RESPONSE OF M2 MACROPHAGES IN A SIMULATED TUMOR MICROENVIRONMENT TO INFECTION WITH VESICULAR STOMATITIS VIRUS

by

REBECCA FULLER

Honors Thesis

Appalachian State University

Submitted to the Department of Biology

in partial fulfillment of the requirements for the degree of

Bachelor of Science

August, 2018

Approved by:

_____

Dr. Maryam Ahmed, Ph.D., Thesis Director

_____

Dr. Darren Seals, Ph.D., Second Reader

_____

Dr. Lynn Siefferman, Ph.D., Biology Honors Director

ABSTRACT

Vesicular Stomatitis Virus (VSV) is a good candidate for oncolytic therapy due to its ability to induce apoptosis in a number of different types of cells. VSV's effect on macrophages has not been studied in-depth. Here, the effects of infection with both wild type (rwt) VSV and matrix (M) protein mutant (rM51R-M) VSV on cytokine production and cell viability of M2 macrophages cultured alone and in co-culture with MDA-MB-231 breast cancer cells were studied. Infection with rM51R-M VSV induced an increase in pro-inflammatory cytokines IL-6 production in co-culture conditions and TNF-α by M2 macrophages cultured alone. Viability of M2 macrophages cultured alone decreased after infection with both types of VSV. In co-culture conditions, cell viability decreased after infection with rwt VSV and increased after infection with rM51R-M VSV. We also set out to determine whether the MDA-MB-231 breast cancer cells or THP-1 monocytes were better labeled with fluorescent dye. Cancer cells were more readily labeled than monocytes. Working concentrations of dye were tested but an adequate concentration was not determined. Our data suggests that rM5R-M VSV can both modulate M2 macrophage phenotypes to a more M1-like phenotype and kill breast cancer cells, making it a suitable candidate for oncolytic therapy.

INTRODUCTION

Breast Cancer

Breast cancer is the second most commonly diagnosed cancer among women, with about 1 in 8 women being diagnosed with invasive breast cancer in her lifetime. Approximately 80% of breast cancer diagnoses are invasive ductal carcinoma, and the 5-year survival rate of women

with Stage IV metastatic cancer is estimated to be as low as 22% ("U.S. Breast Cancer Statistics", *Breastcancer.org*). Current treatments for these invasive types of breast cancer are highly ineffective (Chung & Carlson, 2003). Current treatment options for those suffering with invasive breast cancer include radiation, surgery, and chemotherapy. Radiation and surgery are more localized treatment options, focusing more on the eradication and removal of the primary tumor. Chemotherapy is a systemic treatment that affects all of the cells in the body of the patient, and often elicits many negative side effects including nausea, fatigue, and hair loss. Radiation and surgery are ineffective treatments for invasive breast cancer due to their inability to target sites of distant metastasis. Chemotherapy has more promise as a treatment for invasive breast cancer due to its systemic effects, however it has been shown that chemotherapy is only moderately effective in treating metastatic breast cancer, however does not eradicate all the cancer from the body. It can also be damaging to the patient's body, such that the patient's quality of life decreases as a result of chemotherapy's harmful side effects (O'Shaughnessy, 2005). Therefore, there is critical need for the development of alternative and more effective treatment options for the treatment of cancer, especially metastatic disease.

Metastatic Cancer

Cancer is considered to be metastatic when it migrates from the primary tumor into a distant location in the body (Seyfried and Huysentruyt, 2013). To metastasize, all potential secondary tumors must first invade, disseminate, and subsequently colonize a distal environment. The biological events are orchestrated through intrinsic and extrinsic homeostatic factors and molecular pathways. The invasion–metastasis cascade begins with neovascularization, the transition from epithelial to mesenchymal subtype, and the breakdown of extracellular matrix

and basement membrane. These events increase potent angiogenic factors, such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), increasing tumorigenicity. The degradation of the basement membrane is facilitated by matrix metalloproteinases (MMPs). In breast cancer, deregulation of MMP expression, especially MMP-2 and MMP-9, promotes invasion and metastasis. MMP-mediated degradation of the extracellular matrix releases numerous growth factors and signaling molecules that enable tumor expansion. In addition, the tumor microenvironment (TME) cancer cells find themselves in also contributes greatly to numerous aspects of tumorigenicity, including metastasis. Cancer cells interact with surrounding fibroblast, stromal, immune, and endothelial cells through factors that promote angiogenesis, membrane degradation, epithelial–mesenchymal transition (EMT), and colonization.

Once cancer has metastasized and colonized distant tissues, it becomes much more difficult to treat, such that 90% of cancer fatalities occur as a result of cancer metastasis in the body (Seyfried and Huysentruyt, 2013). Currently, most chemotherapeutic approaches revolve around obstructing established tumors through a combination of surgical resection and adjuvant therapy. These therapies have positive outcomes for breast cancer patients with exclusively primary tumors; however, metastatic cancer, being systemic in nature and resistant to chemotherapeutics, tends to have poorer prognosis. Therefore, understanding the factors that contribute to metastasis are critical for developing effective therapeutics. For my studies, I am interested in investigating the interaction between breast cancer cells and tumor-associated macrophages (TAMs) and how therapies targeting this interaction may modify the course of disease.

Oncolytic Viral Therapies

One therapy that has been proposed to treat metastatic breast cancer is oncolytic viral therapy. Oncolytic viruses selectively replicate in and kill cancer cells without harming healthy cells (Liu et. al., 2003; Balachandran & Barber, 2000). The basis for oncolytic therapies is that cancer cells frequently develop alterations in their immune response components, including the type I interferon (IFN) antiviral response pathway during the process of tumorigenesis. Therefore, cancer cells allow high levels of virus replication resulting in induction of cell death pathways, while normal cells with intact antiviral responses are spared from virus-induced cytopathology. In addition to the natural ability of viruses to kill cancer cells, oncolytic viruses are also being engineered to selectively kill cancer cells over normal cells. There have been promising results from clinical trials in which numerous oncolytic viruses are being used as a therapeutic agents for the treatment of a variety of cancers. For example, an E1B 55 kD gene deleted strain of adenovirus, known as H101, was approved for the treatment of head and neck cancer in China in 2005 in combination with chemotherapy. More recently, T-Vec (talimogene laherparepvec), a second-generation oncolytic modified herpes simplex virus (HSV) that has been engineered to express human granulocyte-macrophage colony-stimulating factor (GM-CSF), was approved as a treatment for melanoma in the US in 2015 (Fukuhara et. al., 2015). Although strides have been made in the approval of such agents for the treatment of cancers, progress in terms of widespread usage has been slow due to multiple limiting factors, including lack of expertise with intratumoral viral injections by oncologists and preference for more familiar cancer therapeutics over viral therapies (Rehman, 2016).

In addition to being used as individual therapeutic agents for cancer treatment, it has also been proposed to use oncolytic viruses in conjunction with other anti-tumor agents as

combination therapies. Studies in specific tumor models have shown that cancer-specific factors may render the tumor resistant to oncolytic therapies alone. Therefore synergistic combination approaches may be necessary to enhance the oncolytic effects of different viruses. For example, one study found that for the treatment of malignant gliomas, adding rapamycin, an immunosuppressant, significantly enhanced the replication of oncolytic poxvirus by suppressing the immune response to the virus. (Lun, 2009). Another study combined a prostate-targeted herpes simplex virus type 1 (HSV-1) with a taxane drug and observed reduced tumor grown *in* vivo. This study also found that the combination therapy induced synergistic cytotoxicity of prostate cancer cells, due to both the HSV-1 and the taxane's abilities to arrest cells in certain stages of the cell cycle, thereby inducing eventual cell death (Passer et. al., 2009). Therefore, it is increasingly being appreciated that specific combination approaches may be more efficacious than single therapies alone.

