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The Effect of Cyclophilin A on Vaccinia Virus Replication in vitro

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Abstract

The host protein cyclophilin A (CypA) has been shown to be necessary for viral replication of several viruses, including human immunodeficiency virus and hepatitis C virus. Previous research done in the Geary laboratory, in collaboration with Pacific Northwest National Laboratory, reported that cyclophilin A remains highly associated with vaccinia virus virions even after successive rounds of purification. The goal of this research is to further investigate how cyclophilin A affects vaccinia virus replication *in vitro* using primary tissues harvested from CypA (-/-) knockout mice to determine the difference in viral growth kinetics. Results showed different viral growth kinetics in different primary tissues. These results indicate that vaccinia virus is capable of replicating in CypA negative cells, but that there may be tissue specific differences that could play a role in *in vivo* pathogenesis.

Introduction

Vaccinia Virus

Vaccinia virus (VACV) is a member of the *Poxviridae* family and is the prototypical member of the orthopoxvirus genus (2). Its virion is characterized by having a complex brick shape and contains 190 kb of linear dsDNA (2). It replicates in the cytoplasm of host cells and its genome encodes the majority of the proteins necessary for replication (2). Its other distinguishing characteristics includes two lateral bodies that give the core a distinct dumbbell shape and surface tubules on the exterior of the outer membrane that have no distinct pattern of arrangement (2).

Historically, VACV is known for its use as a vaccine against Variola virus (2). Variola virus is the causative agent of smallpox, it is highly transmissible between people, and has a 25% fatality rate (2). Variola virus is species-specific to humans and has no known animal reservoirs (2). The disease dates back to ancient times with characteristic skin lesions that can be seen on the face of the mummy of Pharaoh Ramses V, who died in 1157 B.C. (22). Combating smallpox dates back to the 11th century where it was recognized in China and India that scarifying healthy individuals with the scabs from smallpox victims resulted in immunity against smallpox (20). The process was known as variolation and although effective carried the risk of inducing systemic smallpox (20). In 1796, Edward Jenner inoculated a small boy with cowpox and then challenged the child with smallpox (20). The child survived both inoculations and the process of vaccination was born (20). At some point in the history

while cowpox was being used to vaccinate against smallpox, it was replaced by vaccinia virus which has no known natural host (21).

In 1966, the World Health Organization (WHO) started a deliberate campaign to eradicate smallpox (2). Finally, in 1977 the last natural case of smallpox was reported in Somalia, and in 1980 WHO officially announced the eradication of the disease (2). Although the disease was successfully eradicated, known stocks still remain in the United States and Russia. The United States Centers for Disease Control and Prevention still considers Variola virus a category A agent as it is easily transmitted from person to person, has a high mortality rate with a potential for a major impact on public health, could cause public panic along with other social disruptions, and would require special measures to be taken by public health departments (5).

To prepare for a potential emergency, multiple lines of research have been followed to enable an effective and efficient response. Previous research done by Dr. Geary's laboratory, in collaboration with Pacific Northwest National Laboratory, investigated the possibility of using microbial forensics through proteomics on viral samples to identify biological signatures which could indicate production methods and sources for attribution purposes (3). Viruses are obligate intracellular pathogens which creates the potential for protein carryover from host cells and growth media (3). An example of host protein carryover in viral particles can be seen in the influenza vaccine. This vaccine is grown in chicken eggs and has enough residual proteins from the egg that it can trigger an allergic reaction in people with egg allergies (3).

Previous research studying protein signatures in virions have had contradictory results (3). This was most likely due to different approaches used for purification and analysis. To address this, Dr. Geary's laboratory in collaboration with Pacific Northwest National Laboratory compared the proteomics of orthopoxvirus samples that had been been grown in different cell types and subjected to different levels of purification (3). After each step of purification, a fraction of each sample was saved for proteomic analysis and the remainder of the sample was subjected to additional levels of purification (3). Fractions analyzed included crude cell samples, samples physically disrupted by a dounce, two sucrose cushion gradients, and finally an isopycnic gradient (3). Each fraction was analyzed to determine the proteins that remained associated with the virion after each step of purification (3). Proteomics revealed that in crude preparations the majority of proteins were of host cell or of media origin, but the samples that were subjected to higher levels of purification showed that the majority of the proteins associated with the virion were of viral origin (3). Viral samples collected from different cell types, but purified to the same extent, showed different protein signatures (3). However, a few proteins remained associated with the orthopoxviruses virions even after extensive purification (3). One such host protein, cyclophilin A, was conserved across cell lines and remained associated with the orthopoxvirus virion even after isopycnic purification (3).

Cyclophilin A

Cycolphilin A (CypA) is a cytosolic 18kD peptidyl-prolyl cis-trans isomerase (PPIase) protein (1). It is a member of the PPIase family which encompasses several groups of proteins including cyclophilins, immunophilins, FK-506-binding proteins, and

parvulins which are highly conserved across the kingdoms (1). The cyclophilin family has a conserved region of 109 amino acids which constitutes the cyclophilin-like domain of the protein (1). The other domains of the cyclophilin protein typically dictate its cellular compartment (1). For example, in humans, CypA, CypNK and Cyp40 are all located in the cytoplasm, while CypB and CypC are located in the endoplasmic reticulum, and CypD is in the mitochondria (1). PPIase proteins aid in protein folding by enabling the change from the energy favored trans state to the unfavored cis state. This is particularly true for the amino acid proline. It takes a considerable amount of free energy to manipulate the amino acid chain into the sterically unfavored cis state where the side chains of proline and another amino acid are adjacent to one another (1). This creates a rate limiting step in protein folding (1). Proteins with PPIase activity are capable of stabilizing the cis-trans transition state (1).

Cyclophilin A in Viral Replication

CypA has been previously shown to be necessary for replication of some viruses. CypA has been shown to interact with the Gag protein of human immunodeficiency virus 1 (HIV-1) and was later shown to be incorporated into the HIV-1 virion (8, 9). This interaction with CypA, has been shown not to be necessary for viral infection, but is necessary for viral replication (10,11). CypA also interacts with several other components of HIV-1 including CD147, heparins, Viral protein R, and the envelope glycoprotein gp120, although continued research is needed to determine the significance on these interactions (12, 13, 14, 15).

CypA has also been shown to play a crucial role in Hepatitis C virus (HCV) replication and has shown promise as a possible drug target for HCV treatment (17). CypA binds nonstructural protein 5A (NS5A) and nonstructural protein 5B (NS5B) (16). Specifically, domain II of NS5A was shown to have RNA binding capabilities that were dependent on the PPIase activity of CypA (17). Although, CypA has been shown to be necessary for HCV replication, the mechanisms of this interaction are still not fully understood (17).

