MODULATION OF BREAST TUMOR ASSOCIATED MACROPHAGES BY ONCOLYTIC VESICULAR STOMATITIS VIRUS

A Thesis
by
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MODULATION OF BREAST TUMOR ASSOCIATED MACROPHAGES BY ONCOLYTIC VESICULAR STOMATITIS VIRUS

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Abstract

MODULATION OF BREAST TUMOR ASSOCIATED MACROPHAGES BY ONCOLYTIC VESICULAR STOMATITIS VIRUS

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High tumor-associated macrophage (TAM) densities in cancerous breast tissue often correlates with poor clinical outcomes. This can be attributed to the M2 macrophage subtype whose wound-healing and immunosuppressive functions promote cancer cell proliferation, tumor angiogenesis, and metastasis. M2-like TAMs may thus be a suitable target for therapeutics. We are interested in developing oncolytic vesicular stomatitis virus (VSV) as a treatment option for breast cancer. VSV is cytotoxic to many breast cancer cells, including the MDA-MB-231 (MDA231) breast cancer line used in this study. We also have recent data suggesting that VSV converts model M2- like macrophages to the more immunogenic, tumor-fighting M1 profile. To model the behavior of VSV in a simulated breast tumor microenvironment (TME), aggressive MDA231 or non-aggressive T47D breast cancer cells along with model THP-1 macrophages were directly co-cultured and then infected with recombinant wild type (rwt virus) and matrix (M) protein mutant (rM51R-M virus) strains of VSV. In order to determine the macrophage phenotype under these experimental conditions, both the secretion of the M1-associated, pro-inflammatory cytokines IL-6 and TNFα and the M2-associated, anti-inflammatory cytokine IL-10 were monitored by ELISA. MDA231 monocultures secreted IL-6, TNFα, and IL-10, and these cytokine levels were reduced when the MDA231 and macrophages were cultured together. We observed that rwt virus inhibited both IL-6 and TNFα secretion under MDA231 co-culture

conditions, but the effect was not statistically significant. The rM51R-M virus also non-significantly inhibited IL-6 production under these conditions, but enhanced TNFα production. Further, while rwt virus has no effect on IL-10 secretion, rM51R-M virus inhibited it under MDA231 co-culture conditions. Conversely, T47D monocultures did not secrete any of the cytokines measured. However, secretion of IL-6, TNFα, and IL-10 was induced when these cells were cocultured with macrophages. The rwt virus had no effect on IL-6, TNFα, or IL-10 production under co-culture conditions. Similarly, rM51R-M virus also did not affect the secretion of the cytokines tested, except for increasing TNFα production when T47D breast cancer cells were co-cultured with M1 macrophages. Results indicated that infection by VSV, especially rM51R-M virus, significantly increases co-culture secretion of TNFα, a known mediator of macrophage tumoricidal activities, and decreased the secretion of IL-10, a tumor-promoting cytokine, in a dose-dependent manner. This suggests that rM51R-M virus may be able to modulate the cytokine milieu of the TME to promote TAM repolarization to a pro-inflammatory, M1-like phenotype. This repolarization of TAMs has the therapeutic potential to promote destruction of the TME and to induce systemic anti-tumor memory in immune cells.

Acknowledgements

I would like to thank my mentors, Dr. Maryam Ahmed and Dr. Darren Seals, for their patience, support and guidance throughout this project. Thank you to Dr. Andrew Bellemer for his thoughtful input and enthusiasm and to Dr. Howard Neufeld for help and teaching with statistics. I would like to thank Appalachian State University, the Department of Biology, and the Office of Student Research, whose funding made this research possible. Finally, thank you to Dylan Fehl, Jamison Slate, and Rebecca Fuller who have spent many hours with me in lab to propel this project forward to completion.

Dedication

I would like to dedicate this thesis to my father, Mark Jude Lacy, and to my partner Jonathan Michael McCanless. Dad, you planted the seeds of curiosity, critical introspection, and resolve in my young mind, without which I would not have grown into who I am today. Jonathan, you gave those seeds the nourishment they needed and taught me how to use the right tools to allow them to flourish. This is a monument to your combined love and effort. Finally: to my nakama, my tribe: Mary, Stephanie, Nick. You inspire me to be a better version of myself every day. Without each of you and all of you, I could not have made it here. Thank you.

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Foreward

Chapter 2 of this thesis will be submitted to the *Virus Research Journal*. It has been formatted per the style guidelines of that journal.

Chapter 1: Introduction

Breast Cancer/Breast Cancer Therapeutics

Breast cancer is a heterogeneous disease, and patient prognosis depends on the tissue from which the tumor arises, its molecular characteristics, and its microenvironment. Today, breast cancer kills more than 41,000 women annually in the United States alone (Altekruse et al., 2010; American Cancer Society, 2018; Mota et al., 2018) thus resulting in the need for a diverse arsenal of therapeutic options and continued research for new and improved treatment options.

Breast carcinomas originate from epithelial cells among each of the tissue types of the breast, and each breast tumor subtype has unique molecular characteristics that affect patient care and prognosis (American Cancer Society, 2018; Bauer et al., 2007; Foulkes et al. 2010; Millikan et al., 2008). If breast cancer is of highly differentiated epithelial/luminal origin, then prognosis is likely to be good for patients (Bauer et al., 2007; Foulkes et al., 2010; Millikan et al., 2008; Mota et al., 2018). However, if the disease is poorly differentiated and of myoepithelial/basal origin, then the five-year patient survival rate is likely to be low (Bauer et al., 2007; Foulkes et al., 2010; Mota et al., 2018). Breast cancers have also been categorized by molecular subtypes (Fig. 1). Luminal A breast cancers are the most common subtype and have the best prognosis (Bauer et al., 2007; Millikan et al., 2008). Originating as low-grade carcinomas (histologically resembling healthy cells), these cancers express low levels of the cellular proliferation marker Ki-67. They also grow slowly because they are negative for human epidermal growth factor receptor 2 (HER2), a receptor that promotes proliferation and downregulates apoptosis when activated. Additionally, they are estrogen and progesterone receptor positive, and therefore patients harboring Luminal A tumors experience high therapeutic success from therapies that block the effects of these hormones (*e.g.* tamoxifen and fulvestrant) (Cheang et al., 2011; Foulkes et al., 2010). Luminal B breast cancers are also hormone-receptor positive but may also be

HER2 positive. They also express high levels of Ki-67, and as a result grow faster than the Luminal A subtype resulting in slightly worse patient prognoses (Foulkes et al., 2010; Cheang et al., 2011; Mota et al., 2018). Treatment for Luminal B patients involves a combination of hormone therapy, chemotherapy, and HER2 targeting antibodies and drugs (*e.g.* trastuzumab, pertuzumab, lapatinib). HER2-enriched breast cancers, in contrast, are estrogen-receptor and progesterone-receptor negative, so they do not respond to hormone therapy. Due to their high expression of HER2, these breast cancers grow quickly and therefore have worse prognoses than either of the luminal tumor subtypes, though they still benefit from HER2 inhibitors (Altekruse et al., 2010; Foulkes et al., 2010; Mota, 2018).

While much progress has been made in developing effective treatments, there are still many breast cancer subtypes for which current therapeutics are ineffective. Triple-negative/basal-like breast cancers (TNBCs) generally have a high degree of aneuploidy compared to other subtypes and do not respond to hormone or anti-HER2 therapies as they are estrogen receptor, progesterone receptor, and HER2 negative (Irvin and Carey, 2008; Foulkes et al., 2010). They are poorly differentiated, high grade (histologically abnormal compared to healthy cells), overexpress the epidermal growth factor receptor, and typically have high proliferative and invasive potential (Irvin and Carey, 2008; Foulkes et al., 2010). Chemotherapy, the administration of one or more chemical agents (e.g. doxorubicin, taxanes) that promote the death and/or inhibit the growth of cancer cells, is the standard treatment for TNBC. It is frequently paired with surgery (*e.g.* lumpectomy, mastectomy) and radiation. Since these treatments have poor efficacy and severe side effects leading to poor patient adherence, prognosis for TNBC remains poor (Altekruse et al., 2010; Bauer et al., 2007; Colleoni, 2010; Foulkes et al., 2010; Irvin and Carey, 2008). Further, metastatic cancers tend to have other characteristics that make them less responsive to treatment, including more genetic defects, increased immune evasion, and the tendency to colonize critical tissues, such as the brain, lung, liver, and bone (Altekruse et al., 2010; American Cancer Society, 2018; Foulkes et al., 2010;).

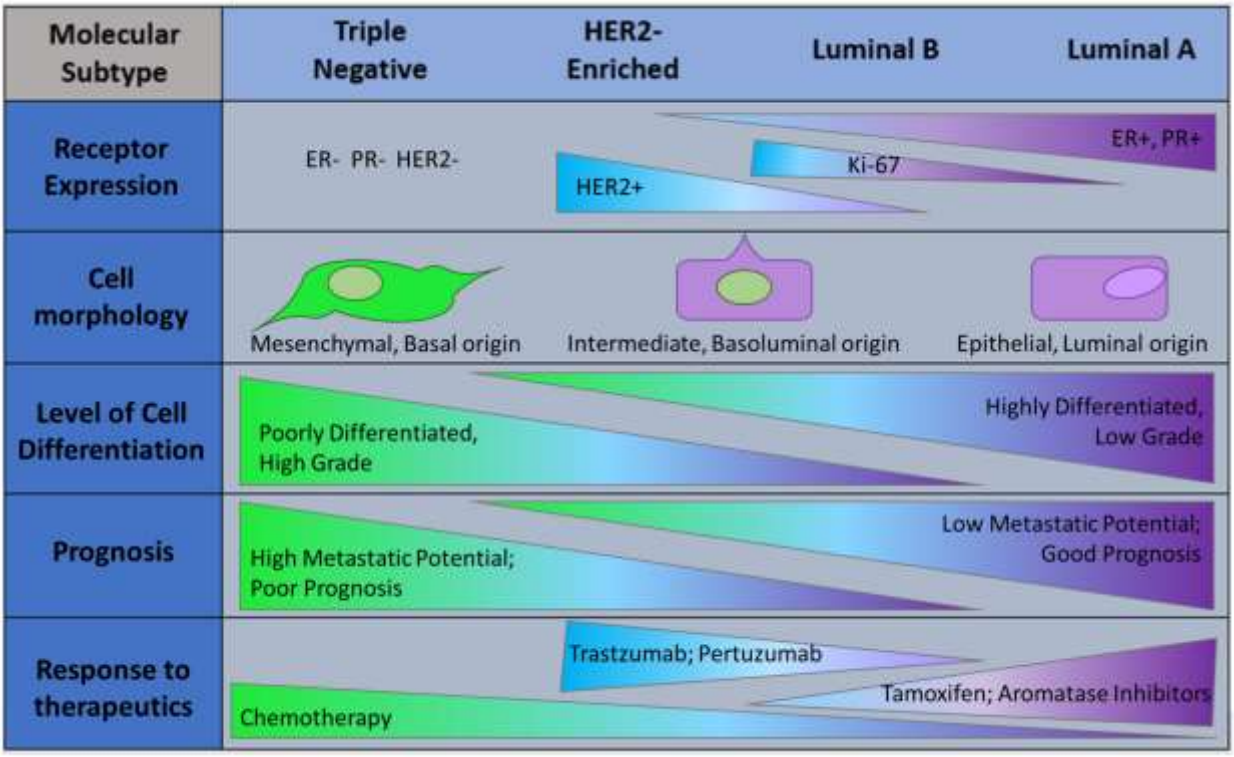

Fig. 1. Breast Cancer Subtypes. Highly differentiated, low grade, hormone receptor positive, luminal tumors (*e.g.* Luminal A and B) have low metastatic potential and good prognoses as such cancers respond well to hormonal therapies. HER2-positive breast cancers are less differentiated and grow more quickly than luminal cancers. They have moderate metastatic potential but respond to monoclonal antibodies, so prognosis is fair. Triple negative breast cancers have a mesenchymal, poorly differentiated phenotype and high invasive potential, and only respond to chemotherapies. They confer the poorest outcomes of the breast cancer subtypes distinguished by their molecular characteristics. Figure was adapted from Wong E and Rebelo J. "Breast cancer pathogenesis and histologic vs. molecular subtypes" Clin Obstet Gynecol. 2011 Mar; 54(1):91-5.

Many breast cancer therapies have limited applications because they only affect one tumor-promoting mechanism. For example, trastuzumab is only effective against HER2-enriched breast cancers while tamoxifen is only effective for estrogen receptor positive cancers. Additionally, some cancers, especially the invasive subtypes, frequently evolve mechanisms to circumvent the action of targeted therapies. Consequently, another opportunity in breast cancer therapeutics is centered around systemic immunotherapy, in which the immune system is modulated to recognize, destroy, and build anti-tumor immunity towards both primary and disseminated tumors. These include therapeutic vaccines, adoptive cell therapy, and oncolytic virotherapies. Therapeutic vaccines elicit a response against tumor-specific antigens, equipping the immune system to detect and eliminate any cells that express those antigens. In adoptive cell therapy, a patient's own T cells are removed, enhanced chemically or genetically, and then reintroduced into the patient. Thus, the more active T cells will be better able to destroy tumor cells. Due to the heterogeneity of breast cancers, however, more generalized therapeutics are currently needed, among them oncolytic virotherapy. One goal of this project was to utilize a virus to target cancer and immune cells to establish long-lasting anti-tumor immunity.

Oncolytic Virotherapies

Oncolytic virotherapy (OVT) refers to the use of viruses to target and destroy cancer cells and, ideally, to promote anti-tumor immunity. Several viruses have demonstrated clinical efficacy in reducing tumor size and extending patient survival in multiple cancer types. As of 2017, over 40 oncolytic viruses were undergoing clinical trials (Lawler et al., 2017). The Food and Drug Administration (FDA) approved the first oncolytic virus for clinical use in the United States in 2015. This virus, Imlygic, is an attenuated strain of herpes simplex virus type I (HSV-1) modified to express granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine that promotes immune cell development and activation. Imlygic has been shown to be a safe and effective therapeutic option for the treatment of melanoma when injected directly into primary cancer lesions (FDA, 2015). It has

recently been shown to be effective against metastatic melanomas and shows promise as a tolerable therapeutic option for even late stage patients (Harrington et al., 2017). Since the approval of Imlygic for clinical use, many more oncolytic viruses have been developed, highlighting the potential of these agents for fighting specific cancers.

In addition to oncolytic herpes virus (a DNA virus) mentioned above, negative-sense single-stranded RNA viruses, especially of the family Paramyxoviridae, are also being investigated as options for OVT. An example is wild-type Newcastle disease virus (NDV), which is cytopathic to many tumor types and has been shown to be safe in early clinical trials (Pecora et al., 2002; Zamarin et al., 2012). Oncolytic measles virus (MeV) strains, such as the Edmonston strain, have shown the most promise in OVT across tumor types. This efficacy results from the frequent overexpression of CD46 by tumor cells, the very receptor that mediates MeV entry. (Msaouel et al., 2009). MeV strains, currently in clinical trials, have shown efficacy in ovarian, hepatic, and lymphatic cancers as well as multiple myeloma and glioblastoma multiforme (Blechacz et al., 2006; Peng et al., 2001; Phuong et al., 2003). Another RNA virus, vesicular stomatitis virus (VSV), which is the focus of my research, is currently in a clinical dose escalation trial for stage IV endometrial cancer (ClinicaTrials.gov trial NCT03120624). Even at high doses, administration of oncolytic vesicular stomatitis virus engineered to express interferon-beta and sodium iodide symporter (VSV-IFNβ-NIS (Voyager-V1™)) results in no persistent viremia nor recoverable infectious virions from buccal swabs, thus highlighting the safety of VSV for use in OVT (Bakkum-Gamez et al., 2018). This virus is also in phase I clinical trials for the treatment of other cancers, including recurrent adult acute myeloid leukemia, recurrent T-cell lymphomas, and recurrent plasma cell myeloma as well as refractory solid tumors (ClinicalTrial.gov trial NCT03017820, trial NCT02923466). However, despite these advances, the mechanisms by which VSV and other oncolytic agents carry out their oncolytic activities are not completely understood, in part due to the complex interactions between cancer cells, viruses, and other components of the tumor microenvironment.

Our goal is to determine the underlying mechanisms by which oncolytic agents like VSV carry out their anti-cancer functions. The basic mechanism governing OVT is illustrated through the lens of a VSV infection in Fig. 2. Specifically, healthy cells with intact antiviral responses are resistant to virus-induced cytopathology due to attenuation of virus replication. Conversely, cancer cells will often support virus replication and are ultimately susceptible to the cytopathic effects induced by viruses. However, the real power of OVT does not lie merely in the ability of viruses to directly kill cancer cells, but in the resulting release of tumor-associated antigens (TAA) that promote immunosurveillance and anti-tumor immunity. Therefore, the natural ability of viruses to target susceptible tissues and the potential for long-lasting immunity against tumors makes OVT an attractive option for the treatment of malignancies (Russell and Barber, 2018).

Vesicular Stomatitis Virus

VSV is a prototype of Mononegavirales, an order consisting of single-stranded, negative-sense, monopartite RNA viruses. Several clinically significant families are in this order, including Filoviridae (Ebola virus, Marburg virus), Paramyxoviridae (measles virus, mumps virus), and Rhabdoviridae (rabies virus, VSV) (Pfaller et al., 2015). The 11kb genome of VSV is linear and codes for five multifunctional proteins: nucleoprotein (N), polymerase cofactor (P), matrix protein (M), glycoprotein (G), and polymerase (L), (Pfaller et al., 2015). After entry into the host cell and an uncoating process, viruses must transcribe their genomes. VSV possesses a negative sense RNA genome, complementary to mRNA. It thus uses an RNA-dependent RNA polymerase (RdRp) for transcription, allowing VSV to convert its genome into positive sense mRNA, which can then be translated by the host ribosomal machinery into proteins (Morin et al., 2013). To initiate transcription, which occurs in the cytoplasm, a complex comprised of the nucleoprotein, polymerase cofactor, and the RdRp must form and associate with the 3’ end of the viral RNA genome (Morin et al., 2013; Ortin and Benito, 2015; Pfaller et al., 2015). Specific sequences signal for the complex to stop transcription after each gene, then reinitiate at

the next gene sequence. This reinitiation signal is inefficient, however, and along with RdRp binding instability, results in genes closer to the 3' end of the genome (nucleoprotein, polymerase cofactor) being transcribed at a higher rate than those closer to the 5' end (glycoprotein, polymerase) (Pfaller et al., 2015; Whelan, 2004;). As more nucleoprotein and polymerase cofactor accumulate, the complex switches its activity from transcribing the genome to replicating it, in which the entire genome is copied in preparation for virion assembly (Pfaller et al., 2015). The new virion proteins and replicated genomes are then packaged by the M protein and are prepared for egress from the host cell.

