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Nowrin Chowdhury April 11, 2017

Replication and Viability of Recombinant Modified Vaccinia Virus Ankara 44/47.1 and 51.1 in Mammalian Cells

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Abstract

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It is very difficult to create vaccines for some viruses, such as human immunodeficiency virus (HIV). Past attempts at vaccines for HIV have failed to stimulate the immune system effectively, leading to insufficient B and T cell activation. Many vaccines use a modified vaccinia virus Ankara (MVA) vector to deliver viral genome targets in order to stimulate the immune system. In 1998, Wyatt *et al* published a new study that showed increased replication of new recombinant MVAs (rMVAs) over the wild-type MVA in rabbit, monkey, and human cell lines. The objective of this study was to establish replication and viability of rMVA 44/47.1 and rMVA 51.1 in mouse, monkey, and human cell lines in order to determine whether they would be good candidates as vaccine vectors. If rMVAs are able to replicate better and delay cell death more effectively, this would allow immune cells more opportunity to recognize viral targets. DF-1 chick embryo fibroblasts, C2C12 mouse myoblasts, Vero monkey kidney epithelial cells, and HeLa human cervical epithelial cells were tested for replication. C2C12 cells, L929 mouse fibroblasts, Vero cells, and HeLa cells were tested for viability with and without an apoptosis causing agent. We find that rMVAs had increased replication over the wild-type MVA in DF-1 cells, C2C12 cells, and HeLa cells, but did not differ significantly in Vero cells. We also find that rMVA 44/47.1 had decreased apoptosis in L929 cells and HeLa cells, and rMVA 51.1 had decreased apoptosis in L929 cells, Vero cells, and HeLa cells. However, these decreases in apoptosis were concurrent with increases in necrosis. Recombinant MVAs could be the answer in creating more effective vaccines, though we must find ways to reduce the necrosis and must better establish these patterns.

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Table of Contents

List of Figures

INTRODUCTION

Background

The introduction of vaccines has completely changed the distribution of human disease among populations across the world. Beginning with Edward Jenner's discovery of using a less pathogenic, but related, virus to prevent smallpox, a new door opened in medicine: disease prevention and eradication (1). Over the years, as scientists discovered more about pathogens and the immune system, vaccines became more sophisticated. For example, the induction of antibodies and cytotoxic T lymphocytes to neutralize threats within the body and the memory retained by the body of the threat has revolutionized the efficacy of vaccines (2). Further, the methods through which researchers designed and synthesized vaccines began to change. For example, the polio vaccine created by Salk was initially introduced as an intramuscular injection that contained a whole virus that was killed with chemicals such as formalin (3). Then, Sabin introduced a live attenuated orally administered vaccine where the genome of the virus contained deletions in order to reduce its pathogenicity (4). Adjuvants, which are compounds added to vaccines such as aluminum hydroxide, were added to enhance immune response to vaccines (2). Eventually, researchers learned to use organisms as vectors to introduce proteins or portions of DNA from the pathogen to the immune system. Vaccine technology continues to grow as researchers learn more about the interaction between the immune system and pathogens.

Even though researchers have created effective vaccines for a wide variety of diseases, there are still many diseases for which a lasting vaccine does not exist. For example, by the end of

2015, 36.7 million people throughout the world were living with HIV (5). Though researchers have gained extensive insight into the virus and treatment options, mankind has been unable to produce an effective vaccine – as a result, the virus continues to spread and the burden of disease remains relatively high. The biggest obstacles in creating a viable vaccine have been the lack of natural sterilizing immunity to HIV-1, as well as its constantly mutating genome (6). In addition, due to their late appearance in the course of infection, broadly neutralizing antibodies tend to have little to no effect (7,8). An ideal HIV vaccine would be safe, easy to administer, inexpensive, and able to activate both humoral (B cells/antibodies) and cellular (CD4 and CD8 T cells) immunity (9). Many vaccine efforts have been marred by insufficient B and T cell activation, creating a need for vectors that can stimulate the immune response even more, without causing harm to the recipient.

Modified Vaccinia Virus Ankara (MVA)

Modified vaccinia virus Ankara (MVA) is a strongly attenuated vaccinia virus (10). It is a member of the *Poxviridae* family, a family of linear, double-stranded DNA viruses (11). These viruses normally replicate within the cytoplasm of the host cell. Once viruses have entered, transcription begins with the early genes, which transcribe tools for viral DNA synthesis (12). Transcription continues with intermediate and late genes, which transcribe all other necessary viral components. The viral DNA is packaged into virions, which mature and exit via fusion with the host cell membrane and detachment into the extracellular space.

In MVA, this replication cycle is severely inhibited due to the process through which MVA was created. MVA was obtained through passaging chorioallantoic vaccinia virus Ankara (CVA) more than five hundred times in primary chick embryo fibroblasts (CEF) (13). Only CEFs, baby hamster kidney 21 cells, and fruit bat cells have been shown to efficiently replicate MVA. However, even with the attenuated replication, many experiments have shown similar, and in some cases greater, immunogenicity of MVA compared to some pathogenic strains of vaccinia virus (14). This can be attributed to the loss of many immune evasion and immunomodulatory traits in MVA, such as the inability to antagonize interferon, a host anti-viral response (15). After the success of using MVA as the smallpox vaccine, researchers began investigating its uses as a vaccine vector for other conditions such as other poxviruses, influenza, measles, and cancer.

The reason for the success of MVA as a vaccine vector lies in its fragmented genome (16). In fact, analysis of the genome of MVA revealed that about 15% of the original chorioallantoic vaccinia virus Ankara genome was missing (17). Most of the missing genes occur in 6 major deletions, causing fragmentation of the genome. There are also smaller mutations and deletions that contribute further to the loss of replication in MVA (17,18). The fragmented genome and many mutations have also led to the limited host range, meaning the virus could only productively replicate in certain animals. As a result, foreign DNA can easily be inserted into the MVA genome and the translated protein can be presented to immune cells that arrive to clear the MVA infection. However, vaccines constructed in this manner can still be improved in terms of immunogenicity generated to the target pathogen in humans (19).

Recombinant MVAs (rMVAs)

In 1998, Wyatt *et al.* published results on the effects of restoration of some of the deletions in the MVA genome (18). Their team transfected cosmids derived from the parental vaccinia virus genome into monkey BSC-1 cells that were infected with MVA to make different recombinant MVAs that now contained previously missing sections of the genome. Most recombinants restored genes on the left side of the genome. The team found that some of these recombinant MVAs were able to form plaques and replicate more effectively than wild-type (WT) MVA in different types of monkey, human, and rabbit cell lines. Some recombinants were able to replicate much more efficiently than others in human and monkey cells, including rMVA 51.1 and rMVA 44/47.1. However, the data regarding replication on mouse cells were not included in the study. In addition, it was not clear how the given recombinants were able to replicate. Nevertheless, in terms of vaccine development for HIV, a vaccinia virus vector that has some replication capacity, but not the pathogenic effects could give the immune response more time to produce and recognize antigens.

Apoptosis

The ability of the rMVAs to replicate could be indicative of a reduction in apoptosis of host cells, as many viruses encode factors that block the apoptosis pathway in order to increase the amount of virus replicated (20). Apoptosis is a mechanism of cell death in which internal or external danger signals cause the cell to transmit signals through a family of cysteine proteases (caspases), which ultimately lead to the destruction of internal elements of the cell, and eventually, the cell shrinks and dies (21). Apoptosis triggered by MVA can be extrinsic or

intrinsic, though the focus of this study is on the intrinsic pathway. The intrinsic apoptosis pathway can be triggered by the lack of certain cellular factors such as growth factors and cytokines, by various viral components, or with staurosporine *in vitro* (22, 23). These cause changes in the mitochondrial membrane, which lead to the release of cytochrome *c*, which then binds to Apaf-1 and pro-caspase-9, forming the apoptosome (Figure 1). Pro-caspase-9 becomes activated caspase-9, which cleaves pro-caspase-3. This activates caspase-3 and leads to internal cell destruction such as DNA breakdown and degradation of cytoskeletal and nuclear proteins. The extrinsic pathway, which is triggered through other factors and goes through different intermediates, activates caspase 8. Caspase 8 cleaves pro-caspase 3, leading to apoptosis. Cells can also undergo necrosis, a cell death pathway in which the cell swells and releases its contents into the microenvironment (22). Delaying apoptosis of an MVA infected cell could be beneficial for a vaccine as it would give immune cells more time to recognize the targeted viral components, such as proteins, increasing the number of immune cells acting.

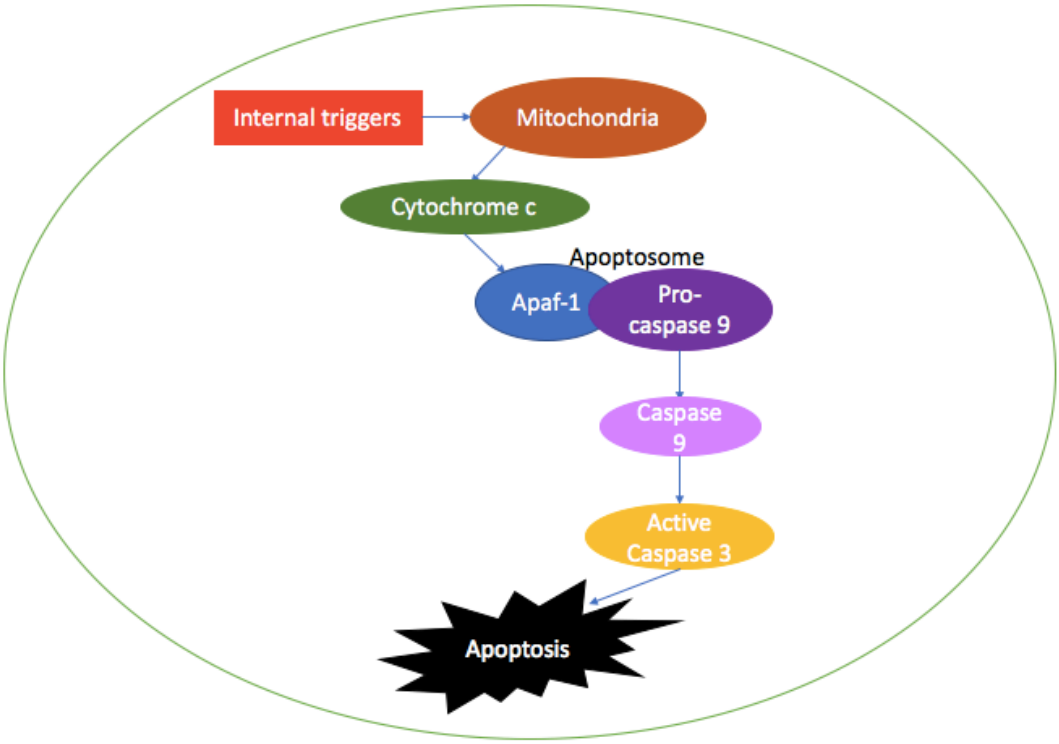


Figure 1. The intrinsic pathway of apoptosis. Cells can initiate different pathways for apoptosis depending on the trigger. MVA triggers both extrinsic and intrinsic pathways. The focus of this paper is the intrinsic pathway, which is artificially induced to a greater extent with staurosporine.