Cyclophilin A in Vaccinia Virus Replication

Previous research done by Castro et al. investigated CypA activity during VACV replication *in vitro* in BSC-40 kidney epithelial cells from the primate *Cercopithecus aethiops* (4, 7). They began by comparing CypA levels in VACV infected cells and non-infected cells. No detectable difference in protein level was found between the two 24 hours post infection (4). This was surprising because VACV shuts down host protein synthesis 1 to 4 hours after infection depending on the multiplicity of infection used (24). To determine why CypA levels remained the same in VACV infected cells as mock infected cells, they compared levels of CypA synthesis after infecting cells with VACV. At two hours post infection, VACV infected cells were still synthesizing CypA protein at the same level as mock infected cells (4). However, 8 hours post infection, CypA synthesis was halted in VACV infected cells while the mock infected cells continued to synthesize the protein (4). This raised the question of how CypA levels can be maintained in VACV infected cells at the same level of mock infected cells 24 hours post infection if CypA synthesis is halted. To address this, rates of CypA degradation were compared by radioactively labeling CypA. Cells that were infected with VACV

retained 100% of their radioactively labeled CypA after 18 hours of infection (4). In comparison, mock infected cells had already degraded 50% of their radioactively labeled CypA (4). This explains how, despite the halt to CypA synthesis, VACV infected cells are able to maintain the same level of CypA as mock infected cells.

Comparisons of CypA location during VACV infection versus mock infected cells was also done using fluorescence confocal microscopy. Samples were stained with DAPI, which binds to DNA, and was used to differentiate between the nucleus, viral factories, and the cytoplasm (4). A fluorescently tagged CypA antibody was used to tag CypA (4). In mock infected cells, CypA was evenly distributed throughout the cytoplasm (4). However, in VACV infected cells, CypA was redistributed to specific sectors of the cytoplasm (4). These sectors had distinct points of DAPI staining indicating the presence of viral DNA (4). These results showed that CypA association with the VACV virion does not occur randomly, but that VACV replication results in the redistribution of CypA to virosomes (4).

Vaccinia Virus Replication in the Presence of Cyclosporin A

To test if CypA is necessary for VACV replication, Castro et al. used cyclosporin A (CsA) to inhibit CypA. CsA is an immunosuppressive drug that is commonly administered in patients to prevent graft rejection (18). The mechanism that leads to immunosuppression is started by CsA binding to the hydrophobic pocket of CypA (1). This structure then binds to calcineurin forming a ternary complex and becomes very resistant to cellular degradation (1). Once calcineurin is bound, its phosphatase activity is inhibited (1). This inhibition stops the nuclear factor of activated

T cells (NF-AT) from being transported into the nucleus and triggering the transcription of key genes, such as interleukin-2 (1).

Castro et al. infected BSC-40 cells with VACV in the presence of 35µM CsA to see if there would be any effect on CypA localization with the virosomes (4). Results showed that in CsA treated cells CypA remained dispersed throughout the cytoplasm and was completely excluded from the virosomes (1). An interesting observation made was that although CypA no longer localized with the virosome in the presence of CsA, granular pockets of CypA were noted in CsA-VACV infected cells. This pattern was not seen in mock infected cells, which always showed equal distribution of CypA throughout the cytoplasm (4). Using AraC, a drug which blocks DNA synthesis, similar staining patterns of granular pockets of CypA were seen, indicating that only early stages of VACV replication are necessary to begin subtle shifts in CypA redistribution (4).

CypA has been shown to be redistributed during VACV infection and that CsA is able to prevent CypA from being redistributed during VACV infection. To determine the effect on VACV replication if CypA was not able to redistribute to the virosome, Damaso et al. analyzed VACV titers in the presence of CsA (25). Their research showed a dose dependent reduction of VACV in response to increasing concentrations of CsA (25).

Project Goals

Previous research showed that VACV replication was inhibited by CsA (25). Due to the redistribution of CypA during VACV infection, it was concluded that CypA was the main target of CsA and thus played a critical role in VACV replication (4). While CsA does have a high affinity for CypA, it is a highly promiscuous drug which also interacts

with numerous other cyclophilins in the cells which may be preventing VACV replication (19). To specifically address the affect of CypA in viral replication, we generated CypA negative cells and infected them with VACV. Replication was assessed by viral growth kinetics using a multistep viral replication analysis and by plaque morphology, size and number. VACV was also grown in CypA negative cells in the presence of CsA to assess if CsA had any further affect on VACV replication even when CypA was not present.

Hypothesis

Vaccinia virus replication will be reduced in cyclophilin A negative cells.

Project Aims

Aim 1: Generate CypA negative cells

Aim 2: Assess VACV replication *in vitro* in CypA positive and CypA negative cells

Aim 3: Assess the affect of CsA on VACV replication in CypA negative cells

Aim 1: Generate Cyclophilin A Negative Cells

Since CypA negative cells were not commercially available it was necessary to generate them. Two methods were attempted to generate CypA negative cells *in vitro.* The first method used short-interfering RNA (siRNA) which utilizes double stranded RNA to target specific cellular mRNA to be degraded by the host cell. This degradation leads to a decrease in gene expression and consequently protein expression. Although this method does not completely knockout the gene of interest, it does silence gene expression significantly and gives an indication of the replication capability of VACV in

the absence of CypA. However, after many attempts to optimize siRNA conditions, there was variability in silencing with no reliable or reproducible results (data not shown).

The second method used to generate CypA negative cells was to establish a colony of CypA negative mice as a source of CypA negative primary cells. The benefit of this method is that CypA is not expressed in any of the cells, so any differences in VACV growth can be attributed to the presence or absence of CypA.

To generate cyclophilin A negative cells, a colony of cyclophilin A negative mice was established. 129.Cg-Ppia^{tm1Lubn}/J (F129) transgenic cyclophilin A heterozygous males was obtained from Jackson Laboratory and breed to F129 wild-type females. These transgenic mice were generated by disrupting the single copy gene of CypA in mouse embryonic stem (ES) cells (23). This was done by creating a plasmid carrying the disrupted CypA gene along with a neomycin resistance gene (23). This plasmid was then incorporated into ES cells via electroporation and finally ES cells were grown in media in the presence of neomycin to select for the desired recombination event (23). Thus ES cells that could grow under selective drug pressure had undergone at least one recombination event (23).

In communications with Dr. Luban who generated these mice, we learned that CypA negative F129 cyclophilin A mice did not produce viable offspring (6). To create a viable colony, F129 transgenic cyclophilin A heterozygous males were crossed with C57BL/6 wild-type females. F129/C57BL/6 females had larger litters and were capable of bringing pups to weaning age, while F129 CypA negative females could not.

Four tissues were selected to establish primary cell cultures: embryos, lung, liver, and kidney. Using several different methods, it was determined that liver and kidney cells were very difficult to generate. Very few cells were adherent to the plate and showed no indication of replicating in cell culture. Lung tissues resulted in adherent cells, although the cells only minimally replicated in culture. Embryonic tissue generated a high number of viable adherent cells, which could replicate. For these reasons, primary lung and embryonic cells were selected to assess VACV replication *in vitro.*

Aim 2: Assess Vaccinia Virus Replication *in vitro*

Viral replication was assessed by a multistep viral replication analysis and by plaque analysis. To determine the kinetics of VACV replication *in vitro,* a multistep viral replication analysis was performed. These samples were then titrated on Vero cells to establish the plaque forming units (PFU) per milliliter (mL) for each sample.