The Host Antiviral Response to VSV Infection

Host cells have evolved mechanisms to target each point in the VSV life cycle in order to inhibit the progression of viral infections. Many of these defense mechanisms are mediated through the type I interferon (IFN) antiviral response. This response is responsible for host antiviral resistance, promotes antigen presentation in macrophages, and induces the production of cytokines that activate T cells, B cells, and natural killer cells. Interestingly, this pathway is partly responsible for the classical activation of M1-type macrophages, including the secretion of the pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα), high phagocytic activity, high anti-tumor activity, and the upregulation of the signal transducer and activator of transcription 1 (STAT1) pathway, the activation of which results in the production of antiviral proteins (Takeda and Akira, 2000). There are three pathways by which type I IFNs (*e.g.* IFNα, IFNβ) are produced, each responding to a different viral component to elicit the production of proteins necessary to limit viral spread. These are the retinoic acid-inducible gene 1 (RIG-1) pathway, the Toll/IL-1 receptor (TIR)-domain-containing adapter-inducing interferon-β (TRIF) pathway, and the interferon regulatory factor 7 (IRF7) pathway.

RNA viruses, such as VSV, induce the RIG-1 pathway in many cell types, including immune cells. Retinoic acid inducible gene I-like receptors (RLRs) are expressed primarily in conventional

dendritic cells, fibroblasts, and hepatocytes and are localized in the cytoplasm of these cells (Kato et al., 2005; Liu and Gu, 2011).

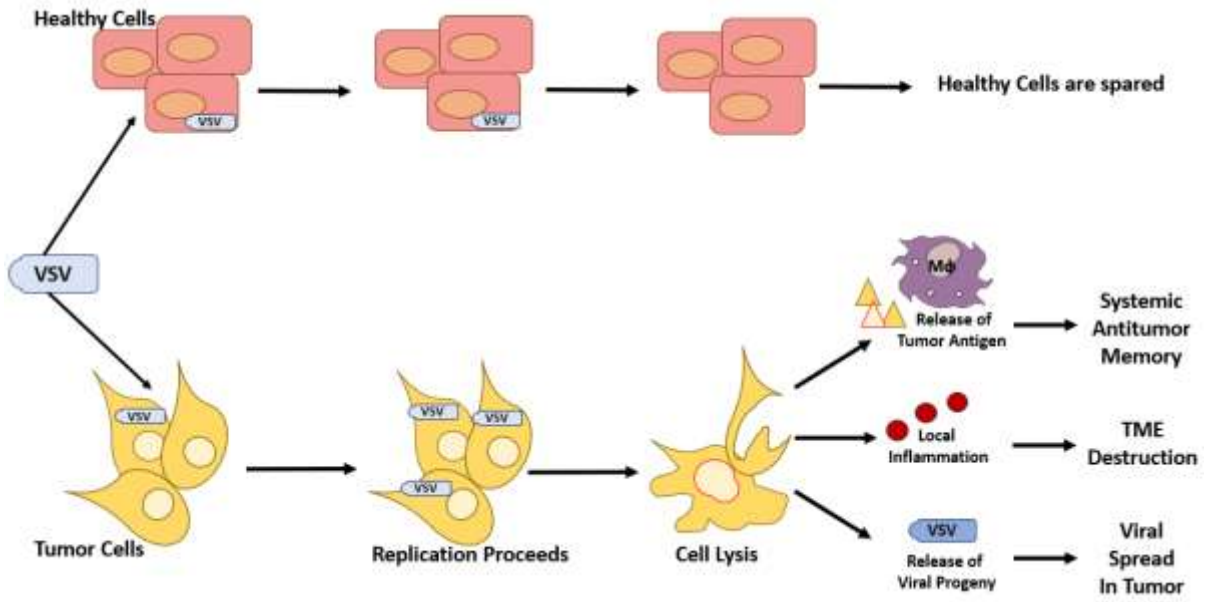

Fig. 2. VSV-based OVT Schema. Oncolytic viruses like VSV are unable to replicate within normal, healthy cells, so these cells are left unharmed. Conversely, VSV is able to infect and replicate within tumor cells resulting in cell lysis. Cell lysis results in the release of tumor antigens into the TME, which can then be phagocytosed and presented by antigen-presenting cells, such as macrophages, T cells, and B cells, to induce anti-tumor memory. Cell lysis also causes local inflammation, which results in the destruction of nearby tumor cells and tumor-associated extracellular matrix, vasculature, and fibroblasts. Viral progeny are also released, and are free to spread throughout the tumor, leading to amplification of TME destruction, systemic anti-tumor memory, and ultimately tumor regression.

RLRs bind to pathogen associated molecular patters (PAMPs), including the short, unmethylated dsRNA molecules that are common products of VSV transcription. Then, RLRs undergo a conformational change that allows activation of RIG-1. RIG-1, a pattern recognition receptor, then associates with melanoma differentiation-associated protein 5 (Mda-5) and together they activate the virus-induced signaling adapter (VISA) (Gerlier and Lyles, 2011; Liu and Gu, 2011; Loo and Gale., 2011). VISA then associates with activated nucleosome assembly protein (NAP1), TANK-binding kinase (TBK1), and inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKKε) to phosphorylate IRF3 and IRF7, which then form a heterodimer and translocate to the nucleus. There, the IRF3/7 heterodimer binds to interferon-stimulated response element 3/7 (ISRE3-7) to produce IFNβ (Gerlier and Lyles, 2011; Liu and Gu, 2011; Loo and Gale, 2011).

The TRIF pathway is induced by viral glycoproteins (G proteins in VSV) that bind Toll-like receptor 4 (TLR4) at the plasma membrane and by unmethylated dsRNAs that bind to TLR3 in internal vesicles. Both TLR4 and TLR3 are primarily expressed in macrophages and hepatocytes. TLR4 undergoes a conformational change that activates the adaptors TRIF-related adaptor molecule (TRAM) and TRIF itself. TLR3 also activates TRIF. Once activated, TRIF activates NAP1, which then is free to associate with TBK1 and IKKε to complete the RIG-1 pathway. Additionally, TRIF/receptor-interacting protein 1 (RIP1) associates with IFNβ promoter stimulator 1 (IPS-1) and Fas-associated protein with death domain (FADD). The complex comprised of TNF receptor associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1) are also phosphorylated, and IRAK1 then dissociates, and the now ubiquitinated TRAF6 is then free to associate with transforming growth factor beta (TGFβ)-activated kinase 1 (Tak1). The double binding of the IPS-1/FADD complex and TAK1 release IKKα from IKKβ. The IKKβ subunit then phosphorylates the IkB/p50/p65 complex, which results in the dissociation of IkB and allows p50/p65 (a.k.a. nuclear factor kappa-light-chain-enhancer of activated B cells; NF-kB) to translocate to the nucleus and initiate the transcription of IFNβ (Kawasaki and Kawai, 2014).

The IRF7 pathway is induced by the binding of unmethylated dsRNAs resulting from VSV replication to TLR9 in the endosomes of plasmacytoid dendritic cells. TLR9 in turn activates myeloid differentiation primary response 88 (MYD88), which dissociates from its complex with IRAK4, IRF7, IRAK1, TRAF6, and IRF5. Once dissociated, IRAK1/TRAF6 feed into the TRIF pathway, while IRF5 and IRF7 are freed to be phosphorylated by the TLR7 cytoplasmic subunit. Once phosphorylated, IRF5 and IRF7 form homodimers and translocate to the nucleus. The IRF5 and IRF7 homodimers bind to interferon-stimulated response elements (ISREs) results in the production of IFNα isotypes (Ning et al., 2011).

The type I IFNs IFNα and IFNβ bind to the IFNAR1 and IFNAR2 receptors in an autocrine and paracrine fashion, resulting in the phosphorylation of two non-receptor tyrosine kinases: Tyk2 and janus kinase 1 (JAK1). These activated kinases phosphorylate the transcription factors STAT1 and STAT2, which then form a complex with IRF9. This complex then translocates to the nucleus to bind to ISRE9 and results in the expression of IFN stimulated genes, the products of which are responsible for the phenotypic changes necessary for cells to fight viral pathogens and quite possibly cancer cells as well (Takeda and Akira, 2000).

Because the type I IFN response is the main antiviral pathway utilized by infected cells and is responsible for attenuating the effects of virus infection, many viruses have evolved mechanisms to suppress this pathway. In the case of VSV, the M protein suppresses the production of host IFN through a global inhibition of host gene expression. This occurs through the inhibition of nuclear-cytoplasmic transport and disruption of host transcription. Therefore, through the function of the M protein, VSV prevents the host cell from mounting an antiviral immune response and results in active virus replication (Black et al., 1993; Whitlow et al., 2006). The importance of the type I IFN response in controlling VSV infection has been demonstrated both *in vivo* and *in vitro*, where in the absence of an intact type I IFN response, VSV is able to replicate and spread more effectively leading to virus-induced cytopathology (Noser et al., 2007).

VSV as an Oncolytic Virus

VSV is a well-studied model system for more pathogenic viruses like influenza virus, Ebola virus and rabies virus. Therefore, the genetic basis for the pathogenesis and replication capacity of VSV is well known. VSV is a natural pathogen of livestock like cattle but has low pathogenicity in humans because of a low pre-existing immunity. VSV also has broad tissue tropism and can be grown and harvested with ease in a laboratory setting (Hastie and Grdzelishvili, 2012). Because VSV has a small, easily manipulatable genome, many OVTs exploit these features through insertion of extra coding regions in the genome and alteration of the viral genes to promote virus replication in infected cells (Hastie and Grdzelishvili, 2012).

Wild type VSV strains are inherently oncolytic (Balanchadran et al., 2001; Ebert et al., 2005; Stojdl, 2000). This is largely because most cancer cells fail to mount an IFN-based antiviral response against VSV (Hastie and Grdzelishvili, 2012; Russell and Barber, 2018). During cancer progression, cells accumulate numerous mutations that provide proliferative and growth advantages to the cell. One of these common mutations is in the type I IFN pathway. The type I IFN pathway is inherently antiproliferative, and its dysregulation reduces apoptotic signaling in tumor cells compared to normal cells. The cost of this advantage is the tumor cell's ability to respond to viral infection or IFN signaling. This leaves them vulnerable to infection and lysis by IFN-sensitive viruses like VSV, and thus makes VSV an attractive candidate as a cancer therapeutic (Stojdl et al., 2000).

Oncolytic VSV Strains

Studies that determine VSV's potential as an oncolytic agent have been ongoing for approximately two decades. During this time, numerous strategies have been explored to enhance the oncolytic activity of VSV. For example, several oncolytic strains of VSV have been developed to

express suicide genes that promote the death of uninfected tumor cells (Hastie and Grdzelishvili, 2012; Porosnicu et al., 2003). Since viral spread within solid tumors is limited, these strategies promote the destruction of uninfected tissues and viral tropism. For example, VSV-TK expresses an additional thymidine kinase gene that kills uninfected/bystander cells with gancyclovir (Fernandez et al., 2002). By inducing intrinsic and extrinsic cell death pathways in nearby, uninfected tumor cells, and by increasing viral tropism for certain cell types, these viruses overcome the limitation of inefficient viral spread.

It is known that OVT results in the propagation of an anti-tumor immune response, as such treatments result in shrinkage of tumors distant from the primary tumor where the virus was initially injected (Kaufman et al., 2016). It has been argued that the stimulation of systemic anti-tumor memory is essential to the long-term therapeutic success of OVT. Activation of T cells, dendritic cells, and CD169-positive macrophages have been implicated in this process (Asano et al., 2011). To enhance these therapeutic effects of oncolytic VSV, immunomodulatory viruses have been engineered. The most impressive of these is the previously mentioned VSV-IFNβ-NIS, currently in clinical trials as an immunotherapy for recurring and metastatic endometrial cancers, multiple myeloma, acute myeloid leukemia, and T-cell lymphoma.

In addition to engineering VSV to express immunomodulatory cytokines, viruses have also been genetically altered to promote immunomodulation, and thus enhance efficacy of OVT. As mentioned above, the M protein of VSV is responsible for shutting down host gene expression and thus the cytopathology and immunosuppression associated with VSV infections (Balachandran, 2004; Black et al., 1993; Kopecky et al., 2001). Consequently, researchers have developed M protein mutants with the inability to suppress the type I IFN response. These M protein mutant viruses have proven to be immunostimulatory and to have increased oncoselectivity and safety compared to wild-type strains of VSV. Their effects generally mimic engineered immunomodulatory viruses, but instead of enhancing only one immunostimulatory effect, M protein mutant viruses simultaneously stimulate many resulting

in an induction of several antiviral pathways (Ahmed et. al, 2003; Kopecky et al., 2001). For example, a variety of M protein mutants have been shown to have increased tropism and oncolysis in ovarian and colorectal cancer cells. Stroidl and coworkers showed that mice treated with AV1 virus, a strain of VSV harboring a methionine to arginine substitution at position 51 (M51R) of the M protein sequence, showed complete remission of their tumors and distant metastases and increased survival compared to mock treated mice (Stojdl et al., 2003). Our laboratory works with a similar M protein mutant VSV known as rM51R-M virus. Previous studies have demonstrated the selectivity of rM51R-M virus for cancer cells over normal cells both *in vitro* and *in vivo* as compared to its isogenic wild type counterpart called rwt virus (Ahmed et al., 2003; Fehl and Ahmed, 2017; Hastie and Grdzelishvili, 2012). The rM51R-M virus showed less toxicity and virulence than rwt virus while effectively targeting cancer cells. Our lab seeks to develop this virus as a potential immunotherapeutic for breast cancer.

The Breast Tumor Microenvironment

It is known that the environment in which the tumor resides, the tumor microenvironment (TME), plays a key role in regulating tumor growth and progression (Hollmén et al., 2015; Ojalvo et al., 2010; Place et al., 2011;). The TME is comprised of a variety of cell types, including tumor cells, stromal cells, and immune cells. The cellular composition of the TME and the interactions of resident cell populations varies in instances of non-metastatic versus metastatic disease, and this has implications for disease outcome (Hollmén et al., 2015; Ojalvo et al., 2010; Place et al., 2011; Schedin et al., 2007). It is known that oncolytic VSV has immunomodulatory effects, but the mechanisms by which it exerts those actions are poorly understood. Direct lysis of cancer cells only accounts for some of these effects, and studies have shown that VSV induces systemic anti-tumor effects by affecting hemopoietic cells, such as T cells and myeloid cells, all which are components of the breast TME. Therefore, a brief discussion of the breast TME is warranted.

Tumors have been described as “wounds that never heal” (Byun and Gardner, 2018). That is, tumor progression proceeds in ways similar to the body’s normal wound-healing mechanisms (Byun and Gardner, 2018; Place et al., 2011; Schedin et al., 2007). These mechanisms include the initiation of factors responsible for vascularization, tissue remodeling, and local immunosuppression, with the TME of metastatic tumors promoting this state more strongly than non-metastatic ones (Byun and Gardner, 2018; Place et al., 2011; Schedin et al., 2007).

Normal wound healing involves a series of tightly regulated events: wound closure, local inflammation, recruitment of immune cells and fibroblasts, and new tissue growth. First, platelet-derived growth factor (PDGF) is secreted and signals blood platelets to the wound site resulting in blood clot formation and wound closure. Next, the clot releases vast quantities of PDGF and TGFβ, which results in the infiltration of immune cells to the wound. Neutrophils and macrophages rid the area of dead tissue and pathogens. During this phase, monocytes produce large amounts of the pro-inflammatory cytokines IL-6 and TNFα to recruit more immune cells to the site, induce apoptosis in damaged cells, and facilitate the transition to the proliferative steps of wound healing. Cells must also begin to secrete the anti-inflammatory cytokine IL-10 as it is necessary to dampen the pro-inflammatory tissue destruction of early response macrophages so that extracellular matrix (ECM) deposition and vascularization can proceed. Infiltrated immune cells and resident tissue cells also produce cytokines and growth factors (including fibroblast growth factor (FGF), epidermal growth factor (EGF), and TGFβ) that prompt the local tissue to proliferate. These signals particularly induce fibroblast migration and proliferation, which in turn deposit the ECM. Additionally, fibroblast along with macrophages prompt vascularization of the new tissue via the secretion of vascular endothelial growth factor (VEGF). Since macrophages are one of the most active immune cells in the wound healing processes that also promote tumor progression, and since they are one of the most abundant cell types in tumor stroma, extensive research has been devoted to characterizing tumor cell-macrophage crosstalk (Hollmén et al., 2015; Ojalvo et al., 2010; Place et al., 2011; Schedin et al., 2007). Further, macrophages

are responsible for mounting the innate immune system's antiviral response and are thus perhaps the cell population of the TME that is most relevant to this study.

Macrophages/Tumor-Associated Macrophages

Monocytes are peripheral blood mononuclear cells generated in the bone marrow from pluripotent stem cells. They are responsible for innate immune functions and for presenting antigens to B and T cells as part of adaptive immunity to pathogens and abnormal cells. Monocytes circulate throughout the blood stream, and then extravasate in response to signals released by tissues (McNab et al., 2015; Mosser and Edwards, 2008). Once extravasated, monocytes differentiate into macrophages to perform a wide range of functions specific to the needs of the infiltrated tissue (Bingle et al., 2002). For instance, macrophages can mediate iron metabolism, dispose of dead cells, defend against pathogens, stimulate wound healing in injured tissues, promote appropriate vasculature growth in development and wound healing, and detect and destroy tumors (Ferrante and Leibovich, 2011; Laoui et al., 2011; Mosser and Edwards, 2008; Sousa et al., 2015). This remarkable phenotypic and functional variation is highly context-dependent and mostly influenced by tissue cues, particularly the cytokine milieu (**Fig. 3**). Due to the high diversity of macrophage phenotypes that exist in tissues, including tumors, it is important to note the distinctions between them.

M0 macrophages are monocytes that have begun the process of differentiation to a terminal macrophage due to exposure to macrophage colony stimulating factor (M-CSF) but are not yet committed to a functionality. M0 macrophages have moderate levels of mannose receptor (CD206), IL-6, and TNFα, and express high levels of CD14, CD163, CD36, CD16, IL-2, and monocyte chemoattractant protein 1 (MCP-1) (Schwende et al., 1996). They also demonstrate enhanced phagocytic activity compared to M1 macrophages, but similar levels as M2 macrophages (Abdullah et al., 2015).

M0 macrophages can be further polarized by cytokines to produce terminally differentiated M1 and M2 macrophages. In the body, classically activated M1 macrophage polarization can occur through exposure to interferon gamma (IFNγ), lipopolysaccharide (LPS), granulocyte macrophage colony stimulating factor (GM-CSF), and TNFα that are secreted by cells in response to pathogens or injury (Martinez and Gordon, 2014; Williams et al., 2016). In order to promote their anti-pathogenic functions, M1 macrophages secrete the pro-inflammatory cytokines IL-10, IFNγ, IL-8, IL-1β, IL-6, chemokine ligand 5 (CCL5), and TNFα. They also show upregulation of the STAT1/2, NF-kB, and mitogen-activated protein kinase (MAPK) signaling pathways (Abdullah et al., 2015; Mosser and Edwards, 2008; Sousa et al., 2015; Takeda and Akira, 2000;). They are CD64- and CD80-positive and secrete reactive nitrogen species to destroy pathogens through the upregulation of iNOS (Abdullah et al., 2015; Martinez and Gordon, 2014; Mosser and Edwards, 2008; Sousa et al., 2015). Through the phagocytosis of pathogens and presentation of antigens on their major histocompatibility complex II (MHC II) molecules, M1 macrophages also bridge innate and adaptive immune responses (Sousa et al., 2015). M1 macrophages are also responsible for the immune system's anti-tumoral response, recognizing tumor-specific irregularities on the surface of cells, phagocytosing them, and presenting the antigens to adaptive immune cells (Williams et al., 2016). Viruses are uniquely equipped to promote an M1 phenotype in macrophages due to their natural ability to induce type I IFN responses in hemopoietic cells, which in turn may promote anti-tumor functioning in such cells, including macrophages.

