# INFLUENCE OF ONCOLYTIC VESICULAR STOMATITIS VIRUS ON MACROPHAGE PHAGOCYTOSIS AND BREAST CANCER CELL GROWTH IN COCULTURE

A Thesis
by
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**Abstract**

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Tumor-associated macrophages (TAMs) are both abundant and effective modulators of breast tumor growth and disease progression. TAMs predominantly manifest as the tumor-promoting M2 subtype, but they can also adopt the more classic, antitumor M1 subtype as well. This research sought to investigate the influence of oncolytic vesicular stomatitis virus (VSV) on M1 and M2 macrophage polarization as revealed by their phagocytic behaviors. We also aimed to explore the reciprocal impact of VSV and macrophages on the growth of an aggressive, so-called triple-negative, MDA-MB-231 breast cancer cell line. To achieve these goals, model monocytes from the THP-1 cell line, were induced to differentiate into M1 or M2 macrophages and then subjected alongside the breast cancer cells in coculture to infection with a recombinant wild-type VSV strain (rwt) or an isogenic matrix (M) protein mutant strain (rM51R-M) of VSV. The rM51R-M strain of VSV, in particular, is known for its ability to promote antiviral responses in infected cells and has been previously shown by our lab to convert M2 THP-1 macrophages to an M1-like phenotype. When assessed for their uptake of latex beads, we noticed that M2-THP-1 macrophages exhibit a 6-fold higher phagocytic

capacity compared to their M1 counterparts, and that this increased to a 10-fold advantage when co-cultured with breast cancer cells. However, infection with the rM51R-M virus diminished the phagocytic activity of M2-THP-1 macrophages back to M1 macrophage phagocytic levels. This effect did not solely arise from the cytopathicity of VSV but may have involved a phenotypic shift in M2 macrophages towards M1-like properties. Regarding effects on MDA-MB-231 breast cancer cell growth, we noticed a remarkable inhibition with cocultured M1 THP-1 macrophages while growth continued to increase with M2 macrophages in a pattern unchanged from breast cancer cells in monoculture. We hypothesized that the M2 macrophages would promote breast cancer cell growth or at least protect the breast cancer cells from VSV infection, but infection with either rwt or rM51R-M virus completely neutralized breast cancer cell growth under all experimental conditions. While our coculture study suggested an important ability of rM51R-M virus to reduce M2 macrophage phagocytic activity to that of M1 levels, a possible indication of macrophage repolarization, it failed to reveal whether such a repolarization could enhance the oncolytic features of this virus. This may reflect less than ideal coculture conditions and alternative strategies are discussed.

**Acknowledgments**

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## Dedication

I take immense pride in dedicating this thesis to those who have showered me with love and unwavering support throughout my journey, both in life as a whole and during my academic pursuit. Foremost and with the utmost sincerity, I owe my deepest gratitude to my mother, Paula Watson, whose unshakable belief in me has been the cornerstone of my growth into the person I am today. Her resilience in the face of adversity, surviving cancer to be with me is nothing short of a miracle, and I thank her and the divine for this gift. Her presence in my life fuels my inspiration to delve into the realm of miracles and science, particularly in the context of cancer. Thanks to my mother and the support of my family, I hold an unshakable belief in the potential for miracles. I am driven to contribute to the scientific advancements that can bring hope, longevity, and happiness to those affected by this relentless disease. I also extend my heartfelt gratitude to my dearest friend, Kasey Curlee, whose presence in my home and my heart has been a constant source of profound enthusiasm and optimism. I am eternally grateful for their unwavering support, which has provided solace and encouragement through life's challenges. To all those who have nurtured my heart, mind, and soul, I offer my heartfelt thanks. And in this journey of gratitude, I must also acknowledge myself. Many years ago, I could not have fathomed the possibility of reaching this chapter in my life, but through self-belief, I have surpassed my expectations. It is through my own determination and resilience that I stand here today, and I am thankful for the strength I have found within myself.

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# CHAPTER 1: INTRODUCTION

## *Cancer Defined*

Cancer broadly describes a group of diseases characterized by uncontrolled cell growth. Cancer can occur in virtually any part of the body and can potentially invade nearby tissues or distant anatomic sites. Cancer is second only to heart disease in the number of deaths worldwide (Siegel et al., 2023).

Cancer is a complex and multifaceted disease, but it retains certain fundamental hallmarks (Hanahan, 2022; Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011). These include sustained proliferation, evasion of growth suppressors, resistance to cell death, replicative immortality, angiogenesis, and invasion/metastasis. Sustained proliferation refers to the unrelenting capacity of cancer cells to divide and grow. This occurs because of oncogene activation/overexpression and tumor suppressor gene silencing. When combined with a newfound resistance to cell death and acquisition of replicative immortality, tumors form. Angiogenesis is the process of forming new blood vessels. This allows cancerous tumors to obtain the nutrients and oxygen they need to survive and thrive. Further activation of invasion and metastasis allows cancer cells to spread from their primary site to other parts of the body, leading to systemic disease. Metastasis accounts for much of the morbidity and mortality associated with this disease (Hanahan, 2022; Hanahan & Weinberg, 2000; Hanahan & Weinberg, 2011)

***The Immune System of the Tumor Microenvironment***

The formation of tumors and the impact of cancer on the body is not relegated to cancerous cells alone. The tumor microenvironment (TME) is comprised of a multitude of cell types (endothelial cells, immune cells, fibroblasts) and extracellular components (growth factors, hormones, cytokines, extracellular matrix proteins) that may all play pivotal roles in how cancer is characterized, prognosed, and treated (Hanahan & Weinberg, 2000; Hao et al., 2012) One of the most critical of these cell types are the immune cells. Cancer cells must grow in the presence of a host immune system designed to seek out and destroy them (Ostrand-Rosenberg, 2008; Rosenberg, 2001). Conversely, the tumor microenvironment is often immunosuppressive by design.

There are two arms of the immune system: innate and adaptive. The innate immune system involves epithelial cells, monocytes, macrophages, dendritic cells, natural killer cells, and other populations of lymphocytes (Adam et al., 2003). These cells recognize unique structural features on the surface of infectious agents via the engagement of immune cell surface receptors. Such engagement triggers the release of cytokines that, in turn, encourage a robust immune response designed to recognize, phagocytose, and/or activate cytotoxic effectors that kill the infectious agents. Other cytokines encourage the repair and regrowth of damaged tissue. The adaptive immune system involves specialized populations of B- and T-lymphocytes that use their own unique receptors (*e.g.*, T-cell receptors) to recognize foreign antigens or produce antibodies

that do the same. This recognition leads to the clonal expansion of specific lymphocyte populations. T cells directly engage their targets to induce cytotoxicity. B cell-produced antibodies coat antigens on target cells, and this opsonization stimulates both antibody-dependent cell-mediated cytotoxicity as well as antibody-dependent cellular-phagocytosis by macrophages and neutrophils (Aderem & Underhill, 1999; Afshar-Kharghan, 2017; Cao et al., 2022; Gonzalez et al., 2018; Markiewski & Lambris, 2009).

How the innate and adaptive immune systems respond to foreign agents from viral, bacterial, and fungal infections is similar to how they confront cancer cells. The immune system responds to overexpressed or mutated antigens on the surface of cancerous cells as foreign agents, spurring the same expansive immunoediting response. Clinical data show that when immune cells infiltrate tumors, the cancer cells become dormant or die, and the patient's prognosis improves (Gannon et al., 2009; Jochems & Schlom, 2011, 2011b; Pagès et al., 2005; Piersma et al., 2007; Sehouli et al., 2011). Conversely, tumor cells become clinically detectable and invasive once they have escaped antitumor immunity (Gavalas et al., 2010a; Gui et al., 2014).

There are several common mechanisms by which cancer cells suppress and avoid the immune system. One is through recruitment of regulatory T-cells (Tregs). Tregs ensure limitations to an overly robust immune response by the host. Tumor-cell secreted growth factors like transforming growth factor beta (TGF-β) help create these Tregs from the very helper T-cells designed to fight cancer (Facciabene et al., 2012; Nishikawa

et al., 2005; Vinay et al., 2015; Wang et al., 2017). Another mechanism of immunoevasion is by downregulation of antigen processing and presentation machinery. Targets of downregulation or inactivation include major histocompatibility complex (MHC) proteins, proteasome subunits like latent membrane proteins (LMP)2 and LMP7, and the transporter associated with antigen processing (TAP) (Garrido et al., 1997). All these mechanisms modulate the ability of cytotoxic T lymphocytes to recognize and kill tumor cells. Immunoevasion is now recognized as an emerging cancer hallmark (Hanahan & Weinberg, 2000, 2011).