Since CypA is not present in the cell culture, we expected that the virus would demonstrate reduced replication in CypA negative primary cell cultures. Therefore, when the titration was performed we would see reduced numbers of plaques in the samples collected from the CypA negative primary cell cultures and high titers of plaques from samples collected from the CypA positive primary cell cultures. However, if CypA is not necessary for VACV replication, we expected to see plaques in the CypA negative samples when they are titrated. VACV would be able to grow in CypA negative cells for instance if another cyclophilin (PPIase) was functionally replacing CypA in the viral replication process. If so, further proteomic analysis will have to be performed in order to identify this other entity.

A plaque assay will be performed in the advent that VACV is able to replicate in CypA negative cells. If CypA is necessary for VACV replication, then we expect to see reduced numbers of plaques on the CypA negative cells. However, if VACV is able to replicate in CypA negative cells, there may be other differences in plaque morphology, number, or size between plaques grown in CypA positive and CypA negative cells. A decrease in plaque number and size should be detectable in CypA negative cells in comparison to CypA positive controls if CypA plays a critical role in VACV replication.

Aim 3: Assess the Effect of Cyclosporin A on VACV Replication in Cyclophilin A Negative Cells

CsA has been shown to play a key role in preventing VACV replication *in vitro* (4). To determine the kinetics of VACV replication in CypA negative cells in the presence of CsA, a viral replication analysis will be performed and samples will be titrated on Vero cells to determine the PFU per mL. From these experiments, we expect to see inhibition of VACV replication in CypA positive cells in the presence of CsA as previously reported by Castro et. al. (4). However, it is difficult to predict how VACV will replicate in CypA negative cells in the presence of CsA. It may have no effect because there is no CypA for CsA to bind to or since CsA is promiscuous, it may bind a different PPIase protein which may alter VACV ability to replicate.

Materials and Methods

Generating Cyclophilin A Negative Mice

129.Cg-Ppia^{tm1Lubn}/J (F129) transgenic cyclophilin A heterozygous males were obtained from Jackson Laboratory and breed to F129 wild-type females. The resulting

heterozygous pups were then breed to generate CypA negative mice. The genotype of these mice was determined by standard PCR methods using primers oIMR3772, oIMR3773, and oIMR3774 ordered from Jackson Laboratory. F129 mice had a low breeding efficiency and CypA negative females had difficulties becoming pregnant and were not able to generate offspring. To create a viable colony, F129 transgenic CypA heterozygous males were crossed with C57BL/6 wild-type females. F129/C57BL/6 CypA negative mice were used to generate primary embryo cell cultures, while F129 CypA negative mice were used to generate primary lung cell cultures.

Generating Cyclophilin A Negative Cells

To generate primary embryonic cells, CypA negative mice were bred and females were sacrificed 14 to 20 days post-conception. Tissues were collected and processed in a BSL-2 biological safety cabinet to ensure sterility. Tissues of interest were collected and placed in individual 100 mm dish filled with 15 mL of 37℃ DMEM without serum. Each tissue was then washed with DMEM in the dish to remove as many red blood cells as possible. For embryonic tissue, the placental sac, placenta, and cranium of the embryo were removed and discarded. Remaining media was discarded and the tissue was finely minced using #10 scalpels. Minced tissues were resuspended in 50 mL of 37℃ DMEM with 12.5 mg collagenase type I from Life Technologies. Samples were then transferred to a spinner flask and placed on a stir plate set at 60 rpm in a 37℃ 5% CO_2 incubator for four hours. After four hours a minimal of 50 mL of DMEM 10% FBS 1xL-glutamine was added to the spinner flask and samples were plated in culture flasks. Additional DMEM 10% FBS 1xL-glutamine was added to bring culture flasks up to a final volume of 25 mL. (Approximately 3 embryos or 5 lungs per 1

T175) Cells were maintained in a 37℃ 5% CO_2 incubator, media was changed every 3 to 4 days (DMEM 10%FBS 1 x L-glutamine), cultures were maintained at about 60-80% confluence, minimal growth was noted in the primary lungs cells, and primary embryo cells were split when necessary.

Multistep Viral Replication Analysis

CypA positive and CypA negative embryonic and lung cells were plated in 12 well plates with 10^5 cells per well 24 hours in advance of inoculation. These were then inoculated with VACV at an MOI of 0.01. Quadruplicate wells were harvested at each time point: 0, 6, 24, 30, 48, 72, and 96 hours post infection. Infected cells and supernatant were harvested by using sterile tips of syringe plungers to scrap the cells off the plate. Supernatant and cells were harvested as one sample and stored at -80℃. Data was non-parametric in nature and was analyzed using the Mann-Whitney Rank Sum Test.

Titration

Samples were titrated on *Cercopithecus aethiops* kidney cell line (Vero cells) to establish the plaque forming units (PFU) per milliliter for each sample. Vero cells were plated 24 hours in advance with 4 x 10^5 cells per well in 12 well plates and maintained in a 37℃ 5% CO_2 incubator. VACV samples collected at all time points were subjected to three freeze-thaws from -80℃ to room temperature. Samples were then subjected to sonication twice at 100% amplitude for 1 minute, with a vortex and quick spin in between. Samples were then centrifuged at 400 x g at 4℃ for 5 minutes to remove any cell particulates from the viral suspension. 10-fold serial dilutions of each sample were

then used to infect Vero cells to determine the PFU per mL. Triplicate wells were infected with each dilution, incubated for 48 hours in a 37℃ 5% CO_2 incubator, and then stained with 0.1 grams crystal violet per 100 mL in 20% ethanol. All plaques were counted and recorded.

Plaque Analysis

CypA positive and CypA negative embryo and lung cells were plated in 12 well plates at a density of 4×10^5 cells per well and maintained at 37℃ in a 5% CO_2 incubator 24 hours before inoculation. Due to differences in infectivity discovered during preliminary experiments (data not shown) primary embryo cells were inoculated with less virus than primary lung cells. Primary embryo cells were inoculated with 10 infectious particles of VACV per well. Primary lung cells were inoculated with 50 infectious particles of VACV per well. Plates were stained at each time point: 0, 6, 24, 30, 48, 72, and 96 hours post infection with 0.1 grams crystal violet per 100 mL in 20% ethanol. The resulting plaques were measured using a Zeiss Axio Observer D1 Inverted Microscope to obtain images and Axio Vision 4.8 was used to measure the resulting plaque size. Plaque size was analyzed using a standard t-test due to its parametric nature. Plaque number data was analyzed using the Mann-Whitney Rank Sum Test due to its non-parametric nature.

Assessing Cyclosporin A (CsA) Toxicity

CsA was dissolved in 100% DMSO and stored at 4℃ at 1000x concentrated stock solutions (stock solutions: 15μM). CypA positive and CypA negative embryonic and lung cells were plated in 12 well plates $4(10^5)$ cells per well 24 hours in advance

and stored in a 37℃ 5% CO_2 incubator. The media was then removed and replaced with media containing 0 µM (0.1% DMSO) or 15 µM CsA. The supernatant and cells were then collected separately. The supernatant was collected directly and the cells were trypsinized, spun down and resuspended in sterile phosphate buffered saline. Cell viability was then assessed with propidium iodide on a flow cytometer. No cellular toxicity was noted at 24 hpi, 48 hpi, or 72 hpi (Data not shown).