M2 macrophages are derived from M0 macrophages via stimulation with IL-14 and IL-13. M2 macrophages are referred to as alternatively activated macrophages and are comprised of several distinct subtypes from their M1 macrophage counterparts. All are CD11b and CD209 positive, secrete IL-13, CCL17, and CCL18, and have increased endocytic and phagocytic activity compared to M1 macrophages. (Abdullah et al., 2015).

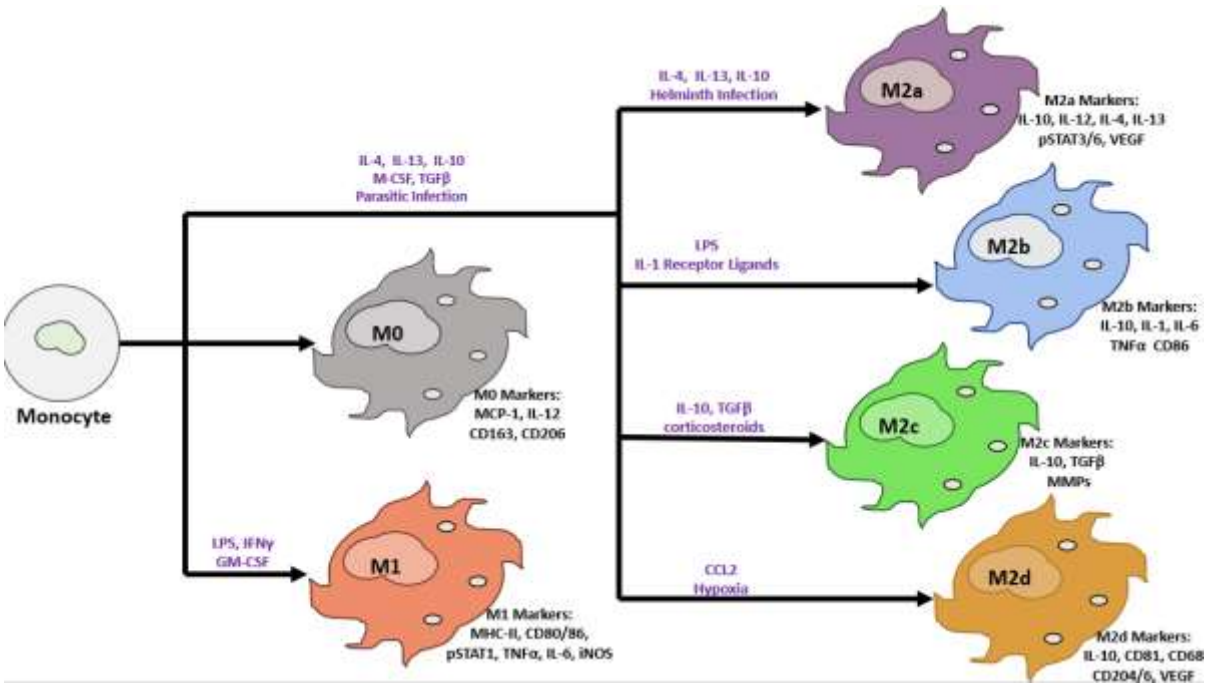

Fig. 3. Macrophage Phenotypic Diversity. Monocytes are polarized to M0 macrophages upon exposure to M-CSF, at which point they express MCP, IL17, CD163, and CD206. M1 macrophages upon stimulation by GM-CSF or pathogen signals (LPS, IFNγ) and will express IL-6, TNFα, phosphorylated STAT1, and iNOS. M2 phenotypes are highly context dependent, though all secrete IL-10. Exposure to helminth signals, IL-4, IL-13, and IL-10, promotes M2a polarization, which is distinguished by the secretion of IL-4, IL-12, and VEGF. Upon exposure to IL-4, IL-13 IL 10, M-CSF and pathogens (*e.g.* LPS), monocytes are polarized to the M2b phenotype. Upon exposure to corticosteroids and TGFβ, M2 macrophages polarize to an M2c phenotype and express/secrete MMPs. The M2d subtype, which occurs from exposure to CCL2 and hypoxia, expresses CD204/206 and secretes VEGF. It is the phenotype most associated with M2-like TAM populations. Polarizing stimuli are highlighted in purple, while markers for each macrophage subtype are in black. Further description of macrophage polarization and phenotype are described in the text.

M2 macrophages are associated with many immune functions, such as wound-healing, parasite defense, and allergenic immune responses. This population is also pro-angiogenic due to their expression of VEGF. The M2 subtypes are designated M2a, M2b, M2c, and M2d and each promotes specialized immune functions in response to the polarizing stimuli present (Martinez and Gordon, 2014; McNab et al., 2015; Mosser and Edwards, 2008;).

M2a macrophages are activated in response to M-CSF, IL-4, and IL-13, and promote allergy development and parasite defense (Martinez and Gordon, 2014; Williams et al., 2016). They retain the M1 macrophage abilities to present antigens on their MHC II molecules but downregulate CD86 expression. Unlike M1 macrophages, M2a macrophages express CD206 and CD163 (Martinez and Gordon, 2014; Sousa et al., 2015). They do not secrete pro-inflammatory cytokines or reactive oxygen species, but instead secrete immunosuppressive IL-4, IL-13, IL-12, and IL-10, polyamines, and VEGF (Martinez and Gordon, 2014; Mosser and Edwards, 2008). They show upregulation of STAT3, which allows M2a macrophages to secrete IL-10, thus dampening the inflammatory effects of M1 macrophages (Takeda and Akira, 2000). M2a macrophages also upregulate the STAT6 signaling pathway (due to IL-4 and IL-13 exposure) to help facilitate their anti-helminth responses (Sousa et al., 2015; Takeda and Akira, 2000). Finally, M2a macrophages are associated with late stage wound-healing through their promotion of scar formation (Wehlong-Henricks et al., 2010).

M2b (“Type II”) macrophages are activated in response to immune complex (IC) and TLR/IL-1R ligands and are involved in propagating signals to B cells (Martinez and Gordon, 2014; Mosser and Edwards, 2008). They express both MHCII and CD86, and secrete low levels of IL-12, high levels of IL-10, and the pro-inflammatory cytokines IL-1, IL-6, and TNFα, thus indicating a phenotype that resembles aspects of both M1 and M2 macrophages. This has led to speculation that this macrophage subtype is an intermediate between the two M1 and M2 extremes (Mantovani et al., 2004; Mosser and Edwards, 2008).

M2c macrophages are polarized in response to IL-10 and glucocorticoids. They are critical to the early stages of wound-healing as they are involved in tissue-remodeling and matrix deposition immune functions (Martinez and Gordon, 2014). They achieve these functions through the secretion of IL-10, TGFβ, and matrix metalloproteinases (MMPs) (Martinez and Gordon, 2014; Sousa et al., 2015).

M2d macrophages also possess both M1-like and M2-like characteristics. They express CCL5, IL-10, CD68, CD206, NOS2, CD81, and CD204 (Chávez-Galán et al., 2015; Martinez and Gordon, 2014; Röszer, 2015; Sousa et al., 2015). M2d macrophages thus express both M1 and M2 associated markers and cytokines. Like M2 macrophages, they express high levels of VEGF and IL-10 and low levels of TNFα. But like M1 macrophages, they express high levels of iNOS, low levels IL-12, and mildly elevated levels of arginase-1 (Ferrante and Leibovich, 2014). Evidence suggests that M2d macrophages begin as an M1-like macrophage engaged in anti-pathogenic activities, but switch to a pro-angiogenic, wound healing phenotype in response to IL-10 and VEGF and the activation of IRF4 (Martinez and Gordon, 2014). This makes sense in the context of the progression of injury and infection. Early on, macrophage populations need to be able to destroy pathogens and remove cellular debris from the tissue, necessitating M1 functionality. As infection resolves, it is necessary that macrophage populations transition to tissue repair, or M2 functionality. If this process goes awry however, human disease, including autoimmunity and cancer, can result (Martinez and Gordon, 2014; Sousa et al., 2015).

Macrophages which have infiltrated tumors are referred to as tumor-associated macrophages (TAMs). TAMs likely have phenotypic heterogeneity based upon the specific characteristics of the TME and their localization within it (Bingle et al., 2002; Pollard, 2004). However, they are often educated by the TME to the M2d subtype where their wound-healing and immunosuppressive activities promote tumor proliferation, cancer cell survival, angiogenesis, and metastasis (Bingle et al., 2002; Ojalvo et al., 2010; Pollard, 2004; Sousa et al., 2015). Many studies have shown that a cancer patient's TAM population is predominately the M2 subtype and this is associated with a poor prognosis. (Bingle

et al., 2002; Byun and Gardner, 2018; Hollmén et al., 2015; Pollard, 2004; Sousa et al., 2015;). Consequently, much research has been devoted to understanding cancer cell-TAM crosstalk so that TAM modulation therapies can be developed (Bingle et al., 2002; Joyce and Pollard, 2009; Ojalvo et al., 2010; Pollard, 2004). One goal, for example, would be to promote repolarization of M2 macrophages to the M1 phenotype.

Breast Cancer/Tumor-Associated Macrophage Crosstalk

To model the interactions between breast cancer cells and TAMs, various cell lines have been adopted. The MCF-7 and T47D cell lines are used to study ER-positive, luminal breast cancers, the BT-20 and SKBR3 cell lines for HER2-enriched breast cancers, and Hs578T and MDA-MB-231 cells lines for TNBC (Hollmén et al., 2015; Mota et al., 2018; Sousa et al., 2015). Additionally, THP-1 leukemia cells have been used to model monocytes, and can be polarized to M0, M1, and M2 macrophages *in vitro*. To generate macrophages, THP-1 monocytes are incubated with phorbol 12-myristate 13-acetate (PMA) for 24 hours. These M0 macrophages can then be further polarized into M1 macrophages by incubation with LPS and IFNγ or into M2 macrophages by incubation with IL-4 and IL-13 (**Fig. 4**). After 48 hours of exposure to the secondary set of polarizing cytokines, these macrophages behave similarly to primary macrophage subtypes, and thus make a good model system for studying macrophage and TAM function (Bosshart and Henzelmann, 2018)**.**

By co-culturing breast cancer cell lines with monocyte/macrophage cell lines, including the THP-1 model cell line used in this study, it is possible to study the ways in which TAMs affect and are affected by their TME, and how that interaction can be modulated to promote tumor stasis or regression. It is known that the physiological characteristics of tumors, including hypoxia, low pH, and the presence of tumor-derived cytokines promote their infiltration by monocytes/macrophages (Lin et al., 2001; Bingle et al., 2002; Hollmén et al., 2015; Sousa et al., 2015) and their education towards the tumor-

promoting M2 phenotype (Bingle et al., 2002; Condeelis and Pollard, 2006; Hollmén et al., 2015 Vasiljeva et al., 2006).

Tumor cell secretion of M-CSF and GM-CSF signal macrophage infiltration, while tumor-cell secretion of IL-4, IL-13, IL-10, and glucocorticoids promote the M2 phenotype (Condeelis and Pollard, 2006; Hollmén et al., 2015; Lin et al., 2001; Sousa et al., 2015). Indeed, primary human monocytes become M2 macrophages when they are cultured in media conditioned by the TNBC cell line MDA-MB-231 (Hollmén et al., 2015; Ward et al., 2015). These monocytes upregulate the mannose receptors CD206 and MRC2 (mannose receptor C Type 2) and other M2 markers (stabilin-1, transglutaminase 2, CD163) and downregulate the M1 activity of reactive oxygen species production (Hollmén et al., 2015; Ward et al., 2015). These results are not observed upon co-culture with the less aggressive ER-positive T47D cell line (Hollmén et al., 2015; Ward et al., 2015).

Having infiltrated the TME, TAMs begin to secrete their own milieu of cytokines which (i) facilitate the polarization of other TAMs to a more M2-like phenotype, and (ii) have positive effects on tumor cell growth and survival. For example, it has been shown that IL-4, which promotes M2 polarization from monocytes, upregulates the expression of cathepsin family proteases that in turn act as mitogens to promote tumor cell proliferation (Gocheva et al., 2010). Similarly, undifferentiated THP-1 monocytes promote the growth of T47D breast cancer cells while THP-1-derived M2 macrophages reduce T47D cell necrosis (Ward et al., 2015). M2 macrophages have also been shown to promote tumor growth in both MDA-MB-231 and MCF7 breast cancer cells (Yang et al., 2016). In fact, M2 macrophages promote cell survival so dramatically that when co-cultured with murine breast cancer cells, they inhibit the cytotoxic action of the anti-mitotic chemotherapy drug Taxol (Hagemann et al., 2004).

In addition to enhancing breast cancer cell growth, co-culture with M2, but not M1, macrophages increased mammosphere formation, a cluster of cancerous stem cells that can form breast tumor-like tissue *in vitro* (Hollmén et al., 2015). The migration of ER-positive breast cancer cell lines

(*e.g.* MCF7, T47D) was also increased under these co-culture conditions (Hollmén et al., 2015). TAMs do contribute to tumor cell invasion and metastasis. Active cathepsins released by such macrophage populations along with MMPs contribute to ECM degradation

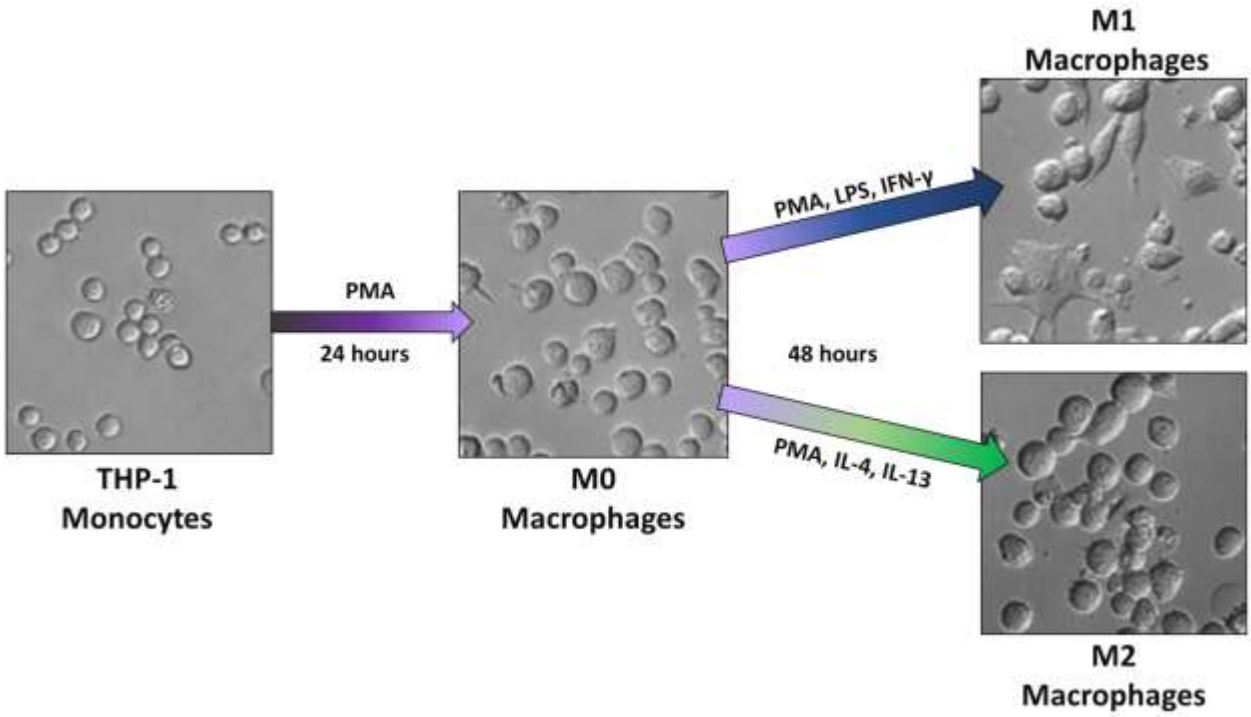

Fig. 4 - THP-1 Differentiation/Polarization Protocol. THP-1 monocytes are differentiated to M0 macrophages by stimulation with PMA for 24 hours. M0 macrophages can be further polarized to M1 macrophages by exposure to IFN-γ and LPS or to M2 macrophages by exposure to IL-4 and IL-13 for 48 hours. *Photo Credit: Megan Polzin (2017).*

(Byun and Gardner, 2018; Goswami et al., 2005; Park et al., 2017; Vasiljeva, et al. 2006). This weakens the ECM and allows for enhanced tumor vascularization and for tumor cell invasion of surrounding tissues. Moreover, the cytokine profile of the breast TME, high in both EGF and M-CSF, promote the formation of invasive cytoskeletal structures in both tumor cells (invadopodia) and macrophages (podosomes) (Williams et al., 2016). Indeed, tumor cells that have direct contact with macrophages use invadopod/podosome machinery to intravasate the blood stream at distant sites and thus complete the process of metastasis (Wykcoff et al., 2007).

Breast cancer cells possess the ability to transition between epithelial and mesenchymal phenotypes and the epithelial to mesenchymal transition (EMT) is considered a critical step in tumor progression and metastasis. This process has been shown to be modulated, in part, by the secretion of TGFβ from M2-like TAMs (Fuxe and Karlsson, 2012; Georgoudaki et al., 2016). Further, MDA-MB-231-educated macrophages begin to express genes involved in the maintenance of stem cell renewal and the EMT (Hollmén et al,, 2015). M2 macrophages enhance the mesenchymal phenotype of aggressive MDA-MB-231 breast cancer cells while M1 macrophages lead to a more epithelial phenotype. (Yang et al., 2016). Conversely, the less aggressive MCF-7 breast cancer cell line acquired a mesenchymal phenotype when co-cultured with M2 macrophages, as indicated by decreased expression of the epithelial cell marker E-cadherin protein and by the increased spindle-like morphology of the tumor cells. M0 and M1 macrophages, however, had no effect on this cell line. This indicates that M2 macrophages promote a more mesenchymal, metastatic phenotype, while M1 macrophages inhibit it (Yang et al., 2016). By inducing M2 polarization in TAM populations, breast cancer cells hijack the wound-healing, M2-like functions of macrophages to enhance their own progression (Byun and Gardner, 2018).