***The Clinical Relevance of Tumor-Associated Macrophages***

Among the immune cells commonly ushered into the TME, tumor-associated macrophages (TAMs) represent the most abundant (Liu & Zeng, 2012; Ostrand-Rosenberg, 2008; Rosenberg, 2001)). TAMs may sometimes comprise as much as 50% of a tumor's mass (Bolat et al., 2006; Chen et al., 2019; Fu et al., 2020; Röszer, 2015). Cancer cell production of platelet-derived growth factor (PDGF) at constitutively high levels acts as a chemoattractant and mitogen for macrophages. Other chemokines like C-C motif chemokine ligand 2 (CCL2), vascular endothelial growth factor (VEGF), macrophage colony-stimulating factor (CSF-1), and interleukin-10 (IL-10) also promote the infiltration of macrophage populations (Hao et al., 2012). For many forms of cancer, including triple-negative breast cancers that are the subject of this thesis, TAM populations bear significant clinical importance. Not only are TAMs abundant in breast

cancer tissue, but their infiltration increases as a function of tumor size and stage. TAMs can be antitumoral, but it is their potential to promote tumor growth, angiogenesis, and metastasis, as well as resistance to cytotoxic therapies by which they are most notorious (Koebel et al., 2007; Melief, 2007; Moffett et al., 2023; 2008; L. Tang et al., 2023; Willimsky & Blankenstein, 2005). Indeed, the density of infiltrating TAM populations negatively correlates with breast cancer 5-year survival rates (Sousa et al., 2015; J. Yang et al., 2015).

It is not surprising that many newfound cancer-fighting strategies aim to reduce TAM populations (Boutilier & Elsawa, 2021a; Edin et al., 2012; Lamagna et al., 2006; Pantano et al., 2013; Theresa Ferrao et al., 2018; Wynn et al., 2013; Yang et al., 2022; Zhu et al., 2017). For example, one group has used sub-cytotoxic levels of the alkylating anticancer drug trabectedin to reduce the production of pro-inflammatory IL-6 and the chemoattractant CCL2, thereby reducing the number of infiltrating monocytes/macrophages to the TME (Allavena et al., 2005; Gnant et al., 2009). Adding anti-angiogenic drugs, such as VEGF inhibitors, as part of established chemotherapies may also reduce TAM numbers, sometimes resulting in increased progression-free survival (Miles et al., 2010; Miller et al., 2007). Bisphosphonates have also been utilized to target aggressive neoplasms such as some breast and bone cancers. One such bisphosphonate, zoledronate, has been found to attack TAMs in a preclinical setting and works as a suitable macrophage-targeted therapy (Junankar et al., 2015; Li et al., 2022; Zang et al., 2019). When testing the efficacy of antitumor drugs, some researchers have

detailed how TAM depletion enables T-cell infiltration thereby allowing for enhancement of the drug's efficacy (Guo et al., 2016; Li et al., 2022).

### *Macrophage Polarization States*

Macrophage taxonomy aims to classify the numerous functions of these mononuclear cells. The complexity and versatility of these cells are vast, and they are continually adaptive to subtle alterations in their microenvironments. The most discussed polarization states for macrophages are the 'classically-activated' M1 and 'alternatively-activated' M2 subtypes. But M2 macrophage is a blanket term for many macrophage subtypes, including M2a (polarized by interleukin-4 (IL-4) and IL-13; function in tissue repair/regrowth), M2b (polarized by immune complexes; regulate antibody-based immune responses and inflammation), M2c (polarized by IL-10, transforming growth factor-β (TGF-β), and glucocorticoids; anti-inflammatory; phagocytic to apoptotic cells), and M2d (polarized by toll-like receptors; angiogenic) (Genin et al., 2015a; Italiani & Boraschi, 2014; Italiani & Boraschi, 2014; Liu & Zeng, 2012; Yang & Zhang, 2017). While it is acknowledged that these different polarization states exist, the efforts in this study have focused on a simplified polarization regime that has been shown to create distinct M1-like and M2-like populations, so the bulk of this introduction focuses on that dichotomy.

As suggested above, M1 and M2 macrophages are differentiated based on their cell surface markers, secretory profiles, and functions (Gordon & Martinez, 2010; Murray et al., 2014). Pro-inflammatory cytokines like interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) and microbial stimuli like lipopolysaccharides (LPS) polarize macrophages to an M1 phenotype. These macrophages are identified by their inflammatory and microbicidal activities. Their production of reactive oxygen species (ROS) and nitric oxide (NO) helps protect the body from viral and bacterial infections (Liu et al., 2021; Liu et al., 2022; Wang et al., 2014). For these same reasons, M1 macrophages also have tumoricidal activity. Anti-inflammatory cytokines like IL-4 and IL-13 often polarize M2 macrophages. However, M2 macrophages can also be made in response to neurotransmitters and neuroendocrine hormones, apoptotic cells, immune cell signaling, and the damage-associated molecular patterns released from infected cells (Wang et al., 2014). In terms of function, M2 macrophages are recognized for their higher phagocytic ability (as compared to M1 macrophages), their role in dampening inflammatory responses, their immunoregulatory actions, and their promotion of tissue repair and remodeling (Murray et al., 2014; Wynn et al., 2013). Some of these functions have been linked to an acceleration of cancer.

Macrophage polarization refers to the activation state of the cell at a singular point in time. Any research on any macrophage subtype must acknowledge the macrophage's ability to shift into alternate subtypes. Polarization states are not fixed. However, this flux can have promising therapeutic applications. For example, the negative context of

M2 macrophages in certain disease states may be altered by simply changing the environment (*e.g.*, back to M1 macrophage-promoting conditions) to improve patient prognosis (Biswas et al., 2012; Edin et al., 2013; Hagemann et al., 2009; Lewis & Pollard, 2006; Ma et al., 2010; Murray & Wynn, 2011; Welsh et al., 2005; van Wu et al., 2020). This might best be appreciated in the context of cancer. The diverse and malleable polarization states of TAMs present another unique immunotherapeutic approach for cancer.

### *Tumor-Associated Macrophage Functions and Related Therapeutic Strategies*

#### *Phagocytosis*

Macrophages are professional phagocytes. Phagocytosis is the ingestion of materials by cells. Phagocytosis by macrophages is critical for the uptake and degradation of senescent or apoptotic cells, wounded tissue debris, and infectious agents. This crucial function supports development, tissue remodeling, immunity, and inflammation. For immunity, phagocytosis by macrophages not only clears pathogens as part of the innate immune system, but it also presents antigens to adaptive immune system cells for more specific and more robust immune responses.

M2 macrophages are considered to be more competent at phagocytosis than their M1 counterparts (Elliott et al., 2017; Wang et al., 2014). For example, phagocytosis of *E.coli*

particles *in vitro* was higher in M2-like primary macrophages versus M1-like macrophages (Schulz et al., 2019). In fact, both the M2a and M2c macrophage subtypes had a significantly higher phagocytic affinity for *E.coli* particles than M1 macrophages (15% and 40% relative to 7% of particles consumed, respectively) (Mendoza-Coronel & Ortega, 2017). In contrast, this research group also showed that M1-like macrophages had a higher ROS production rate than the M2 subtype. This aligns with the rationale that M1 macrophages eliminate pathogens via ROS deployment, whereas M2 phenotypes primarily utilize phagocytosis for clearing cells and particulates in their immediate environment (Boutilier & Elsawa, 2021b; Jaggi et al., 2020; Mosser et al., 2021; Röszer, 2015; Zhang et al., 2016).

