The Effects of Poxvirus E3L on Mutant Influenza Virus Lacking the NS1 Protein

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Candidate for the degree

Bachelor of Sciences

Submitted in partial fulfilment of the requirements for

College Honors

Departmental Distinction in Biology

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The Effects of Poxvirus Protein E3L on Mutant Influenza Virus Lacking the NS1 Protein

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ABSTRACT

Vaccinia virus (VACV) and ectromelia virus (ECTV; also known as "mousepox virus") are double-stranded DNA viruses in the poxvirus family. Influenza virus (Flu) is a single-stranded, segmented RNA virus that infects a wide variety of animal species. Although these viruses display distinct differences in their genome structure, they are able to infect a large variety of species. In order for many viruses like VACV, ECTV, and Flu to replicate and survive in host cells, they must produce immune response modifiers that combat the defense mechanisms of host cells. One major example of such an anti-viral system is the interferon cascade. This system activates protein kinase R (PKR) when a virus is recognized in an infected cell. This pathway shuts down the cellular translation machinery, which is necessary for the production of new infectious virus particles. VACV, ECTV, and Flu express proteins that act to inhibit the downstream effects of PKR in order to restore virus replication capacity. In VACV and ECTV, the protein is called E3L while the Flu version is termed NS1. Since these three viruses have proteins that target the PKR pathway, we wanted to discover if these proteins act in a universal manner or are functionally unique to each virus. In other words, we wanted to determine whether the presence of E3L from either VACV or ECTV E3L could rescue the replication of a mutant Flu virus lacking the NS1 protein (ΔNS1 Flu). To test this idea, the E3L protein from ECTV was first cloned into a mammalian expression plasmid (VACV E3L was obtained from a collaborator). Various types of cultured cells were transfected with the VACV E3L and ECTV E3L followed by infection with delNS1 virus and w.t. Flu. In addition, cells were transfected with the w.t. Flu NS1 protein; essentially, the protein was given back to ΔNS1 Flu as a positive control. In order to evaluate the amount of infection, flow cytometry was utilized to quantify the

expression of the Flu Hemagglutinin (HA) protein at the cell surface **(Figure 1)**. It was hypothesized that the VACV E3L, ECTV E3L, and the w.t. Flu NS1 protein would at least be able to partially rescue replication of the ΔNS1 Flu virus, which cannot itself successfully replicate in cells. After multiple experiments, it was distinguished that the VACV E3L and ECTV E3L does not have a notable affect on rescuing the replication process of the ΔNS1 Flu virus. Although, the E3L protein was unable to rescue the ΔNS1 Flu virus, giving back the NS1 protein to the ΔNS1 Flu virus, rescued the ΔNS1 Flu virus.

INTRODUCTION

Poxviruses belong to the family *Poxviridae*, subfamily *Chordopoxvirinae*, and genus orthopoxvirus and are known to infect an extensive variety of species. The poxviruses contain linear, double-stranded DNA (dsDNA) molecules. The genomes of most poxviruses, which are about 200 kilobases in length, are quite large relative to many other viruses. The viruses are about 300 by 250 by 200 nm in size and can be found in either a single or double envelope form. Unlike most other DNA-containing viruses, the life cycle is entirely contained to the cytoplasm of the host cell [1]. The poxviruses are known for their remarkable capacity to evade host immune responses through encoding varieties of proteins [2].

ECTV, also known as mousepox, is of the orthopoxvirus genus and its natural host is the mouse. ECTV is genetically similar to variola virus (VARV), which is the human smallpox virus, and therefore a useful model in understanding mechanisms of host defense and viral pathogens. The virus infects mice through abrasions on the skin and replicates in the epidermis before spreading to the lymph nodes, blood stream, and other organs. Mice who survive the infection acquire a pustular rash similar to humans infected with VARV [3].

VACV is also of the orthopoxvirus genus and is the most studied of all identified poxviruses. In fact, VACV was utilized by the World Health Organization (WHO) for the VARV vaccine [4]. VACV could have spread from humans to animals, such as cattle. During dry seasons other small animals would roam onto farms where VACV had access to these species and these species served as reservoirs for this particular virus [4]. Unlike the ECTV which spreads throughout the mouse after infection, VACV usually remains restricted to the site of initial infection (e.g. skin) [5].

Similar to poxviruses, influenza A virus (Flu) encodes proteins that inhibit antiviral defense mechanisms in host cells [6]. The flu virus belongs to the *Orthomyxoviridae* group, with a single-stranded RNA (ssRNA) genome consisting of eight different segments [7]. The virus infects mainly waterfowl, but also can infect other animals, like humans. The severity of a Flu infection in humans is influenced by the strain of the virus and the infected population; for example, the virus is generally only lethal in the elderly and the very young [8].

Both poxviruses and Flu encode proteins in which inhibit the interferon (IFN) system. The IFNs are a family of proteins that are released when a virus enters a cell, induces antiviral activity, and protects the cell against a viral infection. Host cells have evolved many sensors that notice that there is something foreign inside the cell [9]. IFNs stimulate antiviral pathways such as double-stranded RNA-dependent protein kinase R (PKR). PKR phosphorylates the eukaryotic translation initiation factor 2 α (eIF2-α), which is essential in host translation. The eIF2-α subunit carries the first amino acid, the start codon, to the ribosomes. When eIF2-α subunit is phosphorylated, the subunit is unable to carry the first amino acid to the ribosomes, and therefore shuts down host translation [10]. Viruses need host translation machinery in order to replicate

and survive inside host cells. Consequently, phosphorylated eIF2-α leads to the inhibition of viral replication, and the virus is unable to replicate within the host cell.