OBJECTIVES

The following represent the experimental objectives of this study:

1. Establish the replication pattern of wild-type MVA (WT MVA), rMVA 44/47.1, and rMVA 51.1 in mammalian cells related to vaccine development.
    a. Determine whether the rMVAs have higher rates of replication in any or all of the cell lines.
2. Establish the viability of rMVA infected cells versus WT MVA infected cells.

MATERIALS AND METHODS

Cell Lines

Mammalian cell lines used in the experiments included the C2C12 mouse myoblast cells, L929 mouse fibroblast cells, Vero monkey kidney epithelial cells, and HeLa human cervical epithelial cells. DF-1 chicken embryo fibroblasts were used to test replication. All cell lines were cultured under sterile conditions and placed in DMEM with 10% fetal bovine serum (FBS), 1% glutamine, 1% penicillin/streptomycin. Cells were incubated at 37°C until confluent in their respective plates. Cells were infected with their respective viruses in DMEM with 2% FBS.

Viruses

The wild-type MVA virus was obtained from the stocks in the Amara lab. The modified viruses (rMVA 51.1 and rMVA 44/47.1) were obtained through the lab of Bernard Moss at the National Institute of Allergy and Infectious Diseases, NIH in Bethesda. The viruses were developed through recombination of cosmids prepared from digested fragments of the parental vaccinia virus Ankara genome with wild-type MVA and purification of the recombinants (18).

Replication Assay

Mammalian cells and DF-1 cells were plated in 12-well Costar plates. Once confluent, cells were infected with wild type virus and each recombinant virus at multiplicity of infection (MOI) of 2 in DMEM with 2% FBS. One group of cells was mock infected. After 1.5 hours, the media was replaced with DMEM with 10% FBS. Cells were collected and frozen at -80°C right after infection and at 3, 6, 10, and 24 hours after infection. Cells were frozen and thawed three times

to lyse the cells and release viral components inside the cells, spun down, and the supernatant was stored in the freezer at -80°C.

Plaque Assay

DF-1 cells were plated in 12-well Costar plates. Once confluent, cells were infected with the virus collected from the replication assay at different dilutions in DMEM with 2% FBS. Each dilution was plated in duplicates. The infection was incubated for 1 hour, after which the media was replaced with methylcellulose media with 2% FBS. After an incubation period of 48 hours, the methylcellulose was removed and the cell monolayer was stained with a crystal violet stain. After washing off the stain, the plaques of viral replication were counted and the average viral titers were calculated and graphed as a growth curve over time. Two-way ANOVAs were used to analyze the data. Fold changes in virus titer over 24 hours were calculated and one-way ANOVAs were used to compare the fold changes between different infections.

Viability Assay

Mammalian cells were plated in 24-well Costar plates. Once confluent, cells were infected with wild type virus and each recombinant virus at MOI of 2 in DMEM with 2% FBS. After 1.5 hours, the media was replaced with DMEM with 10% FBS. Fifteen hours after infection, half of the cells in each infection group were treated with 0.1µM staurosporine in order to induce the intrinsic apoptosis pathway. Untreated and treated cells were harvested at 0, 24, 48, and 72 hours after treatment. Cells were stained with Live/Dead staining (Invitrogen), an MVA-specific antibody

(BEI Resources), and an active-caspase 3-specific antibody (BD Biosciences), and analyzed by flow cytometry.

Analysis of Viability Assay

All data from flow cytometry was analyzed on FlowJo. Gates were created for the cells of interest using forward and side scatter (Figure 2). From there, a quadrant gate of Live/Dead and caspase 3 was formed. For infected cells, once cells were gated, gates were created on MVA-positive cells, which were then quadrant gated for Live/Dead and caspase 3. Percentages of cells in each phase of apoptosis (live, early, and late) as well as necrotic cells were graphed at each time point. Two-way ANOVAs were used to analyze the data.

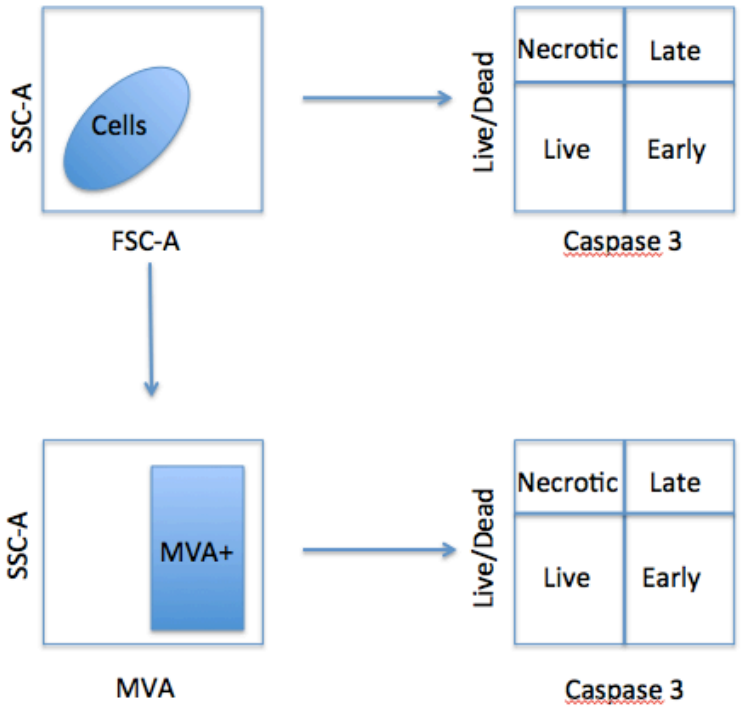


Figure 2. Gating strategy. Cells were gated on using forward and side scatter. Cells were then quadrant gated based on Live/Dead and Caspase 3. Cells were also gated on MVA positive cells, which were further gated based on Live/Dead and Caspase 3 stains.

RESULTS

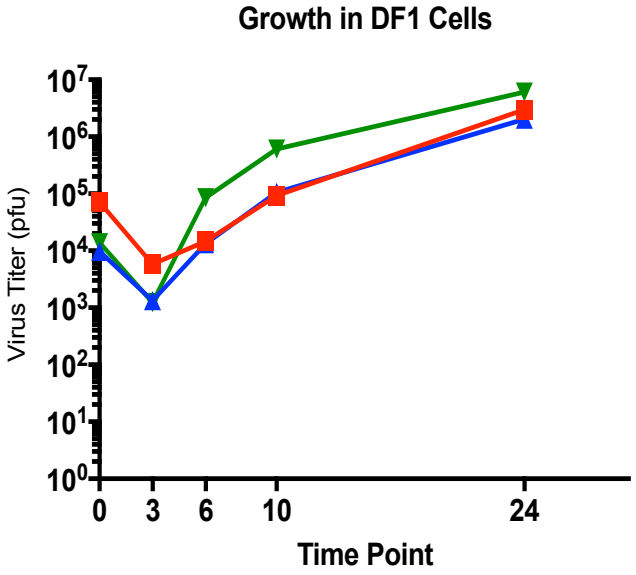
Replication

rMVA 51.1 shows increased replication in DF-1 cells and C2C12 cells

In order to understand the replication of each virus type, an immortalized chicken embryo fibroblast cell line (DF-1) and a mouse myoblast cell line (C2C12) were infected and allowed to replicate for 24 hours. Intracellular virus was extracted at different time points and plaque analysis was done to determine virus titers. In DF-1 cells, from 0-6 h, there was not a significant difference in virus titers of different virus types (Figure 3). However, by 10 h, rMVA 51.1 had significantly higher virus titers than both WT MVA ($p=0.0226$) and rMVA 44/47.1 ($p=0.0268$), indicating increased replication. At 24 h, rMVA 51.1 continued to have significantly higher virus titers than both WT MVA ($p<0.0001$) and rMVA 44/47.1 ($p<0.0001$). Additionally, WT MVA had significantly higher virus titers than rMVA 44/47.1 ($p<0.0001$). Furthermore, the fold change from 0 h to 24 h was calculated for each virus. WT MVA infected cells had a 42.3-fold increase in virus titers over 24 hours, whereas rMVA 44/47.1 infected cells had a 208-fold increase and rMVA 51.1 had a 442-fold increase. There was a significant difference between the fold change in virus titers of WT MVA infected cells and rMVA 51.1 infected cells ($p<0.05$).

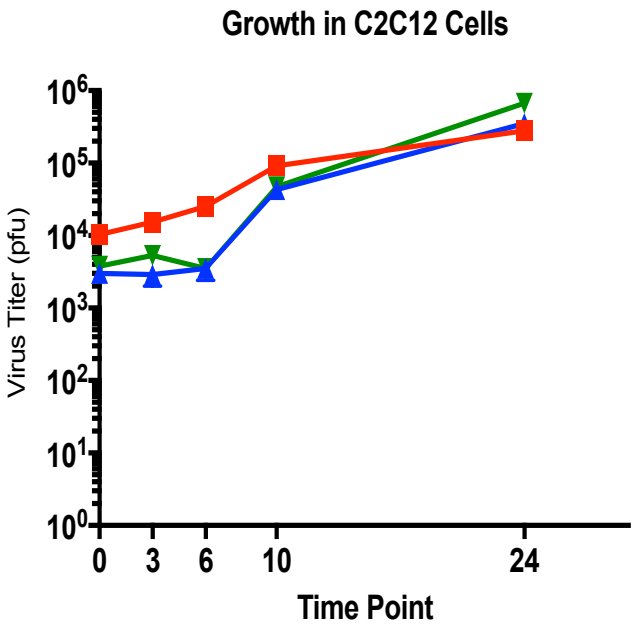
In C2C12 cells, from 0 to 10 hours after infection, there was no significant difference in the replication between the viruses (Figure 4). However, by 24 hours, rMVA 51.1 had significantly higher virus titers than WT MVA ($p<0.0001$) and rMVA 44/47.1 ($p<0.0001$). There was no significant difference in virus titer between rMVA 44/47.1 and WT MVA at any point. In addition, from 0 to 24 hours post-infection, WT MVA infected cells had a 27.2-fold increase in

virus titers, whereas rMVA 44/47.1 had a 117-fold increase and rMVA 51.1 had a 176-fold increase. However, these differences were not significant.