Viral Replication Analysis in the Presence of Cyclosporin A

CsA was dissolved in 100% DMSO and stored at 4℃ at 1000x concentrated stock solutions (stock solutions: 5 µM, 10 µM, and 15 µM). CypA positive and CypA negative embryonic and lung cells were plated in 12 well plates with 10^5 cells per well 24 hours in advance of inoculation and maintained in a 37℃ 5% CO_2 incubator. These were then inoculated with VACV at an MOI of 0.1. Two hours post infection the inoculum media was removed and replaced with fresh media containing: 0 µM (0.1% DMSO), 5 µM, 10 µM, and 15 µM CsA. All samples were run in triplicate. Wells were harvested at 24 hours post infection. Infected cells and supernatants were harvested by using sterile syringe plunger tips to scrap the cells off the plate. Supernatants and cells were harvested as one sample and stored at -80℃. Data was non-parametric in nature and was analyzed using the Mann-Whitney Rank Sum Test.

Results

Multistep Viral Replication Analysis of Vaccinia Virus *in vitro*

In primary embryo cells similar VACV growth rates were observed in both the CypA negative cells and CypA positive cells (fig. 1a-c). In lung primary cell cultures

VACV had a statistically significant reduced growth rate in CypA negative cells from 30 hpi onward in two of the primary cell sets (fig. 2a and 2c). Finally, a difference between primary lung and embryo cells was observed. There was a lag in growth rate and lower titers of VACV in primary lung cells in comparison to primary embryo cells and was independent of the presence or absence of CypA.

Plaque Analysis of Vaccinia Virus Replication *in vitro*

In the plaque assay, plaques formed in both CypA positive and CypA negative primary embryo and primary lung cells. Plaques measured in primary embryo cells showed a statistically significant difference in plaque radius only at 30 hpi (fig. 3a). At time points past 48 hpi there were no well isolated plaques that could be measured due to overlapping plaques generated from secondary infection. In primary lung cells, no plaques were observed until 48 hpi and plaques in CypA negative cells were statistically significantly smaller than plaques measured in CypA positive cells (fig. 3b). Later time points yielded well isolated plaques, but were too large to be measured.

The number of plaques from each plate was also quantified. In primary embryo cells there was a statistically significant difference in plaque number at 24 hpi or 30 hpi (fig. 4a). Plates stained beyond 30 hpi showed an increase in plaques in CypA positive cells, but could not be quantified due to plaque overlap (fig. 5a). However, at 72 hpi it is visually apparent that the monolayer of CypA positive cells had been completely disrupted, while portions of the monolayer were still intact in the CypA negative cells (fig. 5a). Varying sizes of plaques can be seen in primary embryo cells from 48 hpi onward in both CypA positive and negative cells. This is an indication of secondary

infections, which is due to the fact that VACV has several forms of infectious particles. Cell-associated enveloped (CEV) VACV is responsible for the cell-to-cell spread of VACV and results in a single plaque which increases in size over time (26). Extracellular enveloped (EEV) VACV is released from the cell, spreads to a new area of the cell culture and creates a new plaque (27). These plaques start later than the original plaque and are smaller in size than the original plaque. Thus secondary infection can be noted when varying plaque sizes are seen throughout the monolayer.

Plaque number was also assessed in primary lung cells. At 48 hpi, there was not a statistically significant difference in plaque number between CypA positive and negative lung cells (fig.4b). At 72 hpi, there was a statistically significant increase in plaque number in CypA positive cells (fig. 4b). Upon gross examination of the wells distinct plaques can be seen and all are well isolated even at 72 hpi (fig. 5b). There appears to be no significant secondary infection in primary lung cells in either CypA positive or CypA negative cultures as indicated by the uniform size of the plaques suggesting that the plaques formed at approximately the same time post-infection.

Vaccinia Virus Replication in the Presence of Cyclosporin A *in vitro*

Results indicated that in CypA positive cells, as the concentration of CsA was increased, there was a decrease in VACV titer in both primary embryo and lung cells (fig. 6a-b). Similarly, in CypA negative cells there was a decrease in VACV titers observed in a dose dependent manner to CsA (fig. 6a-b).

Discussion

Based on previous *in vitro* studies, it was hypothesized that VACV would exhibit reduced replication in CypA negative cells. Research using proteomics to search for biological signatures showed that CypA remained highly associated with the VACV virion even after being subjected to increasing levels of purification (2). It was also shown *in vitro* that during VACV replication CypA, which is normally evenly distributed throughout the cytoplasm, is redistributed to VACV virosomes (4). To determine if this redistribution was directed by VACV and not an artifact of VACV infection, the authors used CsA to disrupt CypA redistribution during VACV replication (4). Castro et al. showed that when CsA was administered during VACV infection of cells, VACV was no longer able to redistribute CypA (4). Their research also showed that VACV replication was reduced to only residual titers in the presence of CsA, indicating that CypA is a critical host protein for VACV replication (25). However, CsA is known to be a promiscuous drug which can bind several members of the PPIase family (18). To analyze the role that CypA specifically plays during VACV infection, CypA negative primary cells were used to analyze VACV growth in the absence of CypA.

. The multistep viral replication analysis in primary embryo cells revealed that VACV growth is the same in CypA positive and CypA negative cells. In primary lung cells, there is a statistically significant decrease in VACV titers in CypA negative cells from 48 hpi onward in two of the three primary cell sets (fig 2a-c). Results from the data collected from embryo primary cells show that VACV is capable of replicating in the absence of CypA.

VACV growth was further analyzed by comparing primary plaque radii and numbers in CypA positive and negative cells. Results showed that only at 30 hpi was there a statistically significant difference in plaque size in primary embryo cells, with statistically significantly smaller plaques in CypA negative cells (fig. 3a). Interestingly, this trend was not seen at 48 hpi. There was also a statistically significant difference in plaque radius in primary lung cells at 48hpi (fig. 3b). These results indicate a difference in viral spread between the CypA positive and CypA negative cells. This is further supported since there was a statistically significant difference in plaque number in embryo cells at 24 hpi or 30 hpi (fig. 4a). There were also observed differences in the cell monolayer at 72hpi that indicated a decrease in VACV plaques in CypA negative cells (fig. 5a). Similarly in primary lung cells there was a statistically significant difference in plaque number at 72 hpi (fig. 4b).

To analyze these results further, several parameters regarding the primary tissues should be taken into consideration. In the multistep viral growth analysis, the cell density and MOI used were the same in primary embryo and lung cells. However, approximately a log less virus was observed in primary lung cells than in primary embryo cells. In the plaque assay, it was necessary to use five times as much virus to infect primary lung cells than embryo cells in order to establish plaques. Despite this, primary embryo cells had more plaques present at 30 hpi than primary lung cells had at 72 hpi. Finally, lung cells had well isolated plaques at 72 hpi and had no evidence of secondary infection. In embryo cells the monolayer was greatly disrupted and showed evidence of secondary infection in both CypA positive and CypA negative cells.