ECTV, VACV, and Flu encode proteins that enable the viruses to counteract the IFN-induced PKR pathway. The E3L plasmid is approximately 190 amino acids long and has a molar mass of 21.6 kDa and can be found in both the nucleus and cytoplasm in infected cells [11, 12]. E3L contains two functional domain regions: N-terminal and C-terminal. The N-terminal is the Z-DNA and PKR binding domain and the C-terminal is the dsRNA-binding domain [13]. The protein has many functions which include: blocking PKR-induced apoptosis, preventing DNA-dependent activator of interferon regulatory factor (DAI) from interacting with DNA, inhibiting the adenosine-to-inosine editing activity of IFN-induced ADAR and IRF3/7 activation, disabling ISG15 function, and most importantly binding to dsRNA and sequestering it away from PKR, inhibiting PKR activity by preventing 2'-5'-oligoadenylate synthetase [14]. Thus, E3L enables the virus to hijack host translation machinery in order to replicate and produce new virions.

Likewise, Flu encodes a multifunctional protein similar to ECTV and VACV. The multifunctional protein is the NS1 protein. It is approximately 230 amino acids in length, has a molar mass of 26 kDa, and is located on the eighth segment of the Flu genome [8, 15]. The protein location inside the host cell is dependent on several variable factors which include: the viral strain, the expression level of the protein NS1, the cell fixation procedure, the cell line infected and polarity of that cell, and the time post infection. Although protein location is variable, the NS1 protein is mainly found in the nucleus of the infected cell but can also be found in the cytoplasm as well [8]. The protein is nonstructural and is expressed at high levels in an infected cell. The structure can be broken into two functional domains: the N-terminal RNA-binding domain and the C-terminal effector domain. The N-terminal RNA-binding domain is a

symmetrical homodimer with each monomer containing three α-helices, for a total of six dimeric helices [8, 16]. The C-terminal effector domain is also a homodimer with each monomer containing seven β-strands, which are secondary structures [8, 16]. The β-strands are twisted, crescent-like, anti-parallel β-sheet around a long, central α-helix [8]. Furthermore, the NS1 protein consists of eight essential functions. NS1 is involved in viral RNA synthesis, strain-dependent pathogenesis, controls viral mRNA splicing, enhances viral mRNA translation, regulates viral particle morphogenesis, activates phosphoinositide 3-kinase (PI3K), and most importantly, inhibits host IFN antiviral responses [17]. Like E3L, NS1 binds to and sequesters dsRNA and directly binds to PKR, preventing the downstream effects of PKR [18]. Hence, NS1, similar to E3L, enables the virus to hijack host translation machinery in order to replicate and survive.

In a recent study by Guerra *et al.*, they tested the ability for the NS1 protein to compensate for the lack of E3L in VACV. In vitro, the NS1 protein was able to rescue the replication process of the VACV, and therefore the virus was able to survive inside the host cells [19]. In contrast, we attempted to study the reverse of this experiment: Can the ECTV protein E3L rescue the Flu virus that lacks the NS1 protein (delNS1 Flu), and can giving back the NS1 protein to the delNS1 flu virus rescue the virus *in vitro*?

MATERIALS and METHODS

Cell Maintenance:

European rabbit cell line RK13 was maintained in a T-75 flask containing Dulbecco's Modified Eagle Medium (DMEM; Life Technologies), 5% fetal bovine serum (FBS; Gemini BioProducts), and Penicillin-Streptomycin antibiotics (PS; Gemini BioProducts). Cells were

maintained in 37°C incubators (5% CO_2). To guarantee a sterile environment, all work with live cells was performed in a laminar flow hood and all materials used were sterile.

Subculturing:

Cell cultures were subcultured occasionally to maintain the cell line. DMEM medium was removed with a sterile vacuum pipette to ensure that all media was removed because FBS contained in the medium has a natural trypsin inhibitor. Then 4 mL of trypsin was added to the flask. The flask was then placed into a 37°C incubator for five minutes. The trypsin removes cells from each other and from the flask. 6 mL of DMEM medium was added to the flask to inactivate the trypsin. The cells were then washed.

Cell counting:

In order to count the cells, 10 µL of cells was removed from the conical tube and loaded into one chamber of the hemocytometer. The cells were examined using a light microscope under 40x magnification, with a field of view of one square millimeter (mm). Two of the nine square mm squares from each of the chambers were counted to determine the number of cells per 1 mL of medium. To obtain the number of cells per 1 mL, the number of cells were divided in half and then multiplied by the dilution factor and 2000. Cell count was utilized to quantify the amount of cells available to plate.

E3L cloning:

E3L forward primer KozXhol, sequenced 5'-CCA CTC GAG CCG CCA CCA TGG CTA AGA TCT ATA TTG ACG A -3', and E3L reverse primer StopBamHI, sequenced 5' –

CGA GGA TCC GAA TCT AAT GAT GAC GTA ACC AAG AAG -3' were utilized for the restriction digest. Restriction digest contents included 1 µL Xhol (BioLabs), 1 µL BamHI (BioLabs), 4 µL plasmid (pcDNA3.1(-)), 5 µL buffer four (BioLabs), 1 µL 10x BSA (BioLabs) , and 30 µL deionized water. The contents were digested at 37°C. After digestion, contents were gel purified on a 1% agarose gel and then isolated. The gel was purified using PureLink Quick Gel Extraction Kit. Then the DNA was quantified using Qubit® dsDNA BR Assay Kit. Following quantification, ligation took place. Ligation included 4 µL 5x buffer (BioLabs), 100 ng cut DNA, 3:1 PCR DNA, 1 µL ExpressLink™ T4 DNA, and 1 µL deionized water. The contents were incubated at room temperature. Next, a transformation of DH5α bacteria was carried out. Exactly 50 µL bacteria and 5 µL ligation reaction were added to an eppendorf tube and incubated on ice for twenty minutes. The contents were then heat shocked. Then 400 µL of 5.0 SOC media was added to the tube and was shaken for and hour at 37°C. The 250 µL of the contents were plated on an antibiotic and LB plate and incubated at 37°C overnight. A single colony was then picked and placed in serum, in a motion incubator at 37°C over night. Liquid was then collected and a midi prep was completed using PureLink HiPure Plasmid Midiprep Kit.