Research Goal

The importance of TAMs on tumor progression and their response to immunotherapies highlights the need for mechanistic studies of breast cancer cell-TAM crosstalk. Since immunotherapies such as OVT hold such promise for modulating the immune cells of the TME, and because VSV is such a well-characterized oncolytic virus, our lab is focused on characterizing the effects of VSV in this context. Previous experiments from our lab investigated how infection by the rwt and rM51R-M strains of VSV affected THP-1 macrophage viability and phenotype (Polzin, 2017). These experiments showed that rwt kills M2 macrophages (along with M0 macrophages and monocytes), but that M1 macrophages are resistant to infection (Fig. 5). In contrast, infection by rM51R-M increases levels of STAT1 phosphorylation in pre-polarized M2 macrophages, suggesting that infection by rM51R-M induces M2 to M1 repolarization (Fig. 6). Nevertheless, these results fail to consider the complexity of the TME.

The overall goal of this project is to analyze the potential of VSV as a targeted therapy for breast cancer based on both its natural oncolytic properties and its ability to modulate TAM phenotype in a TME setting. We hypothesize that rwt and rM51R-M will reduce M2 macrophage functioning and increase M1-like functioning when model THP-1 macrophages are co-cultured with MDA-MB-231 and T47D breast cancer cells. Specifically, infection with rM51R-M will induce a type I IFN antiviral response to facilitate the secretion of pro-inflammatory cytokines like TNFα and IL-6 that will convert M2 macrophages to an M1 phenotype (Park et al., 2017). Conversely, infection with rwt will induce cell death mechanisms that destroy M2 macrophages, thus altering the cytokine profiles (*e.g.* IL-10) of this simulated TME.

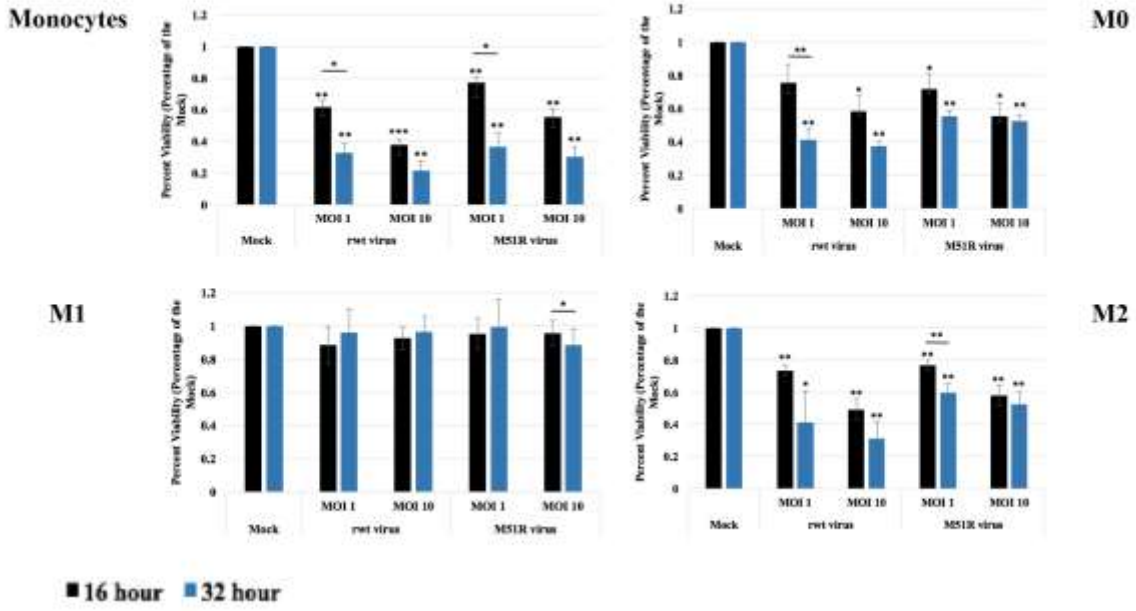

Fig. 5. Effects of VSV on THP-1 Monocyte and Macrophage Viability. Average percentage of viable monocytes, and M0, M1, and M2 macrophages in response to rwt or r-M51R-M (M51R) virus at multiplicities of infection (MOI) of 1 or 10 pfu/cell at 16 (black) and 32 (blue) hours post-infection. Data are the means ± standard deviation of three independent experiments. Statistical analyses were conducted using the Student's T test. * represents statistical differences between mock and viral infections between 16 and 32 hours (Polzin, 2017).

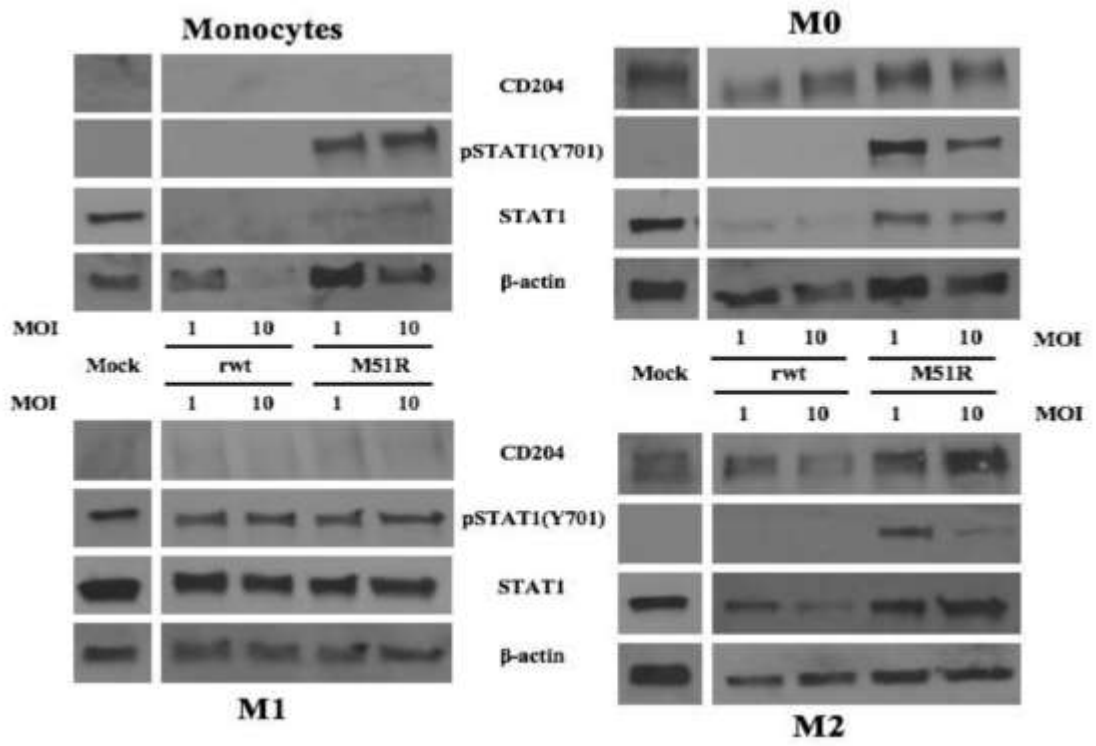

Fig. 6. Effects of VSV on the Polarization Markers pSTAT1 and CD204. Representative western blots depicting changes in polarization markers upon viral infection in monocytes, and M0, M1, and M2 macrophages (Polzin, 2017).

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Chapter: 2

1. Introduction

Breast cancer is the second leading cause of female cancer fatalities with almost 41,000 women succumbing to the disease annually in the United States alone (American Cancer Society, 2018). Most of these deaths are due to metastatic disease as 5-year survival rates drop from over 90% for patients harboring local tumors down to less than 25% for patients with distant ones (American Cancer Society, 2018). The poor prognosis for women with metastatic breast cancer is primarily due to ineffective therapies, especially for cancers that develop resistance to common therapeutic agents. It is therefore critical to develop novel interventions for aggressive/invasive breast cancers.

Oncolytic viruses are promising cancer therapies because they target and destroy susceptible cancer cells while sparing healthy cells (Bartlett et al., 2013; Lawler et al., 2017; Zamarin and Palese, 2012). This tropism by oncolytic viruses is based on genetic defects in cancerous cells that result in diminished antiviral responses. Infected cancer cells then succumb to virus-induced cytopathology, leading to cell lysis and the release of tumor antigens that are made available to antigen presenting immune cells. Tumor antigen presentation, combined with the resulting local inflammation, leads to systemic immune activity against tumors. Therefore, not only are oncolytic viruses an attractive option for the treatment of local cancers but also offer the potential for treatment of metastatic disease.

Several oncolytic viruses, including poliovirus, herpes simplex virus, and vesicular stomatitis virus (VSV), are currently in clinical trials as cancer treatments (ClinicalTrials.gov trial NCT01491893,

trial NCT02031965, trial NCT03120624). Our goal is to develop oncolytic VSV as a therapeutic intervention for breast cancer and to understand how different VSV strains modulate components of the tumor microenvironment (TME). There are numerous advantages for using VSV as an oncolytic agent, including its ability to induce apoptosis in cancer cells, its well-characterized and easily manipulated genome, the lack of pre-existing immunity to VSV in the population, and its low pathogenicity in humans (Hastie and Grdzelishvili, 2012).

Recombinant wild-type (rwt) and matrix (M) protein mutant (rM51R-M) strains of VSV have been tested extensively *in vitro* and *in vivo* for their efficacy in different cancer (Ahmed et al., 2010; Fernandez et al., 2002; Hastie and Grdzelishvili, 2012; Porosnicu et al., 2003; Stojdl et al., 2003). The M protein of VSV is responsible for inhibiting host gene expression in infected cells through inhibition of nuclear-cytoplasmic transport and transcription (Black et al., 1993). These effects culminate in the inhibition of the host type I interferon (IFN) antiviral response, thus allowing VSV to effectively replicate in infected cells. rM51R-M virus has a methionine to arginine substitution at position 51 of the M protein amino acid sequence resulting in a defect in its ability to inhibit the host antiviral response. **(**Ahmed et al., 2003; Black et al., 1993; Kopecky et al., 2001**)**. Therefore, rM51R-M virus represents a safer therapeutic option than rwt virus because the antiviral response stimulates components of the innate immune system. It was previously shown that upon infection with rM51R-M virus, dendritic cells upregulate expression of the co-stimulatory molecules CD80 and CD86 and secreted the cytokine interleukin 6 (IL-6), while those infected by rwt did not. (Ahmed, 2009; Hastie, 2012). By inducing the expression of new host genes, the M protein mutant virus may also be able to enhance anti-tumor immunity, even at distal sites.

As part of the body's innate immune system, macrophages are ubiquitous in the body. Tumors are no exception, and high tumor-associated macrophage (TAM) densities often correlate with poor clinical outcomes, including in breast cancers (Bingle et al., 2002; Byun and Gardner, 2018; Pollard, 2004; Sousa et al., 2015;). Macrophages demonstrate high phenotypic plasticity. The extremes of this

paradigm are represented by the M1 and M2 subtypes. M1 macrophages express the pro-inflammatory cytokines IL-6 and tumor necrosis factor alpha (TNFα), exert anti-microbial functions, and promote tumor killing. Alternatively, M2 macrophages are anti-inflammatory, function in wound-healing, and are tumor-promoting. In the body, macrophages likely function somewhere between these extremes, since macrophages are highly sensitive to polarizing signals from their environment. Signals that result from pathogen invasion, such as type I and type II interferons (IFN) and lipopolysaccharide (LPS), polarize macrophages to an M1 phenotype. Signals that result from tissue damage, like hypoxia, low pH, and IL-10 stimulate macrophages to the M2 phenotype. Breast cancer cells also secrete factors capable of educating infiltrating TAMs to various polarization states. For example, the triple negative breast cancer cell line MDA231 has been shown to secrete high levels of IL-6 and TNFα to facilitate macrophage infiltration, and high levels of IL-10 to promote M2 macrophage polarization (Chuang et al., 2016). M2 polarization in turn promotes tumor proliferation, cancer cell survival, angiogenesis, and metastasis (Bingle et al., 2002; Pollard, 2004; Sousa et al., 2015). For this reason, breast cancers enriched with M2 macrophages in the TME are associated with poor prognosis (Bingle et al., 2002; Byun and Gardner, 2018; Pollard., 2004; Sousa et al., 2015). This is due to M2 macrophage secretion of factors that support tumor cell proliferation (*e.g.* epidermal growth factor), angiogenesis (*e.g.* vascular endothelial growth factor), local immunosuppression (*e.g.* IL-10), and metastasis (*e.g.* matrix metalloproteinases). M2 macrophages are thus an attractive target for therapies (Byun and Gardner, 2018; Goswami et al., 2005; Martinez and Gordon, 2014; Sousa et al., 2015; Wykcoff et al., 2007).

Several experimental cancer therapeutics, including oncolytic virotherapies, have aimed to modulate TAMs to a pro-inflammatory M1 phenotype with the overall goal of overcoming the immunosuppressive TME. (Park et al., 2017; Takeda and Akira, 2000; Tan et al., 2016**)**. Studies have shown that infection of co-cultures of MDA-MB-231 (MDA231) breast cancer cells and pre-polarized human monocyte-derived macrophages by oncolytic measles and mumps viruses resulted in decreased MDA231 viability, regardless of the macrophage polarization state. Moreover, this reduction in cell

viability was not due to viral replication, but resulted from TNFα-mediated apoptosis (Tan et al., 2016). This result, combined with the elevated IL-6 and decreased IL-10 levels in the co-cultures, suggests that macrophages acquire an anti-tumor, pro-inflammatory phenotype as a result of viral infection. Nevertheless, measles and mumps viruses carry risks to patients and for safety concerns require attenuation. In this study, we investigated the potential of VSV as a safer therapeutic option for metastatic breast cancer through modulation of TAMs. We hypothesized that the induction of the type I IFN antiviral response and the secretion of pro-inflammatory cytokines in response to infection with rM51R-M would convert M2-like TAMs to an M1-like phenotype thus allowing for effective anti-tumor immunity. To test this hypothesis, we determined the ability of both rwt and rM51R-M viruses to induce the expression of pro- and anti-inflammatory cytokines in a co-culture system designed to simulate the TME. THP-1-derived monocytes or macrophages were co-cultured with MDA231 or T47D breast cancer cells and infected by either the rwt or rM51R-M strains of VSV. Results indicated that infection by VSV, especially rM51R-M virus, significantly increases co-culture secretion of TNFα, a known mediator of macrophage tumoricidal activities, and decreased the secretion of IL-10, a tumor-promoting cytokine, in a dose-dependent manner. This suggests that rM51R-M virus may be able to modulate the cytokine milieu of the TME to promote TAM repolarization to a pro-inflammatory, M1-like phenotype, potentially promoting destruction of the TME and inducing systemic anti-tumor memory in immune cells.

2. Materials and Methods

2.1. Cell Lines and Viruses

The MDA231 and T47D breast cancer cell lines were propagated in RPMI media (HyClone Laboratories, SH30027.02) supplemented with 10% fetal bovine serum (Atlanta Biologicals, S11150) with (T47D) or without (MDA231) 0.002 % insulin (Gibco by Life Technologies, 41400-045). THP-1

monocytic leukemia cells were propagated in RPMI media supplemented with 10% fetal bovine serum (Atlanta Biologicals, S11150) 0.01 M Hepes (VWR International, VW1481-04), 1% 100x vitamins, 1% Penn-Strep (Corning, 30-002-CI), and beta-mercaptoethanol (MP Biomedicals LLC, 190242). Recombinant wild-type (rwt) virus, the matrix (M) protein mutant (rM51R-M) virus, and rwt and rM51R-M viruses expressing GFP (rwt-GFP and rM51R-M-GFP) were a gift from Dr. Douglas Lyles from Wake Forest University (Winston-Salem, NC) and have been previously described (Whitlow et al., 2006). Viral stocks were prepared in baby hamster kidney (BHK) fibroblasts using methods described previously (Kopecky et al., 2001).

2.2. Cell Viability Assays

The viability of MDA231 and T47D cells in response to infection by rwt or rM51R-M was measured by MTT assay (Trevigen, MTT Cell Proliferation Kit, 4890-25-01). Cells were cultured in 96-well plates at a concentration of $1x10^5$ cells/well, incubated for 14 hours, then infected with rwt or rM51R-M at a multiplicity of infection (MOI) of 1 or 10 plaque forming units (pfu) per cell. After 24 and 48 hours post-infection, cells were assayed for viability per the manufacturer's directions. Data are the result of triplicate measurements from three, independent experiments and are reported as the mean ± standard error relative to mock infections.

2.3. ELISA

To differentiate THP-1 monocytes into macrophages, $5x10^5$ cells/well were seeded in a 24-well plate in complete THP-1 media containing 25nM PMA (Sigma-Aldrich, P1585). The macrophages were then polarized to their respective subtypes by first rinsing with phosphate-buffered saline and then incubating for an additional 48 hours in 0.7 mL of complete THP-1 media supplemented with 25nM PMA for M0 macrophages, with 25nM PMA, 20 ng/mL LPS (Sigma-Aldrich; L5148), and 20 ng/mL IFN (Bio-Legend; 570202) for M1 macrophages, or with 25nM PMA, 20 ng/mL IL-4 (Bio-Legend, 574002), and 20 ng/mL IL-13 (Bio-Legend, 571102) for M2 macrophages. For co-culture, $5x10^5$

MDA231 or T47D breast cancer cells were suspended in 0.3 mL of complete THP-1 media containing the appropriate polarizing cytokines and were added to $5x10^5$ THP-1 monocytes or THP-1 derived M0, M1, or M2 macrophages (1:1 cell ratio). Co-cultures were incubated for 14 hours before infection by rwt or rM51R-M viruses at an MOI of 1 or 10 pfu/cell. Monocultures of THP-1 monocytes and macrophages as well as MDA231 and T47D breast cancer cells were similarly infected by these VSV viruses. Conditioned media were collected 24 hours post-infection and stored in microcentrifuge tubes at -80 C. Conditioned media was subjected to ELISA for IL-6 (BD OptEIA, 555220), TNFα (BD OptEIA, 555212), and IL-10 (BD OptEIA, 555157) per the manufacturer's directions. Data are the result of duplicate or triplicate measurements from three, independent experiments and are reported as the mean ± standard error in units of pg/mL.

2.4. Statistics

Data were subjected to pairwise Student's t-tests in Microsoft Excel to determine statistical significance.

3. Results

3.1. MDA231 and T47D breast cancer cell lines show an extensive variance in the secretion of IL-6, TNFα, and IL-10.

Breast cancer cells secrete factors capable of modifying TAM polarization states. For example, MDA231 breast cancer cells, a triple negative breast cancer cell line, have been shown to secrete high levels of IL-6, TNFα, and IL-10 to promote macrophage accumulation and M2 polarization (Equivel-Velazquez et al, 2015; Sousa et al., 2015). In contrast, T47D breast cancer cells, an ER-positive cell line of luminal origin, have not been shown to secrete these cytokines. In order to verify how these

breast cancer cell lines educate macrophages, it was necessary to measure the levels to which these cytokines of interest were secreted. MDA231 and T47D breast cancer cell monocultures were therefore cultured in 24-well plates at a density of $5x10^5$ cells/well for 38 hours, after which the conditioned media was collected and subjected to an ELISA protocol to establish baseline secretion of the pro-inflammatory cytokines IL-6 and TNFα and the anti-inflammatory cytokine IL-10. MDA231 cells secreted high levels of IL-6 (22,200 pg/mL), while TNFα (2215 pg/mL) and IL-10 (1300 pg/mL) were 10- to 20-fold lower, respectively (Fig. 7). Conversely, T47D cells do not secrete detectable levels of these cytokines (Fig. 7). Our data were thus consistent with previously reported findings.