The literature describing macrophage-mediated phagocytosis of cancer cells reveals more complicated information about the M1 and M2 subtypes. As professional phagocytes, macrophages have the potential to engulf and digest cancer cells in a manner like that of infectious agents. For example, Cao and coworkers showed that in response to the chemotherapeutic drug paclitaxel not only was an M1-polarization state established (upregulation of the M1 marker MHCII and downregulation of the M2 marker CD206), but that it was primarily the M1 macrophages that phagocytosed the cancer cells, including both human colorectal and breast cancer cell lines (Cao et al., 2022). In another study, researchers used a therapeutic anti-CD47 antibody to disengage CD47-overexpressing cancer cells from signal regulatory protein alpha (SIRP-α)-expressing macrophages, thereby freeing up the latter for cancer cell phagocytosis

(Jaiswal et al., 2009; Tian et al., 2022; Tseng et al., 2013). It was under these conditions that the M1 phenotype was also far more phagocytic than the M2 one (Zhang et al., 2016). Finally, in a therapeutic study focusing on a M2 macrophage-specific receptor for phagocytosis called the mannose receptor (CD206 a protein expressed predominantly by M2 macrophages, the researchers introduced a synthetic peptide (RP-182) that induced a conformational switch in CD206 and caused an M2 to M1 phenotypic change, thereby allowing M1 macrophages to phagocytose the cancer cells (Jaynes et al., 2020).

It is studies like these that lend support for the antitumor profile of M1 macrophages. Perhaps to understand more fully how the phagocytic capacity of TAMS specifically impacts cancer, it is crucial to characterize the mechanisms by which phagocytosis occurs. Phagocytes recognize and differentiate between a heterogeneous number of ligands expressed on a target cell's surface (Mosser et al., 2021). This recognition occurs through dedicated receptors that initiate particle internalization and increase the phagocytic rate (Aderem & Underhill, 1999; Gavalas et al., 2010a; Hess et al., 2009; Leidi et al., 2009; Markiewski & Lambris, 2009; Pio et al., 2014; Rőszer et al., 2011)Two common receptors for this purpose are the Fc gamma receptors and the complement receptors.

Fc gamma receptors (FcyRs) specific for the immunoglobin G (IgG) class of antibodies are commonly involved in phagocytosis during the adaptive immune response and are among the most well-studied phagocytic signaling mechanisms (Clynes et al., 1998;

Daëron, 1997; Strzelecka et al., 1997). They function as part of the antibody-dependent cellular phagocytosis (ADCP) of opsonized particles mentioned before. Interestingly, ADCP has excellent clinical efficacy in cancer, and many immunotherapies promote this activity (Cao et al., 2022; Kong et al., 2022; Tian et al., 2022; Tseng et al., 2013; Van Bommel et al., 2018). However, when Liedi and coworkers studied the phagocytosis of B-chronic lymphocytic leukemia cells pre-treated with chemotherapeutic Rituximab antibodies, they noticed that M2 macrophages (polarized with CSF-1) were more efficient phagocytes than M1 macrophages (polarized with granulocyte-macrophage stimulating factor (GM-CSF), LPS, and IFN-γ) (Leidi et al., 2009). This phagocytosis by M2 macrophages was further enhanced by IL-10 treatment (M2c polarization) but inhibited by IL-4 treatment (M2a polarization) suggesting that there may be nuances in the phagocytosis of cancer cells even among different M2 macrophage subtypes, much as there exists for the phagocytosis of *E. coli* particles (Leidi et al., 2009; Mendoza-Coronel & Ortega, 2017).

A second mechanism by which macrophages perform phagocytosis is via complement receptors (CRs). CRs bind to complement factors that become associated with pathogen-associated molecular patterns (PAMPs) or to antibody-bound antigens on the surface of infectious agents or damaged cells. CR-mediated phagocytosis is considered pro-inflammatory in macrophages (Acharya et al., 2020). Despite this, one recent study showed that M1 and M2 murine macrophages differentially express CRs, including CR1/2 for the M1 macrophages and CR4 for the M2 macrophages, and that both subtypes use

them to engage with complement components to mediate phagocytosis (Ghate et al., 2021). However, Fraser and colleagues demonstrated that the complement factor C1q stimulated human monocytes and mouse microglia to reduce production of pro-inflammatory cytokines and increase production of the anti-inflammatory cytokine IL-10, as well as other markers of M2 polarization (Benoit et al., 2012; Fraser et al., 2009). So while M1 and M2 macrophages mediate CR-dependent phagocytosis, there are conditions by which complement factors may promote M2 macrophage polarization and thereby promote phagocytosis by M2 macrophages as well.

In sum, it seems clear that the phagocytosis of cancer cells by macrophages is an important part of antitumor immunity. But while it may seem like this would be a prevailing feature of anticancer M1 macrophages, that is not always the case. For example, M2 macrophages are considered better phagocytes than M1 macrophages, and M2 macrophages, which are considered to have a pro-tumor phenotype, may even phagocytose cancer cells under certain conditions.

*Antigen Presentation*

Antigen presentation is the process by which certain immune cells, upon phagocytosis of pathogens, display the foreign molecules on their surface in concert with MHCI or MHCII molecules for recognition by the adaptive immune system, and particularly for T cells. In the case of cancer, antigen presentation plays a critical role in anticancer immunity

(Mpakali & Stratikos, 2021). Macrophages are an antigen-presenting cell type. As antigen presentation is a pathological response to an infection, it is perhaps unsurprising that it is associated with M2-to-M1 polarization, including an upregulation of MHCII molecules and the proinflammatory cytokines IL-12 and IL-23 (Mills & Ley, 2014). However, there are cases where antigen presentation is carried out by M2 macrophages as well, or by a mix of both macrophage subtypes (Barrio et al., 2012; Mills & Ley, 2014; Mpakali & Stratikos, 2021). It may also be an activity exhibited by TAMs, though the actual subtype involved in that process was not determined (Abès et al., 2010; Asano et al., 2011; Barrio et al., 2012; Gül et al., 2014; Madden et al., 1993; Mpakali & Stratikos, 2021; Muraoka et al., 2019; Wieczorek et al., 2017; Yuen et al., 2020).

*Inflammation*

Inflammation is the biological process by which the body seeks to 'heal' damaged or infected tissue. Inflammation is controlled by monocyte/macrophage populations. These cells migrate to areas of injury and polarize to various phenotypes that serve to fight infection and heal wounds. In doing so, they also release growth factors and cytokines that can drastically alter the local tissue microenvironment (Coussens & Werb, 2002; Mantovani et al., 2008). As mentioned prior, TAMs are common to most cancers. Here, they also impact the inflammatory state, and that, in turn, controls everything from carcinogenesis to angiogenesis. In brief, classically activated M1-macrophages are identified by their pro-inflammatory cytokine secretions (IL-1B, IL-6, IL-12, IL-23, and

TNF-α) and have been reported to have high tumoricidal capacity. As indicated before, M1 macrophages are cytotoxic to tumor cells by releasing ROS and NO (Bernsmeier et al., 2020; Bruns et al., 2015; Kennel & Greten, 2021) and they have a direct role in cytolysis via antibody-dependent cellular phagocytosis (Genin et al., 2015; Ma et al., 2010; Porcheray et al., 2005; Rautela et al., 2018). Thus, M1-type TAMs, as immune cells, would significantly limit tumor growth and disease progression (Franklin et al., 2014; Guo et al., 2016; Lewis & Pollard, 2006b; Salmaninejad et al., 2019). M2 macrophages, alternatively, have an anti-inflammatory phenotype and function to scavenge debris and promote tissue repair. The consensus regarding most TAM populations is that they worsen cancer patient prognosis and survival because they exhibit an M2 phenotype (Muraoka et al., 2019)). This can be appreciated through a study of the THP-1 monocyte cell line as a model for macrophage differentiation and polarization, and the effects of such on the non-invasive MCF-7 and the invasive MDA-MB-231 breast cancer cell lines. Here, M2 polarized macrophages supported MCF-7 breast cancer cells by providing a favorable outgrowth environment, whereas M1 macrophages promoted cellular dormancy while in co-culture (Yang et al., 2016). Highly invasive and mesenchymal MDA-MB-231 cells also experienced attenuation when co-cultured with M1 macrophages, some presenting a mesenchymal-epithelial morphological shift and thereby a more differentiated phenotype.