Collectively these data indicate a difference in viral susceptibility and release. Primary lung cells appear to be less susceptible to VACV infection than primary embryo cells. This was evident by the need to increase the inoculum of VACV in the plaque assay for primary lung cells. Primary lung cells also appear to have a reduction in viral release as seen in the plaque assay where only primary plaques were present. In contrast, secondary plaques were present in primary embryo plaque assays. These differences in viral susceptibility and release could account for the differences seen in the multistep viral growth analysis between primary lung and embryo cells. The log difference in virus could be due to increase release of virus in primary embryo cells. As a preliminary step in addressing this possibility, a multistep viral growth analysis could be performed and cells (containing primarily IMV particles) and the supernatant (containing primarily EEV particles) could be independently assessed to analyze the different VACV virions present.

Viral susceptibility and release in the lung could be a critical factor *in vivo* since orthopoxviruses are typically transmitted by the aerosol route of infection (28). The reduction in viral titer in CypA negative primary lung cells could indicate a reduced susceptibility to VACV infection. However, the primary embryo data should be considered also since VACV could replicate readily in CypA negative cells. Further work *in vivo* is necessary to determine the biological significance of these findings. Due to the differences between primary lung and embryo cells, careful consideration should be taken of route of infection *in vivo,* as different routes can have different courses of pathogenesis (29).

Previous work done *in vitro* analyzing VACV growth in the presence of CsA showed only residual levels of VACV titers (25). Results shown in figures 1 and 2 indicate that in both primary lung cells and primary embryo cells VACV was capable of replicating in CypA negative cells, despite the differences in final viral load. This indicates that there is a more complicated mechanism to VACV replication and that VACV is not dependent on CypA to replicate. Due to the promiscuous nature of CsA, the possibility that CsA could be interacting with multiple proteins that are combining to inhibit VACV replication was considered. To evaluate this possibility, VACV was grown in the presence of increasing concentrations of CsA. With the administration of increasing concentrations of CsA in CypA positive cells, there was a decrease in VACV titers, as expected. In CypA negative cells, as increasing concentrations of CsA were administered there was also a decrease in VACV titers. This suggests the CsA is interacting with protein(s) other than CypA which aid in VACV replication and whose mechanisms are yet to be elucidated. There are fourteen cyclophilin paralogues in human cells, one or even multiple of which could be acting as a functional replacement for CypA in CypA negative cells during viral replication. To assess this possibility further, viral progeny grown in CypA negative cells could be analyzed with proteomics to determine if another PPIase protein is incorporated into the virion in lieu of CypA.

In conclusion, this work demonstrated that VACV can replicate in the absence of CypA, but a statistically significant reduction in viral load was observed in primary lung cells. The biological significance of this finding remains to be determined. If the absence of CypA reduces VACV growth to the point where the immune system has time to respond to the infection, then there should be a reduction in the disease state. However,

if the reduced VACV growth is able to supersede the immune response, then there would likely be no difference in pathogenesis. To elucidate the mechanism of VACV replication in the absence of CypA, further proteomic analysis would need to be performed to see what other proteins may be interacting with VACV and allowing its replication.

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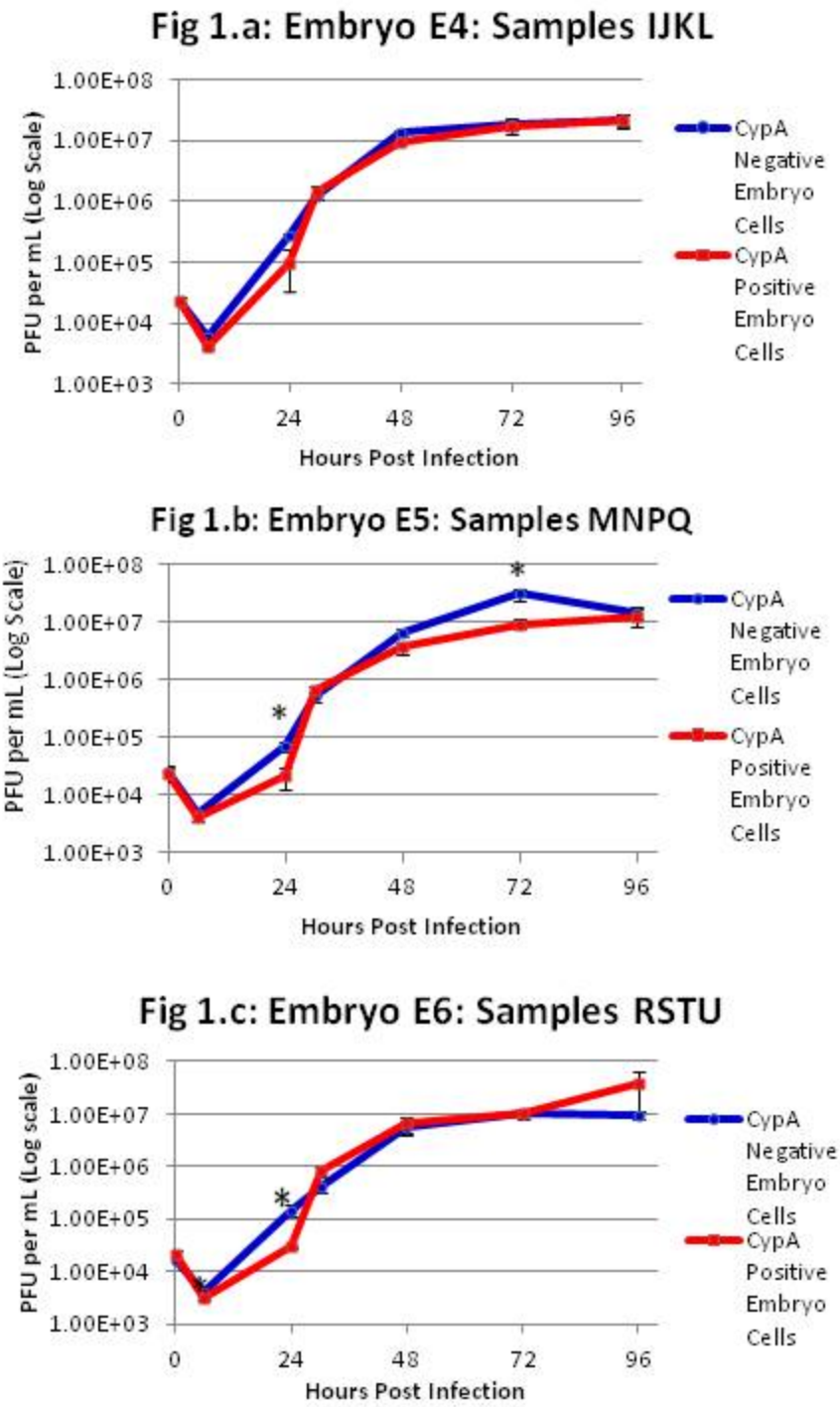
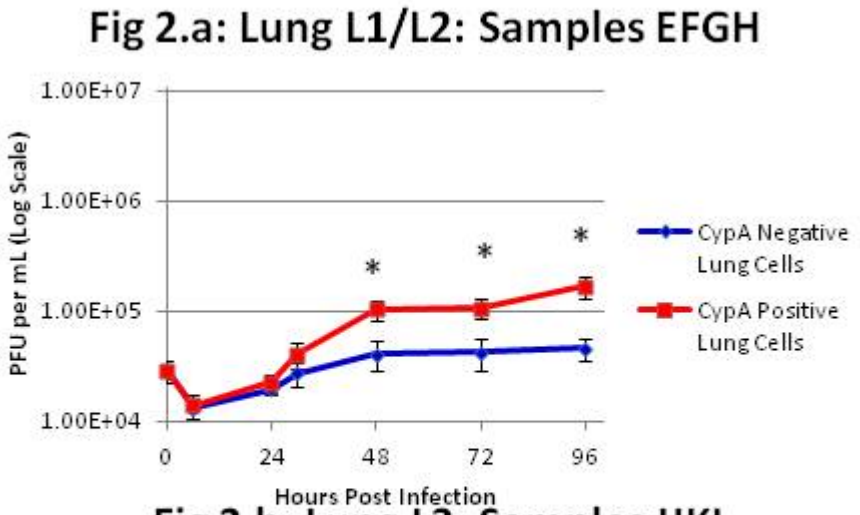
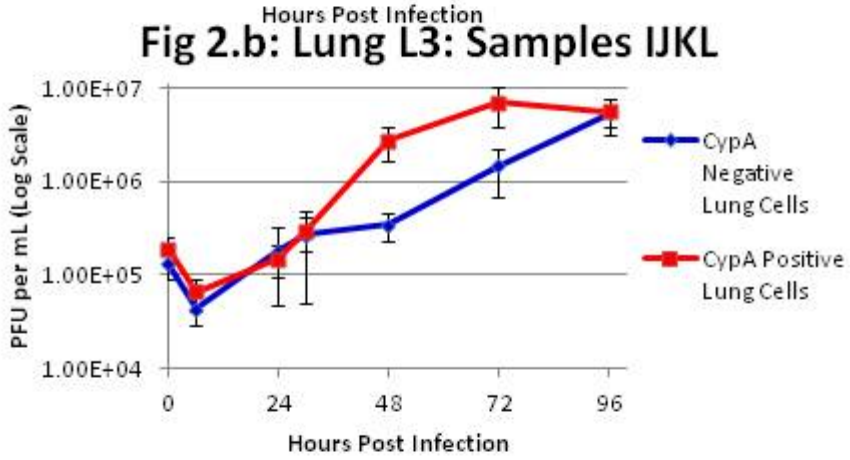