Virus	Fold Change (24 h)
WT MVA	42.3
rMVA 44/47.1	208
rMVA 51.1	442

Figure 3. Growth in DF-1 cells. By 10 hours post-infection in DF-1 cells, rMVA 51.1 had significantly higher virus titers than both rMVA 44/47.1 and WT MVA, and by 24 hours, WT MVA had significantly higher titers than rMVA 44/47.1 ($p<0.05$). To assess replication, virus titers were calculated at each time point through plaque assays. Fold change represents the increase in virus titer at 24 h based on the 0 h titer. Data points represent the mean of two samples and error bars represent the standard error of the mean.



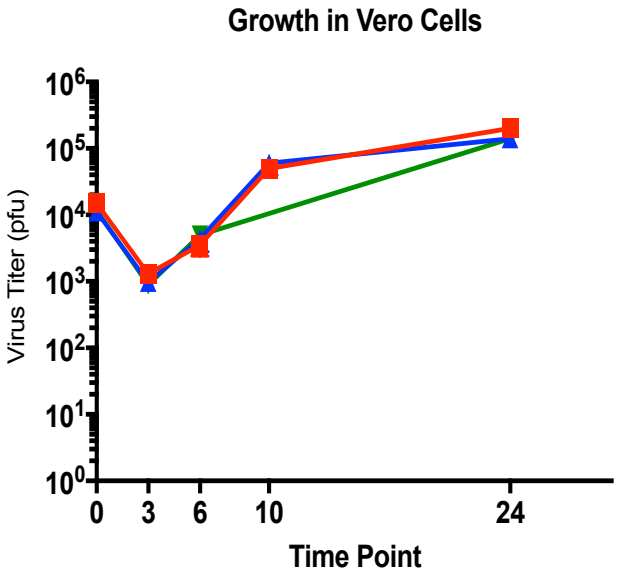
Virus	Fold Change (24 h)
WT MVA	27.2
rMVA 44/47.1	117
rMVA 51.1	176

Figure 4. Growth in C2C12 cells. By 24 hours post-infection, rMVA 51.1 had significantly higher virus titers than both rMVA 44/47.1 and WT MVA in mouse myoblast cells ($p<0.05$). To assess replication, virus titers were calculated at each time point through plaque assays. Fold change represents the increase in virus titer at 24 h based on the 0 h titer. Data points represent the mean of two samples and error bars represent the standard error of the mean.

WT MVA shows higher rates of replication in Vero cells

In order to understand the replication of each virus in a non-human primate cell line, monkey kidney epithelial (Vero) cells were infected and allowed to replicate for 24 hours. Intracellular virus was extracted at different time points and plaque analysis was done to determine the level of virus titers. Virus titers for rMVA 51.1 could not be determined at 10 h. By 24 h, WT MVA had significantly higher virus titers, and therefore rates of replication, than both rMVA

44/47.1 ($p<0.0001$) and rMVA 51.1 ($p=0.0004$) (Figure 5). There was no significant difference between rMVA 44/47.1 and rMVA 51.1. In terms of growth over 24 h, WT MVA had a 13.5-fold increase in virus titer, rMVA 44/47.1 had a 12.6-fold increase, and rMVA 51.1 had a 12.0-fold increase. There were no significant differences between these changes.



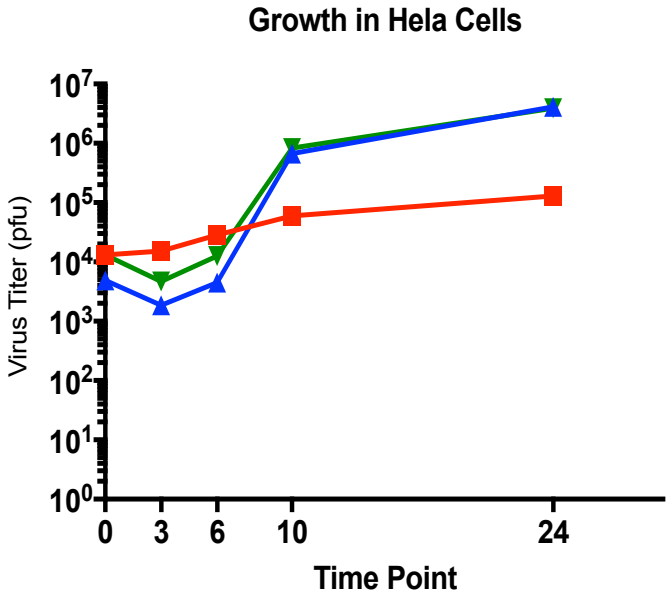
Virus	Fold Change (24 h)
WT MVA	13.5
rMVA 44/47.1	12.6
rMVA 51.1	12.0

Figure 5. Growth in Vero cells. WT MVA had significantly higher rates of replication than rMVAs in monkey kidney epithelial cells by 24 hours ($p<0.05$). To assess replication, virus titers were calculated at each time point through plaque assays. Fold change represents the increase in virus titer at 24 h based on the 0 h titer. Data points represent the mean of two samples and error bars represent the standard error of the mean.

rMVAs show higher rates of replication than WT MVA in HeLa cells

In order to understand the replication of each virus type, immortalized human cervical epithelial (HeLa) cells were infected and allowed to replicate for 24 hours. Intracellular virus was extracted at different time points and plaque analysis was done to determine virus titers.

By 24 h, both rMVA 44/47.1 and rMVA 51.1 have significantly higher virus titers than WT MVA ($p<0.0001$ for both) (Figure 6). On the other hand, there was no significant difference between the two recombinants. The overall growth over 24 hours is depicted by the fold change. WT MVA infected cells had a 9.92-fold increase in virus titers from 0 h to 24 h. On the other hand, rMVA 44/47.1 infected cells had a 837-fold increase and rMVA 51.1 infected cells had a 298-fold increase. Based on fold changes, rMVA 44/47.1 had significantly larger titers than WT MVA ($p<0.05$).



Virus	Fold Change (24 h)
WT MVA	9.92
rMVA 44/47.1	837
rMVA 51.1	298

Figure 6. Growth in HeLa cells. Recombinant MVAs have significantly higher rates of replication in HeLa cells than WT MVA by 24 hours ($p<0.05$). To assess replication, virus titers were calculated at each time point through plaque assays. Fold change represents the increase in virus titer at 24 h based on the 0 h titer. Data points represent the mean of two samples and error bars represent the standard error of the mean.

Viability

No differences in viability among C2C12 cells infected with rMVA at late time points

In order to compare the viability of infected cells, mouse myoblast cells were infected with each virus 1.5 hours, treated with staurosporine, extracted at various time points, and stained for Live/Dead, MVA, and caspase 3. Since MVA could no longer be detected at 24 h in these cells, all cells were used to determine percentiles. By 48 h post-treatment, all untreated and treated cells were dead (Figures 7 and 8). There was a significant difference between the initial (0 h) percentage of cells in the early phase of apoptosis in both untreated and treated cells with rMVA 51.1 and those in WT MVA ($p=0.0404$, $p=0.0415$ respectively). There was no significant difference in total caspase activity between different infections in untreated and treated groups (Figure 9).

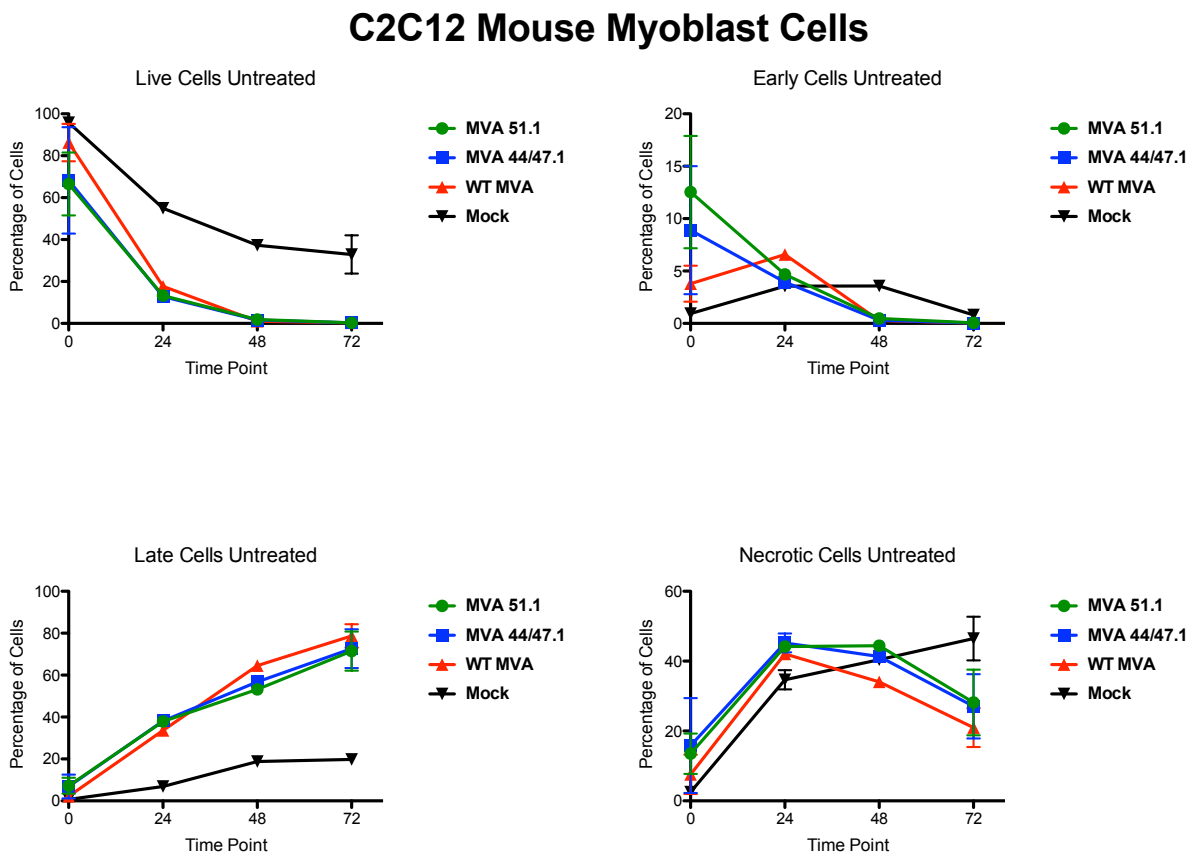


Figure 7. Viability in C2C12 cells. Untreated infected mouse myoblasts showed similar amounts of viable and apoptotic cells at all time points except for the initial time point. To assess viability, the percentage of viable, apoptotic and necrotic C2C12 mouse myoblast cells were quantified. There were no significant differences between infections for the frequencies of live cells, late apoptotic cells, and necrotic cells. At 0 h, there were a significantly higher percentage of cells in the early apoptotic phase that were infected with rMVA 51.1 than WT MVA ($p<0.05$). However, this difference is not statistically significant by 24 h. Data points represent the mean of two samples and error bars represent the standard error of the mean.