3.2. M1 THP-1 macrophages secrete significantly more IL-6, TNFα, and IL-10 than M2 macrophages.

Macrophages also vary in the cytokines they secrete. M1 macrophages secrete the pro-inflammatory cytokines IL-6 and TNFα in accordance to their response to an infection. M2 macrophages, in contrast, secrete IL-10, a potent immunosuppressive cytokine. To continue addressing the baseline cytokine secretion profiles of the cells used in this study, monocultures of THP-1 monocytes and pre-polarized M0, M1, and M2 macrophages were cultured in 24-well plates at a density of $5x10^5$ cells/well for 38 hours, after which the conditioned media was collected and subjected to an ELISA. In agreement with expectations, the magnitude of IL-6 and TNFα secretion roughly correlated with the degree of M1 macrophage functionality (Fig. 8A and B). M1 macrophages secreted 5217 pg/mL TNFα, while M2 macrophages secreted 20.6 pg/mL, an ~250-fold difference overall (Fig. 8B). Similarly, M1 macrophages secreted 1018 pg/mL IL-6 and M2 macrophages secreted only 12.46 pg/mL, an ~100-fold difference. Non-polarized M0 macrophages secreted moderate levels of TNFα (698 pg/mL) and IL-6 (72.96 pg/mL) (Fig. 8B and A). In contrast to expectations, M1 macrophages expressed far more IL-10 (992 pg/mL) than the background levels secreted by the other cells. In this

study, THP-1 monocytes did not secrete detectable levels of IL-6 or TNFα and only background levels of IL-10.

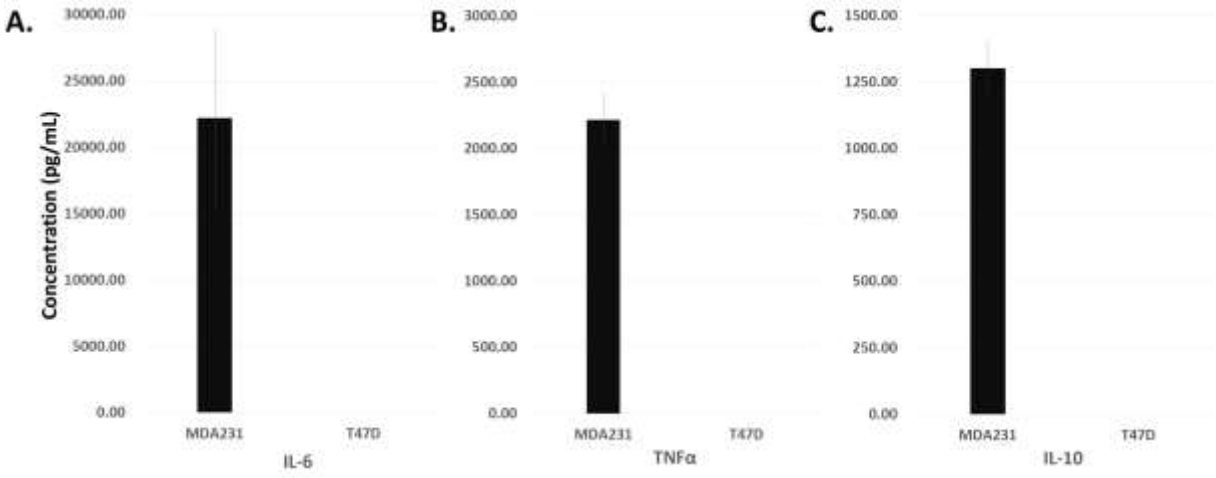

Fig. 7. IL-6, TNFα, and IL-10 Secretion by MDA231 and T47D Breast Carcinoma Cells. MDA231 and T47D breast cancer cells were cultured in 24-well plates at $5x10^5$ cells/well for 38 hours, after which the conditioned media was collected and subjected to an ELISA protocol for IL-6 (A), TNFα (B), and IL-10 (C). Data are the result of duplicate measurements from three, independent experiments and are reported as the mean ± standard error. Note that these cytokines were undetectable in the T47D conditioned media.

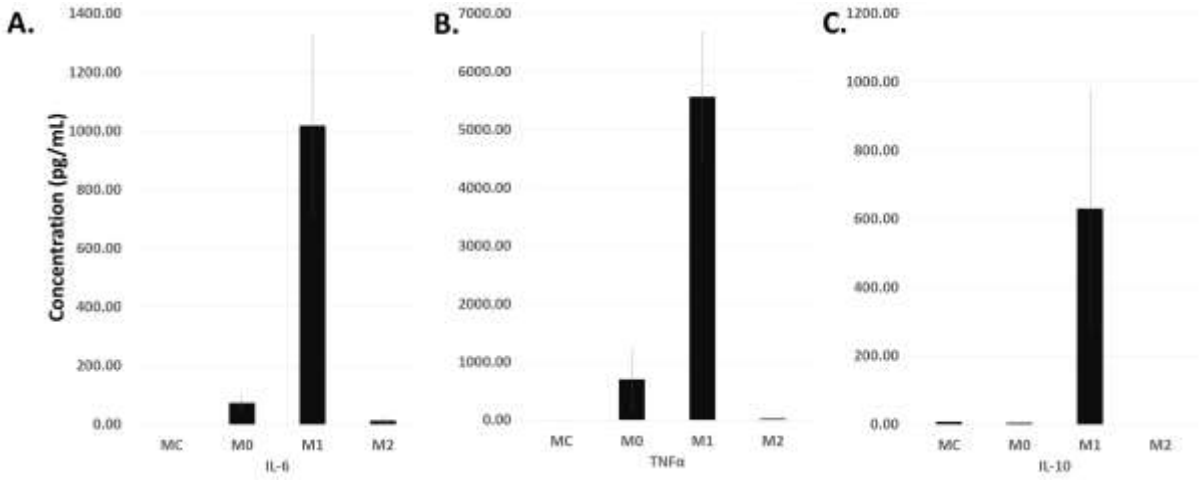

Fig. 8. IL-6, TNF*α*, and IL-10 Secretion by THP-1 Monocytes and Macrophages. THP-1 monocytes (MC) and pre-polarized M0, M1, and M2 macrophages were cultured in 24-well plates at $5x10^5$ cells/well for 38 hours, after which the conditioned media was collected and subjected to an ELISA protocol for IL-6 (A), TNFα (B), and IL-10 (C). Data are the result of duplicate measurements from three, independent experiments and are reported as the mean ± standard error.

3.3. Co-cultures with THP-1 monocytes/macrophages dampens cytokine secretion by MDA231 cells and accentuates cytokine secretion by T47D cells.

To better reflect the conditions of the breast TME, we next created and characterized co-culture systems between breast cancer and monocyte/macrophage cell lines. THP-1 monocytes and pre-polarized M0, M1, and M2 macrophages were cultured in 24-well plates at a density of $5x10^5$ cells/well. Then, MDA231 and T47D breast cancer cells were added at a 1:1 ratio ($5x10^5$ cell/well) and co-cultured for 38 total hours, after which the conditioned media was collected and subjected to an ELISA.

The total secreted levels of IL-6 by MDA231 breast cancer cells were the least affected by co-culture with THP-1 monocytes and macrophages (Fig. 9A, D, G, and F). Declines were seen with monocyte co-cultures and with M0 and M1 macrophages compared to MDA231 monocultures as well, but none of these differences were statistically significant. This was also true for the slight increase imposed by co-culture with M2 macrophages. Co-cultures generally had a dampening effect on MDA231 TNFα secretion (Fig. 9B, E, H, and K). Compared to MDA231 monocultures, TNFα levels declined upon co-culture with M0 ($p=0.015$) and M1 ($p=0.034$) macrophages, was trending toward a decrease with monocytes (albeit insignificant statistically) but was virtually unaffected by co-culture with M2 macrophages. Finally, like TNFα, the total levels of IL-10 secreted by MDA231 breast cancer cells also experienced some declines with co-culture (Fig. 9C, F, I, and L). This was statistically significant with monocytes ($p=0.007$) and M0 macrophages ($p=0.006$), but only trended towards a decrease with M1 and M2 macrophages.

Another way to think about this data set is to compare cytokine secretion among the different types of co-cultures (*i.e.* tumor microenvironments). For example, the MDA231/M0 macrophage co-cultures secreted more IL-6 than the MDA231/M1 macrophage co-cultures ($p=0.115$) (Fig. 9D and G). Conversely, MDA231/M1 macrophage co-cultures secreted more TNFα than the MDA231/M0 macrophage co-cultures ($p=0.034$) (Fig. 9H and E). And finally, both MDA231/M1 macrophage and

MDA231/M2 macrophage co-cultures exceeded MDA231/M0 macrophage co-cultures in IL-10 production ($p=0.020$ and $p=0.014$, respectively) (Fig. 9I, L, and F).

Problematically, MDA231 breast cancer cells have a robust cytokine secretion profile for IL-6, TNFα, and IL-10 (Fig. 7). Moreover, the effect of THP-1 monocyte or macrophage co-culture were somewhat variable and did not seem to follow any established hypothesis regarding the potential anti-tumor effects of M1 macrophages or pro-tumor effects of M2 macrophages (Fig. 9).

In contrast to MDA231 cells, T47D cells secreted low levels of IL-6, TNFα, and IL-10 (Fig. 7), and co-culture conditions often resulted in a strong induction. For example, IL-6 secretion increased for every co-culture condition relative to T47D monocultures, particularly for monocytes ($p=0.020$) and M2 macrophages ($p=0.004$) (Fig. 10A, D, G, and J). Moreover, T47D/M2 macrophage co-cultures secreted far more IL-6 than T47D/monocyte ($p=0.003$) and T47D/M0 macrophage ($p=0.049$) co-cultures (Fig. 9J, A, and D). Like IL-6, all of the co-culture conditions increased TNFα secretion relative to T47D monocultures too. This was particularly true for monocytes ($p=0.002$) and M1 macrophages ($p=0.040$) (Fig. 9B and H). Moreover, the T47D/M1 macrophage co-cultures secreted more TNFα than T47D/monocyte co-cultures ($p=0.0452$) (Fig. 9H and B). Finally, the co-culture of T47D breast cancer cells with THP-1 macrophages increased IL-10 secretion, particularly for M0 ($p=0.017$) and M1 ($p=0.007$) macrophages (Fig. 9F and I). The highest levels of IL-10 secretion were seen with T47D/M1 macrophage co-cultures, which was significantly higher than T47D/M0 ($p=0.007$) and T47D/M2 ($p=0.005$) macrophage co-cultures (Fig. 9I, F, and L). Like T47D monocultures, T47D/monocyte co-cultures failed to secrete detectable levels of IL-10 (Fig. 9C). It is interesting to speculate whether T47D breast cancer cells may be more immunogenic to THP-1 monocytes and macrophages or perhaps it is the case that the T47D cells respond to the THP-1 monocytes and macrophage by exhibiting a more aggressive cytokine profile similar to the more aggressive MDA231 TNBC cell line.

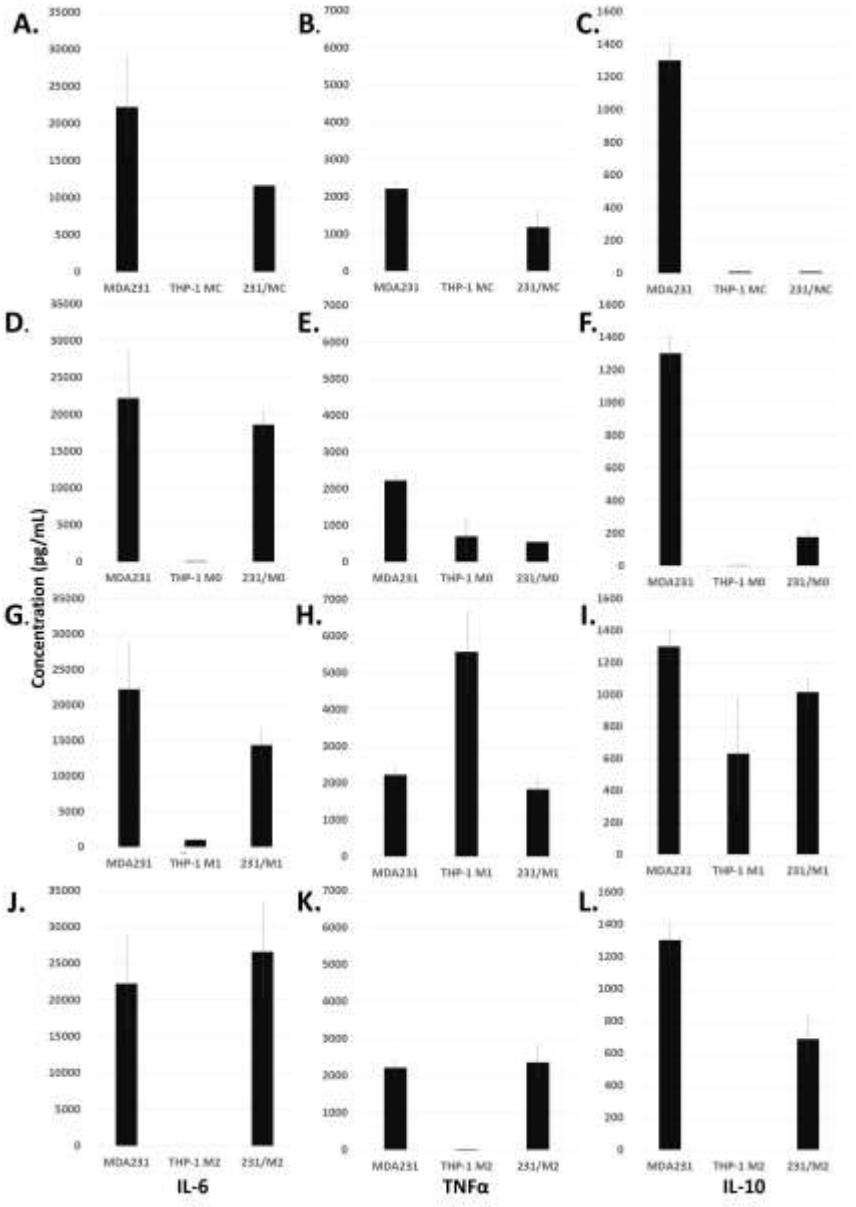

Fig. 9. IL-6, TNFα, and IL-10 Secretion by MDA231 Breast Cancer Cell/THP-1 Monocyte and Macrophage Co-cultures. THP-1 monocytes (MC) and pre-polarized M0, M1, and M2 macrophages were co-cultured in 24-well plates at a 1:1 ratio with MDA231 breast cancer cells ($5x10^5$ cells/well each) for 38 hours, after which the conditioned media was collected and subjected to an ELISA protocol for IL-6 (A,D,G,J), TNFα (B,E,H,K), and IL-10 (C,F,I,L). Data are the result of duplicate measurements from three, independent experiments and are reported as the mean ± standard error. Mono-culture data was previously reported in **Fig. 7** and **Fig. 8**.

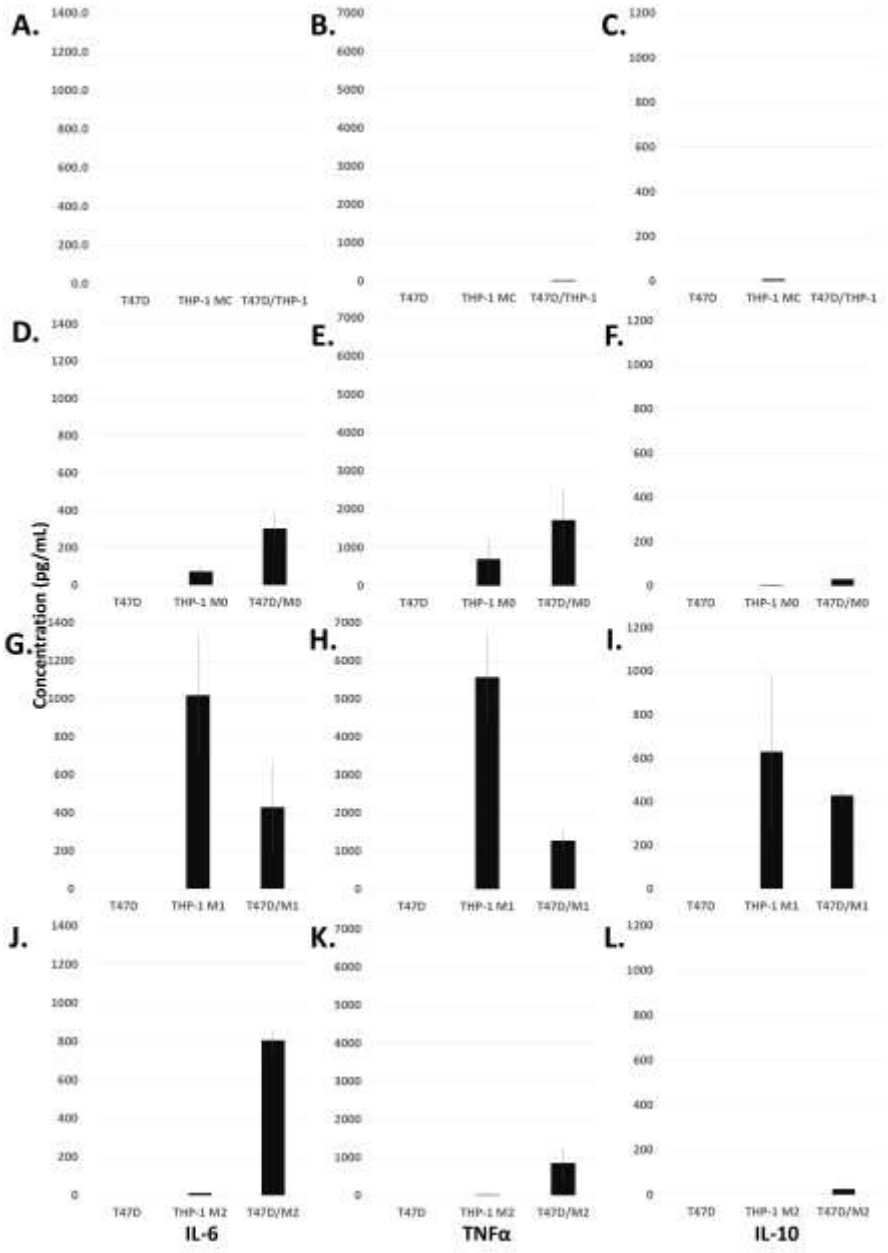

Fig. 10. IL-6, TNFα, and IL-10 Secretion by T47D Breast Cancer Cell/THP-1 Monocyte and Macrophage Co-cultures. THP-1 monocytes (MC) and pre-polarized M0, M1, and M2 macrophages were co-cultured in 24-well plates at a 1:1 ratio with T47D breast cancer cells ($5x10^5$ cells/well each) for 38 hours, after which the conditioned media was collected and subjected to an ELISA protocol for IL-6 (A,D,G,J), TNFα (B,E,H,K), and IL-10 (C,F,I,L). Data are the result of duplicate measurements from three, independent experiments and are reported as the mean ± standard error. Mono-culture data was previously reported in **Fig. 7** and **Fig. 8**.