Vesicular Stomatitis Virus

One virus currently being studied for use as an oncolytic agent is vesicular stomatitis virus (VSV). VSV, an enveloped virus in the family *Rhabdoviridae*, has a negative single-stranded mRNA genome. It is pathogenic to cattle, with infection producing lesions and sores in the mouths and on the hooves of cows (Ahmed & Lyles, 1998). However, VSV is typically nonpathogenic in humans, exhibiting flu-like syndromes in rare cases (Ahmed & Lyles, 1998). VSV is a good candidate as an oncolytic agent due to its ability to induce apoptosis in numerous types of cancer cells. The virus enters cells with the help of clathrin-coated vesicles and is able to replicate in a wide variety of cell types. In the process of virus replication, the wild type (wt) strain of the virus inhibits the host IFN antiviral response through the function of the viral matrix

(M) protein, which prevents the host cell from transcribing the products necessary to recognize and defend against it. Specifically, studies have shown that M protein of VSV inhibits new host gene expression through inhibition of host transcription and nuclear-cytoplasmic transport of host messages, with the effect of inhibiting the expression of antiviral gene products (Ahmed et. al, 2003).

rM51R-M virus is a M protein mutant strain of VSV that has a methionine to arginine switch at amino acid 51 of the M protein. This mutation renders the virus defective in inhibiting host gene expression, thus allowing the host cell to mount an IFN response and express gene products with antiviral activity. Previous studies have highlighted the selectivity of M protein mutant viruses as selective oncolytic agents with the ability to induce innate immune responses. In one study, virus was more quickly cleared from the nasal mucosa of mice that were inoculated with rM51R-M VSV when compared to mice that were inoculated with rwt VSV. The mutant strain of VSV was also unable to spread to the central nervous systems of the mice, unlike rwt VSV (Ahmed et. al., 2008), thus demonstrating its safety in vivo. In addition to being a safer oncolytic agent than wild-type strains of VSV, rM51R-M virus has also been shown to exhibit immunostimulatory properties. One study found that rM51R-M virus was able to induce maturation of myeloid dendritic cells after infection through stimulating IFN expression and expression of cytokines involved in dendritic cell maturation. Additionally, the dendritic cells that matured through infection with rM51R-M virus were found to be competent in priming T-cells, such that they proliferated more robustly and had greater effector function than T-cells primed with lipopolysaccharide, a bacterial endotoxin (Ahmed et. al, 2006). Its ability to induce an immunostimulatory response and the fact that it is safer than wt VSV make rM51R-M VSV a good candidate as an oncolytic agent.

As mentioned previously, my study involves investigating the effects of VSV on breast cancer. The breast cancer cells we utilize for our studies (MDA-MB-231 cells) have defects in their IFN response pathway, thus they are unable to respond or defend themselves against viral infection. As a result of their inability mount an effective antiviral response, the virus is able to replicate within the cancer cells, induce apoptotic and other cell death mechanisms, and spread to neighboring cells. The viral progeny are thus equipped to infect and lyse other cancer cells in the tumor mass. In addition to releasing viral progeny, a main component of oncolytic therapies is the ability of viruses to kill cancer cells to release tumor antigens that can be sampled and identified by infiltrating immune cells. This creates a cancer-specific immunogenic response to promote the targeting and killing of cancer cells not only at the primary tumor site, but at metastatic distal sites.

One immune cell type that samples antigens in the environment for processing and presenting is the macrophage. Macrophages are phagocytic lymphocytes found both in bodily tissues and in the bloodstream in the form of white blood cells. They are derived from monocytes, which are found in the blood and polarize into macrophages after exposure to certain cellular products and cytokines. There are numerous types of macrophages in the body, with the M1 and M2 types being the main focus of my project. M1 macrophages are pro-inflammatory and are responsible for defending against intracellular pathogens as well as cancers. M2 macrophages have an anti-inflammatory function and are primarily responsible for wound healing and protecting the body from extracellular pathogens such as parasites (Chávez-Galán et al., 2015). Along with other immune cell types, macrophages are a main component of the tumor microenvironment. Macrophages found within the primary tumor are known as tumor-associated macrophages (TAMs). A large number of M1 macrophages in the tumor microenvironment is

correlated to a better prognosis amongst invasive triple-negative breast cancer patients, while a high number of M2 macrophages in the tumor microenvironment is correlated with a poor prognosis. Therefore, targeting M2 macrophages within the tumor microenvironment is currently a focus for numerous types of cancer therapeutics.

TAMs play an important role in the tumor microenvironment. During early tumor progression, M1-polarized macrophages have high tumoricidal activity and work to aid the immune system in disrupting tumor activity and growth. However, during late-stage tumor progression, macrophages are induced into an M2-like phenotype. M2 macrophages have several functions within the tumor microenvironment, such that they aid the advancement of tumor progression. M2-like TAMs assist in angiogenesis of vasculature into the tumor and immunosuppression of cells around the tumor (Chanmee et al., 2014). M2 macrophages also play a role in the metastasis of cancer and the migration of cancer cells from the primary tumor to distant sites in the body. They do this by forming a complex that includes the M2 macrophage, a cancer cell, and an endothelial cell. This complex is able to extravasate through the blood vessel wall and migrate to distant tissues in the body, including the liver and the lungs. Therefore, M2 macrophages represent a target for therapeutics due to their contributions in tumorigenesis. Current therapeutics are focused on eliminating or converting M2 macrophages to a more inflammatory macrophage phenotype with anti-cancer properties, such as M1 macrophages.

Previous research in our laboratory suggests that, when infected with oncolytic VSV, M2 macrophages can be educated to convert to an M1-like phenotype, perhaps through induction of the type I IFN response by rM51R-M virus (Polzin, 2017). However, it is unknown how M2 macrophages will respond to VSV in the context of a tumor microenvironment. M1 macrophages secrete pro-inflammatory cytokines, including IL-6 and TNF-alpha, whereas M2 macrophages

secrete anti-inflammatory cytokines, including TGF-beta and IL-10. (Carswell et. al., 1975) Therefore, the production of specific cytokines in culture is indicative of the ability of macrophages to undergo phenotypic switching in response to therapies.

The overall goal of my project is to determine the effect of therapies with oncolytic VSV on M2 macrophages and breast cancer cells in a simulated tumor microenvironment. For these studies, THP-1 cells differentiated to M2 macrophages were co-cultured with MDA-MB-231 breast cancer cells and infected with a wild-type (wt) strain of VSV, rwt virus, as well the M protein mutant virus, rM51R-M virus. Cytokine secretion and cell viability was determined following infection as an indication of these cell populations to respond to the different VSV strains. Rwt virus, containing the wt M protein, is a potent inhibitor of host gene expression, while the M protein mutant virus is defective at this function and thus, allows the expression of genes in infected cells including those involved in inducing an antiviral response. I hypothesize that upon infection with VSV, the secretion of cytokines IL-6 and TNF-α will be upregulated in samples containing M2 macrophages alone and co-culture samples. This result may be more prominent in cells infected with rM51R-M virus due to its ability to induce innate immune responses. I also hypothesize that, in co-culture, M2 macrophages and breast cancer cells will be more resistant to cell death induced by infection with VSV. In addition, I predict that cells will be more sensitive to killing by the wt strain of VSV, rwt virus, due to its ability to suppress the host antiviral response. Our results showed that infection with rM51R-M virus induced an upregulation of inflammatory cytokine production both in samples containing M2 macrophages alone as well as in co-culture samples. Results also indicated that rwt VSV is more cytotoxic to cells than mutant virus, and that cells are more resistant to both strains of VSV in co-culture when compared to the macrophages alone. Therefore, we can conclude that the rM51R-M virus

may be able to induce a phenotypic switch in M2 macrophages to a more M1-like phenotype, and that the mutant VSV is a promising agent for oncolytic virotherapy.