The relationship between inflammation, cancer, and macrophage phenotype does not always comply with the aforementioned M1/M2 paradigm. For example, M1

macrophages frequently secrete pro-inflammatory cytokines (e.g., IL-6 and TNF-α) that can, in turn, activate nuclear factor-(NF)-κB signaling, a strong promoter of inflammation-associated mutations leading to cancers (Mantovani et al., 2008; Biswas et al., 2006; Pikarsky et al., 2004; Tak & Firestein, 2001). Chronic inflammation is now considered an emerging hallmark of carcinogenesis and tumor promotion (Hanahan & Weinberg, 2000, 2011).

*Growth, Angiogenesis, and Metastasis*

It has long been suggested that TAMs promote tumor growth, angiogenesis, and metastasis, and that this is associated with poor patient prognosis (Asano et al., 2011; Chen et al., 2017; Edin et al., 2012; Jochems & Schlom, 2011; Kawai et al., 2008). Among the growth factors produced by M2 macrophages, vascular endothelial growth factor (VEGF) is noteworthy for its function as a potent mitogenic stimulant for endothelial cells. This leads to the angiogenic switch that significantly increases intertumoral microvessel density (Bolat et al., 2006; Coussens et al., 2000; Min et al., 2021). TAMs also promote vascular growth through TGF-β and platelet-derived growth factor (PDGF) production. TGF-β also supports the epithelial-mesenchymal transition (EMT) (Chen et al., 2019; Coussens & Werb, 1996; Zeng et al., 2019). During an EMT, cells lose their adherence to other cells, their polarity, and become motile. While this transition is vital to wound healing and tissue development, cancer cells utilize it to increase their invasive/metastatic potential. PDGF, in turn, can stimulate fibroblast growth, which

creates a supportive extracellular matrix that also promotes tumor growth and invasion. Apart from platelets, TAMs are the only other cells of blood origin capable of producing PDGF (Uutela et al., 2004). There is one study showing that M1 macrophages can stimulate EMTs. Established in a study with THP-1-derived macrophages of the M1 phenotype, Bednarczyk and coworkers showed that many factors associated with the M1 phenotype, such as NF-κB, SNAIL, SLUG, vimentin, and β-actin, could all alter breast cancer cell morphology, increasing their migration, and favoring metastasis (Bednarczyk et al., 2018). Other growth factors and cytokines like IL-8, IL-1B, IL-6, TGF-β, TNF-α, and exosomes have also been implicated in the same detrimental EMT process supported by M1-type macrophages. Biswas and associates also demonstrated that the inflammatory M1 macrophage phenotype of ovarian cancers may support tumor metastasis. In this case, M1-conditioned media, when applied to the ovarian cancer cells, promoted tumor cell migration, whereas the M2-conditioned media did not (Biswas et al., 2006b). We can therefore see that even the historically beneficial M1 macrophage phenotype may support carcinogenesis and perhaps metastasis under certain conditions (Cohen et al., 2015; Danlos et al., 2023; Greten et al., 2004).

***Oncolytic Virotherapy***

Ongoing research, including clinical trials, continuously broadens our understanding of the TME's role in therapeutic strategies. Traditional cancer treatments include radiation and chemotherapy. Radiation therapy distributes ionizing radiation to localized tumor

sites and depends heavily on the accumulation of deleterious mutations within the cancerous tissue. Chemotherapy involves the distribution of systemically delivered anticancer drugs that induce cytotoxicity. Both approaches may have high toxicities and lower efficacy against metastatic disease and drug-resistant cancers. (Baskar et al., 2012; Ohuchida et al., 2004; Tang et al., 2023). Since one of the hallmarks of cancer is immunosuppression, there has been a movement towards immunotherapeutic strategies for cancer treatment. One promising immune-based therapy lies in oncolytic viruses (OV)—*i.e.*, oncolytic virotherapy (OVT) (Apolonio et al., 2021). OVTs are based on viruses with innate or bioengineered properties that enable infection of and replication within cancer cells, and selectively over healthy cells. These therapies primarily rely on the direct lysis of cancer cells following infection, but in recent years, there has been increasing interest in the immunomodulatory effects of OVs. TAMs are of particular interest in this regard as OVs may alter their presence and/or phenotype, thereby converting an immunosuppressed TME into an immunostimulatory one (Chaurasiya et al., 2018; Hofman et al., 2021; De Matos et al., 2020; Prestwich et al., 2008). When OVs infect tumor cells, lysis ensues, releasing new infectious viral progeny. Other 'danger signals' are also released, including tumor cell debris, tumor-associated antigens (TAAs), and both pathogen-associated and damage-associated molecular patterns (Bai et al., 2019; Davola & Mossman, 2019; Harrington et al., 2010; De Matos et al., 2020; Peng et al., 2019). These, in turn, stimulate the innate and adaptive immune systems, including TAMs, which will locally respond within the TME.

### *Macrophages as a Barrier to OVTs*

There are several ways that macrophages respond to OVs. First, they may pose a barrier to the virus by quickly clearing virus particles and virally infected cells from the body. In 2018, the FDA approved a herpes simplex virus (HSV)-based OVT (oHSV-1) to treat melanoma after successful clinical trials. Delwar and coworkers wanted to see if patients with glioblastoma multiforme (GBM) might also be suitable for this type of therapy (Delwar et al., 2018). The answer was yes, but only with cancer cells in isolation. Microglia/macrophages hindered the oncolytic efficacy of oHSV-1 against U87 human GBM cells. Indeed, the dose-dependent inhibition of viral titers with the addition of microglia was attributed to an internalization of the virus via phagocytosis in such a way as to block further viral infections. Given such results, several groups have explored macrophage depletion as a means of increasing virus delivery (Galmbacher et al., 2010; Shashkova et al., 2008). This aligns with cancer immunotherapy methods that seek a similar depletion scheme for high abundance, M2-type TAMs (Jakeman et al., 2015; Morahan et al., 1985; Shashkova et al., 2008; Sinclair & Sissons, 1996). This approach would decrease a cell type that supports tumorigenesis while also stimulating a more robust viral infection.

***Macrophages as a Target of OVTs***

Rather than a barrier, several OVTs have seen changes in macrophage polarization during an infection. One group has developed HSV as an effective OV by incorporating a transgene expressing a full-length CD47 antibody (OV-αCD47-G1) (Tian et al., 2022). CD47 is often called a 'don't eat me' signal. When expressed on the surface of cancer cells, its binding to SIRPα on macrophages suppresses phagocytosis. Here, the researchers showed that infection of A2780 human ovarian cancer cells with the engineered virus led to a nearly 10-fold increase in macrophage-mediated phagocytosis over a control virus, thus providing proof-of-principle in their studies. *In vivo,* they found that OV-aCD47-G1 prolonged mouse survival by inhibiting tumor progression. They also discovered that the expression of CD86 (a marker for M1 macrophages) increased within the TME, while CD206 (a marker for M2 macrophages) decreased. There was also a dramatic increase in the transcription of macrophage cytokine genes, such as IL-1b, IL-6, IL-10, IL-12A, and the nitric oxide synthase enzyme NOS2, all of which are markers for the presence of M1 macrophages. This suggests that the engineered virus might also have the potential to modulate TAM phenotypes (Tian et al., 2022).

Another group using oncolytic herpes simplex virus 2 (HSV-2) explored the effects of a combination therapy involving the virus (OH2) and anti-SIRPα in a mouse colon and breast cancer model *in vitro* and *in vivo* (Kong et al., 2022). To explore the feasibility of

their combination therapy, they examined whether an anti-SIRPα antibody would block the CD47-SIRPα axis in macrophages. Their results suggested that using this antibody removed the antagonistic effect of CD47 on phagocytosis. In addition, tumor cell lysates following OH2 infection induced RAW264.7 macrophage polarization towards a CD68-positive M1 phenotype, thus confirming a standard polarization change in macrophages in response to many OVs. *In vivo,* the combination therapy with OH2 and anti-SIRPα antibody was more dramatic than OH2 alone, with smaller tumors and increased survival. It also increased CD86-positive M1 macrophage (and CD16-positive natural killer cell and CD8-positive cytotoxic T cell) infiltration, particularly into the central mass of the tumor. This combination treatment caused the TME to go from M2 macrophage-abundant/anti-inflammatory to M1 macrophage-abundant/pro-inflammatory), thus increasing antitumor immunity (Kong et al., 2022).