3.4. MDA231 breast cancer cells are more sensitive to the cytopathic effects of VSV than T47D breast cancer cells.

This project ultimately seeks a better understanding of how VSV impacts TAMs, and approaches this through an analysis of cytokine secretion profiles in breast cancer cell/macrophage co-cultures. The baseline secretion of IL-6, TNFα, and IL-10 has been established in monocultures of MDA231 and T47D breast cancer cells, THP-1 monocytes, and pre-polarized M0, M1, and M2 THP-macrophages. Before assessing the impact of VSV on cytokine secretion, we first wanted to establish the impact of VSV on cell viability. Our lab has previously performed cell viability assays on mono-cultures of THP-1 monocytes and pre-polarized macrophages and the data indicate that infection with VSV reduces the viability of THP-1 monocytes and M0 and M2 macrophages, but not M1 macrophages (Polzin, 2017). Since VSV has natural oncolytic ability, it was necessary to determine viability in the wake of a VSV infection in our breast cancer cell lines. MDA231 and T47D breast cancer cells were seeded in a 96-well plate at a density of $1x10^5$ cells/well in triplicate. When cells approached approximately 80% confluency, they were infected with rwt or rM51R-M viruses at MOIs of 0.1 and 10 pfu/cell. Cells were then subjected to an MTT cell viability assay 24 and 48 hours post infection.

Infection of MDA231 breast cancer cells with both rwt and rM51R-M viruses resulted in a dose- and time-dependent decrease in cell viability (Fig. 11A). For example, infection of MDA231 breast cancer cells with rwt virus at an MOI of 0.1 pfu/cell for 24 hours resulted in a 3% decrease in cell viability, and this increased to a 35% decrease at an MOI of 10 pfu/cell. Furthermore, there was no significant difference between rwt and rM51R-M viruses at killing MDA231 cells, except that rM51R-M virus at an MOI of 0.1 pfu/cell killed more MDA231 cells at 48 hours post infection compared to rwt virus at the same MOI ($p=0.042$). In contrast, T47D breast cancer cells showed only minor decreases in viability to VSV infection. The greatest effect resulted in an ~17% decrease in viability after a 24 hour infection with rwt virus at an MOI of 10 pfu/cell, but even this condition was not statistically different from mock-infected cells (Fig. 11B). These results indicate that MDA231 breast

cancer cells are susceptible to killing by VSV at the observed time points, while T47D cells are not. Such differences must be taken into consideration when addressing how VSV affects the cytokine profile of breast cancer/monocyte and macrophage co-cultures.

3.5. *Synchronous infection of co-cultures of MDA231 breast cancer cells and M2 macrophages with rM51R-M virus results in a shift to a more pro-inflammatory cytokine profile*

Once baseline cytokine profiles had been established for the breast cancer and monocyte/macrophage cell lines in this study, both in mono- and co-cultures, we sought to determine how infection with the rwt and rM51R-M strains of VSV would modulate the expression of these cytokines. We hypothesized that infection with VSV would result in an increase in the pro-inflammatory cytokines IL-6 and TNFα and a decrease in the anti-inflammatory cytokine IL-10, thus suggesting that these viruses have the natural ability to disrupt breast cancer cell-macrophage cross-talk via the induction of cell death (rwt virus) and/or antiviral (rM51R-M virus) pathways. To this end, THP-1 monocytes and pre-polarized M0, M1, and M2 macrophages were cultured in 24-well plates at a density of $5x10^5$ cells/well. Then, MDA231 and T47D breast cancer cells were added at a 1:1 ratio ($5x10^5$ cells/well) and co-cultured for 14 hours. The co-cultures were then infected with rwt and rM51R-M viruses at an MOI of 1 and 10 pfu/cell for 24 hours, after which the conditioned media was collected and subjected to ELISA protocol for detection of IL-6, TNFα, and IL-10.

As reported before, MDA231 breast cancer cells secrete high levels of the pro-inflammatory cytokine IL-6, the THP-1 monocytes and macrophage do not secrete IL-6, and the changes imposed by co-culture produced intermediate results (Fig. 9). None of the differences, however, between MDA231 monocultures and co-cultures were statistically significant. Moreover, in no case did VSV change IL-6 secretion in a significant way, and this was true regardless of the culture conditions (monoculture vs. co-culture), the cells being cultured (MDA231 cells and/or THP-1 monocytes/macrophages), the strain of VSV (rwt or rM51R-M), or the multiplicity of infection (1 or 10 pfu/cell). In contrast to the relative unresponsiveness of MDA231 breast cancer cells, T47D cells did alter IL-6 secretion under certain

experimental conditions. For example, while neither T47D breast cancer cells nor THP-1 monocytes and M2 macrophages secreted significant levels of this cytokine, the rM51R-M strain of VSV greatly enhanced IL-6 secretion in both T47D and THP-1 M1 macrophage monocultures. Still, while under co-culture conditions, neither rwt nor rM51R-M virus significantly altered the expression of IL-6 relative to mock-infected cells (Fig. 12A, D, G, and J).

As with the IL-6 data described above, co-cultures generally had a dampening effect on the secretion of the pro-inflammatory cytokine TNFα in MDA231 cells (Fig. 9B, E, H, and K). However, upon infection of co-cultures with rwt or rM51R-M viruses, we observed changes in TNFα production that were dependent on the macrophage population. Infection of MDA231/monocyte and MDA231/M1 macrophage co-cultures, for example, resulted in decreased TNFα production, regardless of viral strain (Fig. 13B and H). In contrast, there was an increase in TNFα secretion in M0 and M2 co-cultures (Fig. 9E and K). This increase was statistically significant in the case of infection with rM51R-M virus at an MOI 10 pfu/cell. The effect of rM51R-M virus on inducing expression of a proinflammatory and immunogenic cytokine like TNFα is not surprising given the inability of this virus to inhibit host gene expression. In fact, numerous reports have documented the potential of rM51R-M virus at promoting antiviral and anti-tumor immunity. Furthermore, the increased levels of TNFα upon infection of M2 co-cultures with rM51R-M virus also suggest a shift to a more pro-inflammatory profile. Unlike MDA231 cells, T47D cells were generally more responsive to VSV infection (Fig. 10). This was especially true for rM51R-M virus, which greatly increased the production of TNFα in T47D monocultures (Fig. 13B, E, H, and K).

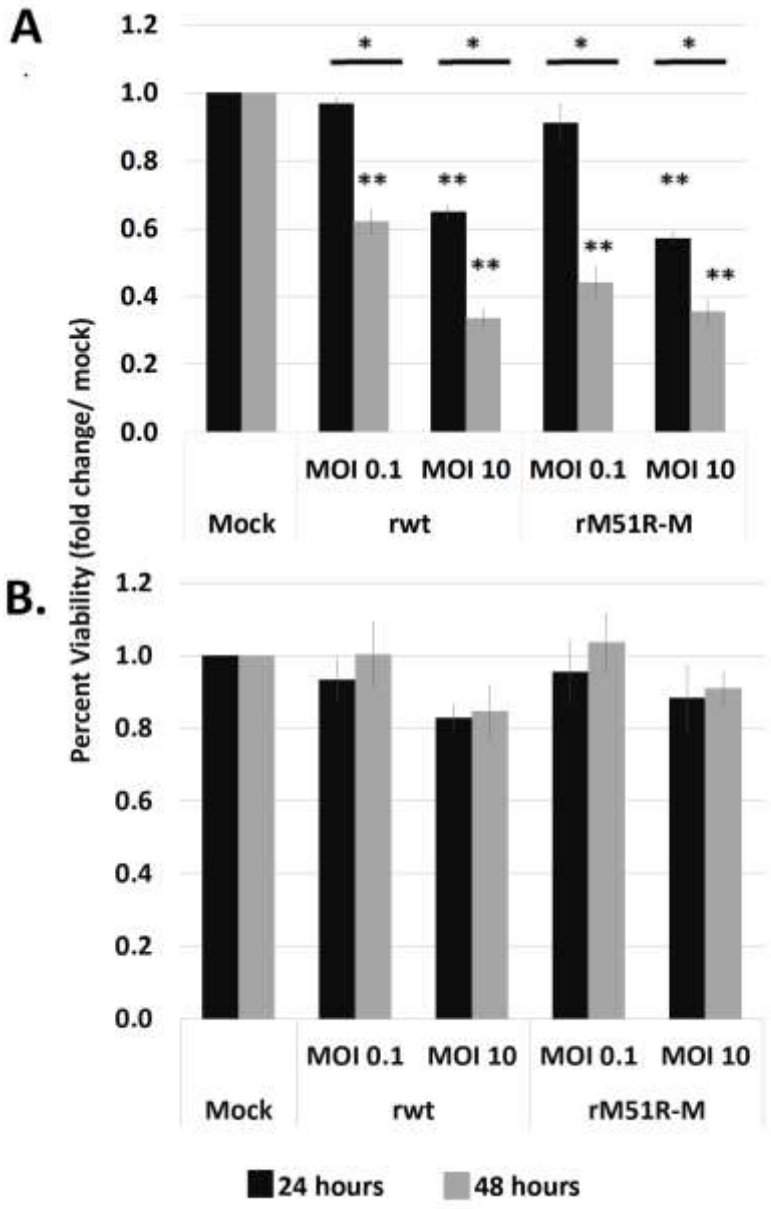

Fig. 11. Effect of VSV Infection on MDA231 and T47D Breast Cancer Cell Viability. MDA231 (A) and T47D (B) breast cancer cells were cultured in a 96-well plate at $1x10^5$ cells/well for 14 hours, then infected with rwt and rM51R-M at MOIs of 0.1 and 10 pfu/mL. Cell were subjected to an MTT assay 24 and 48 hours post infection. Data are the result of triplicate measurements from three, independent experiments and are reported as the mean ± standard error. *, $p<0.05$; **, $p<0.01$

However, under co-culture conditions with THP-1 monocytes and M0 and M2 macrophages, we observed minimal changes in the secretion of TNFα in response to virus infection (Fig. 10B, E, and K). Interestingly, infection with rM51R-M virus enhanced TNFα production over mock conditions in M1 co-cultures, especially when infected at an MOI of 1 pfu/cell (Fig. 10H). These results indicate that while T47D cells are responsive to intracellular stimuli such as viral infections, their responsiveness is diminished in conjunction with macrophages.

The final cytokine we analyzed was the anti-inflammatory cytokine IL-10, a known marker of M2 pro-tumor macrophages (Fig. 9C, F, I, and L ; Fig. 10C, F, I, and L). MDA231 cells secreted high levels of IL-10, but levels declined in co-culture with THP-1 monocytes and M0 macrophages. This decline was not apparent in co-culture with M1 and M2 macrophages. However, upon infection of MDA231/M1 macrophage and MDA231/M2 macrophage co-cultures with VSV, there was a dose-dependent decrease in IL-10 production, which was statistically significant in the case of infection with rM51R-M virus at an MOI of 10 pfu/cell (Fig. 14I and L). Interestingly, this decrease in IL-10 levels was not observed in MDA231 monocultures. In contrast, T47D cells produced undetectable levels of IL-10, but the levels of this cytokine increased upon co-culture with M0 and M2 macrophages. In addition, although not statistically significant, infection of M0 co-cultures with either virus strain reduced IL-10 levels relative to those observed in mock-infected co-cultures (Fig. 10F). A similar effect was seen in in T47D/M2 macrophage co-cultures infected with rwt virus (Fig. 10L). Taken together, our data indicate that VSV strains have the potential to diminish IL-10 production in M2 macrophages, perhaps as a mechanism to promote a pro-inflammatory environment in response to virus infection.

In summary, we were able to show that while infection with VSV does result in changes in the cytokine profiles of T47D breast cancer cells co-cultured with THP-1 monocytes and pre-polarized M0, M1, and M2 macrophages, generally these results were not significant. This contrasts with the MDA231 co-culture data, the cytokine profiles of which are statistically changed as a result of infection with VSV, especially rM51R-M virus. The proportional changes associated with results in co-culture are

summarized in Fig.s15 and 16. For MDA231 cells (Summary Fig. 15), we see that while IL-6 secretion was not affected by VSV, rM51R-M virus significantly increased secreted levels of TNFα in M2 macrophages and decreased secreted levels of IL-10 in both M1 and M2 macrophages. These changes were not observed in T47D cells (Summary Fig. 16), a result that may not be surprising given the overall resistance of these cells to infection and killing by both strains of VSV. Specifically, if cytokine secretion is dependent on the ability of the virus to gain access to cells and modulate gene expression, perhaps the ability of VSV to access T47D cells in the context of a co-culture environment is further limited. In contrast, the greater sensitivity of MDA231 cells to virus infection would render them subject to VSV-induced modulation of cellular programming.

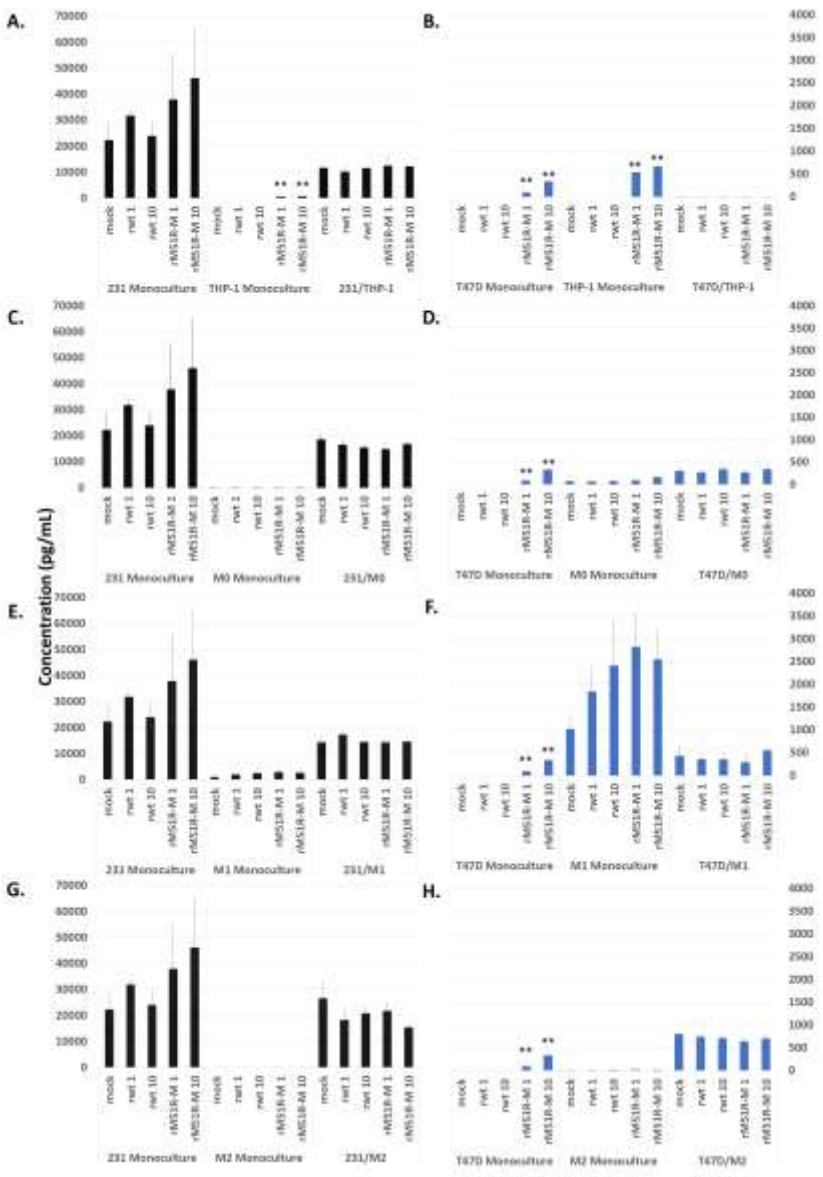

Fig. 12. rwt and rM51R-M viruses do not impact IL-6 production in co-cultures of breast cancer cells and THP-1 macrophages. THP-1 monocytes (A, B) and pre-polarized M0 (C, D), M1 (E, F), and M2 (G, H) macrophages were co-cultured at 1:1 ratio with MDA231 (A, C, E, G) and T47D (B, D, F, H) breast cancer cells for 14 hours after which cultures were infected with rwt and rM51R-M viruses at MOIs of 1 and 10 pfu/cell for 24 h. Culture supernatants were analyzed for secreted IL-6 by ELISA. Data are the result of triplicate measurements from three, independent experiments and are reported as the mean ± standard error. Note. There were no significant differences between mock-infected co-cultures and those infected with rwt or rM51R-M viruses.

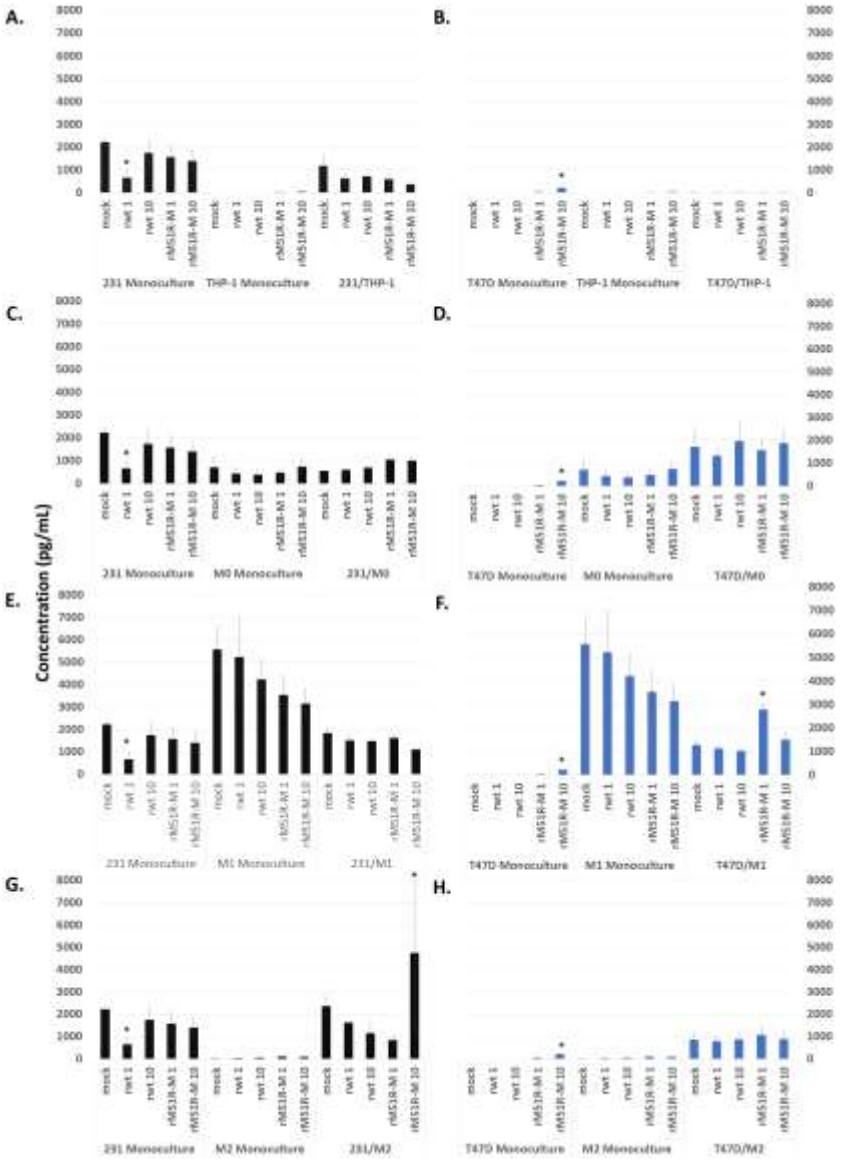

Fig. 13. Infection of co-cultures of breast cancer cells and THP-1 derived macrophages by rM51R-M results in increases TNFα secretion. THP-1 monocytes (A, B) and pre-polarized M0 (C, D), M1 (E, F), and M2 (G, H) macrophages were co-cultured at 1:1 ratio with MDA231 (A, C, E, G) and T47D (B, D, F, H) breast cancer cells for 14 hours after which cultures were infected with rwt and rM51R-M viruses at MOIs of 1 and 10 pfu/cell for 24 h. . 24 hours post infection, the culture media was collected, centrifuged, and stored at -80 C. Culture supernatants were analyzed for secreted TNFα by ELISA. Data are depicted as the average and standard error of three, independent experiments performed in duplicate and analyzed by Student's T-test. ($p < 0$ .05 is indicated by *).