METHODS

Cell lines and viruses

The MDA-MB-231 breast cancer cells were obtained from the laboratory of Dr. Darren Seals. They were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) in 100mm plates and incubated at 37°C + 5% CO_2. The THP-1 human monocytic cell line was cultured at a baseline concentration of 200,000 cells/mL in RPMI containing antibiotics and supplements (100x vitamins, 5 mL Penn/Strep, 5 mL of 1 M Hepes, 1.75 µL 2-mercaptoethanol, 50 mL FBS) and incubated at 37°C + 5% CO_2. Recombinant wild-type (rwt) virus and the matrix (M) protein mutant (rM51R-M) virus were generous gifts from Dr. Doulas Lyles from Wake Forest University Baptist Medical Center (Winston-Salem, NC) and have been described previously (Whitlow et al., 2006). Virus stocks were prepared in BHK cells using methods described previously (Kopecky et al., 2001). Specifically, BHK cells were grown in 6-well plates and infected with virus stock at different dilutions. At 1 hour, sterile 2X Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% FBS was added in equal parts to 2% agar and added to the cells. Following 24-48 hours of incubation, plates were refrigerated for one hour following the addition of 3.7% formaldehyde to each well. Formaldehyde and agar was removed, and crystal violet was added to each well for visualization of plaque formation and quantitation of viral titers in PFU (plaque forming units)/ml.

Monocyte polarization to M2 macrophage phenotype

THP-1 monocytes were placed in 24-well plates at a concentration of 500,000 cells/mL of media, such that there were 500,000 cells in each well. Phorbol myristate acetate (PMA) was added to each well at a concentration of 1μL/mL and the cells were left to polarize into M0 macrophages for 24 hours. After 24 hours, the cells were examined to ensure that they had "settled" onto the bottom of the plate. After polarization to M0 macrophages, the old media was pulled off. Interleukin-4 (IL-4), Interleukin-13 (IL-13), PMA, and RPMI were combined in a test tube at a concentration of 1 μL of cytokines per 1 mL of RPMI. 700 μL of this cytokine-RPMI solution were added to each well and incubated for 48 hours to generate M2 macrophages. Figure 1 depicts our strategy for polarization of THP-1 cells to the different macrophage subsets.

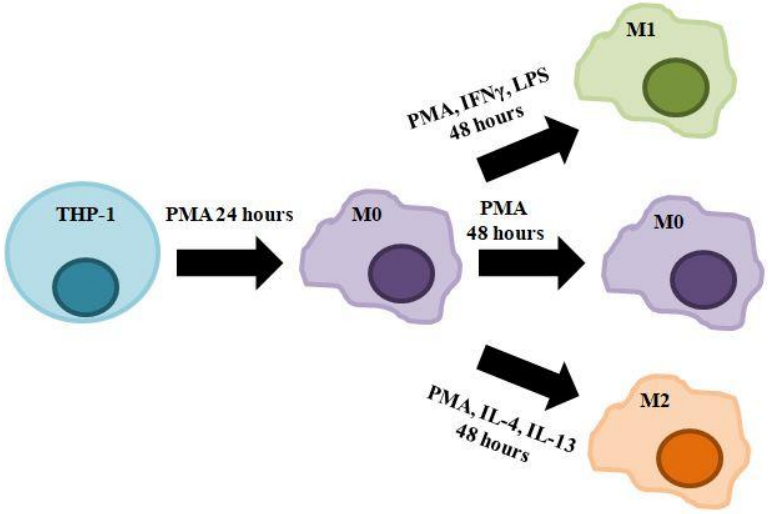


Figure 1. Macrophage polarization procedure.

Co-culture of breast cancer cells and macrophages

After polarization of THP-1 cells to M2 macrophages, an equal number of MDA-MB-231 breast cancer cells were added to M2 macrophages in 24-well plates such that 500,000 breast cancer cells were placed in each well. Polarizing cytokines were also added to the wells at a concentration of 1 μL/mL of breast cancer cells added. Cells were allowed to incubate for 4 h

and then infected with rwt or rM51R-M virus for 24 hours. Supernatant was collected and stored at -80°C until use. For controls, M2 macrophages and MDA-MB-231 cells were infected alone, to determine the effect of the viral strains on cytokine levels in the absence of co-culture. Cytokine levels were determined with the use of ELISAs following the manufacturer's protocol (BD OptEIA) and as outlined by Ahmed et al. (Ahmed, Puckett, Armilli, Braxton, Mizel and Lyes, 2010). The procedure for co-culture of breast cancer cells with macrophages is depicted in Figure 2.

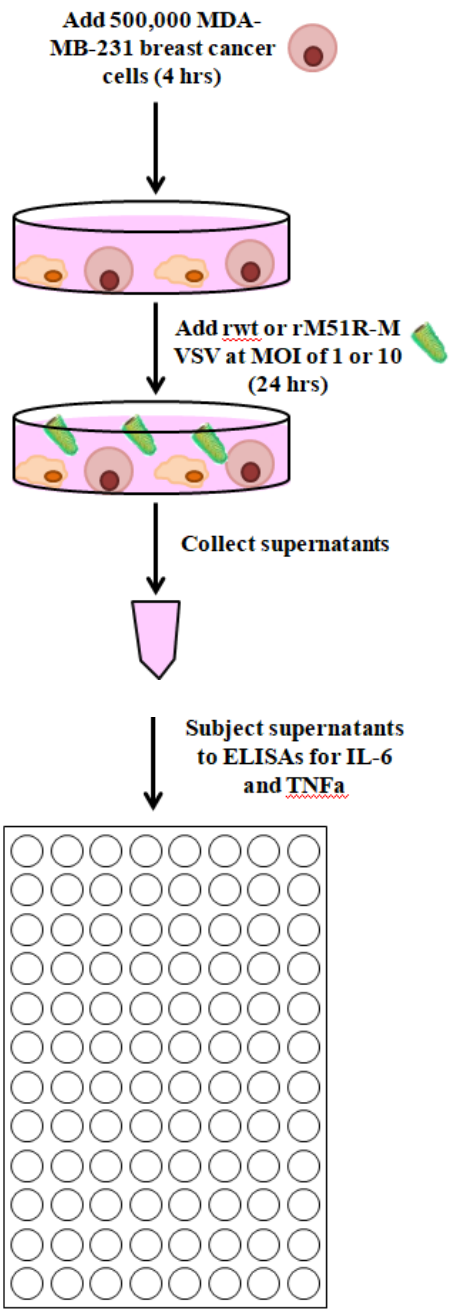


Figure 2. Co-culture of M2 macrophages and MDA-MB-231 breast cancer cells.

Determination of cell viability:

1. Trypan Blue Exclusion:

Cells were co-cultured as described previously in the methods. After co-culture, cells were lifted with Accutase. 10 µL of sample were combined with 10 µL of trypan blue dye and homogenized by pipetting up and down. 10 µL of this mixture was placed into a hemocytometer and the live and dead cells were counted.

2. Cell tracker

THP-1 cell staining with CellTracker Violet

THP-1 cells were harvested by centrifugation and supernatants were aspirated. Cells were resuspended in pre-warmed CellTracker™ Working Solution and incubated for 15–45 minutes at 37°C + 5% CO_2. After centrifugation, labeled cells were removed from the CellTracker™ Working Solution, resuspended in RPMI + 10% FBS, and dispensed into a 6-well plate. Cells were imaged directly after staining using the 4′,6-diamidino-2-phenylindole(DAPI) excitation filter for the CellTracker™ Violet probe.

MDA-MB-231s, M2 macrophages staining:

Cells were cultured in a 6-well plate. After reaching 80% confluency, the culture media was removed. Pre-warmed CellTracker™ Working Solution was added to dish and incubated for 15–45 minutes at 37°C + 5% CO_2. CellTracker™ Working Solution was removed and 3 mL of RPMI+ 10 % FBS was added to each well. Plate was imaged using fluorescent microscopy with the 4′,6-diamidino-2-phenylindole (DAPI) excitation filter for the CellTracker™ Violet probe directly after labeling, then again 24 hours post-labeling.