In the same vein as the previous studies, F. Cao and coworkers engineered vaccinia virus to secrete a chimera of the SIRPα ectodomain and the Fc portion of an antibody as a means of blocking the CD47/SIRPα-signaling axis to promote phagocytosis of cancer cells (F. Cao et al., 2021). They tested this virus on osteosarcomas. As in the other studies, the OV was an effective killer of these cancer cells, both *in vitro* and *in vivo*. In an immune-competent mouse model, they found that their engineered vaccina virus increased the infiltration of myeloid cells, particularly M2 macrophages, into the TME. Usually, the recruitment of M2 macrophages to a tumor would have a negative influence. However, inhibiting the CD47/ SIRPα blockade of phagocytosis enabled the

M2 macrophages to target and kill infected tumor cells in these studies (Cao et al., 2021).

In further investigations into how OVs might modulate macrophage phenotypes, research performed by Kwan used a bioengineered version of HSV-1 with a 'therapeutic safety net' lacking infected cell protein 34.5 (ICP34.5), the primary neurovirulence factor for the virus (Kwan et al., 2021). This makes the virus even more selective for cancer cells and less toxic to the host. Working with breast tumors *in vivo*, the authors showed reduced tumor growth and fewer lung metastases following intravenous virus administration. Three doses of the virus led to 100% survivability in the mice. However, these effects were not as pronounced when macrophages were eliminated from the mice by administering the macrophage apoptosis-inducing drug clodronate. The authors also noticed that HSV-1 was able to infect macrophages *in vitro*. This resulted in increased in M1 markers (CD80, CD86), pro-inflammatory cytokines (IL-6, IL-12, TNF-α), and NO, and decreased in M2 markers (CD64, CD163, CD206). Here again, it seems that reprogramming of TAMs yielded a TME that was more M1-like, thereby promoting an anticancer immune profile within the tumors (Kwan et al., 2021).

It seems that the phenotype of macrophages relates to the efficacy of HSV-1 treatment, as another lab discussed in a paper by Liu and coworkers (Liu et al., 2021). This group engineered a strain of HSV-1 that lacked both ICP34.5 and ICP47, the latter of which is an antigen presentation inhibitor that reduces immune activation of cytotoxic T-

lymphocytes. The engineered virus still activated T-cells and after an established infection of uveal melanoma cell lines, the virus reduced tumor growth at the local injection site and in another distantly located tumor. They also identified large areas of inducible NOS-positive M1 macrophages (alongside natural killer and dendritic cells) within the virally infected tumors (Liu et al., 2021).

In their studies, Van Den Bossche and coworkers used an adenovirus Delta-24-RGD (Gül et al., 2014). This virus harbors a 24-base pair deletion in the early region 1A (E1A) gene and therefore only replicates in cells with a dysfunctional retinoblastoma (pRb) tumor suppressor pathway, a common feature of cancer cells. Infection of pre-polarized, primary human macrophages with this viral strain reduced the M2 marker CD164 and increased the M1 marker CD64. Pro-inflammatory cytokines (TNF-α, IL6, IFN-γ) were similarly upregulated, and these same cytokines could be detected in the cerebrospinal fluid of GBM patients being clinically treated with the virus. Finally, and most promisingly, a patient who had received Delta24-RGD treatment 26 months before the paper's publication, underwent a second tumor resection due to recurrence of the cancer. Intertumoral macrophages in this patient demonstrated an increased presence of CD64 and a decreased presence of CD206. This result suggests that the treatment with Delta-24-RGD had a pro-inflammatory effect leading to prolonged phenotypic changes to the TAMs. The authors also discovered that the TAMs phagocytosed infected tumor cells. They suspect this was carried out by the prevailing M1 macrophage populations in those tumors (Gül et al., 2014).

### *Vesicular Stomatitis Virus as a OVT*

Vesicular stomatitis virus (VSV) is a non-pathogenic, enveloped, negative-strand RNA virus of the family Rhabdoviridae. It possesses an 11-kilobase genome that encodes five proteins: nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large polymerase protein (L) (Abdelmageed & Ferran, 2020; Barber, 2004; Chakraborty et al., 2009; Letchworth et al., 1999; Sung et al., 2021). VSV can infect nearly all cell types, but productive infections are rare in healthy cells due to the antiviral responses mediated by type I IFNs (Gaudier et al., 2002; Stojdl et al., 2003). However, since many cancer cells harbor defects in the IFN response, VSV can infect and selectively target cancer cells, making it an effective OV (Ayala-Breton et al., 2012; Jebar et al., 2015).

Several modified versions of VSV have been created to test their effectiveness as OVT agents. For example, one group has focused on engineering VSV in such a way as to target inhibitors of apoptosis (IAPs), a group of proteins expressed by cancer cells that block the intrinsic apoptotic signaling pathway (Tang et al., 2022). One such virus expresses the IAP inhibitor Smac (VSV-S). Tang and colleagues showed an elevation of cancer cell apoptosis, reduced tumor growth, and higher survival (with 50% survival increasing from 15 to 35 days). The researchers also saw profound changes in the TME, including reductions in myeloid-derived stem cells and macrophages. CD206-positive

M2 macrophages were a part of this reduction, but CD86-positive M1 macrophages stayed the same. The researchers suggested that infection with VSV-S induced an acute inflammatory response and a favorable immunosuppressive TME.

***Studies with rM51R-M Virus***

Among the most promising variants of VSV for OVT are those that engage the antiviral response in infected cells. Some of these variants harbor mutations in the M protein. The wild-type M protein is what allows VSV to avoid innate antiviral immunity by directly inhibiting the transcription of host mRNAs as well as their nuclear-to-cytoplasmic transport. This inhibition attenuates the synthesis of IFN and other crucial pro-inflammatory proteins (Blondel et al., 1990; Clinton et al., 1978; Lichty et al., 2004; Raux et al., 2010). In our lab, we use the rM51R-M mutant strain of VSV. rM51R-M virus contains a methionine to arginine substitution at position 51. This mutation renders rM51R-M defective in inhibiting host gene expression and, therefore, capable of inducing the expression of genes involved in the body's antiviral responses, including the type I IFN response, in infected cells. rM51R-M virus was developed to limit viral replication to tumor cells with a modified type I IFN signaling axis and is generally regarded as a safer alternative to the wild-type virus (*e.g.*, rwt virus) (Ahmed & Lyles, 1997; Ahmed et al., 2008; Ahmed et al., 2004; Black et al., 1993; Blondel et al., 1990). rM51R-M virus retains interest as an OVT because it has also shown an ability to kill multiple types of cancers, including those of breast tissue (Ahmed et al., 2003, 2004b,

2010). Other VSV mutants, such as VSV-IFN-β, also activate a type I IFN response, and have reached early stages of clinical trials for the treatment of hematopoietic neoplasms and advanced solid tumors (Garmaroudi et al., 2022; Lin et al., 2023; Moglan et al., 2023; Porosnicu et al., 2022; Shalhout et al., 2023; Velazquez-Salinas et al., 2017; Yun et al., 2022).

In recent research from our lab, a green-fluorescent protein (GFP)-expressing rM51R-M virus was tested for its effect on THP-1 macrophage populations (Polzin et al., 2020). M2 THP1 macrophages were susceptible to viral infection as over 20% of cells exhibited positive GFP fluorescence, indicating cells with actively replicating virus. This also led to a 50% loss of viability in the M2 macrophages. Meanwhile, M1 THP-1 macrophages, already induced to an antiviral state, exhibited no signs of viral replication or loss of viability. The M2 THP1 macrophages also exhibited signs of repolarization upon infection with rM51R-M virus. This was demonstrated, in part, by an increase in phosphorylated signal transducer and activator of transcript 1 (pSTAT1) and cluster of differentiation 80 (CD80), a surface marker involved in immune regulation. STAT1 is a transcription factor associated with IFN-γ-induced M1 polarization. Upon phosphorylation, dimerization, and translocation to the nucleus, STAT1 induces the expression of other M1 markers, such as CD68, CD86, IL-6, iNOS, and TNF-α. The repolarization data partly aligned with experiments on podosome development in macrophages. Podosomes are actin-rich cell surface protrusions that degrade the extracellular matrix in invasive cell types like macrophages. Infection with VSV reduced

podosome numbers in M2 macrophages, which may be interpreted as a loss in their migratory capabilities. The rwt strain of the virus, in particular, reduced podosome numbers to such a degree that they resembled the levels observed in M1-type macrophages. Thus, in both form and function, VSV infection impacted macrophages, thereby suggesting a beneficial development for the virus as a potential OVT (Polzin et al., 2020a). In a theoretical study by Almuallem et al., the authors utilized a mathematical model to describe new hypotheses regarding the impact of VSV on TAMs based on the Polzin paper (Almuallem et al., 2021). Their mathematical models suggested, among other things, that (i) virus-induced repolarization of macrophages might reduce tumors, though it might not eliminate them, and (ii) viral infection rates of the tumor cells might delay tumor relapse (Almuallem et al., 2021). Further *in vitro* or *in vivo* studies involving breast cancer/macrophage cell lines might address some of these hypotheses more directly.