Flu infection:

DMEM was suctioned from each well. The wells were gently washed with 2 mL "Flu buffer", which is 1x PBS with some BSA, two times, suctioning off buffer after each wash. Then 0.5 mL flu buffer was added to each well. Virus was then individually added to its respective well (w.t flu 1 $TCID_{50}$ per cell and ΔNS1 flu 1 $TCID_{50}$ per cell). The plate was placed in a 37°C incubator for thirty minutes. Afterwards, 1.5 mL DMEM was added to each well and the plate was placed in the incubator overnight.

Transfection:

In a micro centrifuge tube #1 125 µL OPTI-MEM (IX) and 8 µL lipofectamine 3000 reagent, was added and then vortexed. In a separate micro centrifuge tube #2 2500ng DNA (empty plasmid 2.7 µL, NS1 plasmid 2.6 µL, VACV E3L plasmid 2.1 µL, or ECTV E3L 3.6 µL), 5 µL P3000 Reagent, and 125 µL OPTI-MEM (1X) was added and then vortexed. Contents from micro centrifuged tube #2 were transferred to micro centrifuge tube #1, vortexed, and then incubated at room temperature for five minutes. Then all the tube contents were added to one well. The plate was placed in a 37°C incubator overnight.

HA and NS1or E3L stain:

DMEM was suctioned from each well and then 300 µL of trypsin was added to each well and then the plate was placed in a 37°C incubator for five minutes. Approximately 700 µL of DMEM was added to each well. The cells from each well were collected and placed in individual flow cytometer tubes. Then 2 mL of FACS buffer was added to each tube. The cells were washed. The liquid was decanted. 2.0 µL α hemagglutinin (HA) was added to each tube and then incubated at room temperature, in the dark for twenty-five minutes. After incubation was completed 2 mL of FACS buffer was added to each individual tube and then cells were washed. Then the liquid was decanted from each tubes and 1 µL anti-mouse IgG FITC was added to all tubes. Tubes were incubated at room temperature, in the dark for twenty-five minutes. When incubation was completed 2 mL FACS buffer was added to each tube and then the cells were washed. The liquid was then decanted and 250 µL of cytofix/cytoperm was added to each tube. Then the tubes were incubated at room temperature, in the dark for twenty minutes. After

incubation was completed 2 mL of perm wash buffer (PWB), was added to each tube and then the cells were washed. The liquid was decanted and 1 µL α-NS1 (or 2 µL α-E3L) was added to each tube. Then the tubes were incubated at room temperature, in the dark for thirty minutes. After incubation was completed 2 mL of PWB was added to each tube and then the cells were washed. The liquid was decanted and 1 µL anti-mouse IgG2a APC was added to each tube and incubated at room temperature, in the dark for thirty minutes. After incubation, 2 mL PWB was added to the cells were washed. The liquid was then decanted and 400 µL paraformaldehyde (PFA) was added to the tubes. The data was then collected using flow cytometry.

E3L stain:

DMEM was suctioned from each well. 300 µL of trypsin was added to each well and then the plate was placed in a 37°C incubator for five minutes. 700 µL of DMEM was added to each well. The cells from each well were collected and placed in individual flow cytometer tubes. 2 mL of PWB was added to each tube. The cells were washed. The liquid was decanted and 2 µL α-E3L was added to each tube. Then the tubes were incubated at room temperature, in the dark for thirty minutes. After incubation was completed 2 mL of PWB was added to each tube and then the cells were washed. The liquid was decanted and 1 µL anti-mouse IgG2a APC was added to each tube and incubated at room temperature, in the dark for thirty minutes. After incubation, 2 mL PWB was added to the tubes and then the cells were washed. The liquid was then decanted and 400 µL PFA was added to the tubes. The data was then collected using flow cytometry.

HA surface stain:

DMEM was suctioned from each well and then 300 µL of trypsin was added to each well and then the plate was placed in a 37˚C incubator for five minutes. Approximately 700 µL of DMEM was added to each well. The cells from each well were collected and placed in individual flow cytometer tubes. Then 2 mL of FACS was added to each tube. The cells were washed. The liquid was decanted and 2 µL α-HA (a mouse antibody against HA) was added to each tube. Then the tubes were incubated at room temperature, in the dark for twenty-five minutes. After incubation was completed 2 mL of FACS was added to each tube and then the cells were washed. The liquid was decanted and 1 µL anti-mouse IgG FITC was added to each tube and incubated at room temperature, in the dark for twenty-five minutes. After incubation, 2 mL FACS was added to the tubes and then the cells were washed. The liquid was then decanted and 400 µL PFA was added to the tubes. The data was then collected using flow cytometry.

RESULTS

When RK13 cells were not transfected with the ECTV E3L plasmid, only had one population with no expression of E3L. **(Figure 2a)**. However, when RK13 cells were transfected with the ECTV E3L plasmid, there were two visible populations: a population with little to no expression of the E3L protein and a population with detectable E3L protein. The population with E3L protein expression was successfully transfected with the E3L plasmid (**Figure 2b**). When the cells were not transfected with the ECTV E3L plasmid, the population had no E3L expression, whereas the cells that were transfected were successfully expressing the E3L protein **(Figure 2c)**.