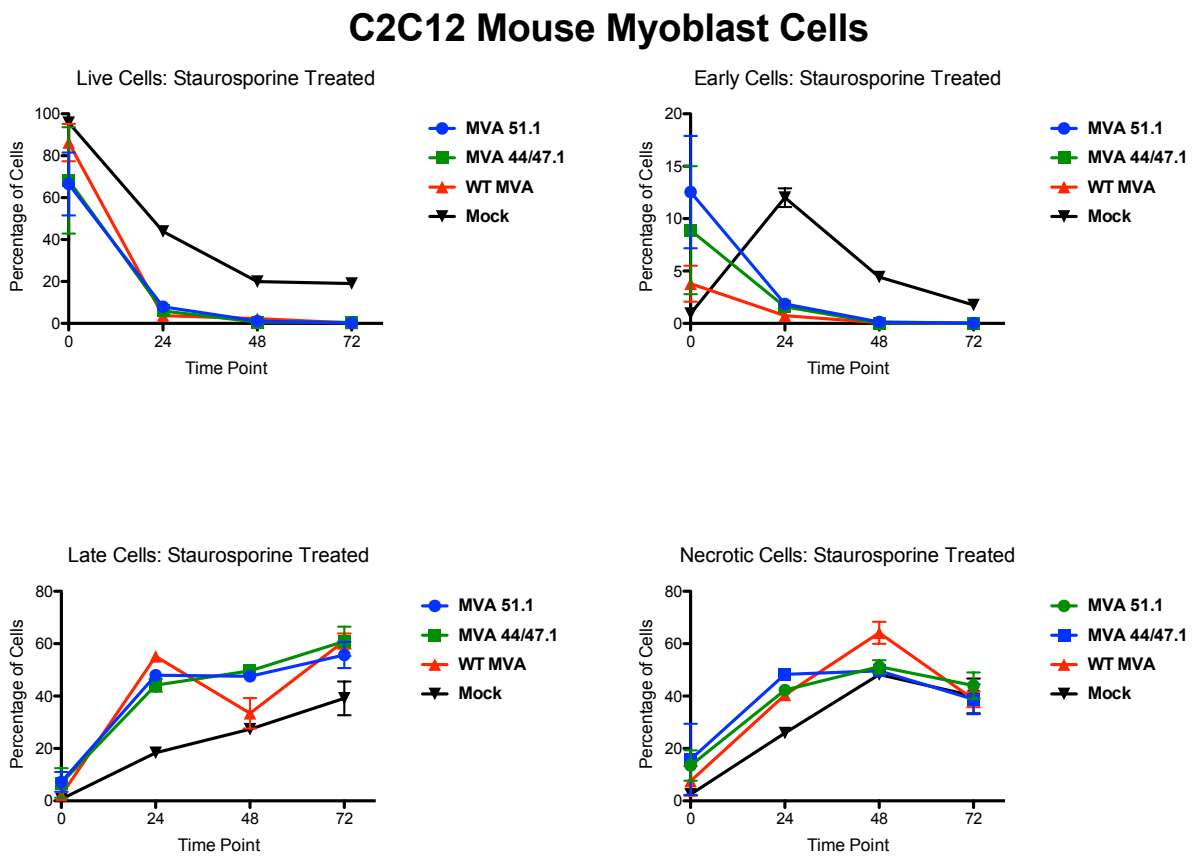


Figure 8. Viability in treated C2C12 cells. Staurosporine treated, infected mouse myoblasts showed similar amounts of viable and apoptotic cells at almost all time points. To assess viability, the percentage of viable, apoptotic and necrotic C2C12 mouse myoblast cells were quantified after cells were treated with staurosporine. There were no significant differences between infections in live cells, and necrotic cells. At 0 h, there were a significantly higher percentage of cells in this phase that were infected with rMVA 51.1 than WT MVA ($p<0.05$). However, this difference is negligible by 24 h. In addition, there is a significantly higher percentage of cells in late apoptosis that are infected with rMVA 44/47.1 compared to those infected with WT MVA ($p<0.05$). Data points represent the mean of two samples and error bars represent the standard error of the mean.

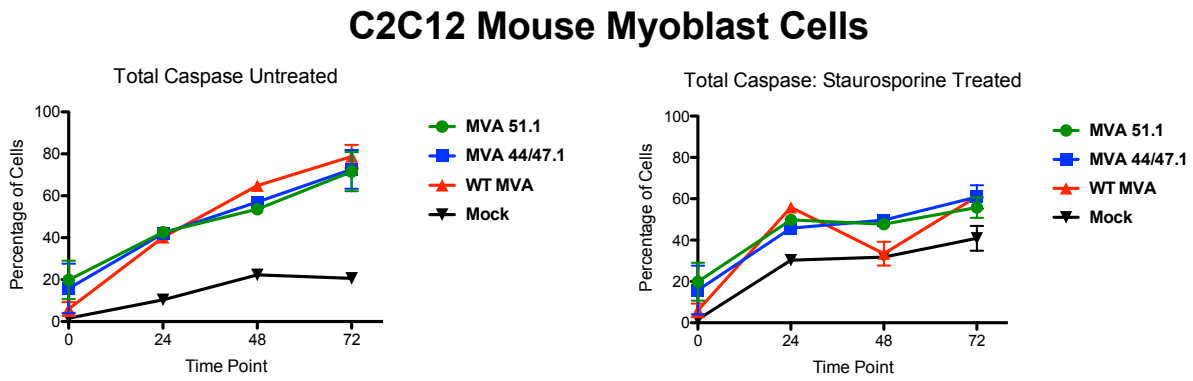


Figure 9. Caspase 3 activity in C2C12 cells. Untreated and treated infected cells showed similar amounts of total caspase activity at each time point. To assess viability, the percentages of infected C2C12 mouse myoblast cells with caspase 3 activity (indicating apoptosis) were quantified in untreated cells and staurosporine treated cells. There were no significant differences in caspase 3 activity at any time point between any of the infections. Data points represent the mean of two samples and error bars represent the standard error of the mean.

rMVAs may delay induced apoptosis in L929 cells, though rMVA 51.1 may induce more necrosis

In order to compare the viability of infected cells, mouse fibroblast cells were infected with each virus, some were treated with staurosporine, extracted, and stained for Live/Dead, MVA, and caspase 3. By 48 h in untreated cells, rMVA 51.1 infected cells have a significantly lower percentage of live cells and significantly higher cells in the late apoptosis phase than WT cells ($p=0.0127$ and $p=0.0061$, respectively) (Figure 10). In addition, by 48 h rMVA 51.1 infected cells have a significantly higher percentage of late apoptotic cells than rMVA 44/47.1 ($p=0.0006$).

These differences are negligible by 72 h. On the other hand, in cells induced to undergo apoptosis by staurosporine, by 24 h, WT MVA infected cells have a significantly lower percentage of live cells and a significantly higher percentage of late apoptotic cells than rMVA 44/47.1 ($p=0.0018$) (Figure 11). WT MVA infected cells also have a significantly higher percentage of late apoptotic cells than rMVA 51.1 at 24 h ($p=0.0002$). As a result at 24 h in staurosporine treated cells, WT MVA infected cells show a significantly higher percentage of cells with caspase 3 activity than rMVA 44/47.1 and rMVA 51.1 ($p= 0.005$ and $p<0.0001$, respectively) (Figure 12). Nevertheless, at 24 h in staurosporine treated cells, rMVA 51.1 have significantly higher percentages of necrotic cells than both WT MVA infected cells and rMVA 44/47.1 infected cells ($p=0.0056$ and $p=0.0012$, respectively) (Figure 11). By 48 h, these differences are all insignificant.

