoboken: John Wiley and Sons, Inc. p 157.
2. Buller ML, Palumbo GJ. Poxvirus pathogenesis. Microbiological Reviews. 1991; 55(1): 80-122.
3. Parham, P. 2009. The immune system. 3rd ed. New York: Garland Science. p 9.
4. Parham, P. 2009. The immune system. 3rd ed. New York: Garland Science. p 11.
5. Parham, P. 2009. The immune system. 3rd ed. New York: Garland Science. p 140.
6. Hammarlund E et al. Monkeypox virus evades antiviral CD4 and CD8 T cell responses by suppressing cognate T cell activation. PNAS. 2008; 105(38): 14567-14572.
7. Alzhanova D et al. T Cell Inactivation by Poxviral B22 Family Proteins Increases Viral Virulence. PLoS Pathogens. 2014; 10(5).
8. Reynolds SE, Moss B. Characterization of a large, proteolytically processed cowpox virus membrane glycoprotein conserved in most chordopoxviruses. Virology. 2015; 483: 209-217.
9. Reynolds SE, Earl PL, Minai M, Moore I, Moss B. A homolog of the variola virus B22 membrane protein contributes to Ectromelia virus pathogenicity in the mouse footpad model. Virology. 2017; 501: 107-114.