RK13 cells that were not transfected with ECTV E3L were infected with w.t. Flu. The cells were stained for HA, a protein that goes to the surface of the infected cell during the virus

life cycle. The cells that had surface expression of HA were the cells successfully infected with Flu. The RK13 control cells, not infected or transfected were stained for HA, and the cells were HA-negative and therefore were negative for the flu infection. Therefore, the cells that were infected with the w.t Flu were successfully infected, as expected **(Figure 3, Figure 5a)**. RK13 cells transfected with the ECTV E3L plasmid and then infected with the w.t. Flu were stained for HA and had two populations. The one population was HA-negative, indicating no Flu infection and the other population was HA positive, indicating a successful Flu infection **(Figure 4)**.

Additionally, RK13 cells that expressed ECTV E3L gene, without needing a transfection were infected with w.t. Flu. The infected cells had a population that was HA-positive, whereas the other population of cells that were not infected were HA-negative **(Figure 5b)**. The control population of the non-transfected and non-infected RK13 plain cells was HA-negative **(Figure 6a)**. When RK13 plain cells were infected with w.t. Flu, there was two populations. A population remained outside of the gated area (HA-negative) and a population of 42.41% into the gated area, which suggests the cells were successfully infected and HA-positive/flu-infected **(Figure 6b)**. When infected with ΔNS1 Flu, there were two populations. One population remained HA-negative and a population of 19.90% shifted into the gated area, which depicts they were successfully HA-positive **(Figure 6c)**. RK13 cells that expressed ECTV E3L gene were also infected with w.t. Flu and ΔNS1 Flu. When infected with w.t. Flu, there were two visible populations: HA-negative and the other population of 34.06% was HA-positive **(Figure 6e)**. When infected with ΔNS1 Flu, there were two populations: HA-negative and the other population of 15.56% was HA-positive **(Figure 6f)**.

RK13 plain cells that were not transfected or infected, the control group, had one population that were HA-negative and therefore had no flu infection **(Figure 7a)**. RK13 plain

cells that were not transfected and infected with ΔNS1 Flu had two populations. The large population was HA-negative and the small population, approximately 2.13%, was HA-positive **(Figure 7b)**. Cells that were transfected with an empty plasmid and then infected with the ΔNS1 Flu had two populations. The large population remained outside the gated area, HA-negative, whereas a population of 2.03%, was HA-positive **(Figure 7c)**. About 5.82% of cells that were transfected with the NS1 plasmid and then infected with the ΔNS1 Flu shifted into the gated area, indicating the cells were HA-positive **(Figure 7d)**. Approximately 2.69% cells that were transfected with the VACV E3L plasmid and then infected with the ΔNS1 Flu shifted into the gated area, representing HA-positive cells **(Figure 7e)**. Roughly 2.62% of cells transfected with the ECTV E3L plasmid and then infected with the ΔNS1 Flu were HA-positive **(Figure 7f)**.

DISCUSSION

The ECTV virus protein E3L was cloned and then transfected into RK13 plain cells. In order to test whether the ECTV E3L clone was viable and if the cells were successfully transfected, non-transfected and transfected cells were assessed for E3L expression. The control group, the non-transfected cells, was negative for the E3L protein, whereas the transfected cells had two populations: a population that was E3L-negative and a population that that was E3L-positive. Therefore, the ECTV E3L plasmid is viable and the transfection is a successful process.

Thereafter, RK13 plain cells were infected with the w.t. Flu virus and compared to the control, non-infected RK13 cells. In order to test whether the host cells are infected with the flu virus, a HA stain is necessary. HA is a protein that goes to the surface of an infected host cell during the life cycle of the Flu virus. Therefore, HA is utilized to quantify the amount of virus-infected cells. The flu-infected cells had a population of HA-positive cells indicating that the

cells were flu infected, compared to the control group, that were HA-negative. This indicates that the flu virus was able to replicate inside the host cells. Additionally, RK13 plain cells were transfected with the ECTV E3L plasmid and therefore the cells successfully express the ECTV E3L protein. When infected with the w.t. Flu, keeping in mind that the w.t. Flu possesses the NS1 protein; the cells have two distinct populations. The smaller population was HA-negative, which indicates that the virus was unable to replicate in these transfected cells. On the other hand, the other population was HA-positive. This indicates that some of the w.t. Flu virus was able to replicate and survive in these ECTV E3L transfected cells. These results are intriguing because in a study by Guerra *et al*., the NS1 protein was able to rescue a mutant VACV lacking the E3L protein *in vitro* (19). In figure 4, since there are two distinct populations, this suggested that the ECTV transfection is hindering the replication process of the w.t. Flu.

In order to understand if the transfection was the causative factor for the second population that was similar to the control group, RK13 cells that stably express ECTV E3L were obtained. The control, plain RK13 cells were not infected and the population were HA-negative, whereas the RK13 plain cells infected with w.t. Flu were HA-positive. RK13 cells that constitutively express E3L were then infected with the w.t. Flu virus. Compared to the control group, RK13 + E3L cells, the infected cells were HA-positive and infected. There was only one distinct population and therefore the ECTV E3L transfection could possibly be the cause of the two distinct populations in Figure 4. One explanation is that the transfection turns on the IFN system prior to the infection. Therefore, the host cells are getting a head start on viral protection and therefore the virus is unable to infect many cells. Cells that readily express the E3L protein do not have the pretreatment that triggers the IFN system because there was no introduction of foreign DNA via transfection prior to Flu infection.