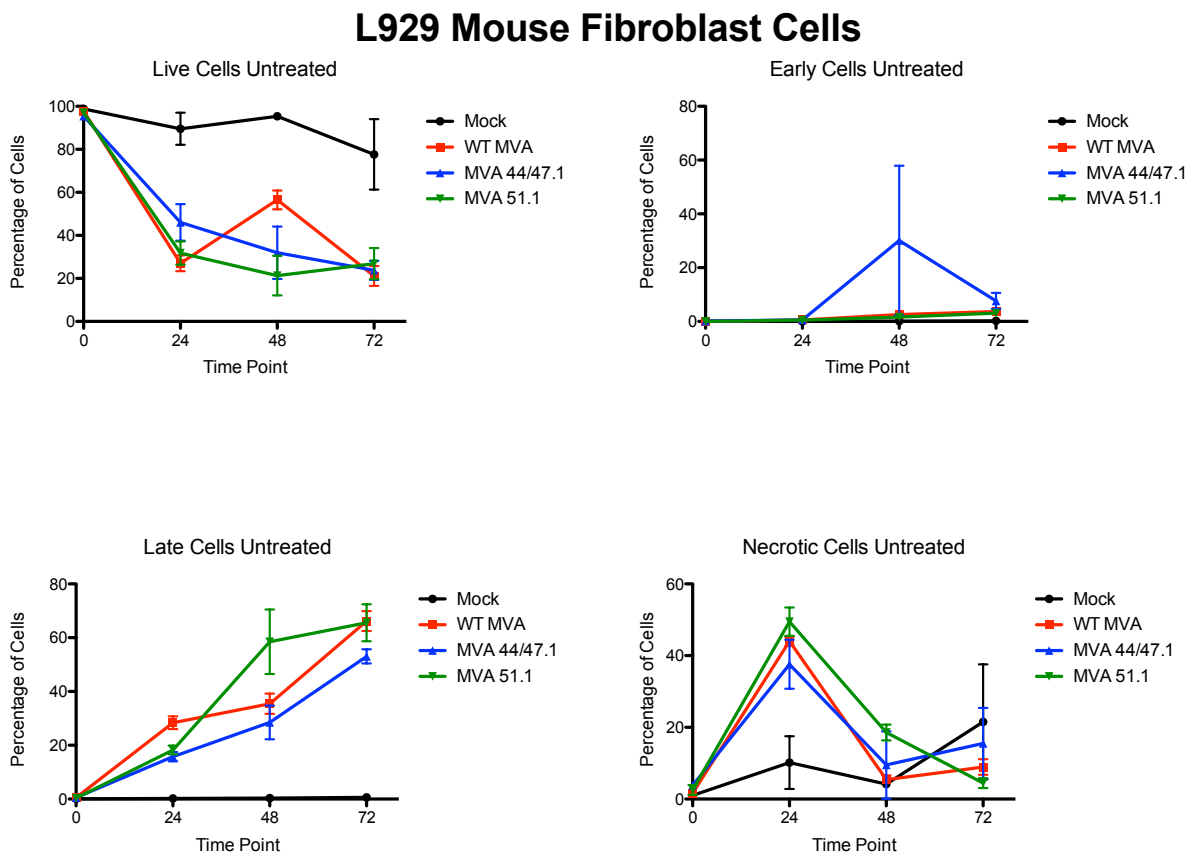


Figure 10. Viability in L929 cells. By 48 hours post-treatment, rMVAs show increased cell death by apoptosis in untreated cells. To assess viability, the percentage of viable, apoptotic and necrotic L929 mouse fibroblast cells were quantified. At 48 h, rMVA 51.1 had significantly fewer live cells and significantly higher frequencies of late apoptotic cells than WT MVA ($p<0.05$). Also, rMVA 44/47.1 had significantly fewer late apoptotic cells than rMVA 51.1 ($p<0.05$). Data points represent the mean of two samples and error bars represent the standard error of the mean.

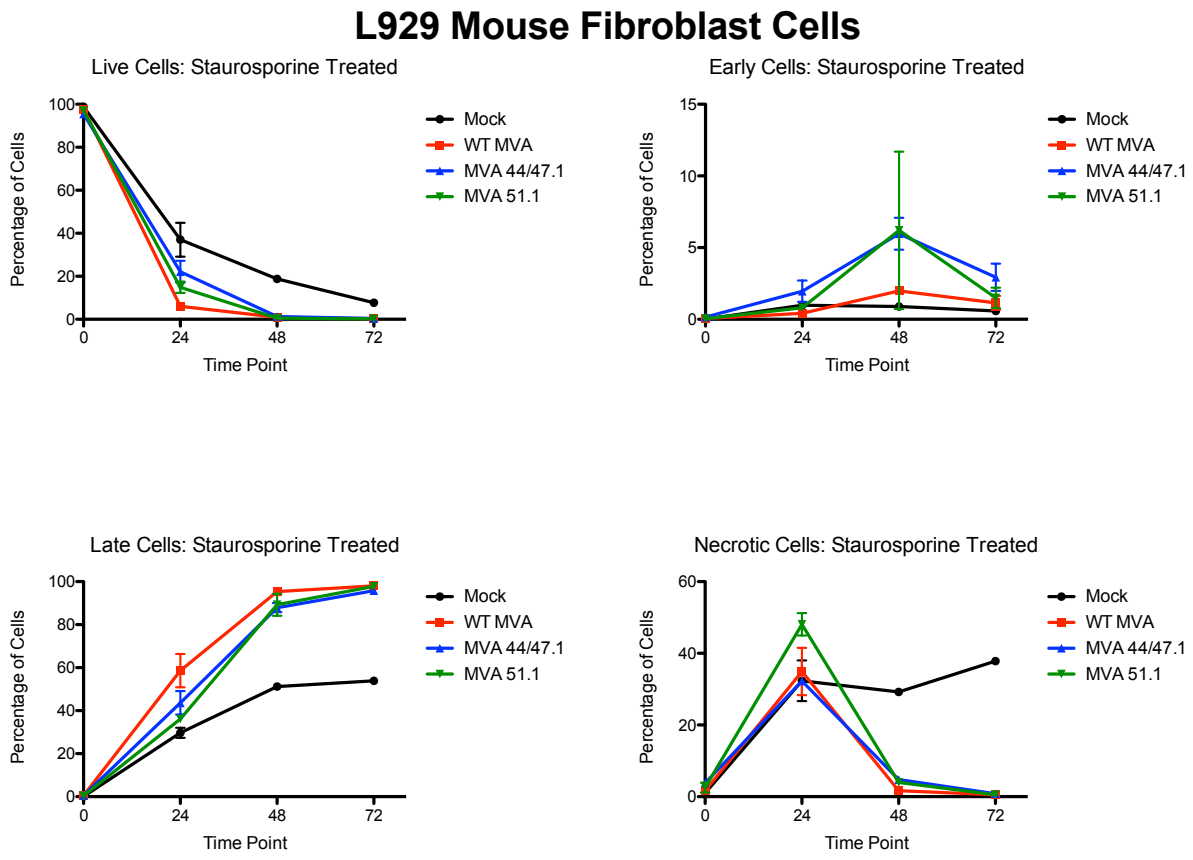


Figure 11. Viability in treated L929 cells. rMVA 44/47.1 shows significantly less cell death at 24 hours than other L929 infected cells. To assess viability, the percentage of viable, apoptotic and necrotic L929 mouse fibroblast cells were quantified after cells were treated with staurosporine. At 24 h, WT MVA had a significantly smaller proportion of live cells than rMVA 44/47.1 ($p<0.05$) and a significantly greater proportion of late apoptotic cells than both rMVA 44/47.1 and rMVA 51.1 ($p<0.05$). rMVA 51.1 also shows significantly higher percentages of necrotic cells than WT MVA and rMVA 44/47.1 ($p<0.05$). Data points represent the mean of two samples and error bars represent the standard error of the mean.

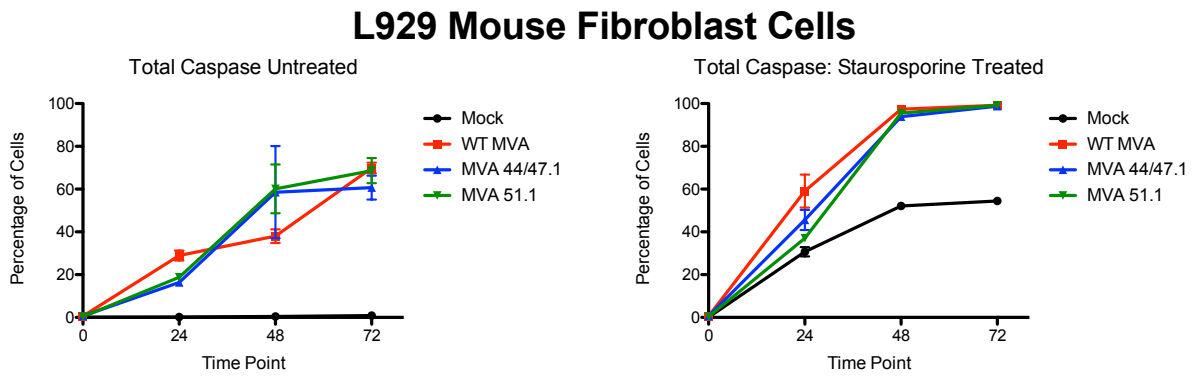


Figure 12. Caspase 3 activity in L929 cells. WT MVA infected show significantly more apoptosis by 24 hours in apoptosis induced cells than rMVAs. To assess viability, the percentages of infected L929 mouse fibroblast cells with caspase 3 activity were quantified in untreated cells and those treated with staurosporine. At 24 h in treated cells, WT MVA had a significantly higher percentage of apoptotic cells than those infected with rMVA 44/47.1 and rMVA 51.1 ($p<0.05$). There were no significant differences in caspase 3 activity in untreated cells. Data points represent the mean of two samples and error bars represent the standard error of the mean.

rMVA 44/47.1 has increased cell death at earlier time points compared to other infections in Vero cells, whereas rMVA 51.1 shows a slight delay in apoptosis in Vero cells

In order to compare the viability of infected cells, monkey kidney epithelial cells were infected with each virus and treated with staurosporine, extracted, and stained for Live/Dead, MVA, and caspase 3. In untreated cells at 24 and 48 h, rMVA 44/47.1 has a significantly lower percentage of live cells than WT MVA ($p=0.0026$ and $p<0.0001$, respectively) and rMVA 51.1 ($p=0.0001$ and $p<0.0001$, respectively) (Figure 13). In addition, at 24, 48, and 72 h, rMVA 44/47.1 had a

significantly higher percentage of necrotic cells than both WT MVA ($p=0.0045$, $p=0.0018$, and $p=0.0004$, respectively) and rMVA 51.1 ($p=0.0202$, $p<0.0001$, and $p<0.0001$, respectively). Furthermore, at 24 h rMVA 51.1 shows significantly lower levels of early apoptotic cells than rMVA 44/47.1 ($p=0.0196$), and somewhat lower levels than WT cells, though this is not significant. In untreated cells, at 24 h, rMVA 51.1 had significantly lower total caspase 3 activation levels than both WT MVA ($p=0.0314$) and rMVA 44/47.1 ($p=0.0035$), but by 72 h, this relationship reversed and rMVA 51.1 had significantly higher total caspase 3 levels than both WT MVA ($p=0.0162$) and rMVA 44/47.1 ($p<0.0001$) (Figure 15). Also, at 48 h, rMVA 44/47.1 showed higher levels of caspase 3 activity than WT MVA ($p=0.0016$), but by 72 h, this relationship also reversed and rMVA 44/47.1 had lower caspase 3 activity than WT MVA ($p=0.0002$). In apoptosis induced cells, there were less drastic differences overall. At 48 h, rMVA 44/47.1 had significantly lower percentages of live cells than WT MVA ($p=0.0291$) and rMVA 51.1 ($p=0.0017$) and had significantly higher caspase 3 activity than rMVA 51.1 ($p=0.006$) (Figures 14 & 15).