***Objectives***

This study endeavored to identify the impact of VSV on a very aggressive triple-negative breast cancer cell line when using a simulated TME of cocultured 'TAMs.' Triple-negative breast cancer (TNBC) is a subtype of breast cancer characterized by the absence of three specific receptors commonly found on the surface of cancer cells: the estrogen receptor (ER), the progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2). Due to this absence, TNBC is not effectively treated with hormone-based

therapies or targeted therapies that work against HER2-positive breast cancers, making it a challenging and aggressive form of the disease. The lack of these receptors limits treatment options for TNBC, predominantly to chemotherapy, which can have significant side effects. Therefore, researchers have turned to immunotherapies as a potential avenue for treatment (Neve et al., 2006; Standish et al., 2008; Wagner et al., 2019; Wang et al., 2017). Immunotherapies harness the power of the patient's immune system to recognize and attack cancer cells. In the case of TNBC, there is a strong focus on developing immunotherapies that can stimulate the immune response against these triple-negative tumors. Research in this area aims to discover immune-based strategies that can effectively target and treat TNBC, potentially offering more tailored and less toxic treatment options for patients with this challenging subtype of breast cancer (Hollmén et al., 2015; Ma et al., 2020; Pe et al., 2022; Stewart et al., 2012; Troester et al., 2009). The development of immunotherapies for TNBC is highly relevant to cancer research as it addresses a critical unmet need in the field of oncology and holds promise for improved outcomes and reduced side effects for patients with this aggressive form of breast cancer.

One aim was to identify the direct impact of VSV infections, including both the rwt and rM51R-M strains, on pre-polarized macrophage phagocytic function in mono- and coculture with MDA-MB-231 breast cancer cells. Phagocytosis in this case would be a surrogate for M1 and M2 macrophage phenotype, as the latter subtype exhibits much greater phagocytic activity than the former. We also aimed to monitor the growth of

MDA-MB-231 cells in mono- and coculture with each pre-polarized THP-1 macrophage population—again following infection with rwt and rM51R-M viruses. We hypothesized that classically-activated THP-1-derived M1 macrophages would prohibit the growth of MDA-MB-231 breast cancer cells while alternatively-activated THP-1 M2 macrophages would stimulate their growth. We also hypothesized that the susceptibility of M2 macrophages to infection with rM51R-M virus would compromise their viability, function, and/or polarization state, reverting them to M1-like macrophages, such that the breast cancer growth initially supported by these macrophages would be concomitantly reduced.

# CHAPTER 2: MATERIALS AND METHODS

## *Agonists*

Stock solutions for all agonists used in THP-1 differentiation/polarization were prepared in advance and stored frozen at -80°C. A 1mM stock solution of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) was diluted 1:40 in dimethyl sulfoxide (DMSO) (Company, Part#) to generate a 1000X, 25µM stock solution. IL-4 (BioLegend, #574002), IL-13 (BioLegened, #571102), and IFN-γ (BioLegend, #570202) were purchased as 50µg or 100µg units and reconstituted in sterile, distilled water to a concentration of 200µg/ml according to manufacturer instructions. LPS (Sigma-Aldrich, #L5418), purchased as a 1mg/ml stock, was diluted in 0.05% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) to a concentration of 200µg/ml. Working 1000X stock solutions of 20µg/ml were then made for all cytokines by diluting 10-fold in 0.05% BSA/PBS and freezing as 10µl aliquots to minimize freeze/thaw effects.

## *Cell Culture*

THP-1 monocytes were cultured in RPMI media (Fisher Scientific, MT10040CV) supplemented with 10% fetal bovine serum (FBS) (R&D Systems, S11150) and 0.05mM 2-mercaptoethanol at 37°C and 5% $CO_2$. THP-1 cultures were passaged every 3-4 days, so the cell concentration stayed between 2 and 10 x $10^5$ cells/ml. THP1 monocytes were

differentiated into adherent macrophages under standard cell culture conditions with 25nM PMA for 24 hours. This was followed by their polarization using 20ng/mL LPS, 20ng/mL IFN-γ, and 25nM PMA for M1 macrophages, or 20ng/mL IL-4, 20ng/mL IL-13, and 25nM PMA for M2 macrophages for an additional 48-hour culture period. MDA-MB-231 breast cancer cells were cultured in RPMI media supplemented with 10% FBS at 37°C and 5% $CO_2$. These cells are typically passaged with 0.25% trypsin and 2.21mM EDTA (trypsin-EDTA; Millipore Sigma, 59417C) every three days at a 1:10 dilution.

***Virus***

Recombinant strains of VSV (rwt, rM51R-M) were a generous gift from Dr. Douglas Lyles of the Wake Forest University School of Medicine (Black et al., 1993; Lawson et al., 1995; Whelan et al., 1995; Whitlow et al., 2006). Viral stocks were prepared by infecting baby hamster kidney (BHK) fibroblasts for 24 hours before having the media spun down, collected into cryovials, and stored at -80°C. Subsequent viral infections were carried out at an MOI (multiplicity of infection) of 0.1 or 10 plaque-forming units (pfu) per cell.

***Cell Tracker™ Violet Stock Solutions***

Stock solutions of 10mM Cell Tracker™ Violet (ThermoFisher Scientific, #C10094) were created by dissolving 0.1mg of dry powder in 30µL of dimethyl sulfoxide (10mM). Working solutions of 10µM Cell Tracker™ Violet were made by diluting 10µL of the

10mM stock Cell Tracker™ Violet stock solution into 90µl of RPMI media and dividing it into 10µl aliquots. Both stock and working solutions were stored at -80°C.

***Cell Tracker™ Violet-labeling of Breast Cancer Cells***

MDA-MB-231 cells were lifted with trypsin-EDTA, diluted with 5mL of THP-1 culture media, and counted in the presence of a 1:1 dilution of 0.4% trypan blue using a hemocytometer. 1.5 x $10^5$ cells were distributed into individual wells of a 24-well plate in a final volume of 400µl THP-1 media and supplemented with 4uL of 10µM Cell Tracker™ Violet (100nM final concentration) for 30 minutes at 37°C. Once incubated with the dye, the cells were washed twice with sterile PBS as a last preparation for coculture with THP-1 macrophages.