Following, RK13 plain cells were either untransfected or transfected with the ECTV E3L plasmid and then infected with either the w.t. Flu or the ΔNS1 Flu. The control group, non-transfected and non-infected RK13 plain cells had essentially no HA expression, indicating that the cells were not infected with the virus. Plain RK13 cells that were infected with the w.t. Flu had two populations, a non-infected and infected population. The infected population consisted of approximately 42.41% cells, which indicates the cells were successfully infected with the w.t. Flu. The RK13 plain cells where then transfected with the E3L protein, but were not infected. There were two visible populations. The majority of the population was outside of the gated area, indicating that the cells were HA-negative and flu-negative. A population of approximately 0.37% (0%) shifted into the gated that indicates that the virus was replicating and HA-positive. When the plain RK13 cells were transfected with the ECTV E3L protein and then infected with the w.t. Flu, there were two distinct populations. The one population was the HA-negative, non-infected cells. The virus was unable to replicate and survive in this population. The population of 34.06% shifted into the box, and therefore the cells were infected with the virus, HA-positive. The virus was able to replicate and survive in these cells. The quantity of infected cells dropped approximately 8%, which indicates that the ECTV E3L protein is not as affective as the NS1 protein in the virus replication process.

The NS1 protein demonstrates at least eight essential functions, such as viral RNA synthesis, strain-dependent pathogenesis, controlling viral mRNA splicing, enhancing viral mRNA translation, regulating viral particle morphogenesis, activating phosphoinositide 3-kinase (PI3K), and inhibiting host IFN antiviral responses [17]. The NS1 protein has many more effects for the flu virus than does the E3L protein for the VACV and ECTV. The E3L protein functions include blocking PKR-induced apoptosis, preventing DNA-dependent activator of interferon

regulatory factor (DAI) from interacting with DNA, inhibiting the adenosine-to-inosine editing activity of IFN-induced ADAR and IRF3/7 activation, disabling ISG15 function, and most importantly binding to dsRNA and sequestering it away from PKR, inhibiting PKR activity by preventing 2'-5'-oligoadenylate synthetase [14]. Since these functions are similar, yet differ greatly, the E3L protein may not be able to compensate for the NS1 protein because it does not have the necessary functions. Therefore, when the E3L protein binds and sequesters dsRNA, the NS1 protein is unable to bind to the dsRNA. Thus, E3L prevents the detection of the virus by the host cell, but is unable to carry out complete replication processes that the NS1 protein is able to do for the flu virus. Hence, when cells are transfected with the E3L protein and then infected with the w.t. Flu, the E3L and NS1 protein are competing against each other to bind to the dsRNA and when the E3L protein binds to the dsRNA, it does not have the necessary functions to carry out the NS1 protein functions.

Moreover, when plain RK13 cells were infected with the ΔNS1 Flu, 19.90% of the cells were infected and able to replicate in these cells. Compared to the w.t. Flu, there was approximately a 20% drop of infection, which depicts that the lack of the NS1 protein hinders the replication and survival of the ΔNS1 Flu in host cells. Furthermore, when the RK13 plain cells were transfected with the ECTV E3L protein and then infected with the ΔNS1 Flu, approximately 15.56% of the cells were HA-positive. The percentage of infected cells was actually lower in the transfected cells than the non-transfected cells. This indicates that the transfection is turning on the IFN system prior to the infection, and that the E3L protein is unable to compensate the ΔNS1 Flu for the loss of the NS1 protein.

Additionally, RK13 plain cells were transfected with various plasmids to understand the various affects different proteins have on the virus. The control, RK13 plain cells that were not

infected with the ΔNS1 Flu, had approximately 0% of HA-positive cells. RK13 plain cells that were not transfected, but infected with the ΔNS1 Flu, 2.13% of cells were infected. The small quantity of infected cells is expected because the ΔNS1 Flu does not have the protein to sequester the dsRNA from the PKR pathway. RK13 cells that were transfected with an empty plasmid and infected with the ΔNS1 Flu had approximately 2.03% of cells infected. The quantity of infected cells decreased because the plasmid is turning on the IFN pathway before infection, and therefore the cells are prepped for the viral infection. Additionally, when the RK13 plain cells were transfected with the NS1 protein, the protein was given back to the virus, approximately 5.82% of cells were infected. The NS1 protein was able to recover about 3% of the viral infection compared to the ΔNS1 Flu infection on plain RK13 cells. This indicates that given back the NS1 protein at least partialluy the virus. When RK13 plain cells were transfected with the VACV E3L plasmid and then infected with the ΔNS1 Flu, approximately 2.69% of the cells were infected. Similarly, RK13 plain cells were transfected with the E3L protein and then infected with the ΔNS1 Flu and about 2.62% of cells were infected. The VACV and ECTV protein E3L is able to rescue the ΔNS1 Flu minimally, compared to the NS1 protein. Again, the E3L protein and NS1 protein has many commonalities, but also have distinct functionalities (19).

In conclusion, the ECTV E3L protein is able to rescue the ΔNS1 Flu minimally (if at all) and when the NS1 protein is given back to the ΔNS1 Flu, the replication process is somewhat restored. E3L protein has many functions that are similar to the NS1 protein, but the proteins do have differences (19). These differences are enough to resist the rescue of the ΔNS1 Flu by the E3L protein. The E3L protein can bind to the dsRNA of the Flu virus, but is unable to aid the virus through its replication cycle and therefore is unable to rescue the virus, which contradicts the study by Guerra and colleagues, in which the NS1 protein was able to rescue the VACV

lacking the E3L protein. Therefore, the NS1 protein performs more essential functions for the Flu virus than the E3L protein performs for the ECTV and VACV.

ACKNOWLEDGEMENTS

I would like to thank Dr. Adam Hersperger for not only being my faculty advisor, but for his guidance and persistent support throughout my Virology research. I would also like to thank Dr. Hersperger for the wealth of knowledge he has instilled in me-and for all the time and effort he put in to further my collegiate experience. Finally I would like to thank Dr. Andrew Samuelsen and Dr. Frieda Texter for their contributions as my departmental and college readers.