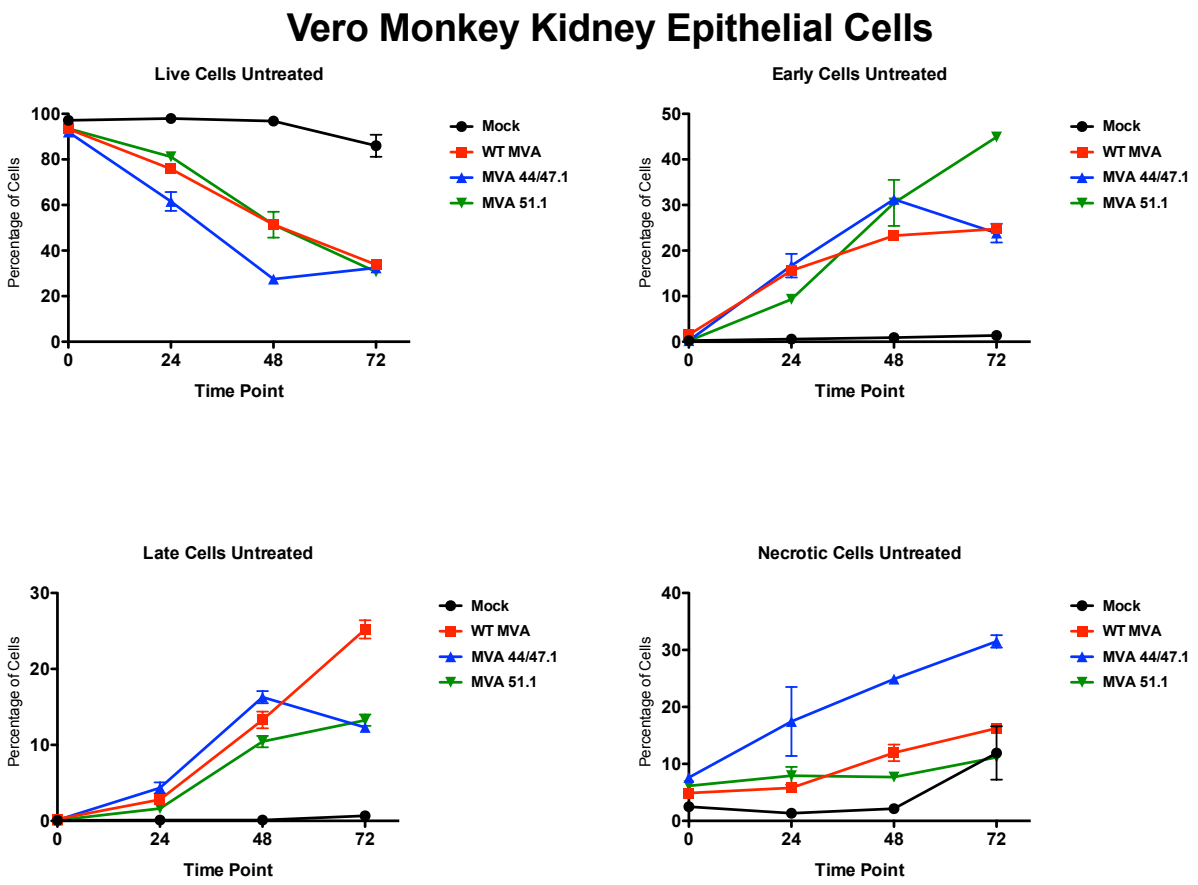


Figure 13. Viability in Vero cells. rMVA 44/47.1 infected cells had increased cell death over other MVA infected cells at most time points, while rMVA 51.1 shows a slight delay in apoptosis at 24 hours. To assess viability, the percentage of viable, apoptotic and necrotic Vero monkey kidney epithelial cells were quantified. At 24 and 48 h, rMVA 44/47.1 had significantly lower percentages of live cells than WT MVA and rMVA 51.1 ($p<0.05$). At 24, 48, and 72 hours, rMVA 44/47.1 had significantly higher percentages of necrotic cells than WT MVA and rMVA 51.1 ($p<0.05$). Finally, rMVA 51.1 had significantly less early apoptotic cells than rMVA 44/47.1 and somewhat less than WT MVA, though this was not significant. Data points represent the mean of two samples and error bars represent the standard error of the mean.

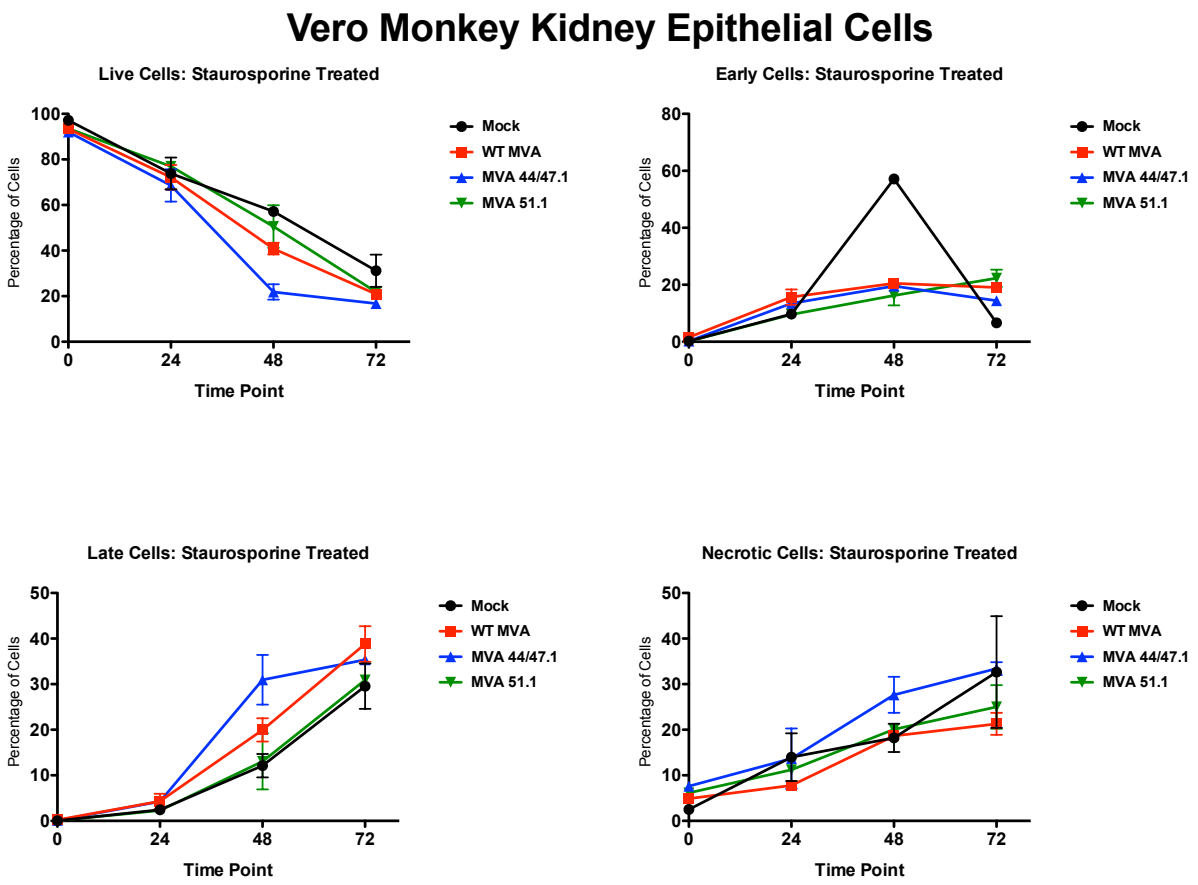


Figure 14. Viability in treated Vero cells. rMVA 44/47.1 infected Vero cells had significantly less viable cells than any other infection group by 48 hours in staurosporine treated cells ($p<0.05$). To assess viability, the percentage of viable, apoptotic and necrotic Vero monkey kidney epithelial cells were quantified after cells were treated with staurosporine. Data points represent the mean of two samples and error bars represent the standard error of the mean.

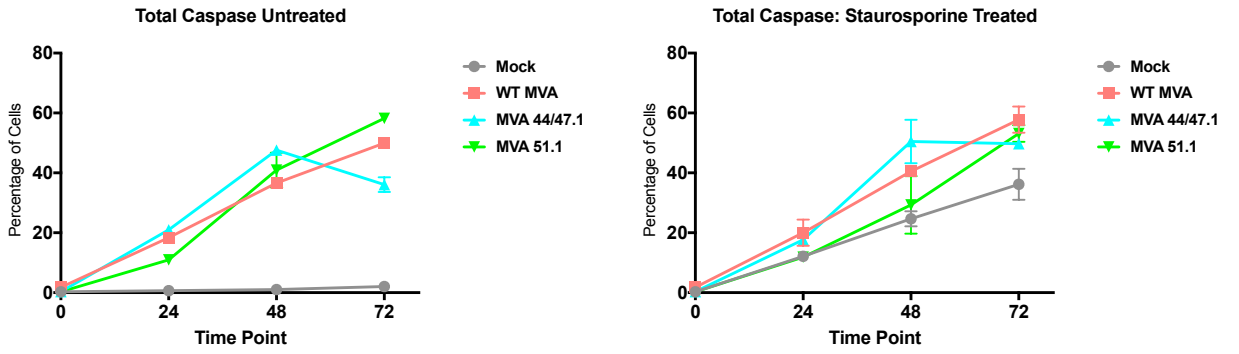


Figure 15. Caspase 3 activity in Vero cells. rMVA 51.1 infected cells show a slight delay in apoptosis compared to other infection groups at 24 h, whereas rMVA 44/47.1 showed increased apoptosis. To assess viability, the percentages of infected Vero monkey kidney epithelial cells with caspase activity were quantified in untreated cells and those treated with staurosporine. rMVA 51.1 had significantly less total caspase activity than either infection group in untreated at 24 h ($p<0.05$). However, this relationship reversed by 72 h. rMVA 44/47.1 had significantly higher caspase activity than WT at 48 h ($p<0.05$), but this relationship also reversed by 72 h. In treated cells, at 24 h, rMVA 51.1 had significantly less total caspase activity than rMVA 44/47.1 ($p<0.05$). Data points represent the mean of two samples and error bars represent the standard error of the mean.