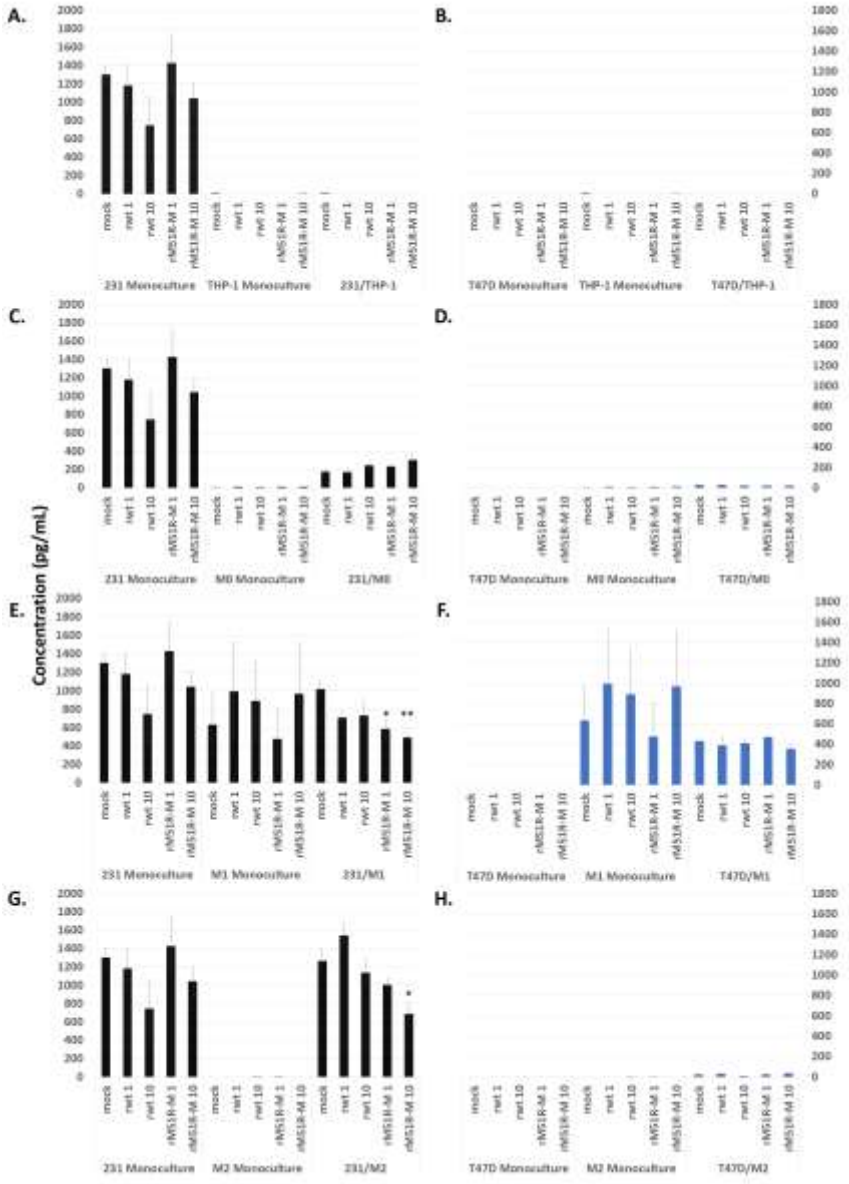

Fig. 14. MDA231, but not T47D breast cancer cells co-cultured with M1 and M2 macrophages showed significant decreases in IL-10 secretion upon infection by rM51R-M. THP-1 monocytes (A, B) and pre-polarized M0 (C, D), M1 (E, F), and M2 (G, H) macrophages were co-cultured at 1:1 ratio with MDA231 (A, C, E, G) and T47D (B, D, F, H) breast cancer cells for 14 hours after which cultures were infected with rwt and rM51R-M viruses at MOIs of 1 and 10 pfu/cell for 24 h. Culture supernatants were analyzed for secreted IL-10 by ELISA. Data are depicted as the average and standard error of three, independent experiments performed in duplicate. and analyzed by Student's T-test compared to mock-infected co-cultures. ($p < 0.05$ is indicated by *; $p < 0.01$ is indicated by **).

a.

Cytokine	Virus	rwt		rM51R-M	
	Mock	MOI 1	MOI 10	MOI 1	MOI 10
IL-6	0%	-13%	-2%	+7%	+5%
TNFα	0%	-48%	-41%	-51%	-71%
IL-10	0%	-100%	-100%	-100%	-100%

b.

Cytokine	Virus	rwt		rM51R-M	
	Mock	MOI 1	MOI 10	MOI 1	MOI 10
IL-6	0%	-15%	-17%	-20%	-10%
TNFα	0%	+9%	+26%	+92%	+82%
IL-10	0%	-2%	+40%	+31%	+72%

c.

Cytokine	Virus	rwt		rM51R-M	
	Mock	MOI 1	MOI 10	MOI 1	MOI 10
IL-6	0%	+20%	0%	0%	0%
TNFα	0%	-20%	-20%	-13%	-41%
IL-10	0%	-30%	-28%	-43%*	-52%**

d.

Cytokine	Virus	rwt		rM51R-M	
	Mock	MOI 1	MOI 10	MOI 1	MOI 10
IL-6	0%	-32%	-22%	-18%	-43%
TNFα	0%	-31%	-52%	-65%	+200%*
IL-10	0%	+22%	-11%	-21%	-46%*

Fig. 15. Summary of MDA231 co-culture cytokine data. Co-cultures of MDA231 cells with (A)THP-1 monocytes, (B) M0, (C) M1, and (D) M2 macrophages were infected with rwt or rM51R-M viruses and analyzed for secretion of IL-6, TNFa and IL-10 by ELISA. Changes in cytokine levels (pg/mL) following virus infection relative to mock-infected cells were determined. Data are the average of 3 experiments and analyzed by Student's T-test compared to mock-infected co-cultures. ($p < 0.05$ is indicated by *; $p < 0.01$ is indicated by **).

a.

Cytokine	Virus	rwt		rM51R-M	
	Mock	MOI 1	MOI 10	MOI 1	MOI 10
IL-6	0%	-25%	+57%	+25%	-34%
TNFα	0%	-9%	-5%	0%	-21%
IL-10	0%	n/a	n/a	n/a	n/a

b.

Cytokine	Virus	rwt		rM51R-M	
	Mock	MOI 1	MOI 10	MOI 1	MOI 10
IL-6	0%	-11%	+12%	-11%	+13%
TNFα	0%	-24%	+14%	-9%	0%
IL-10	0%	-9%	24%	-33%	-33%

c.

Cytokine	Virus	rwt		rM51R-M	
	Mock	MOI 1	MOI 10	MOI 1	MOI 10
IL-6	0%	-18%	-18%	-33%	+28%
TNFα	0%	-12%	-20%	+220%*	+17%
IL-10	0%	-10%	+10%	-10%	-18%

d.

Cytokine	Virus	rwt		rM51R-M	
	Mock	MOI 1	MOI 10	MOI 1	MOI 10
IL-6	0%	-8%	-12%	-21%	-13%
TNFα	0%	-8%	0%	+26%	+3%
IL-10	0%	+23%	-58%	-3%	41%

Fig. 16- Summary of T47D co-culture cytokine data. Co-cultures of T47D cells with (A)THP-1 monocytes, (B) M0, (C), M1, and (D) M2 macrophages were infected with rwt or rM51R-M viruses and analyzed for secretion of IL-6, TNFα and IL-10 by ELISA. Changes in cytokine levels (pg/mL) following virus infection relative to mock-infected cells were determined. Data are the average of 3 experiments and analyzed by Student's T-test compared to mock-infected co-cultures. ($p < 0.05$ is indicated by *; $p < 0.01$ is indicated by **).

4. Discussion

The basis of oncolytic virotherapy with VSV relies upon tumor cells harboring defects in their IFN signaling pathways that accumulate as a result of their transformation to malignancy (Jhwar et al., 2017). Such defects in the antiviral response pathways in tumor cells render them sensitive to infection with VSV and subsequent activation of apoptotic pathways leading to cell death. Data shown here shows that upon infection with both rwt and rM51R-M viruses, MDA231 breast cancer cells were susceptible to the cytopathic effects of VSV infection, suggesting that this cell line may have defects in its ability to mount an appropriate type I IFN antiviral response (Fig. 11A). Furthermore, they were differentially responsive to VSV as indicated by in their variable ability to produce the cytokines, IL-6, TNFα and IL-10 following infection with VSV (Fig. 12A, C, E, and G; Fig. 13A, C, E, and G; Fig. 14A, C, E, and G). Specifically, MDA231 cells produced high levels of IL-6 and TNFα, which were unaffected by infection with VSV. Conversely, T47D breast cancer cells showed less reductions in cell viability upon infection with rwt and rM51R-M viruses, indicating they are at least partially resistant to the cytopathic effects induced by these strains. In addition, T47D monocultures were induced to secrete the pro-inflammatory cytokines IL-6 and TNFα upon infection with the immunogenic rM51R-M virus, but not the immunosuppressing rwt virus (Fig. 12B, D, F, and H; Fig. 13B, D, F, and H). Taken together, the results indicate that T47D breast cancer cells likely retain their IFN antiviral response, conferring resistance to the effects of virus infection. Future experiments should confirm cell line susceptibility with the use of VSV-GFP and viral titer experiments.

Our results show that M1 and M0 macrophages secreted moderate to high levels of the pro-inflammatory cytokines IL-6 and TNFα and moderate levels of IL-10, while monocytes and M2 macrophages secreted background levels of IL-10 (Fig. 8). These results partially confirm the respective phenotypes of each of the populations. Previous work showed that M1 macrophages secrete high levels of IL-6 and TNFα, that M0 macrophages secrete moderate amounts, while monocytes and M2 macrophages do not secrete these cytokines and this was confirmed by our data (Abdullah et al.,

2015; Montavani et al., 2004). Contrary to the literature, however, M2 macrophages did not exhibit robust IL-10 secretion, but M1 macrophages did. It is interesting to speculate as to why this pattern occurred. Baseler and co-workers showed that in order to meet the high energy demands dictated by LPS-induced glycolysis, M1 macrophages secrete IL-10 (Baseler et al., 2016). Polarized to an M1 phenotype by exposure to LPS, the mouse bone marrow-derived macrophages in this study secreted about 3200 pg/mL TNFα, confirming their M1 polarization status, and about 1100 pg/mL IL-10. Therefore, it is possible that the THP-1 M1 macrophages used here also upregulate their production of IL-10 in order to dampen LPS-induced inflammation and thus achieve metabolic homeostasis. On the other hand, all M2 macrophages should secrete IL-10, and the M2 macrophages here did not. Some studies show that longer incubation with IL-4 and IL-13 may be necessary to fully polarize an M0 to an M2 macrophage and that THP-1 monocytes may not adequately mimic M2a macrophages (those polarized by IL-4 and IL-13) (Shiratori et al., 2017). Further, while it was not expected that M0 macrophages should secrete as much pro-inflammatory cytokines as the M1 macrophages in this study, some protocols indicate that PMA alone is enough to polarize THP-1 monocytes to an M1-like phenotype (Tedesco et al., 2018). It is also possible that THP-1 stocks were harboring mycoplasma infection or were in some other way contaminated, and thus not as responsive to polarizing cytokines.

Data presented here shows that THP-1 monocytes upregulate the secretion of the pro-inflammatory cytokines IL-6 and TNFα upon infection with the immunogenic rM51R-M virus, but not the immunosuppressing rwt virus (Fig. 12A and B; Fig. 13A and B; Fig. 14A and B). Together with previous data showing the THP-1 monocytes upregulate the M1-marker pSTAT1 upon infection with rM51R-M, this data verifies that rM51R-M may be capable of inducing M1-like functioning in naïve THP-1 monocytes (Polzin, 2017). Similar results were seen for M2 pro-tumor macrophages, suggesting that viruses such as rM51R-M virus may be used to re-wire the functioning of macrophages within the tumor microenvironment. The phenotypic plasticity of macrophages is well documented and has thus made macrophages attractive targets for cancer therapies (Biswas and Mantovani, 2010). Further,

studies have successfully induced repolarization in primary, model, and tumor-educated macrophages through the use of viral agents. For example, primary M2 polarized murine bone marrow derived macrophages that were exposed to IFNγ were successfully repolarized to an M1 phenotype, as evidenced by increased IL-6 and TNFα and decreased IL-10 secretion (Stout et al., 2005). Similarly, Mylonas and co-workers demonstrated that macrophage repolarization occurs *in vivo* (Mylonas et al., 2009). Researchers implanted mice with adult nematodes to induce M2 polarization of macrophages, which was confirmed through the upregulation of IL-4 dependent markers like Arg-1. The macrophages were then exposed to LPS and IFNγ, and began to demonstrate NO activity, confirming a partial repolarization to an M1 phenotype.

Other researchers have demonstrated the efficacy on oncolytic viruses in TAM repolarization. For example, Masemann and co-workers used oncolytic influenza to promote the destruction of non-small-cell lung cancer (NSCLC) tumors and demonstrated that the effect was partially due to the virus' modulatory effects on alveolar macrophages (Masemann et al., 2018). NSCLC tumors promote an anti-inflammatory TME by promoting M2 polarization in their TAM population. Infection with the oncolytic influenza virus resulted in the repolarization of these TAMs to an M1 phenotype as evidenced by increased MHCII and iNOS expression and increased virus in the alveolar macrophages of tumor bearing mice after infection vs uninfected mice. Similarly, Tan and co-workers co-cultured MDA231 breast cancer cells with human monocyte-derived macrophages and infected cultures with oncolytic measles and mumps viruses (Tan et al., 2016). Treatment with both viruses resulted in MDA231 cell killing, which was enhanced in the presence of these macrophages. The increased therapeutic efficacy was determined to be due to increased macrophage associated tumoricidal mediators, such as TNFα and NO, which occurred regardless of initial macrophage polarization state. This indicates that human monocyte-derived macrophages in this system are induced to behave in an M1, anti-tumor manner upon infection with measles and mumps viruses.

One of the main goals for our therapies is to enable VSV to modulate the phenotype of M2 macrophages to more M1-like states. In our study, THP-1 derived M2 macrophages in our study were induced to secrete the pro-inflammatory cytokine TNFα upon infection with rM51R-M virus (Fig. 13G). The lack of any observed significance with the cytokine IL-6 (Fig. 12) could be due to the susceptibility of M2 macrophages to killing by VSV, thus preventing M2 macrophages the opportunity to more fully express inflammatory cytokines (Polzin, 2017). As evidence of this possibility, we observed that the more cytopathic rwt virus was not able to induce expression of these cytokines. Taken with previous data showing that M2 macrophages infected by rM51R-M upregulate the M1 marker pSTAT1 (Polzin, 2017), it is possible that surviving M2 macrophage could strongly upregulate the production of IL-6 and TNFα, repolarizing them to an M1 phenotype. To confirm this hypothesis, future microscopy or flow cytometry studies will determine which cells in the culture are infected with VSV, which are undergoing cell death, and which express markers specific to M1 macrophages.

Our co-culture studies allowed us to start analyzing the complex interactions between cancer cells and different macrophage populations, as well as between virus and the tumor microenvironment. MDA231 breast cancer cells and THP-1 monocytes and M0 macrophages infected with VSV did not significantly alter their cytokine secretion profiles. This is likely because MDA231 breast cancer cells, THP-1 monocytes, and M0 macrophages are susceptible to VSV-induced cytopathic effects. However, infection of MDA231/M1 co-cultures with rM51R-M virus resulted in a significant decrease in IL-10 levels. Since M1 macrophages are already stimulated to express pro-inflammatory and anti-viral factors (Martinez and Gordon, 2014), it is likely that infection with the immunogenic rM51R-M virus does not increase their secretion of proinflammatory cytokines but may dampen the secretion of anti-inflammatory IL-10. Most interestingly, rM51R-M virus was able to induce TNFα secretion in co-cultures of MDA231 breast cancer cells and M2 macrophages, while decreasing levels of IL-10 (Fig. 13G; Fig. 14G). IL-10 is a potent immunosuppressive cytokine. When abundant in the TME, tumors may evade detection and destruction by immune cells due to the ability of IL-10 to dampen the secretion

of IL-6 and TNFα and thus these functions in these cells (Sheikhpour et al, 2018). Decreases in IL-10 levels in the TME could result in a modulation away from this immunosuppression and towards activation of immune cells, including TAMs to an anti-tumor state. Further, TNFα is a known macrophage-associated anti-tumor mediator, the secretion of which results in the induction of cell death mechanisms in tumor cells (Nishara et al., 1995). TNFα also inhibits the generation of tumor-promoting M2-like TAMs (Kratochvill et al.ref, 2015). Since M2 TAMs in this system are being induced to secrete more TNFα, we can hypothesize that they are potentially being polarized away from an M2 phenotype and gaining M1 functioning, specifically the ability to induce cell death in MDA231 cells. Together, the data suggest that rM51R-M virus has the capacity to modulate M2-like, MDA231-educated TAMs to become more pro-inflammatory (M1-like), further lending evidence to our phenotypic switch hypothesis.