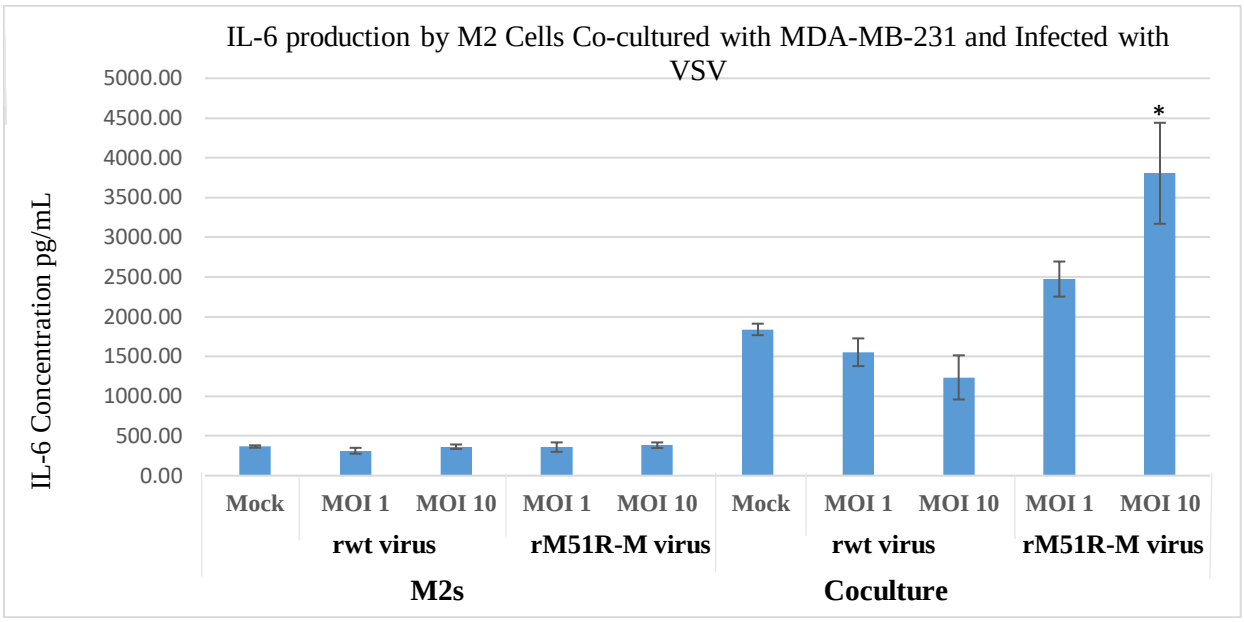
RESULTS

Il-6 production increases in co-culture conditions after infection with mutant VSV

Previous studies in our laboratory have shown that M2 macrophages are susceptible to infection and killing by both rwt and rM51R-M viruses (Polzin, 2017). In addition, results suggest that infection of M2 macrophages by VSV educates the macrophages into a more M1-like macrophage phenotype (Polzin, 2017) as seen by the expression of cytokines and cell surface markers indicative of the M1 state. What is not known is how VSV impacts macrophages, as well as tumor cells, in the context of a tumor microenvironment. To determine whether oncolytic VSV has the ability to modulate macrophages and breast cancer cells in a simulated tumor microenvironment, THP-1 cells differentiated into M2 macrophages were co-cultured with MDA-MB-231 cells and infected with rwt and rM51R-M viruses at MOIs of 1 and 10 pfu/cell. An MOI of 1 indicates that each cell in culture will be exposed to one plaque-forming unit (pfu) or virus particle in the culture. However, in actuality, when infected at an MOI of 1, only 45-65% of cells in culture will be infected with at least one virus particle. This method of infection allows us to observe how a virus replicates and spreads within a culture. An MOI of 10 indicates that 10 virus particles are added to a culture for each cell in that culture. With an MOI of 10, virtually 100% of the cells in the culture are infected. This method of infection allows us to observe how the cells respond to a synchronous infection with virus. Following infection for 24h, supernatants were collected and subjected to ELISA for the detection of secreted IL-6. M1-like macrophages are capable of producing IL-6 in response to exposure to endogenous pathogens, and thus, this cytokine serves as a marker for M1 macrophages. Figure 3A shows IL-6 levels induced by M2 macrophages alone and in direct co-culture with MDA-MB-231 breast cancer cells with and without infection with VSV. Results showed that minimal IL-6 is produced by

M2 macrophages, and that neither rwt nor rM51R-M virus is capable of stimulating production of IL-6 by these macrophages. However, MDA-MB-231 cells produce higher levels of IL-6 on their own (Figure 3B) and neither virus alters those levels. Interestingly, when macrophages are co-cultured with breast cancer cells, overall levels of IL-6 decrease as compared to those produced by breast cancer cells alone. In addition, infection by rM51R-M virus elicits an increase in IL-6 production under co-culture conditions. These results indicate that infection with rM51R-M VSV could cause M2 macrophages to upregulate M1 markers, in this case IL-6, when these macrophages are co-cultured with MDA-MB-231 breast cancer cells. This suggests that the virus could be educating the M2 macrophages to a more M1-like phenotype in response to infection, such that they secrete IL-6 as an immune response to infection with the virus. The absence of an observable increase in IL-6 production by M2 macrophages alone when infected with mutant virus, compared to the significant increase observed in co-culture conditions after infection with mutant VSV suggests that the presence of cancer antigen could influence IL-6 secretion by M2 macrophages. Indirect co-cultures with cancer antigen and M2 macrophages would elucidate the validity of this hypothesis.