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APPENDIX

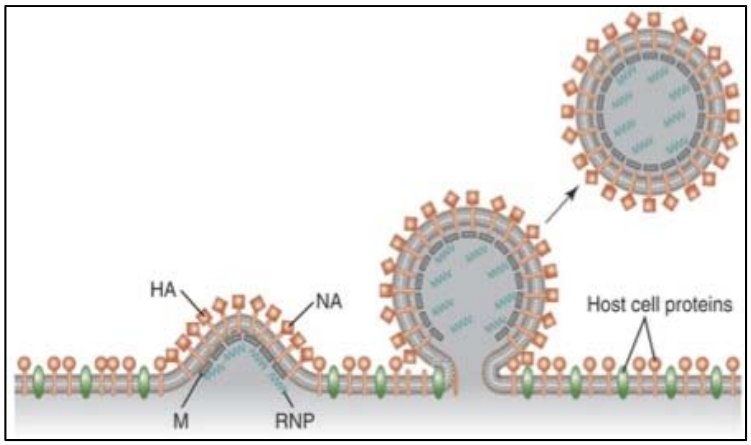

Figure 1: Hemagglutinin (HA) protein that goes to the surface of flu infected cells during the flu life cycle.

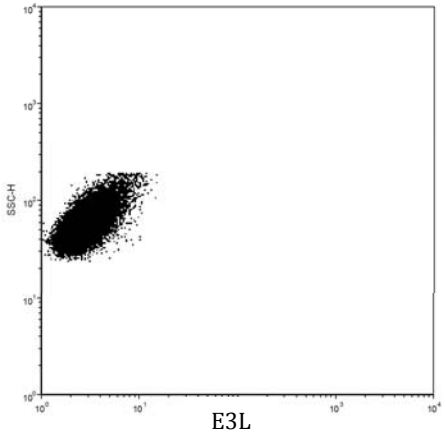

Figure 2a: Control, non-transfected RK13 cells, stained for ECTV E3L.

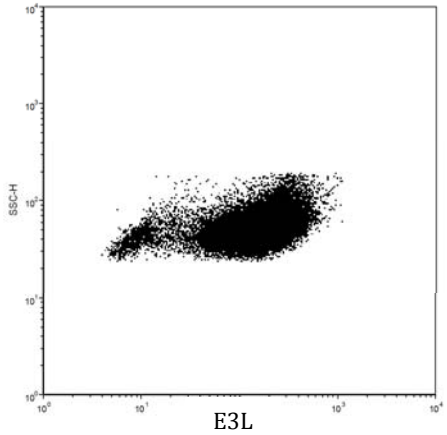

Figure 2b: ECTV E3L transfected cells, stained for E3L.

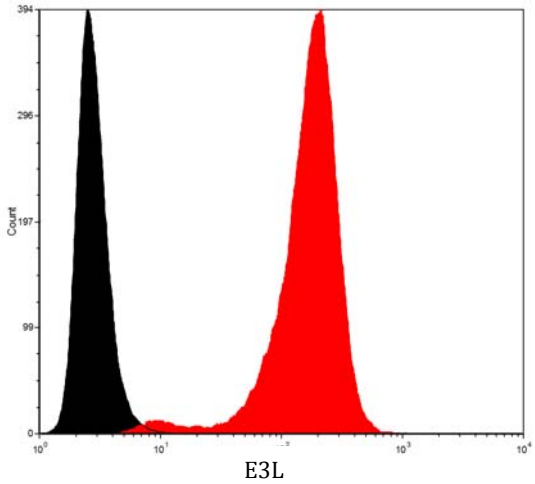

Figure 2c: Histogram comparing ECTV E3L transfected cells (red) vs. non-transfected cells (black).

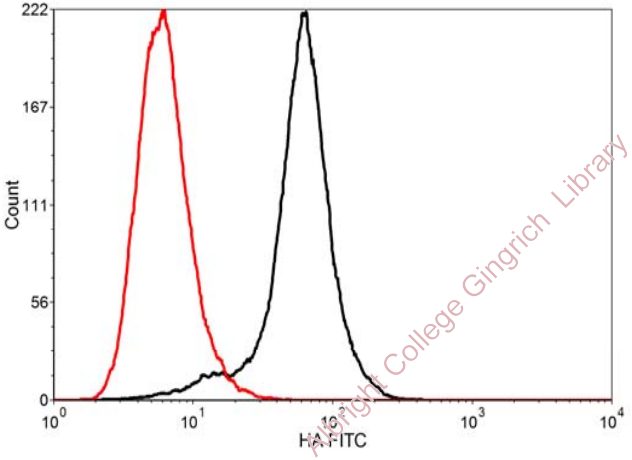

Figure 3: Histogram comparing RK13 plain cells infected with w.t. Flu (black) vs. uninfected control (red).

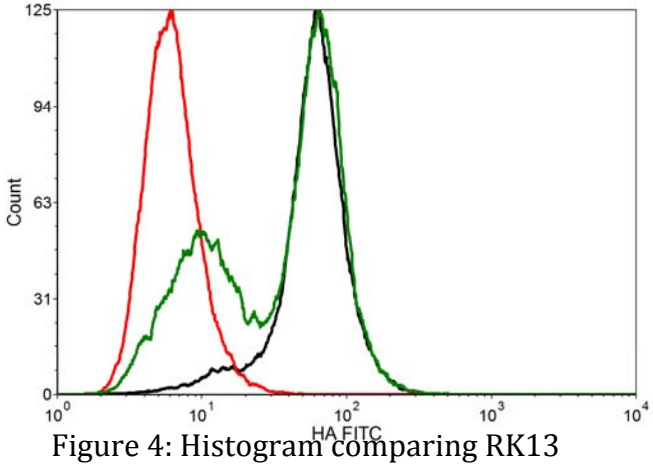

Figure 4: Histogram comparing RK13 plain cells infected with w.t. Flu (black) vs. uninfected control (red) vs. transfected cells with ECTV E3L and infected with w.t. Flu (green).