Figure1: Multistep Viral Replication Analysis in primary embryo cells. Three sets of primary embryo cells of both CypA positive and negative were harvested separately and run independently (Fig 1a-c). A multistep viral replication analysis at an MOI of 0.01 was performed over a 96 hour period and quadruplicate samples were harvested for each genotype at each time point. Viral titers were assessed by titration. Data analyzed using the Mann-Whitney Rank Sum test * indicates a statistical significance of $P \leq 0.05$. Error bars indicate the standard error of the mean.





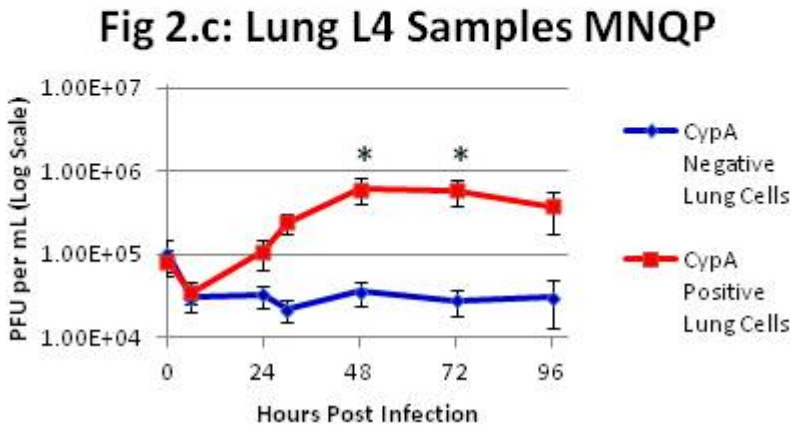
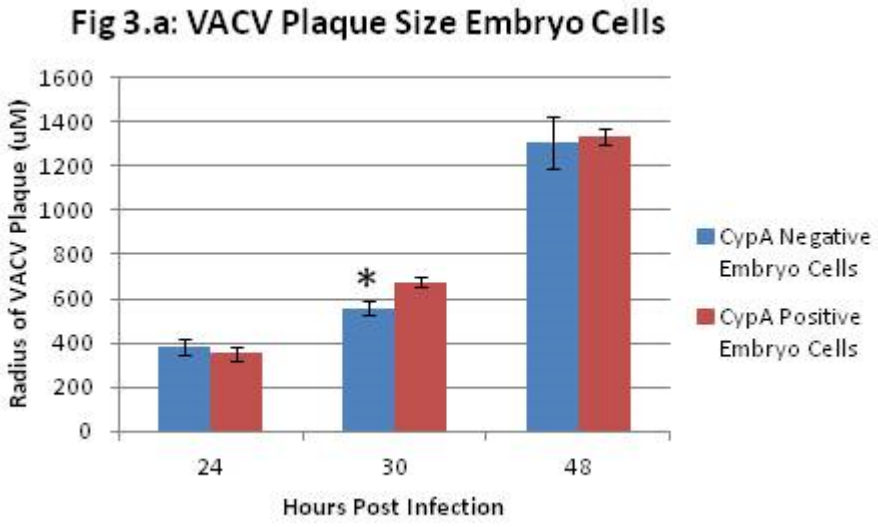


Figure 2: Multistep Viral Replication Analysis in Primary Lung Cells. Three sets of primary lung cells of both CypA positive and negative were harvested separately and run independently (Fig 1a-c). A multistep viral replication analysis at an MOI of 0.01 was performed over a 96 hour period and quadruplicate samples were harvested for each genotype at each time point. Viral titers were assessed by titration. Data analyzed using the Mann-Whitney Rank Sum test * indicates a statistical significance of $P \leq 0.05$. Error bars indicate the standard error of the mean.