***Phagocytosis of Latex Beads***

The phagocytic activity of cells was determined using fluorescent red carboxylate-modified polystyrene latex beads (diameter, 2.0µm)(Sigma-Aldrich, #L3030). THP-1 monocytes were seeded in a 24-well plate at 1.5 x $10^5$ cells/well (for monocultures) or at 7.5 x $10^4$ cells/well (for cocultures), then polarized into M1 or M2 macrophages as described above. Meanwhile, Cell Tracker™ Violet-labeled MDA-MB-231 breast cancer cells were lifted with trypsin-EDTA, diluted with 5mL of THP-1 culture media, and counted in the presence of trypan blue as described above. Cells were then adjusted to

a final concentration of 1.5 x $10^5$ cells/400µl/well (monocultures) or 7.5 x $10^4$ cells/400µl/well (cocultures) using THP-1 culture media containing PMA and the appropriate polarizing cytokines. These labeled MDA-MB-231 cells were seeded in 24-well plates alone (monocultures) or used to replace the media in the M1 or M2 macrophage-containing wells to complete the 1:1 seeding ratio (cocultures). Phagocytosis assays conducted under mock conditions were incubated for 18 hours at 37°C. Phagocytosis assays conducted in the presence of VSV were pre-equilibrated for 2 hours at 37°C before the cells were infected with rwt or rM51R-M virus at an MOI of 0.1 pfu/cell for the next 16 hours. The culture media was then replaced with THP-1 media supplemented with a 500uL 0.005% suspension of latex beads for all assay conditions and then incubated for an additional 6 hours at 37°C. The cells were then washed 4 times with 500uL of 1X sterile PBS before vacuum aspiration and replacement with 500µL THP-1 media. Phagocytic activity was measured in live cells imaged at 10X using an Olympus IX-81 inverted fluorescent microscope, a DP80 camera, and cellSens software (3 images/condition). Images were processed with Photoshop (Release 23.5.1) to generate composite images of the phase contrast and fluorescent channels and to count cells. Phagocytic activity was the ratio of the total number of cells that ingested at least one latex bead over the total number of cells capable of phagocytosis (*i.e.*, macrophages). Phagocytosis by MDA-MB-231 cells was only counted under monoculture conditions.

***Breast Cancer Cell Growth Assays***

The growth of Cell Tracker™ Violet-labeled MDA-MB-231, breast cancer cells, was measured in monoculture or in co-culture with pre-polarized M1 or M2 THP1 macrophages at 37°C from 2-38 hours. In one series of experiments (MDA Lift), the labeled MDA-MB-231 cells were lifted from dishes, counted, and reseeded into 24-well plates with or without pre-polarized macrophages such that the seeding density was 1.5 x $10^5$ cells/well (monocultures) or 7.5 x $10^4$ cells/well/cell type (cocultures), all in a final volume of 400µl. Later, cell growth assays were conducted such that the pre-polarized macrophages were lifted and reseeded into 24-well plates with labeled MDA-MB-231 cells (Mac Lift). Briefly, 1.0 x $10^7$ THP-1 monocytes were seeded into 6.5cm cell culture dishes in a volume of 3mL and polarized into M1 or M2 macrophages as described above. Note that THP1- cells were plated at a significantly higher count than needed for the experiment to account for the losses that occur later during lifting. Once cells were polarized to an M1 or M2 phenotype, they were washed twice with sterile PBS and treated with 1.5 mL of Accutase (Millipore, #SCR005) for 1 hour at 37°C. The detached cells were then aliquoted into 1.5 mL microcentrifuge tubes, spun at 1000 revolutions per minute for 5 minutes, and the Accutase solution in the supernatant was replaced with 1mL of THP-1 media for counting on a hemocytometer. M1 or M2 THP-1 macrophages were adjusted in THP-1 media containing PMA and the appropriate polarizing cytokines before overlaying onto the labeled MDA-MB-231 cells in a 24-well plate at a 1:1 seeding ratio (*i.e.*, 7.5 x $10^4$ cells/well/cell type), all in a final volume of

400µl. Once cultures were established for 2 hours, the cells were infected without (mock) or with rwt or rM51R-M virus at an MOI of 0.1 or 10 pfu/cell. Live cell phase contrast and fluorescent microscopy images were taken at 2, 8, 14, 26, and 38 hours (5 images/condition) using the Olympus IX-81 inverted fluorescent microscope at 20X magnification, DP80 camera, and cellSens software. Images were processed with Photoshop (Release 23.5.1) to generate composite images of the phase contrast and fluorescent channels. Breast cancer cells were counted in each image using the open-source tool DotDotGoose (version 1.6.0) based on positive dye fluorescence and morphology.

***Statistical Analysis***

All statistics used to evaluate data were processed using MiniTab™ Statistical Software, Version 21.1.0 (2023). All fold change datasets were transformed using $Log_2$ to achieve equal variance and account for ratio data. A two-way Analysis of Variance (ANOVA) with Tukey's post-hoc Analysis was used for pairwise comparisons between individual conditions for phagocytic activity experiments. Two-way ANOVA with Repeated Measures, where time was the dependent variable, was used with Tukey's post-hoc analysis for pairwise comparisons between conditions in phagocytosis assays and cancer cell growth experiments. Unless otherwise stated, analyses compare interactions of macrophage subtype versus virus strain with time as a dependent variable. All reported

statistically significant findings indicate a p-value of $\leq$ 0.05 within a 95% confidence interval.

# CHAPTER 3: RESULTS

## Part 1. Macrophage Phagocytosis

Oncolytic virotherapies reduce tumor burden by killing cancer cells and promoting antitumor immunity, but how TAMs impact such viral infections is less clear. To address this issue, we sought to determine the potential modulatory effects of VSV infection within a simulated tumor microenvironment *in vitro* containing pre-polarized M1 or M2 macrophages and cocultured breast cancer cells. We have previously seen modulatory effects on THP-1 macrophage populations following infection with rM51R-M virus, a mutant VSV strain that fails to shut down host antiviral responses and is thereby a comparatively safer therapy relative to its wild-type counterpart (rwt virus). This conclusion was based on the observation that M2 THP-1-derived macrophages infected with the rM51R-M strain of VSV express the M1 markers pSTAT1, CD80, and TNF-α (Polzin et al., 2020). We also saw that the 10.8-fold higher phagocytic capability of M2 THP-1 macrophages towards fluorescent *E.coli* bioparticles was reduced to M1 phagocytic levels following rM51R-M infection (Simmons, 2021). Together, these data suggest a possible repolarization of pro-tumor M2 macrophages into antitumor M1 macrophages following rM51R-M infection. M2 macrophages, relative to M1 macrophages, were also more sensitive to the cytopathic effects of this virus (Polzin et al., 2020). All of these previous results were seen with THP-1 macrophages in monoculture. To better address the therapeutic capabilities of VSV, we now sought to

investigate how viral infections were impacted by THP-1 macrophages when cocultured with the triple-negative breast cancer cell line MDA-MB-231. MDA-MB-231 cells are a highly invasive epithelial cancer cell line originally isolated from a 51-year-old woman with metastatic breast cancer. This cell line does not express pro-tumor hormone (estrogen, ER; progesterone, PR) or growth factor (epidermal; HER2) receptors making it representative of triple-negative breast cancers, the most aggressive form of breast cancer known and a cancer with few viable therapeutic options. These cancers can become even more aggressive when macrophages are recruited to the TME. To accurately simulate these breast tumor conditions, we seeded THP-1 macrophages and/or MDA-MB-231 breast cancer cells into 24-well plates at $1.5 \times 10^5$ cells/well. For cocultures, the seeding ratio was 1:1 such that there were $7.5 \times 10^4$ macrophages and cancer cells (but still $1.5 \times 10^5$ total cells) in the well. The latter was done in order to maintain a similar 60-70% confluence of cells during viral infections, which better allows the virus to reach and infect cells within our model. It also permits the MDA-MB-231 cells to proliferate without overwhelming the non-proliferating macrophage populations.