rMVAs undergo decreased apoptosis, but increased necrosis in HeLa cells

In order to compare the viability of infected cells, human cervical epithelial cells (Hela) were infected with each virus and treated with staurosporine, extracted, and stained for Live/Dead, MVA, and caspase 3. Mock infected cells at the 72 h time point could not be included due to contamination. In untreated cells, at 48 h, rMVA 51.1 had significantly lower percentages of live cells than WT MVA ($p=0.0246$) and rMVA 44/47.1 ($p=0.0297$), though this difference became

negligible by 72 h (Figure 16). At 0 and 24 h, WT MVA had higher percentages of early apoptotic cells than both rMVA 44/47.1 ($p=0.0489$ and $p<0.0001$, respectively) and rMVA 51.1 ($p=0.0236$ and $p<0.0001$, respectively). At 48 h, WT MVA also had significantly higher percentages of early apoptotic cells than rMVA 44/47.1 ($p=0.0101$), but the difference between WT MVA and rMVA 51.1 became negligible. Additionally, at 24, 48, and 72 h, WT MVA had significantly higher levels of late apoptotic cells than rMVA 44/47.1 ($p=0.0007$, $p=0.0055$, and $p=0.0038$) and at 48 h, rMVA 51.1 had significantly higher levels of late apoptotic cells than rMVA 44/47.1 ($p=0.0156$). Furthermore, at 24 and 48 h, WT MVA had significantly lower percentages of necrotic cells than rMVA 44/47.1 ($p=0.0113$ and $p=0.0249$, respectively) and rMVA 51.1 ($p=0.0009$ and $p=0.0037$, respectively). Finally, in untreated cells at 24, 48, and 72 h, WT MVA infected cells had significantly more total caspase activity than rMVA 44/47.1 ($p<0.0001$, $p=0.001$, and $p=0.0022$, respectively) and rMVA 51.1 ($p<0.0001$, $p=0.0054$, and $p=0.008$, respectively) (Figure 18). The pattern in staurosporine treated cells was similar. At 72 h, WT MVA had significantly lower levels of live cells than rMVA 44/47.1 ($p<0.0001$) and rMVA 51.1 ($p=0.0178$) (Figure 17). At 24 h, WT MVA had higher levels of early apoptotic cells than rMVA 44/47.1 ($p<0.0001$) and rMVA 51.1 ($p=0.0047$). At this time point, rMVA 51.1 also had significantly higher percentages of early apoptotic cells than rMVA 44/47.1 ($p=0.0091$). At 72 h, WT MVA had significantly higher percentages of early apoptotic cells than rMVA 51.1 ($p=0.0096$), but had no significant difference when compared with rMVA 44/47.1. At 24, 48, and 72 h, WT MVA consistently had significantly larger percentages of cells in the late apoptotic phase than rMVA 44/47.1 ($p<0.0001$ for all time points) and rMVA 51.1 ($p<0.0001$, $p=0.0005$, and $p<0.0001$, respectively). Also, at 48 and 72 h, rMVA 51.1 had a significantly larger portion of cells that were in the late

apoptosis phase than rMVA 44/47.1 ($p=0.0285$ and $p<0.0001$, respectively). Further, at 0 and 24 h, WT MVA showed significantly lower percentages of necrotic cells than both rMVA 44/47.1 ($p<0.0001$ and $p=0.0184$, respectively) and rMVA 51.1 ($p=0.0081$ and $p<0.0001$, respectively). At 48 and 72 h, WT MVA had significantly lower percentages of necrotic cells than rMVA 51.1 ($p=0.0184$ and $p=0.0180$, respectively), though there was no significant difference between WT MVA and rMVA 44/47.1 at these time points. Lastly, at 0, 24, 48, and 72, WT MVA showed significantly higher levels of caspase 3 activity than rMVA 44/47.1 ($p<0.0001$, $p<0.0001$, $p=0.0002$, and $p<0.0001$, respectively) and rMVA 51.1 ($p=0.0058$, $p=0.0002$, $p=0.0281$, and $p<0.0001$, respectively) in staurosporine treated cells (Figure 18).

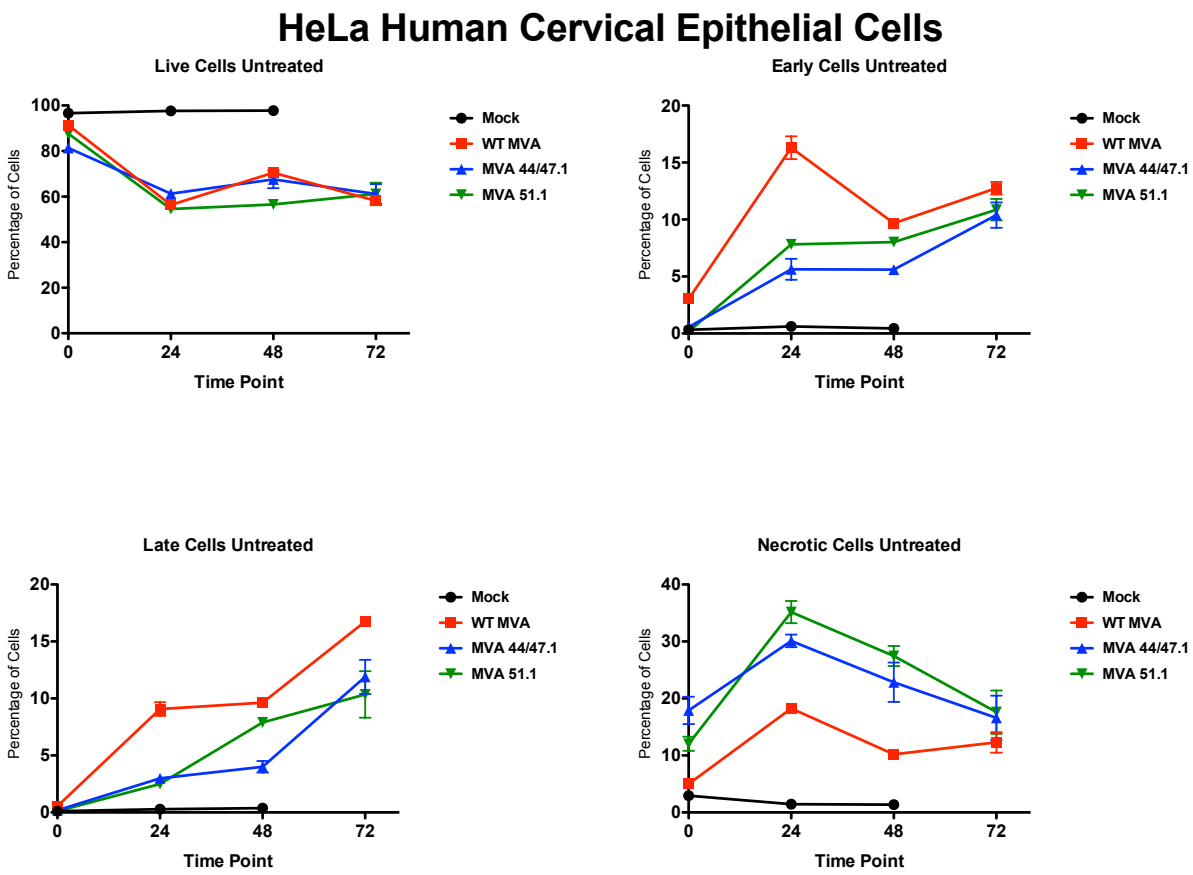


Figure 16. Viability in HeLa cells. rMVA infected Hela cells have a lower proportion of untreated cells undergoing apoptosis than WT MVA infected cells, but have higher levels of necrotic cells. To assess viability, the percentage of viable, apoptotic and necrotic HeLa human cervical epithelial cells were quantified. At 0 and 24 h, WT MVA had significantly higher percentages of cells in the early phase of apoptosis than either of the recombinants, and at 48 h, significantly higher than rMVA 44/47.1 ($p<0.05$). At 24 and 72 h, WT MVA also had significantly higher percentages of cells in the late phase of apoptosis than either of the recombinants, and at 48 h, significantly higher than rMVA 44/47.1 ($p<0.05$). At 24 and 48 h, rMVAs had significantly higher percentages of cells than that of WT MVA undergoing necrosis

($p<0.05$). Data points represent the mean of two samples and error bars represent the standard error of the mean.

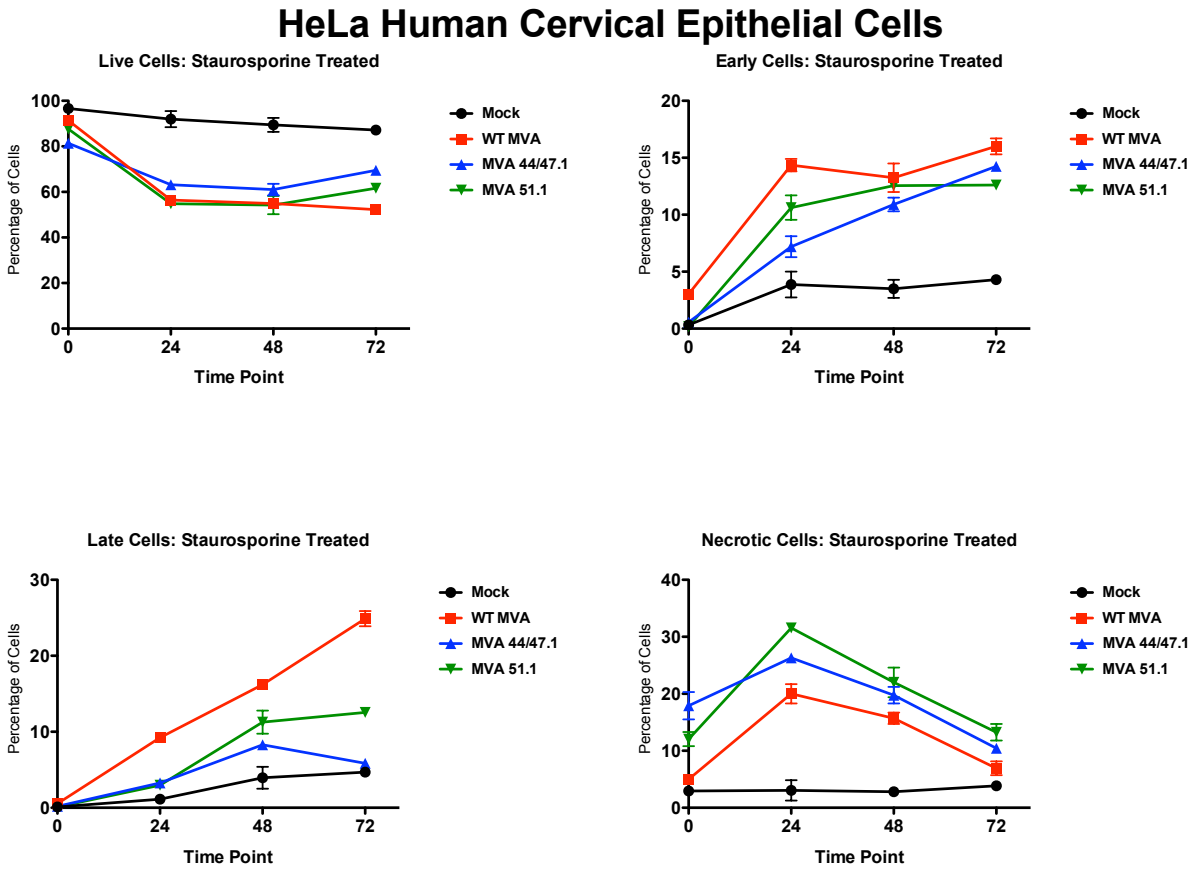


Figure 17. Viability in treated HeLa cells. Even with an apoptosis inducing agent, rMVA infected cells still show a smaller proportion of cells undergoing apoptosis and larger proportion of cells undergoing necrosis than WT MVA infected Hela cells. To assess viability, the percentage of viable, apoptotic and necrotic HeLa human cervical epithelial cells were quantified after cells were treated with staurosporine. At 72 h, there were significantly larger percentages of live cells infected with rMVAs than those with WT MVA. Also, at 24 h, there were significantly more WT MVA infected cells undergoing early apoptosis than the rMVA