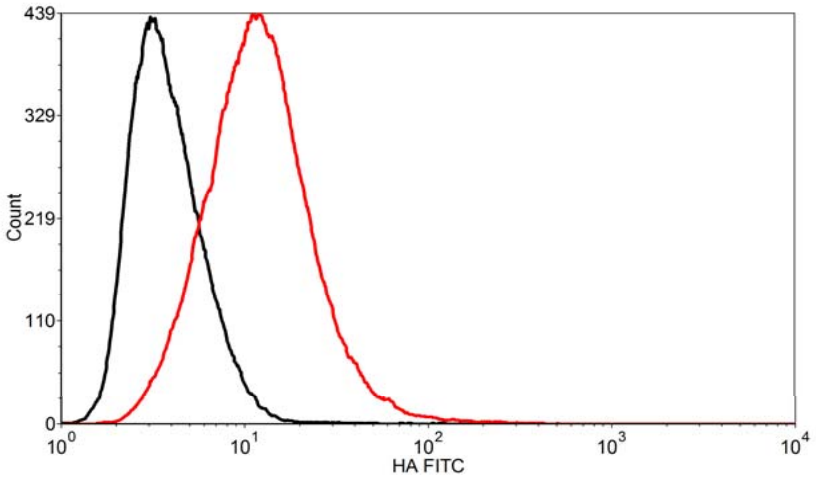

Figure 5a: Histogram comparing RK13 plain cells uninfected (black) vs. w.t. Flu infected (red).

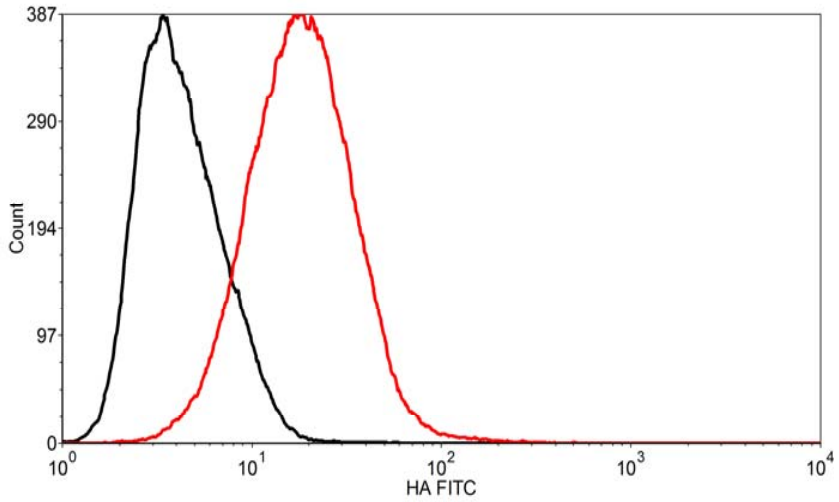

Figure 5b: Histogram comparing RK13 cells expressing theVACV E3L protein uninfected (black) vs. w.t. Flu infected

RK13 plain no virus

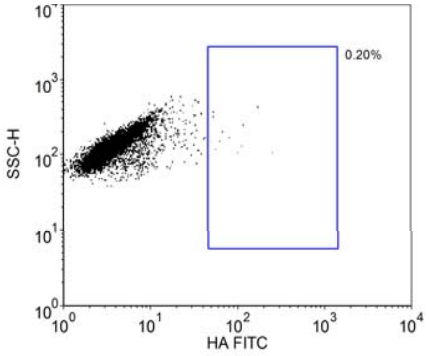

Figure 6a: Control, non-transfected, uninfected RK13 plain cells stained for HA.

RK13 plain + wild-type Flu

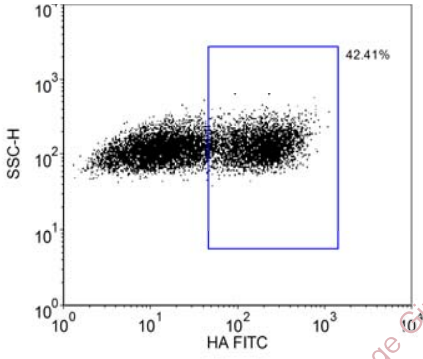

Figure 6b: RK13 plain cells infected with w.t. Flu and stained for HA.

RK13 plain + ΔNS1 Flu

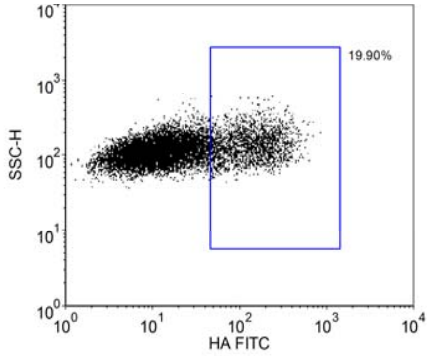

Figure 6c: RK13 plain cells infected with ΔNS1 Flu and stained for HA.

RK13+E3L no virus

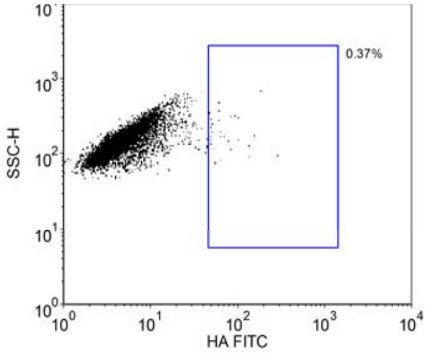

Figure 6d: Control, non-transfected, uninfected RK13 cells expressing ECTV E3L and stained for HA.