A



B

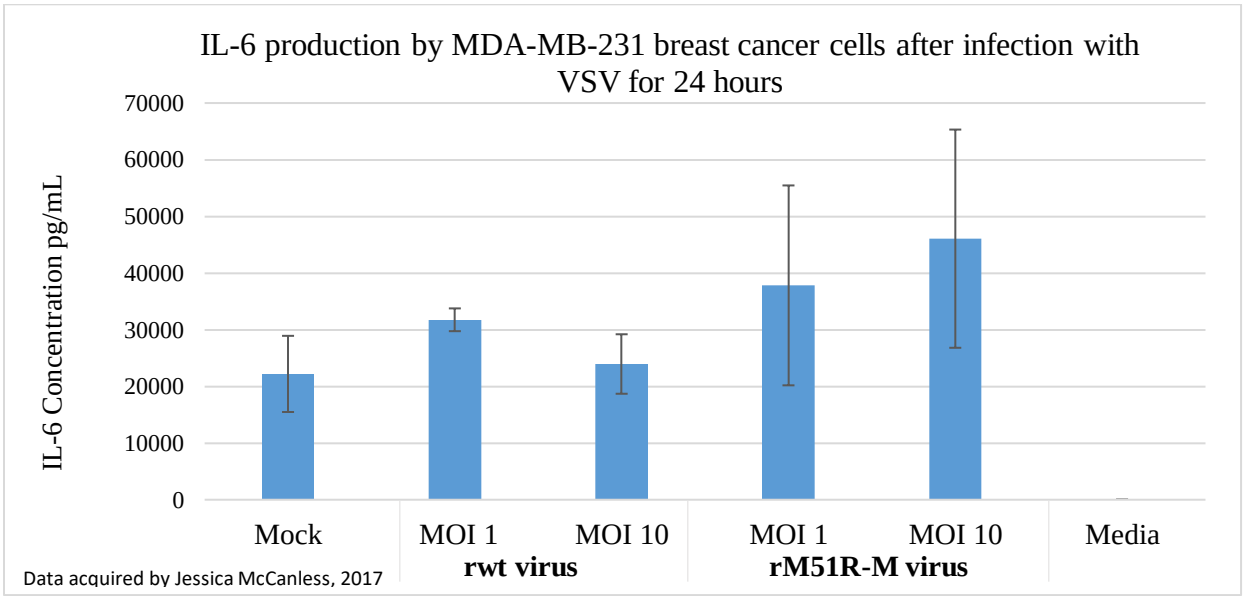


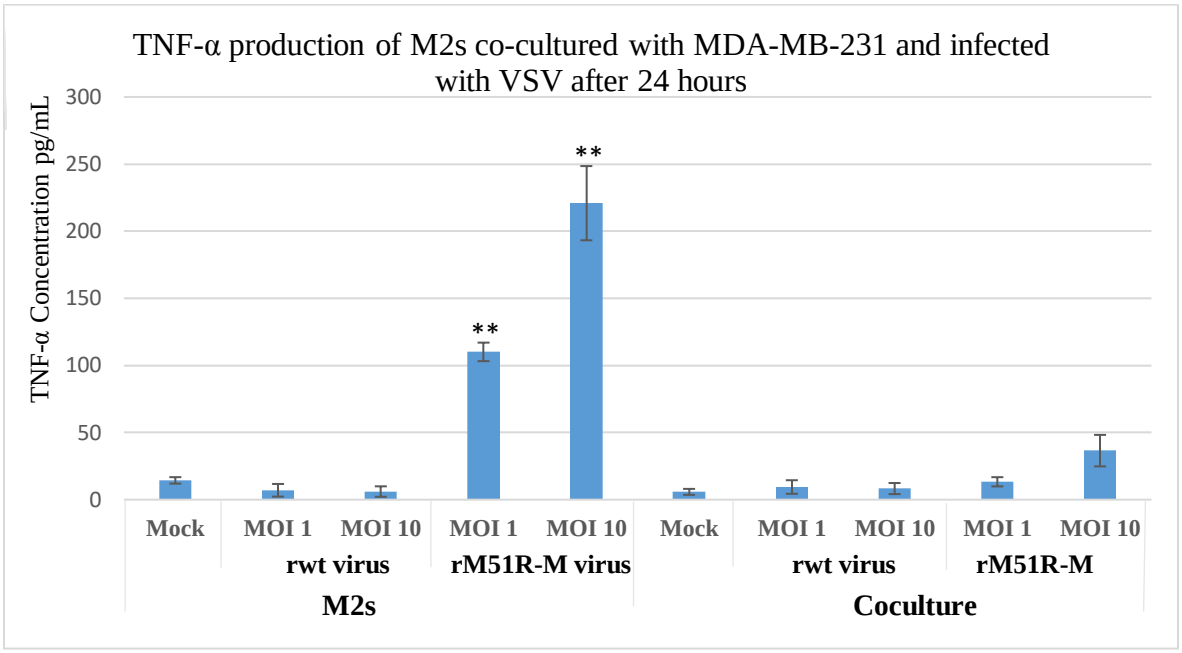
Figure 3. IL-6 production by MDA-MB-231 cells, M2 Macrophages and Co-culture after 24 hr infection with VSV. Production of IL-6 (pg/mL) by M2 macrophages and co-culture wells (A) and MDA-MB-231 cells (B) after infection with either rwt or rM51R-M viruses at an MOI of 1 or 10 pfu/cell for 24 hours. Data are the means and standard deviations of 3 independent

experiments. Cells in Figure A were grown in media without serum, while those in Figure B were grown in media with serum.

TNF-α production by M2 macrophages alone increases after infection with rM51R-M virus

Another cytokine that serves as a marker for M1 macrophages is TNF-α. TNF-α has been associated with enhancing the polarization towards M1 macrophages and inhibiting the M2 phenotype. As before, MDA-MB-231 cells were co-cultured with M2 macrophages and infected with VSV. Secreted TNF-α was determined by ELISA at 24 h post-infection (Figure 4A). Results indicated that low levels of TNF-α were produced by M2 macrophages, as expected. However, rM51R-M virus significantly increased TNF-α levels when infected at both MOIs. In contrast, rwt virus did not stimulate TNF-α production in these cells. MDA-MB-231 cells alone did produced higher levels of TNFa as compared to M2 macrophages and VSV did not induce changes in levels of this cytokine. Similar to results in figure 3 with IL-6, overall levels of TNFa decreased under co-culture conditions. However, under co-culture conditions, rM51R-M virus induced TNF-α production, but levels were lower than those obtained from M2 cells infected alone with rM51R-M virus. These data indicate that the breast cancer cells could potentially secrete factors that inhibit the production of TNF-α by M2 macrophages after infection with rM51R-M virus under co-culture conditions. These data also indicate that cells in co-culture could be more resistant to infection by rM51R-M than cells cultured alone. Further research is currently being conducted in our lab to determine whether or not this is the case.

A



B

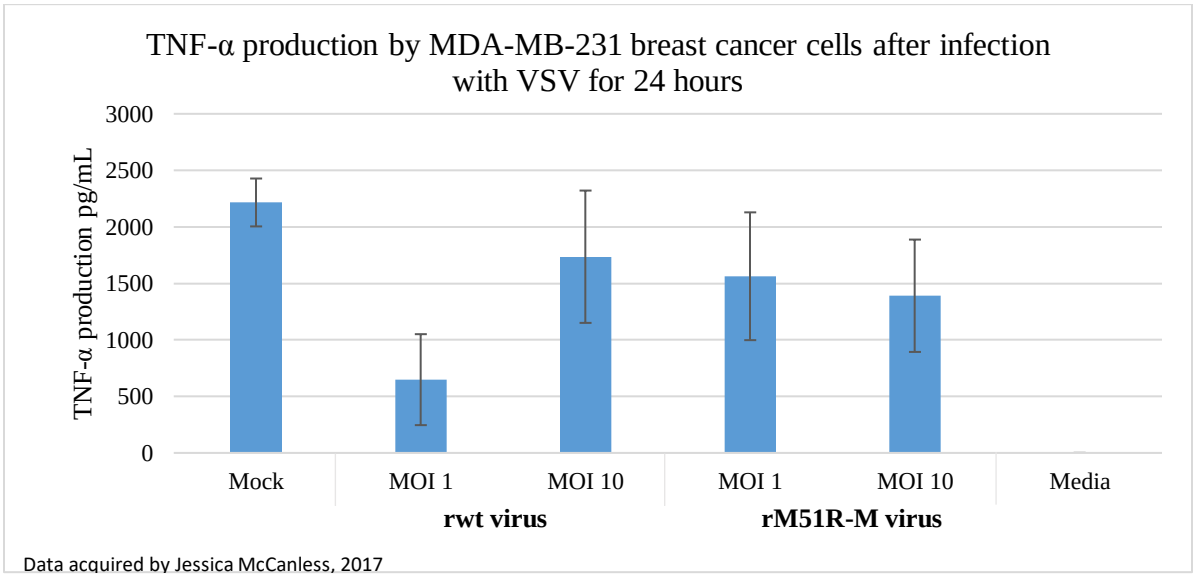


Figure 4. TNF-α production by MDA-MB-231 cells, M2 Macrophages and Co-culture after 24 hr infection with VSV. Production of TNF-α (pg/mL) by M2 macrophages and co-culture

(A) wells and MDA-MB-231 cells grown in media without serum (B) after infection with either rwt or rM51R-M VSV at an MOI of 1 or 10 pfu/cell for 24 hours. Data are the means and standard deviations of 3 independent experiments. Cells in Figure A were grown in media without serum, while those in Figure B were grown in media with serum.