infected cells, and at 24, 48, and 72 h, there are significantly more WT MVA infected cells undergoing late apoptosis than any rMVA infected cells ($p<0.05$). However, at 0 and 24 h, the rMVAs have significantly higher percentages of cells undergoing necrosis than WT MVA, and at 48 and 72 h, rMVA 51.1 had significantly higher percentages of cells undergoing necrosis than WT MVA ($p<0.05$). Data points represent the mean of two samples and error bars represent the standard error of the mean.

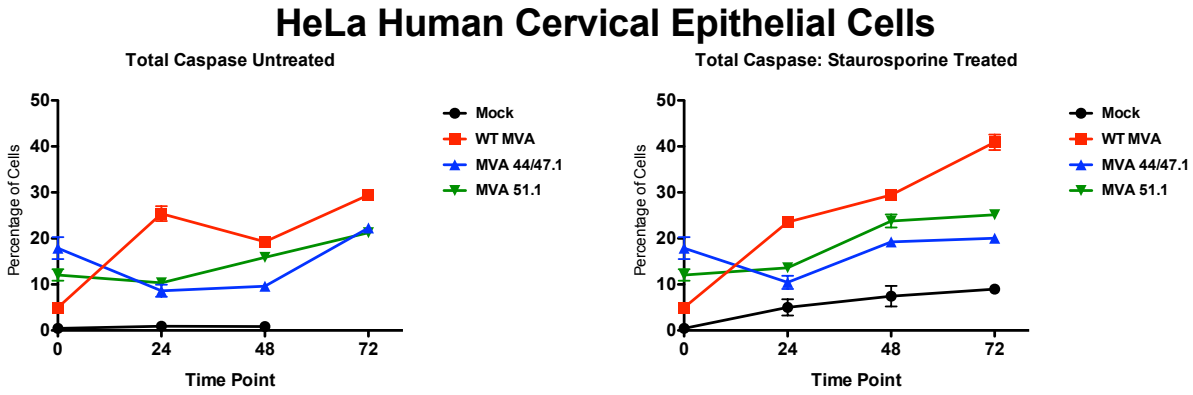


Figure 18. Caspase 3 activity in HeLa cells. rMVAs are better at deterring apoptosis in both untreated and staurosporine treated Hela cells. To assess viability, the percentages of infected HeLa cells with caspase 3 activity were quantified in untreated cells and those treated with staurosporine. In both untreated and treated cells, WT MVA infected cells had significantly more caspase activity at 24, 48, and 72 h than the rMVA infected cells ($p<0.05$). Data points represent the mean of two samples and error bars represent the standard error of the mean.

CONCLUSION

Recombinant MVAs were tested against WT MVA in order to assess whether they had an increased replicative capacity and ability to maintain viability in mammalian cell lines. In the C2C12 mouse myoblast cell line, replication was greatly increased by both rMVAs over WT MVA, and this was slightly more in rMVA 51.1 than rMVA 44/47.1 (Figure 19). However, by 24 hours, there was almost no difference in apoptosis and necrosis levels in these cells. In the L929 mouse fibroblast cells, there was a decrease in apoptosis at 24 h in both rMVAs, but rMVA 51.1 had an increase in necrosis at this time point. In the Vero monkey kidney epithelial cells, all viruses showed similar replication patterns, and WT MVA had slightly higher rates of replication. Nonetheless, in these cells, rMVA 51.1 showed a decrease in apoptosis at 24 h, whereas rMVA 44/47.1 showed an increase. rMVA 44/47.1 also had increased necrosis over the other two viruses. Finally, in the HeLa human cervical epithelial cells, the rMVAs showed enhanced replication, with rMVA 44/47.1 replicating the most robustly of all. However, HeLa cells also had the pattern of decreased apoptosis accompanied by increased necrosis.

Cells and Viruses	Virus Titer Fold Change (24 h)	Early Apoptosis (% Cells, 24 h)	Late Apoptosis (% Cells, 24 h)	Necrotic (% Cells, 24 h)
C2C12				
WT MVA	27.2	6.57	33.7	42.1
rMVA 44/47.1	117	3.95	38.1	45.2
rMVA 51.1	176	4.67	37.9	44.2
L929				
WT MVA	NA	0.54	28.4	44.0
rMVA 44/47.1	NA	0.60	15.8	37.6
rMVA 51.1	NA	0.50	18.3	49.5
Vero				
WT MVA	13.5	15.6	2.82	11.6
rMVA 44/47.1	12.6	16.7	4.35	17.5
rMVA 51.1	12.0	9.31	3.27	7.92
HeLa				
WT MVA	9.92	16.3	9.08	18.3
rMVA 44/47.1	837	5.64	3.00	30.1
rMVA 51.1	298	7.82	2.50	35.2

Figure 19. Overall replication and cell death patterns of recombinant and WT MVAs at 24 hours in mammalian cell lines.

DISCUSSION

Vaccines are an essential part of preventative medicine—however vaccines for some diseases, such as HIV, can be very difficult to create (6,7). Past efforts to design an effective vaccine have been inhibited by insufficient B and T cell response (7,9). In order to overcome this issue, researchers may be able to increase the immunogenicity of the vector used to deliver HIV antigens (9,19). In a viral vector model for vaccination, this could mean increasing the amount of viral particles and limiting cell death so that immune cells can have more time and opportunities to recognize target antigens. MVA, a viral vector commonly used for vaccine development, has a fragmented genome and limited replication in human cells. However, if researchers can increase replication in human cells, we could possibly increase the immunogenicity of the vector.

Using rMVA viruses from the Moss lab which had large portions of the fragmented MVA genome reinserted into them, we aimed to determine the replication capacity of these viral vectors in mammalian cells and also determine their ability to delay apoptosis, or cell death (18). We first endeavored to understand the replication of the recombinant viruses compared to the WT virus in the cells from mammals that are often used during vaccine development: mice, monkeys, and humans (24). Previous studies on these recombinant viruses indicated increased replication of rMVA 44/47.1 and rMVA 51.1 in human, monkey, and rabbit cells (13, 18). Data on replication of these viruses in mice was not provided. Although the data on replication in human cells were confirmed, this study found alternative results regarding replication in monkey cells. Vero cells showed a reduction in replication with the rMVAs over

the WT MVA (Figure 5). This could be due to small sample size (n=2) or errors in lysing of cellular material. More studies would be needed to clarify the discrepancy. We also found that rMVA 51.1 established greater replication in C2C12 mouse myoblast cells than WT MVA, but the rMVA 44/47.1 did not (Figure 4). This may be due to reinsertions in the genome that encoded machinery to allow for greater replication. Overall, these results show that the reinsertions into the genome of the rMVAs have somewhat increased their host range by allowing greater replication in some mammalian cells. However, the host range may still be somewhat limited and more studies would need to be conducted in order to confirm these results.

In the second part of the study, we endeavored to determine whether the enhanced replication of the recombinant viruses correlated with enhanced survival of infected cells, as is often reported (20). However, in the context of the C2C12 mouse myoblast cells, this hypothesis was not corroborated. Although the rMVA 51.1 showed increased replication in these cells, infection with all viruses showed similar viability, apoptosis, and necrosis levels, indicating that the proteins encoded by the inserted gene fragments had no effect on cell signals for apoptosis and necrosis in C2C12 cells. On the other hand, these results may be flawed, as viruses were difficult to detect after 24 hours. These tests in another mouse cell line, L929, produced different results. Interestingly, in the untreated and treated L929 cells, rMVA 51.1 showed decreased levels of apoptosis at 24 h compared to WT MVA, but this difference became reversed or negligible by 48h (Figure 12). rMVA 44/47.1 also had similar results when treated with staurosporine, the apoptosis inducer. This indicates there may be some delay of apoptosis

in these cells early in its life cycle, but this effect diminishes within two days. In addition, an increase in the percentage of cells undergoing necrosis (Figure 11) coincided with the decrease in apoptosis, which supports the notion that although the apoptosis pathway is blocked by viral components, other components of the virus could still be causing the infected cells to die. Ultimately, this diminishes the usefulness of delaying apoptosis in these cells. A more exaggerated, but similar result was seen with the HeLa cells. Vero cells seemed to show somewhat of a delay in apoptosis at 24 h, especially in the treatment group, but this was not accompanied with the same increase in necrosis. On the other hand, rMVA 44/47.1 seemed to be more toxic in Vero cells than any other infection group, possibly due to insertions that are recognized by the cellular factors (such as interferon receptors) and trigger cell death (11). Finally, in the human cell line (HeLa) the rMVAs also showed enhanced replication over WT MVA, especially rMVA 44/47.1. In future studies, more time points should be examined between 0-48 h in order to better understand the delay in apoptosis seen around 24 hours.

Ultimately, both rMVA 44/47.1 and rMVA 51.1 have the potential to serve as vectors for vaccines as they have an expanded host range and may be able to delay apoptosis in the early phase of infection, but will normalize to the wild-type within 72 hours. Nevertheless, more needs to be understood on the balance of necrosis and apoptosis in cells infected with these viruses. Studies would need to be conducted in order to understand what is triggering the necrosis pathway and how that could potentially be stopped. Furthermore, replication of rMVA 44/47.1 and rMVA 51.1 in monkey cells needs to be established concretely, as any vaccine trial

for HIV must include testing on primates. Additionally, safety factors of increasing the host range and reinserting large segments of vaccinia virus genome need to be addressed *in vivo*.

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