RK13+E3L + wild-type Flu

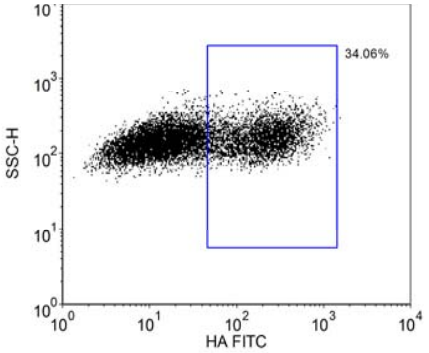

Figure 6e: RK13 cells expressing ECTV E3L infected with w.t. Flu and stained for HA.

RK13+E3L + ΔNS1 Flu

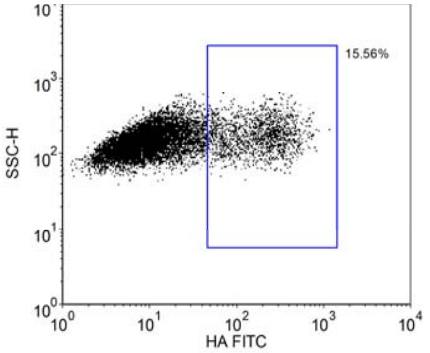

Figure 6f: RK13 cells expressing ECTV E3L infected with ΔNS1 Flu and stained for HA.

uninfected control

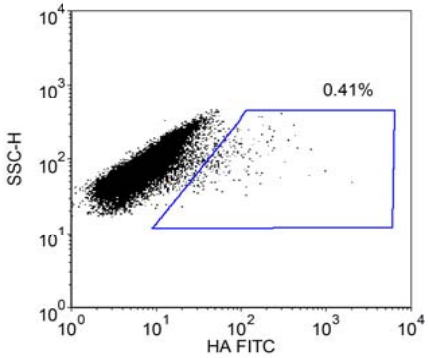

Figure 7a: Control, non-transfected, uninfected RK13 plain cells and stained for HA.

ΔNS1 Flu alone (no transfection)

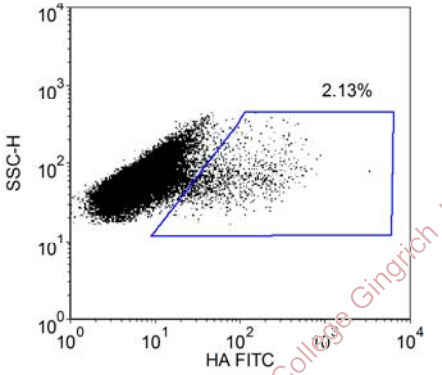

Figure 7b: RK13 plain cells infected with ΔNS1 Flu and stained for HA.

ΔNS1 Flu + empty plasmid

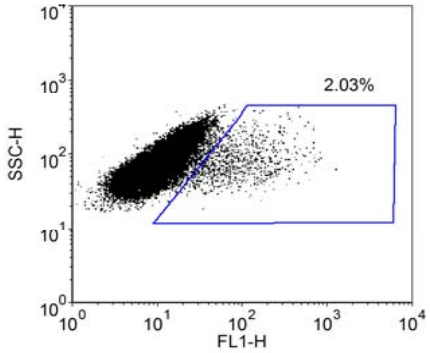

Figure 7c: RK13 plain cells transfected with an empty plasmid and infected with ΔNS1 Flu and stained for HA.

ΔNS1 Flu + NS1 plasmid

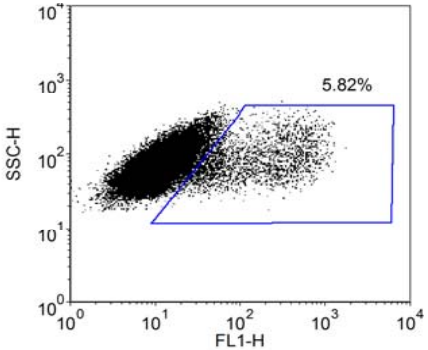

Figure 7d: RK13 plain cells transfected with the NS1 plasmid and infected with ΔNS1 Flu and stained for HA

ΔNS1 Flu + VACV E3L plasmid

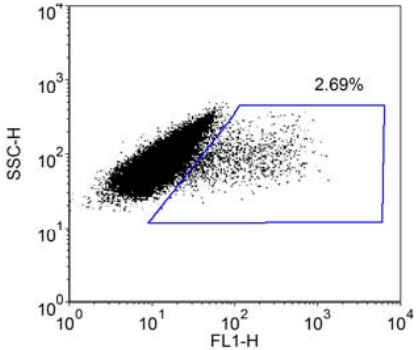

Figure 7e: RK13 plain cells transfected with the VACV E3L plasmid and infected with ΔNS1 Flu and stained for HA

ΔNS1 Flu + ECTV E3L plasmid

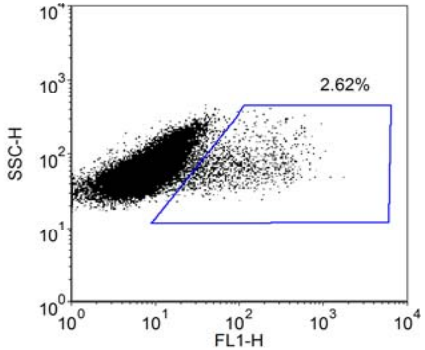

Figure 7e: RK13 plain cells transfected with the ECTV E3L plasmid and infected with ΔNS1 Flu and stained for HA