Cells in co-culture are more resistant to cell death induced by VSV than cells cultured alone

Previous work in our lab has shown that M2 macrophages and MDA-MB-231 cells alone are susceptible to infection and killing by VSV. What is not known is whether under co-culture conditions, cells remain susceptible to killing by VSV, or if they are resistant to VSV infection due to a 'field-effect' within the simulated tumor microenvironment. Furthermore, determination of the viability of cells within the microenvironment may provide insight into the mechanisms by which VSV modulates macrophage populations during oncolytic therapies. To start to determine how VSV impacts the viability of cells in the simulated tumor microenvironment, M2 macrophages alone, and in co-culture with MDA-MB-231 cells were infected with rwt and rM51R-M viruses for 24h. Cells were harvested and exposed to trypan blue to determine cell viability. Figure 5 shows the percentage of surviving cells following VSV infection relative to those under mock-infection conditions.

Preliminary results from one experiment indicate that a greater number of M2 macrophages died after infection with rwt virus when compared to the cell death observed following infection with rM51R-M virus. This was the case when cells were infected at either MOI of 1 or 10 pfu/cell. We also observed a decrease in cell viability in the co-culture wells after infection with rwt virus at both MOIs. Finally, our results showed that a decrease in cell death occurred in co-culture conditions after infection with rM51R-M virus as indicated by an increase in viability as

compared to mock-infected cells. Although these studies do not distinguish how VSV affected individual cell populations (macrophage or breast cancer) within the co-culture, future studies are aimed at dissecting the response of specific cells within the simulated tumor microenvironment.

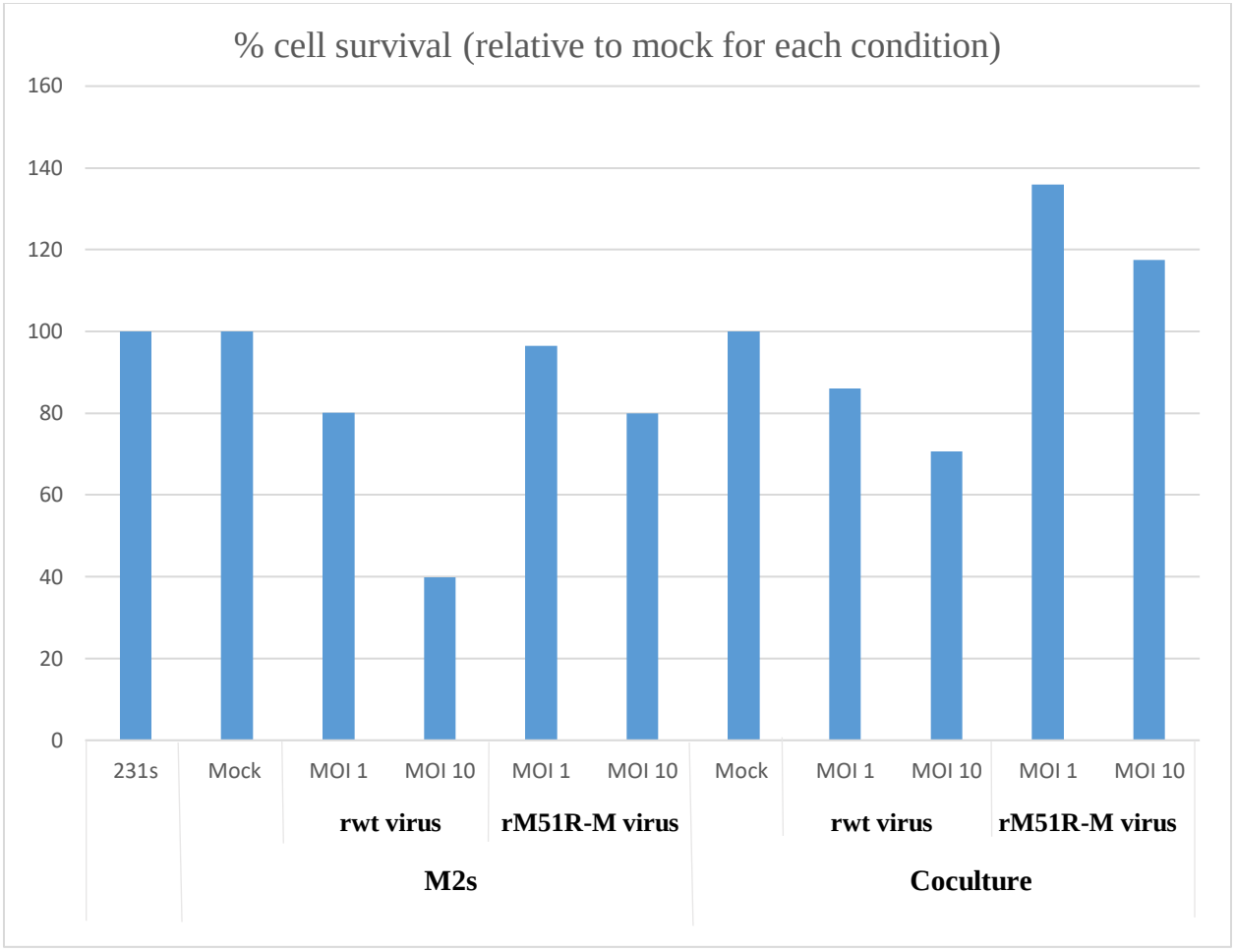


Figure 5. The Effects of VSV on Viability of M2 macrophages and MDA-MB-231 breast cancer cells alone and in direct co-culture. Viability of total cells within the co-culture was tested through trypan blue exclusion. Data are expressed as the percentage of cell survival within the total cell populations (MDA-MB-231, M2 macrophages). Data shown represents one experiment.

MDA-MB-231 breast cancer cells are able to be labeled by CellTracker™ Violet

One means to determine how cells within the co-culture population are responding to infection with VSV, is by distinguishing cells within the co-culture through labeling and then measuring the impact of virus infection on specific populations. To do this, I attempted to label cells with CellTracker™ violet. CellTracker™ Violet is a fluorescent dye that can be used to stain living cells. It is retained in the membranes of cells for up 72 hours and through several generations. In order to properly stain the cells for fluorescent imaging, we first had to determine which cell type -THP-1's, M2 macrophages, or MDA-MB-231 breast cancer cells- would be the most appropriate for labeling. We first labeled THP-1 monocytes with the CellTracker™ Violet at concentrations ranging from 0.5µM-3µM. When they were imaged, fluorescence could not be detected (data not shown). Upon further inspection, it was found that the dye was causing the cells to die at the higher of the concentrations. Therefore, higher concentrations of CellTracker™ were not tested on this cell line. The MDA-MB-231 breast cancer cells were incubated in concentrations of cell tracker ranging from 3µM-5µM. Upon imaging, very dim fluorescence was observed (Figure 6), however the signal was not strong enough to track the cells in culture. Upon inspection, the cells looked healthy and did not show signs of stress due to the dye. Higher concentrations will be tested in the future to determine which concentration of CellTracker™ dye is optimal for labeling the cells.