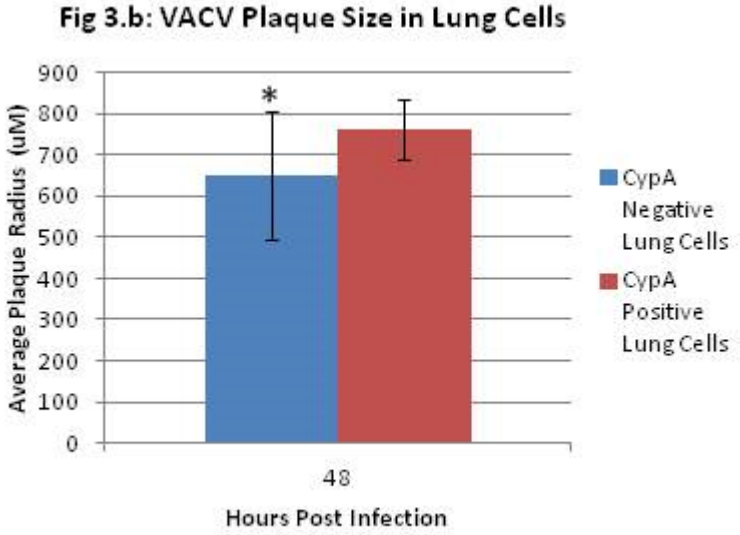
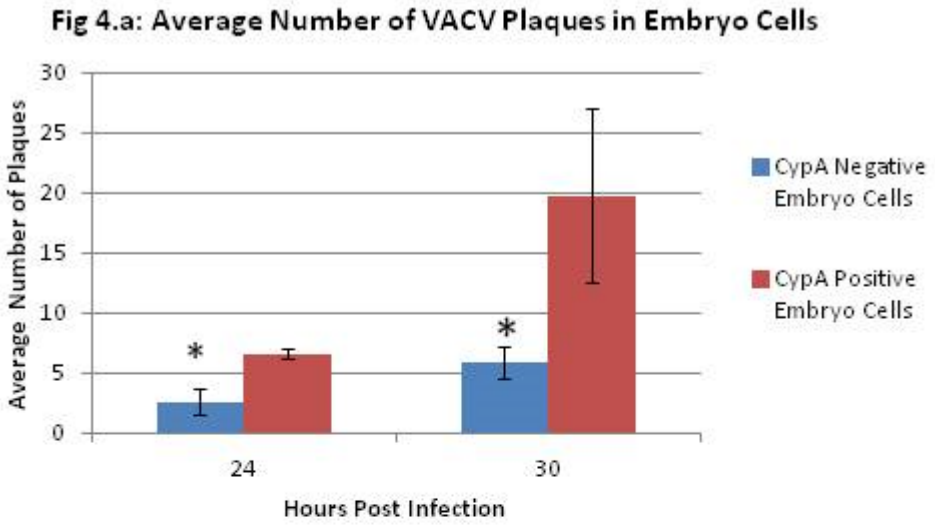


Figure 3 Vaccinia Virus Plaque Size. a) Three sets of primary embryo cells of both CypA positive and negative were harvested separately and run independently. A plaque assay was performed using 10 PFU per well. Samples were run in quadruplicate. Plates were stained over a 96 hour time periods with 0.1 grams crystal violet per 100 mL in 20% ethanol. The resulting plaques were then measured by using a Zeiss Axio Observer D1 Inverted Microscope to obtain images and Axio Vision 4.8 were used to measure the resulting plaque size. Plaques stained beyond 48 hpi were not well isolated and could not be measured) Two sets of primary lung cells of both CypA positive and negative were harvested separately and run independently. A plaque assay was performed using 50 PFU per well. Samples were run in quadruplicate. Plates were stained over a 96 hour time period with 0.1 grams crystal violet per 100 mL in 20% ethanol. The resulting plaques were then measured by using a Zeiss Axio Observer D1 Inverted Microscope to obtain images and Axio Vision 4.8 was used to measure the resulting plaque size. Plaques stained beyond 48 hpi were too large to be measured on the Zeiss Axio Observer D1 Inverted Microscope. Data analyzed with a standard t-test * indicates a statistical significance of $P \leq 0.05$. Error bars indicate the standard error of the mean.



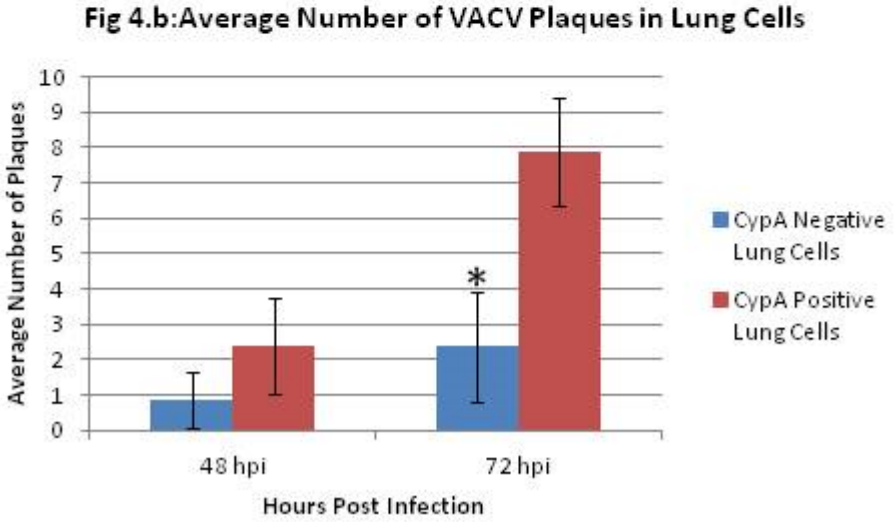


Figure 4 VACV Plaque Numbers. a) Three sets of primary embryo cells of both CypA positive and negative were harvested separately and run independently. A plaque assay was performed using 10 PFU per well. Samples were run in quadruplicate. Plates were stained over a 96 hour time period with 0.1 grams crystal violet per 100 mL in 20% ethanol. b) Two sets of primary lung cells of both CypA positive and negative were harvested separately and run independently. A plaque assay was performed using 50 PFU per well. Samples were run in quadruplicate. Plates were stained over a 96 hour time period with 0.1 grams crystal violet per 100 mL in 20% ethanol. Data analyzed using the Mann-Whitney Rank Sum test * indicates a statistical significance of $P \leq 0.05$. Error bars indicate the standard error of the mean

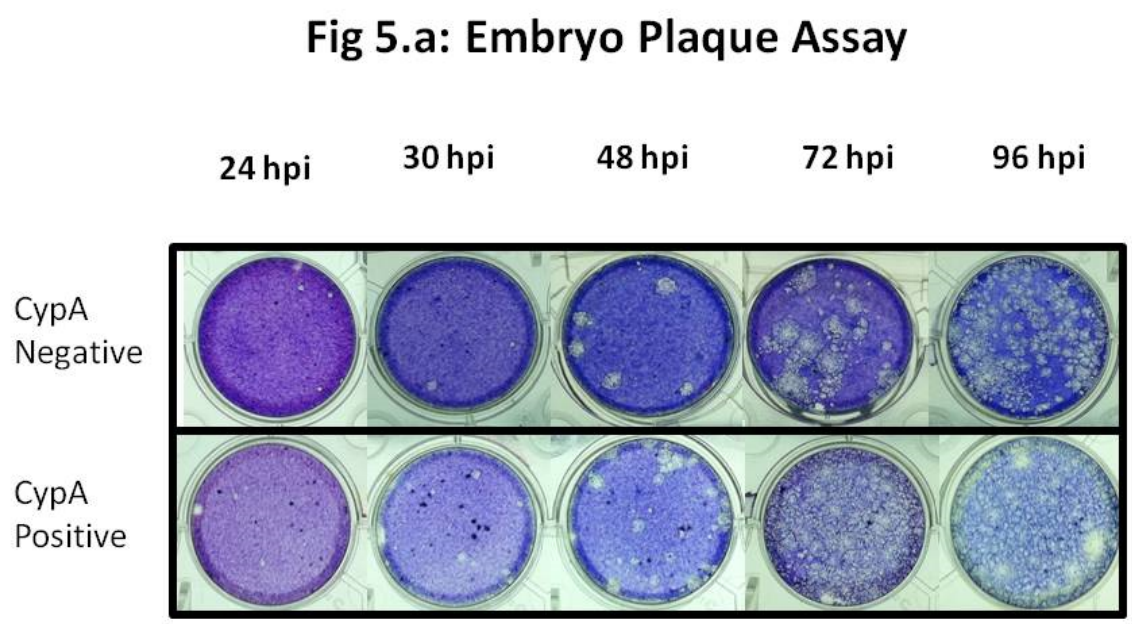


Figure 5a Representative gross Image of Embryo Plaque Assay. a) Three sets of primary embryo cells of both CypA positive and negative were harvested separately and run independently. A plaque assay was performed using 10 PFU per well. Samples were run in quadruplicate. Plates were stained over a 96 hour time periods with 0.1 grams crystal violet per 100 mL in 20% ethanol.