An unexpected result we observed was that even though co-cultures of T47D breast cancer cells and THP-1 monocytes, M0 macrophages, and M2 macrophages secreted both IL-6 and TNFα, none of these cell types secreted these cytokines in monoculture (Fig. 10). There are two possible explanations for this data. Firstly, it could be that T47D cells in co-culture with THP-1 monocytes and macrophages acquire a more invasive phenotype, as evidenced by these cells acquiring a MDA231-like cytokine profile. Alternatively, it could be that T47D breast cancer cells may be immunogenic to monocytes, inducing them to become pro-inflammatory and M1-like. In addition, co-cultures of T47D breast cancer cells and THP-1 monocytes and all macrophage subtypes did not significantly alter their secretion of cytokines following infection with either rwt or rM51R-M viruses (Fig. 13B, D, F, and H; Fig. 14B, D, F, and H; Fig. 15B, D, F, and H). These results suggest that monocytes or macrophage populations may be upregulating their antiviral responses upon exposure to T47D breast cancer cells, again leading to more M1-like phenotypes. This may also explain why hormone receptor positive breast cancers, as exemplified by the T47D cell line, have better patient prognoses, even though they have TAMs (Mota et al., 2017). Specifically, macrophages seem to respond to these cells by upregulating M1-like

functions, leading to increased immune surveillance, recognition, and destruction of such breast tumors. Such breast cancer cells would not be able to survive circulation in the blood stream or away from the primary tumor site, preventing the establishment of disseminated tumors.

In summary, we show that rM51R-M virus was able to alter the cytokine secretion profile of MDA231 breast cancer cells co-cultured with M2 macrophages, such that we observed significant increases in the levels of TNFα and decreases in the levels of IL-10 (Fig. 16). This suggests that rM51R-M virus has the capacity to induce changes to M2 pro-tumor macrophages and convert them to an anti-tumor, pro-inflammatory state. Although we have not conclusively determined whether these changes induce the complete conversion of M2 macrophages to an M1-like state, all evidence thus far suggests that possibility. Therefore, our data support the propriety of targeting M2-like TAMs to become more M1-like as a therapeutic option for metastatic breast cancer. In contrast to rM51R-M virus, rwt virus, with its ability to kill M2 macrophages, does not induce these changes, most likely due to its ability to inhibit host gene expression. This does not mean that rwt virus would not be a viable option for oncolytic therapies, but given the safer nature of rM51R-M virus, as well as its ability to stimulate innate immunity, it may be the preferred viral agent in this case. Another important result that we obtained from this study is that co-culturing non-invasive T47D breast cancer cell line with THP-1 monocytes and macrophages induces an anti-tumor, pro-inflammatory cytokine secretion profile which is resistant to the effects of virus infection. This suggests that targeting M2-like invasive breast TAM populations to facilitate TAM repolarization could help inhibit the metastasis of such tumors. Wound-healing assays of these co-cultures should be investigated to verify whether T47D and MDA231 breast cancer cell and THP-1 monocyte and pre-polarized macrophage co-cultures show invasive behavior and whether infection by rwt and rM51R-M inhibits the invasive behavior of these cells. Future studies will focus on further confirming this repolarization process and determining the mechanisms by which each cell type in co-culture responds to infection with rwt and rM51R-M viruses.

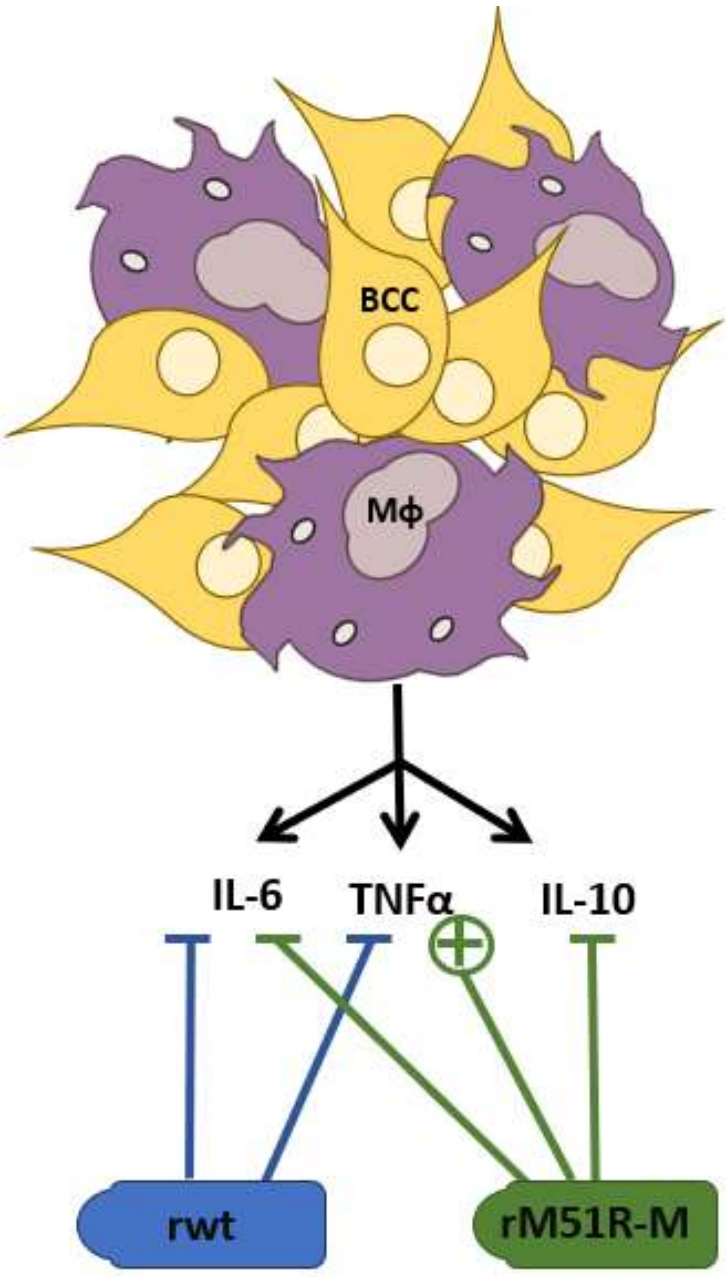

Fig. 16. Infection of co-cultures of MDA-MB-231 breast cancer cells and THP-1 derived M2 macrophages by rM51R-M, but not rwt, amplifies the production of TNFα and inhibits the secretion of IL-10. The modulation of the TME by rM51R-M is likely due to its ability to stimulate an anti-viral response in infected M2 macrophages. Infection by rwt results in non-significant decreases in IL-6 and TNFα so its TME modulatory effects are most likely due to the virus's cytotoxic effects.

Breast Cancer Monocultures	Comparison		Cytokine			Comparison		Cytokine		
			IL-6	TNFα	IL-10			IL-6	TNFα	IL-10
	T47D monoculture	Mock	#DIV/0!	#DIV/0!	#DIV/0!	MDA231 monoculture	Mock	1.000	1.000	1.000
		rwt 1	#DIV/0!	#DIV/0!	#DIV/0!		rwt 1	0.245	0.026	0.664
		rwt 10	#DIV/0!	#DIV/0!	#DIV/0!		rwt 10	0.847	0.486	0.178
		rM51R-M 1	0.025	0.089	#DIV/0!		rM51R-M 1	0.454	0.540	0.736
		rM51R-M 10	8.96E-05	0.021	#DIV/0!		rM51R-M 10	0.307	0.201	0.275

Macrophage Monocultures	Comparison		Cytokine			Comparison		Cytokine			Comparison		Cytokine			Comparison		Cytokine		
			IL-6	TNFα	IL-10			IL-6	TNFα	IL-10			IL-6	TNFα	IL-10			IL-6	TNFα	IL-10
	M1 macrophage monoculture	Mock	1.000	1.000	#DIV/0!	M1 macrophage monoculture	Mock	1.000	1.000	1.000	M0 macrophage monoculture	Mock	1.000	1.000	1.000	THP-1 monocyte monoculture	Mock	#DIV/0!	#DIV/0!	1.000
		rwt 1	0.831	0.730	#DIV/0!		rwt 1	0.256	0.826	0.607		rwt 1	0.848	0.666	0.403		rwt 1	#DIV/0!	#DIV/0!	0.134
		rwt 10	0.893	0.383	0.374		rwt 10	0.247	0.408	0.673		rwt 10	0.948	0.586	0.537		rwt 10	#DIV/0!	#DIV/0!	0.127
		rM51R-M 1	0.288	0.101	0.374		rM51R-M 1	0.097	0.227	0.768		rM51R-M 1	0.701	0.712	0.139		rM51R-M 1	0.000	0.250	0.127
		rM51R-M 10	0.436	0.451	#DIV/0!		rM51R-M 10	0.110	0.139	0.640		rM51R-M 10	0.126	0.960	0.015		rM51R-M 10	0.000	0.083	0.191

Breast Cancer Monoculture Compared to Co-cultures	Comparison		Cytokine			Comparison		Cytokine			Comparison		Cytokine			Comparison		Cytokine		
			IL-6	TNFα	IL-10			IL-6	TNFα	IL-10			IL-6	TNFα	IL-10			IL-6	TNFα	IL-10
	T47D Monoculture to T47D/M2 Co-culture	Mock	1.000	1.000	1.000	T47D Monoculture to T47D/M1 Co-culture	Mock	1.000	1.000	1.000	T47D Monoculture to T47D/M0 Co-culture	Mock	1.000	1.000	1.000	T47D Monoculture to T47D/THP-1 Co-culture	Mock	1.000	1.000	#DIV/0!
		rwt 1	0.582	0.887	0.728		rwt 1	0.797	0.655	0.700		rwt 1	0.794	0.667	0.654		rwt 1	0.606	0.696	#DIV/0!
		rwt 10	0.320	0.990	0.356		rwt 10	0.788	0.489	0.787		rwt 10	0.788	0.841	0.208		rwt 10	0.564	0.833	#DIV/0!
		rM51R-M 1	0.162	0.737	0.973		rM51R-M 1	0.665	0.023	0.374		rM51R-M 1	0.772	0.829	0.072		rM51R-M 1	0.808	0.664	#DIV/0!
		rM51R-M 10	0.388	0.967	0.478		rM51R-M 10	0.643	0.650	0.241		rM51R-M 10	0.782	0.886	0.245		rM51R-M 10	0.684	0.174	#DIV/0!

Comparison		Cytokine			Comparison		Cytokine			Comparison		Cytokine			Comparison		Cytokine		
		IL-6	TNFα	IL-10			IL-6	TNFα	IL-10			IL-6	TNFα	IL-10			IL-6	TNFα	IL-10
MDA231 Monoculture to MDA231/M2 Co-culture	Mock	1.000	1.000	1.000	MDA231 Monoculture to MDA231/M1 Co-culture	Mock	1.000	1.000	1.000	MDA231 Monoculture to MDA231/M0 Co-culture	Mock	1.000	1.000	1.000	MDA231 Monoculture to MDA231/THP-1 Co-culture	Mock	1.000	1.000	1.000
	rwt 1	0.354	0.235	0.266		rwt 1	0.398	0.484	0.071		rwt 1	0.519	0.387	0.948		rwt 1	0.582	0.678	0.072
	rwt 10	0.464	0.287	0.576		rwt 10	0.908	0.264	0.192		rwt 10	0.293	0.230	0.345		rwt 10	0.883	0.465	0.072
	rM51R-M 1	0.571	0.056	0.201		rM51R-M 1	0.996	0.530	0.042		rM51R-M 1	0.267	0.098	0.348		rM51R-M 1	0.652	0.363	0.072
	rM51R-M 10	0.106	0.588	0.056		rM51R-M 10	0.952	0.651	0.011		rM51R-M 10	0.467	0.094	0.206		rM51R-M 10	0.613	0.308	0.072

Fig. 17. P-value results of pairwise student's t-tests comparing infected cultures to mock infected cultures. Average concentrations of each cytokine, IL-6, TNFα, and IL-10, of cultures infected with rwt and rM51R-M virus at an M.O.I. of 1 and 10 p.f.u/cell were compared to the averages of mock infected cultures using a Student's t-test. Compared data are the result of three independent experiments.

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Chapter 3

Summary Limitations, and Future Directions

Our recent data suggests that VSV converts M2 macrophages to the more immunogenic, tumor-fighting M1-like profile. MDA231 monocultures secreted IL-6, TNFα, and IL-10, and these cytokine levels were unchanged when the two cell types were cultured together. We observed that rwt virus inhibited both IL-6 and TNFα secretion under co-culture conditions. The rM51R-M virus also inhibited IL-6 secretion under co-culture conditions but enhanced the secretion of TNFα. In contrast, rwt virus had no effect on IL-10 secretion while rM51R-M virus inhibited it under co-culture conditions. Our results suggest that rM51R-M virus may modulate the immunosuppressive cytokine profile of TAMs, potentially allowing for immune surveillance and destruction of the TME and tumor cells.

Our data demonstrates the potential propriety of targeting TAMs as a therapeutic option for metastatic breast cancer. Monocultures of T47D breast cancer cells, THP-1 monocytes, M0 macrophages, and M2 macrophages all showed the induction of pro-inflammatory cytokines IL-6 and TNFα upon infection with VSV, while M1 macrophages did not. Co-cultures of T47D with any monocyte or macrophage subtype showed high concentrations of IL-6 and TNFα compared to monocultures and infection by VSV did not change concentrations significantly. This suggests that TAMs of hormone receptor positive breast cancers such as T47D may be able to recognize abnormalities of such tumors, and upregulate their pro-inflammatory, M1-like functions in response. Therefore, these types of cancers may have a TME that does not promote tumor cell proliferation and survival, angiogenesis and immunosuppression (Gocheva et al., 2010). The immune system is therefore able to control such tumors and keep them from progressing to metastasis.

It stands to reason that since VSV is able to re-program macrophages as well as cancer cells, it would be interesting to determine if the virus has the ability to alter the invasive potential of cancers.

Since invasive cancers are associated with poor patient prognoses, a main area of investigation is the development of therapeutics to target metastatic cancers or prevent cancers from progressing to metastases. In order to investigate whether infection by VSV affects the invasive potential of cells, wound healing assays or Boyden chamber assays could be performed. In a wound healing assay, breast cancer cells and THP-1 monocytes and macrophages would be seeded into a 60mm cell culture dish at a 1:1 ratio and allowed to incubate for 14 hours. A pipette tip would be used to scratch cells of the bottom of the dish and the resulting scratch would be measured. Co-cultures would then be infected by rwt and rM51R-M virus at MOIs of 1 and 10 pfu/cell. 24 hours post infection, the scratch would be re-measured and its width compared to the original width. Since adherent and invasive cell types will migrate across a plate to be near to other cells, these assays would allow us to distinguish the invasive behavior of our cancer cells in conjunction with different populations of macrophages. If VSV inhibits the migratory of these co-cultures, then the width of the scratches in virus infected cultures would be larger than mock infected ones.

In a Boyden chamber assay, two chambers are separated by a membrane. Labeled breast cancer cells and THP-1 monocytes or macrophages would be seeded at a 1:1 ratio into the top chamber containing ECM components and incubated with media containing a chemoattractant in the bottom chamber for 14 hours. Cells would be infected by rwt and rM51R-M virus at an MOI of 1 and 10 pfu/cell. Cells that are able to migrate towards the chemoattractant would be able to pass through to the bottom side of the membrane and could be counted. If VSV inhibited the invasive potential of these co-cultures, then there would be significantly fewer cells on the bottom of the membrane in virus infected co-cultures than mock infected ones. Furthermore, adding virus to this system would give us insight into whether VSV modulates the metastasis-promoting functions of M2 macrophages and provide the basis for moving into an animal model of metastatic breast cancer (Gocheva et al., 2010).

Since this preliminary study suggests that MDA231/M2 macrophage co-cultures alter their TMEs away from a pro-cancer, immunosuppressive state in response to infection by VSV, future

studies are required to confirm the phenotypic changes in VSV-infected M2 macrophages and co-cultures to test the hypothesis that the type I interferon (IFN) antiviral response pathway is involved in this phenotypic switch. Ahmed and co-workers showed that rM51R-M virus, but not rwt virus, induces the maturation of myeloid dendritic cells, and that these cells increase their expression of CD80, CD 86, IL-12, IL-6, and type I IFN (Ahmed et al., 2006). Since macrophages have similar responses to viral infections as DCs, it is reasonable to assess similar markers in macrophages to determine macrophage repolarization to an M1 phenotype.

Supernatants from such monocultures and co-cultures should be subjected to an ELISA protocol assessing changes in the secretion of IFNα and IFNβ, which are produced as a result of VSV infection and subsequent activation of the JAK/STAT pathway. The propriety of this assay is supported by previous data from this lab and elsewhere shows that THP-1 derived M2 macrophages activate STAT1, a known M1 marker, in response to infection by VSV, especially rM51R-M (Polzin 2017, Takeda and Akira 2000). Thus, Western Blot Analysis should be used to assess for the activation of STAT1 in co-cultures of THP-1 derived macrophages and MDA231 and T47D breast cancer cell lines. If IFNα/β secretion and phosphorylated STAT1 levels increase, this would provide strong support for the M2 to M1 phenotypic switch hypothesis (Mantovani et al., 2004; Toshchakov et al., 2002). Additionally, Flow cytometry should be used to measure changes in cell surface marker expression to confirm the phenotypic switch hypothesis, using CD86+CD80+ and CD163+CD206+ for M1 and M2 polarization, respectively (Chávez-Galán et al., 2015; Gordon and Taylor, 2005).

It is also not yet clear which cells are responsible for changes in cytokine secretion, nor which cells are dying as a result of viral infection. The pattern of changes in cytokine secretion of co-cultures of MDA231 and monocytes and macrophages upon viral infection most closely resembles the pattern of changes of the corresponding macrophage type. Thus, we hypothesize that while both MDA231 breast cancer cells and all non-M1 macrophages are susceptible to killing by VSV, it is the macrophages that are responsible for the induction of the type I IFN pathway. To confirm this, future studies require

the use of fluorescent microscopy to determine which cells allow for replication of VSV and which are dying as a result of infection by VSV. MDA231 cells should be labeled using a Fluorescent CellTracker Dye then trypsinized and added to pre-polarized macrophages seeded in a 6-well culture dish and allowed to incubate for 14 hours, then infected by rwt-GFP and rM51R-M-GFP viruses. Propidium iodide should be added to asses for viability and live image fluorescent microscopy images should be taken at appropriate time points post infection, likely 16, 24, and 32 hours. This approach will allow us to determine which cells are supporting VSV replication and which are dying in co-culture conditions. Intracellular cytokine staining would also provide high resolution as to which cells are altering their cytokine production in response to infection by VSV.

If our M2-to-M1 repolarization hypothesis is confirmed and it is established that rM51R-M does inhibit invasion of co-cultures of MDA231 breast cancer cells and M2 macrophages, then animal studies can be considered. An orthotopic 4T1 mouse model would be ideal, since direct implantation of tumors into the mammary fat pad allows for the elucidation of breast tissue-specific interactions with the immune system and responses to infection by rwt and rM51R-M viruses.

In conclusion, our data has shown that infection with VSV potentially induces TAM repolarization from a tumor-promoting M2 phenotype to a tumoricidal M1 phenotype. This research supports further investigation into the use of VSV as a targeted therapeutic for TAMs. Most promising, this research indicates that it is the rM51R-M virus that is responsible for the most promising TAM modulatory effects, thus indicating that the potential of a safe therapeutic option for even late stage patients.

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Vita

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