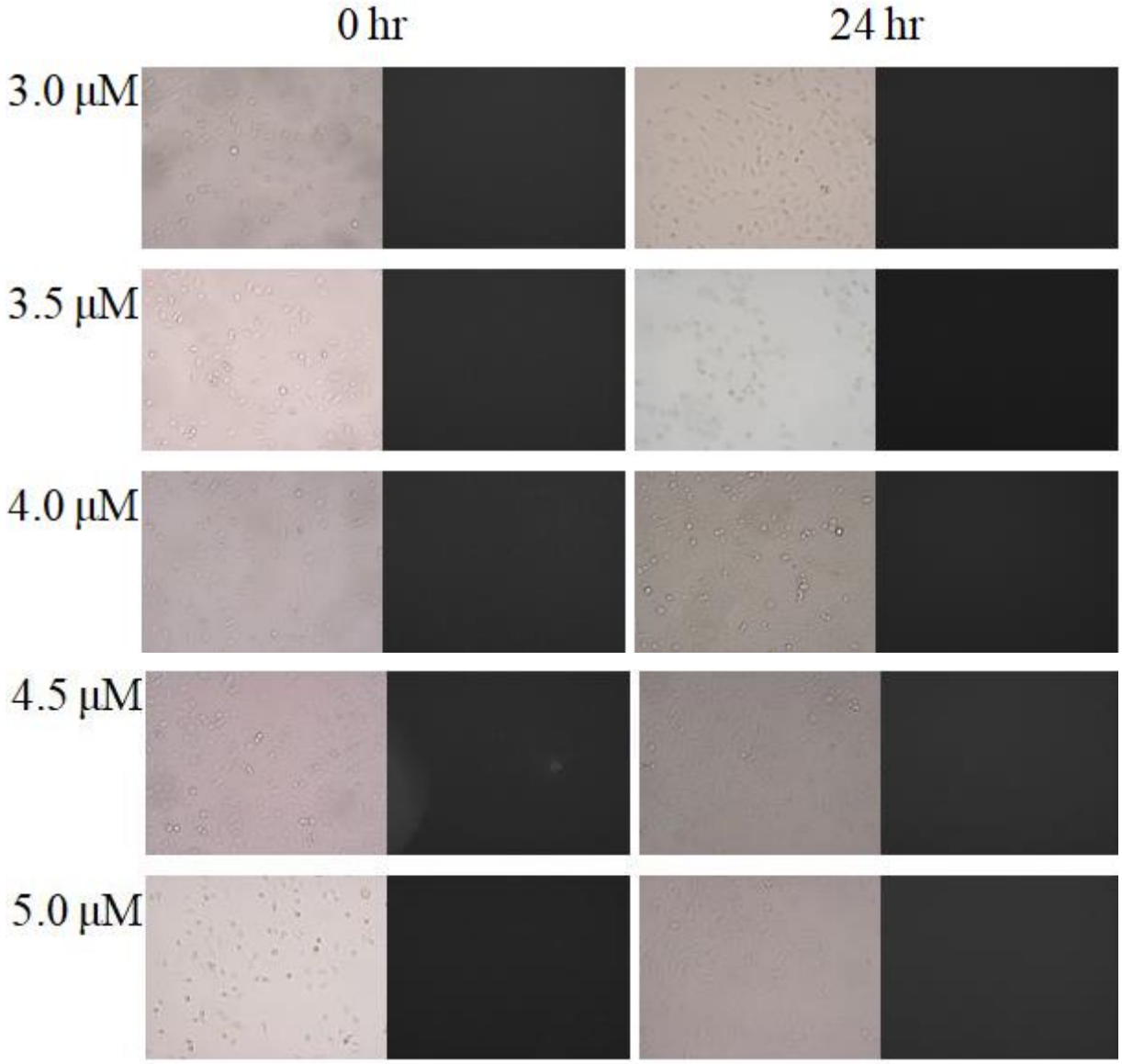


Figure 6. Labeling MDA-MB-231 breast cancer cells with various concentrations of CellTracker™ Violet dye. Brightfield and fluorescent imaging of MDA-MB-231 breast cancer cells after incubation with various concentration of CellTracker™ Violet. Cells were imaged immediately after incubation and 24 hours after incubation with dye. Fluorescence was imaged with 4′,6-diamidino-2-phenylindole (DAPI) filter.

DISCUSSION

The results of this study have shown the ways by which rM51R-M vesicular stomatitis virus interacts with components of a simulated tumor microenvironment. One of the responses that elucidates VSV's interactions with TME components is cytokine secretion by these components after infection with virus. We observed increased IL-6 production in co-culture conditions infected with mutant VSV compared to those of untreated co-cultures. We also observed increased TNF-α production co-culture wells treated with rM51R-M virus over untreated co-culture wells. Higher levels of IL-6 and TNF-α within the TME indicate that the environment is pro-inflammatory, while lower levels are an indication of an anti-inflammatory TME. An anti-inflammatory TME encourages metastasis of the tumor, while a pro-inflammatory TME encourages suppression of tumor growth and inhibition of metastasis. The observable increase in pro-inflammatory cytokines in our co-culture wells after infection with mutant VSV indicates that mutant VSV has the potential to modulate the TME to a more pro-inflammatory state. This modulation to a pro-inflammatory state could potentially discourage metastasis, both at the primary tumor site and at distal sites with secondary tumors. Modulation of the TME from an anti-inflammatory state to a more pro-inflammatory state also suggests that M2 macrophages are undergoing a phenotypic switch to more M1-like macrophages after infection with rM51R-M virus, and are therefore producing pro-inflammatory cytokines as a result of this switch. Other oncolytic viruses have been shown to potentially encourage a phenotypic switch in M2 macrophages. One study found evidence that M2 macrophages display phenotypic alterations toward a more M1-like macrophage in co-culture with MDA-MB-231 breast cancer cells after infection with attenuated measles and mumps viruses (Tan et. al., 2016).

Previous research conducted in our lab suggests that M2 macrophages possess phenotypic plasticity, such that, upon exposure to virus, they have the ability to convert to a more M1-like state by upregulating M1 macrophage markers such as STAT1/pSTAT and downregulating pathways associated with M2 function, including CD204 expression. (Polzin, 2017). Under normal circumstances, M2 macrophages do not produce TNF-α as an effector cytokine. However, M1 macrophages do produce TNF-α as an effector cytokine once polarized to the M1 phenotype. Our data indicates that M2 macrophages infected with rM51R-M virus produce significantly more TNF-α than those that were not infected. This supports the implication of the previous data, that M2 macrophages may switch to a more M1-like phenotype upon infection with mutant VSV (Polzin, 2017). This could be due to the virus's ability to induce a type I IFN response in cells that it infects. The inability of the M protein mutant VSV to inhibit the host cell's IFN response make the mutant virus uniquely qualified to encourage this phenotypic switching of M2 anti-inflammatory macrophages to a more pro-inflammatory M1-like macrophage. Further research is being conducted in our lab to determine whether or not this is the case.

Looking at cell viability (Figure 5), there is more cell death from infection with rwt virus in both the M2 wells and the co-culture conditions when compared to that of rM51R-M virus. This suggests that rwt is more lethal to cells due to its ability to take over host transcription factors and inhibit protein synthesis, which is consistent with data from other studies (Ahmed et. al, 2003). It is also clear that more cell death took place in the M2 wells compared to that in the co-culture conditions. This suggests that the cells are more resistant to VSV when in co-culture conditions. This could be due to the M2's ability to encourage survival of cancer cells within the

tumor microenvironment. Cancer is a disease whose goal is to resist cell death and proliferate indeterminately. The M2s could be engaging in cytokine cross-talk with the cancer cells that would encourage the survival of the cancer cells and make them more resistant to infection with virus. However, this data is the product of only one experiment, so further research will be conducted to determine if this is the case.

Our results showed that MDA-MB-231 breast cancer cells were more effectively labeled with the CellTracker™ Violet than the THP-1 human monocytic cell line (data not shown). THP-1 cells were incubated in concentrations of CellTracker™ Violet ranging from 0.5 µM-3µM. When analyzed using fluorescent microscopy, the signal from the cells was too dim for all of the concentrations. Furthermore, upon inspection it was discovered that the THP-1's were being killed by the higher concentrations of the dye, so it was decided that the MDA-MB-231 breast cancer cells would be more effectively labeled.

The optimal concentration for the CellTracker™ Violet dye has not yet been determined. Concentrations of dye ranging from 3µM-5µM were tested, however the signal given off by the cells after incubation in these concentrations was not strong enough to produce a clear image. In the package directions, the highest recommended working concentration of dye is 25 µM. Our concentrations may simply not have been concentrated enough to induce a strong enough fluorescence in the cells. Further testing of higher concentrations will be performed to determine the optimal concentration of dye.

In conclusion, my results showed that mutant VSV is able to induce the secretion of pro-inflammatory cytokines in co-culture conditions. These results imply that the strength in using VSV as an oncolytic agent lies not only in its ability to directly kill cancer cells, but also manipulate macrophages within the tumor microenvironment. Therefore, it is important to further study the underlying mechanism of this phenomenon and better understand the implications this information has on the use of VSV an oncolytic agent.