Figure 5.b: Lung Plaque Assay

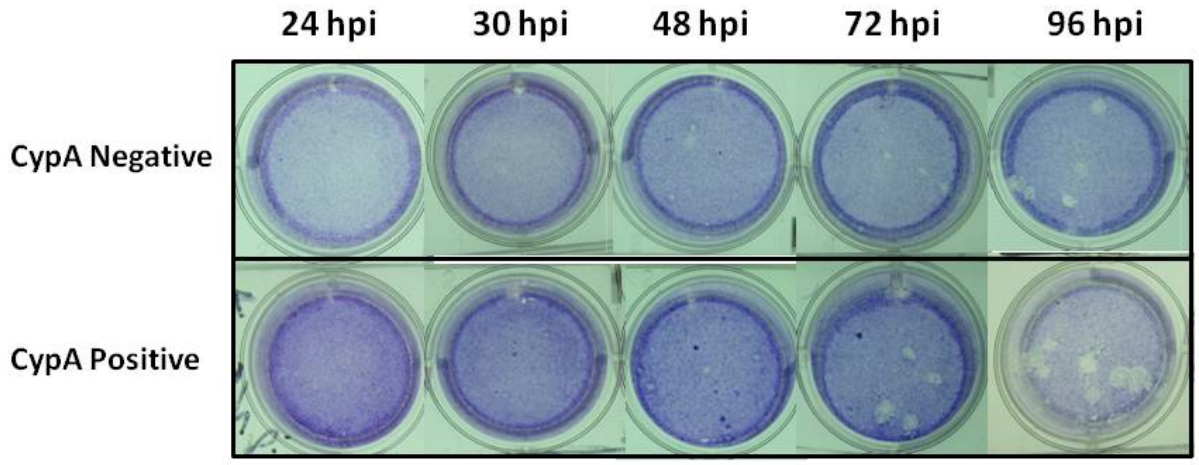


Figure 5b Representative gross Image of Lung Plaque Assay. b) Two sets of primary lung cells of both CypA positive and negative were harvested separately and run independently. A plaque assay was performed using 50 PFU per well. Samples were run in quadruplicate. Plates were stained over a 96 hour time period with 0.1 grams crystal violet per 100 mL in 20% ethanol.

Fig 6.a: VACV Growth in Embryo Cells in the Presence of CsA

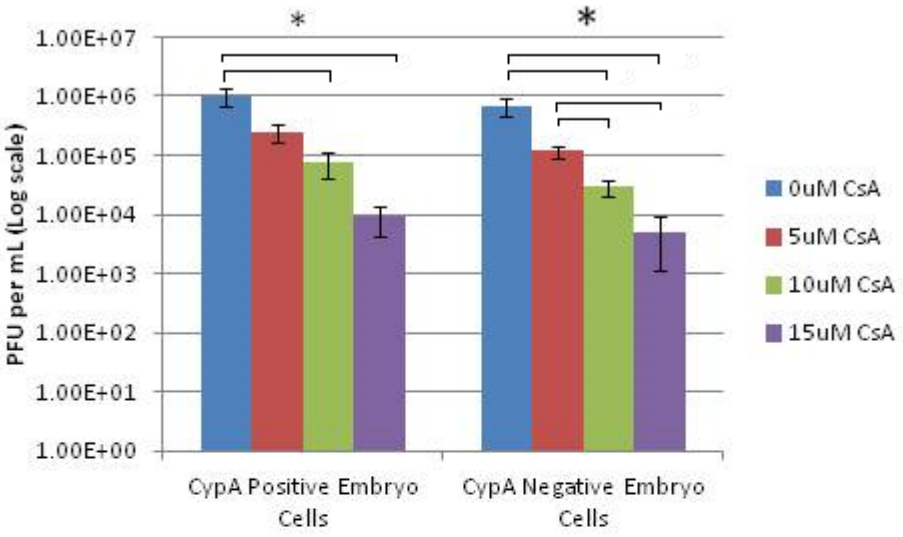


Fig 6 b: VACV Growth in Lung Cells in the Presence of CsA

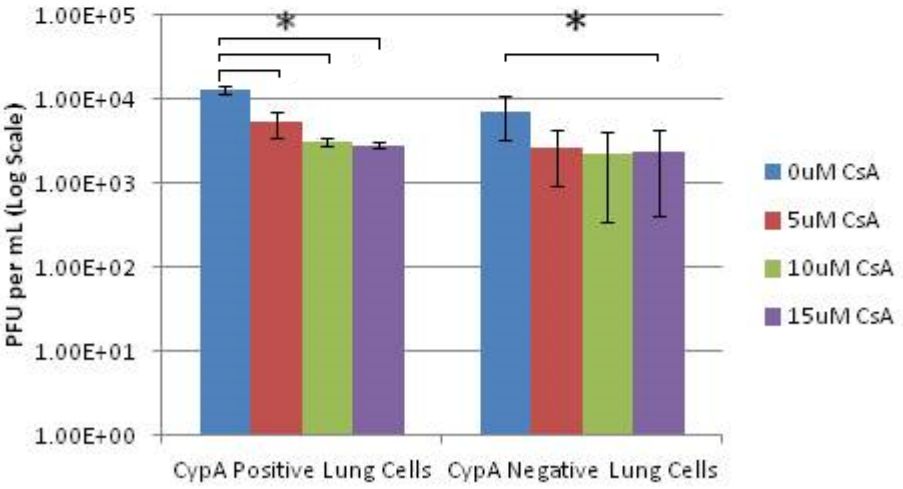


Figure 6 VACV Grown in the Presence of CsA. a) Three sets of CypA positive and negative primary embryo cells were harvested separately and run independently. An MOI 0.1 was used and samples were run in quadruplicate and harvested at 24 hpi. Viral titers were assessed by titration b) Two sets of primary lung cells of both CypA positive and negative were harvested separately and run independently. An MOI 0.1 was used and samples were run in quadruplicate and harvested at 24 hpi. Viral titers were assessed by titration. Data analyzed using the Mann-Whitney Rank Sum test * indicates a statistical significance of P≤0.05. Error bars indicate the standard error of the mean.