FUTURE DIRECTIONS

An important step in elucidating the ways in which VSV interacts with the tumor microenvironment is determining how well VSV is able to infect M2 macrophages in co-culture conditions. Previous research in our lab suggests that M2 macrophages are sensitive to infection by VSV when cultured alone (Polzin, 2017), however their susceptibility to VSV in co-culture has not been determined. To continue to investigate this issue, we will label MDA-MB-231 breast cancer cells with CellTracker^{TM} Violet, then co-culture them with M2 macrophages and infect cells with VSV that has been mutated to express green fluorescent protein (GFP). When cells are infected with the GFP VSV, they fluoresce GFP when imaged with fluorescent microscopy. Labeling MDA-MB-231 breast cancer cells will allow us to differentiate infected breast cancer cells from infected M2 macrophages and count them to determine the percent infected.

Finally, we will want to investigate the affect that infection by rM51R-M virus has on the viability of the two cell types in co-culture conditions. VSV is an oncolytic virus, such that cancer cells burst after prolonged infection. This lysis releases cancer cell antigen into the tumor

microenvironment and can be picked up by immune cells in the TME. These immune cells can then present the antigen to other immune cells throughout the body, creating a systemic immune response to the cancer. Therefore, it is important to determine if the cancer is resistant to killing by VSV in a simulated TME. Future experiments will involve repeating these co-cultures after labeling one of the cell with a fluorescent probe and staining for viability after infection.

REFERENCES

Ahmed, M., et al. "Ability of the Matrix Protein of Vesicular Stomatitis Virus To Suppress Beta Interferon Gene Expression Is Genetically Correlated with the Inhibition of Host RNA and Protein Synthesis." Journal of Virology, vol. 77, no. 8, 2003, pp. 4646–4657., doi:10.1128/jvi.77.8.4646-4657.2003.

Ahmed, M., et al. "Immune Response in the Absence of Neurovirulence in Mice Infected with M Protein Mutant Vesicular Stomatitis Virus." Journal of Virology, vol. 82, no. 18, 2008, pp. 9273–9277., doi:10.1128/jvi.00915-08.

Ahmed, M., Lyles, D., 1998. Effect of vesicular stomatitis virus matrix protein on transcription directed by host RNA polymerases I, II, and III. J. Virol. 72, 8413-8419.

Ahmed, Maryam, et al. "Matrix Protein Mutant of Vesicular Stomatitis Virus Stimulates Maturation of Myeloid Dendritic Cells." PubMed, Journal of Virology, 1 Mar. 2006, www.ncbi.nlm.nih.gov/pmc/articles/PMC1395366/.

Balachandran, Siddharth, and Glen Barber. "Vesicular Stomatitis Virus (VSV) Therapy of Tumors." IUBMB Life, vol. 50, no. 2, 2000, pp. 135–138., doi:10.1080/713803696 .

Carswell, E. A., et al. "An Endotoxin-Induced Serum Factor That Causes Necrosis of Tumors."Proceedings of the National Academy of Sciences, vol. 72, no. 9, 1975, pp. 3666–3670., doi:10.1073/pnas.72.9.3666.

Chanmee, Theerawut, et al. “Tumor-Associated Macrophages as Major Players in the Tumor Microenvironment.” Cancers, vol. 6, no. 3, 2014, pp. 1670–1690., doi:10.3390/cancers6031670.

Chung, Cathie T., and Robert W. Carlson. “Goals and Objectives in the Management of Metastatic Breast Cancer .” The Oncologist, 1 Dec. 2003, theoncologist.alphamedpress.org/content/8/6/514.long.

Duque, Guillermo Arango, and Albert Descoteaux. “Macrophage Cytokines: Involvement in Immunity and Infectious Diseases.” Frontiers in Immunology, vol. 5, 2014, doi:10.3389/fimmu.2014.00491.

Fukuhara, Hiroshi, et al. “Oncolytic Virus Therapy: A New Era of Cancer Treatment at Dawn.”Cancer Science, vol. 107, no. 10, 2016, pp. 1373–1379., doi:10.1111/cas.13027.

Hastie, E., and V. Z. Grdzelishvili. “Vesicular Stomatitis Virus as a Flexible Platform for Oncolytic Virotherapy against Cancer.” Journal of General Virology, vol. 93, no. Pt_12, 2012, pp. 2529–2545., doi:10.1099/vir.0.046672-0.

Liu BL, Robinson M, Han ZQ, et al. ICP34.5 deleted herpes simplex virus with enhanced oncolytic, immune stimulating, and anti-tumour properties. Gene Ther. 2003;10(4):292–303.

Lun, X. Q., et al. “Efficacy of Systemically Administered Oncolytic Vaccinia Virotherapy for Malignant Gliomas Is Enhanced by Combination Therapy with Rapamycin or Cyclophosphamide.” Clinical Cancer Research, vol. 15, no. 8, 2009, pp. 2777–2788., doi:10.1158/1078-0432.ccr-08-2342.

O’Shaughnessy, Joyce. “Extending Survival with Chemotherapy in Metastatic Breast Cancer.” The Oncologist, 1 Oct. 2005, theoncologist.alphamedpress.org/content/10/suppl_3/20.short.

Ottolino-Perry, Kathryn, et al. “Intelligent Design: Combination Therapy With Oncolytic Viruses.” Molecular Therapy, vol. 18, no. 2, 2010, pp. 251–263., doi:10.1038/mt.2009.283.

Passer BJ, Castelo-Branco P, Buhrman JS, Varghese S, Rabkin SD., and Martuza RL. Oncolytic herpes simplex virus vectors and taxanes synergize to promote killing of prostate cancer cells. Cancer Gene Ther. 2009;16:551–560.

Racaniello, Vincent. “Multiplicity of Infection.” Virology Blog, 20 Jan. 2011, www.virology.ws/2011/01/13/multiplicity-of-infection/.

Raggi, Federica, et al. “Regulation of Human Macrophage M1–M2 Polarization Balance by Hypoxia and the Triggering Receptor Expressed on Myeloid Cells-1.” Frontiers, 7 Sept. 2017, www.frontiersin.org/articles/10.3389/fimmu.2017.01097/full.

Rehman, Hasan, et al. “Into the Clinic: Talimogene Laherparepvec (T-VEC), a First-in-Class Intratumoral Oncolytic Viral Therapy.” Journal for ImmunoTherapy of Cancer, BioMed Central, 20 Sept. 2016, jitc.biomedcentral.com/articles/10.1186/s40425-016-0158-5.

Russell, Stephen J., and Kah-Whye Peng. “Viruses as Anticancer Drugs.” Trends in Pharmacological Sciences, vol. 28, no. 7, 18 June 2007, pp. 326–333., doi:10.1016/j.tips.2007.05.005.

Seyfried, T.N., Huysentruyt, L.C., 2013. On the origin of cancer metastasis. Crit. Rev. Oncog. 18, 43–73.

Stojdl, David F, et al. “VSV Strains with Defects in Their Ability to Shutdown Innate Immunity Are Potent Systemic Anti-Cancer Agents.” Cancer Cell, vol. 4, no. 4, Oct. 2003, pp. 263–275., doi:10.1016/s1535-6108(03)00241-1.

Tan, Darren Qiancheng, et al. “Macrophage Response to Oncolytic Paramyxoviruses Potentiates Virus-Mediated Tumor Cell Killing.” European Journal of Immunology, vol. 46, no. 4, 2016, pp. 919–928., doi:10.1002/eji.201545915.

Yu, W, and H Fang. “Clinical Trials with Oncolytic Adenovirus in China.” CiteSeerX, U.S. National Library of Medicine, Mar. 2007, www.ncbi.nlm.nih.gov/pubmed/17346105.