***M2 macrophages are better phagocytes than M1 macrophages.***

Our first experiment was to address the baseline phagocytic capabilities of pre-polarized M1 or M2 THP-1 macrophages with or without cocultured MDA-MB-231 breast cancer cells under mock conditions without virus. Cells were equilibrated in culture for 18

hours, followed by an exchange with fresh media containing a 0.005% suspension of fluorescent latex beads for another 6 hours. Following this period, live cell images were taken by fluorescent microscopy (Figures 1-3), which were analyzed quantitatively in Figure 4. To visually distinguish the macrophages from the breast cancer cells, we labeled the latter cell type with Cell Tracker™ Violet dye. Cell Tracker™ Violet is a fluorescent dye that localizes to the cytoplasm and exhibits retention through at least 72 hours of culturing. Previous work has indicated good staining but little cytotoxicity by the dye at 10μM concentration (Simmons, 2021). Figure 1 dramatizes the visual differences between the labeled breast cancer cells and the unlabeled macrophages in the composite phase contrast/fluorescent microscopy images. Figure 1A shows that MDA-MB-231 breast cancer cells essentially do not phagocytose latex beads on their own. This is consistent with the epithelial origin of this breast carcinoma cell line. Given that result, all subsequent analyses of the latex bead assay were quantified using the following specifications: (i) the total number of cells capable of phagocytosis and (ii) the percentage of cells that ingested a minimum of one latex bead. This meant that for the cocultures, the breast cancer cells were not counted under any experimental condition as they did not actively phagocytose latex beads. All counts and percentages presented in the data are of phagocytosis-capable cells only, *i.e.*, the macrophages (Figure 4). In contrast to the MDA-MB-231 breast cancer cells, both THP-1 macrophage populations, as professional phagocytes, performed statistically better in this assay. For the M1 THP-1 macrophages, phagocytosis could be seen in 3.9% of the cells in monoculture (Figures

A
B
C
D
E
MDA-MB-231
M1
MDA-MB-231 + M1
M2
MDA-MB-231 + M2
MDA-MB-231 ZOOM
M1 ZOOM
MDA-MB-231 + M1 ZOOM
M2 ZOOM
MDA-MB-231 + M2 ZOOM

**Figure 1. Phagocytosis of Latex Beads by M1 and M2 Macrophages in Mono- or Coculture with MDA-MB-231 Breast Cancer Cells under Mock Conditions.** Pre-polarized THP-1 macrophages in mono- or 1:1 coculture with Cell Tracker™ Violet-labeled MDA-MB-231 breast cancer cells (white) were incubated for 18 hours, and then exposed to fluorescent latex beads (red) for 6 hours. Representative, composite, live cell images (left) are matched with zoomed images (right) of the demarcated regions and highlight breast cancer cells (blue arrows), M1 macrophages (yellow arrows), M2 macrophages (red arrows), and phagocytosing cells (teal circles). [Figure 1 is on the previous page.]

1B and 4). Phagocytosis by M2 macrophages was statistically higher with 23.7% of all cells ingesting at least one bead (Figure 1D and 4). In many cases, these M2 macrophages phagocytosed multiple beads per cell (Figure 1D). Together, this represents an approximately 6-fold higher phagocytic ability of M2 macrophages over M1 macrophages and is consistent with what had been observed previously by our lab for these macrophage populations using *E. coli* bioparticles (Simmons, 2021).

***The presence of cocultured breast cancer cells enhances macrophage phagocytic capacity.***

When MDA-MB-231 cells were co-cultured with THP-1 macrophages for 18 hours prior to performing the phagocytic bead assay, phagocytosis by macrophages showed a remarkable increase. This can be seen for M1 (Figure 1C) and M2 (Figure 1E) macrophages. The increase from 3.9% of M1 macrophages in monoculture to 5.4% in coculture was not statistically significant (Figure 4). However, the increase from 23.7% of M2 macrophages in monoculture to 58.5% in coculture was significant and

represented the most robust phagocytic activity observed in all experimental conditions. These results demonstrate that a simulated tumor model can be established *in vitro* in such a way as to differentiate cancer cell and macrophage populations all while discerning whether macrophages are ingesting latex beads. These data also suggest functional changes in macrophage populations, and in particular M2 macrophages, that is induced by cross-talk with cancer cells even during a relatively brief 24-hour coculture period.

***VSV strains diminish macrophage phagocytic capacity in both mono- and coculture conditions.***

Given that both mono- and coculture models successfully allow for visualization of macrophage phagocytosis (Figure 1), we next tested the functional impact of VSV on the phagocytic capacity of THP-1 macrophages. For this assay we chose an MOI of 0.1 pfu/cell, an asynchronous infection regime that tests not only how infection impacts phagocytosis directly, but also how communication between cells in response to the infection impacts this activity. Figures 2 (modeling rwt virus infections) and 3 (modeling rM51R-M virus infections) feature images of phagocytosing cells under the established conditions of the assay noting that infection by each VSV strain was initiated 2 hours into the 18-hour mono- or coculture incubation period prior to adding the latex beads.

Neither the rwt nor the rM51R-M strains of VSV completely eliminated phagocytosis of latex beads by either macrophage population in this study (Figures 2 and 3). However, both viral strains trended towards an inhibition of that activity, and that was most dramatically seen with rM51R-M virus. Indeed, infection with rM51R-M virus always led to statistically significant decreases in phagocytosis, both in M1 and M2 macrophages, and under both monoculture and coculture conditions. For M1 macrophages, this reduction was 6.5-fold in monoculture and 6.8-fold in coculture (Figure 4). For M2 macrophage, it was 5.6-fold in monoculture and 5.5-fold in co-culture. These reductions in phagocytosis following rM51R-M virus infection of M2 macrophages brought it down to the phagocytic levels of M1 macrophages under mock conditions. A similar result had been previously observed by this viral strain on M2 THP-1 macrophage phagocytosis of *E.coli* bioparticles (Simmons, 2021).These trends make the phagocytic capabilities of M2 macrophages look more like that of M1 macrophages and lend support for the repolarization of this macrophage population following rM51R-M virus infection.

In contrast to the changes seen with rM51R-M virus, M1 macrophages showed only slight, and statistically insignificant, reductions in phagocytosis following infection with

**Figure 2. Phagocytosis of Latex Beads by M1 and M2 Macrophages in Mono- or Coculture with MDA-MB-231 Breast Cancer Cells following Infection with rwt Virus.** Pre-polarized THP-1 macrophages in mono- or 1:1 coculture with Cell Tracker™ Violet-labeled MDA-MB-231 breast cancer cells (white) were pre-incubated for 2 hours before infection with rwt virus (MOI 0.1 pfu/cell) for an additional 16 hours. Cells were then exposed to fluorescent latex beads for 6 hours. Representative, composite, live cell images (left) are matched with zoomed images (right) of the demarcated regions and highlight breast cancer cells (blue arrows), M1 macrophages (yellow arrows), M2 macrophages (red arrows), and phagocytosing cells (teal circles). [Figure 2 is on the next page.]

A
B
C
D
E
MDA-MB-231
M1
MDA-MB-231 + M1
M2
MDA-MB-231 + M2
MDA-MB-231 ZOOM
M1 ZOOM
MDA-MB-231 + M1 ZOOM
M2 ZOOM
MDA-MB-231 + M2 ZOOM

MDA-MB-231
M1
MDA-MB-231 + M1
M2
MDA-MB-231 + M2
A
B
C
D
D
MDA-MB-231 ZOOM
M1 ZOOM
MDA-MB-231 + M1 ZOOM
M2 ZOOM
MDA-MB-231 + M2 ZOOM

**Figure 3. Phagocytosis of Latex Beads by M1 and M2 Macrophages in Mono- or Coculture with MDA-MB-231 Breast Cancer Cells following Infection with rM51R-M Virus.** Pre-polarized THP-1 macrophages in mono- or 1:1 coculture with Cell Tracker™ Violet-labeled MDA-MB-231 breast cancer cells (white) were pre-incubated for 2 hours before infection with rM51R-M virus (MOI 0.1 pfu/cell) for an additional 16 hours. Cells were then exposed to fluorescent latex beads for 6 hours. Representative, composite, live cell images (left) are matched with zoomed images (right) of the demarcated regions and highlight breast cancer cells (blue arrows), M1 macrophages (yellow arrows), M2 macrophages (red arrows), and phagocytosing cells (teal circles). [Figure 3 is on the previous page.]

rwt virus (1.6-fold in monoculture and 3-fold in coculture) (Figure 4). The results for M2 macrophages, however, were different. For M2 macrophage monocultures, there was virtually no change in the percentage of phagocytic cells, *i.e.*, from 23.7% under mock conditions to 25.8% with rwt virus. However, for the M2 macrophages cocultured with MDA-MB-231 cells, where the mock condition had the extraordinarily high of 58.5% of the cells undergoing phagocytosis, this reduced to a statistically significant 27.2% following rwt infection. While this drop was not as far as that which was induced by rM51R-M virus infection, it did reduce to M2 macrophage monoculture levels, essentially neutralizing the accentuation that MDA-MB-231 coculturing had provided.

***VSV infection-dependent reductions in macrophage phagocytic capacity are not due to the cytopathic effects of VSV.***

The ability of VSV infection to reduce macrophage phagocytic capacity might be simply the result of the cytopathic capabilities of the virus. That is, macrophages might

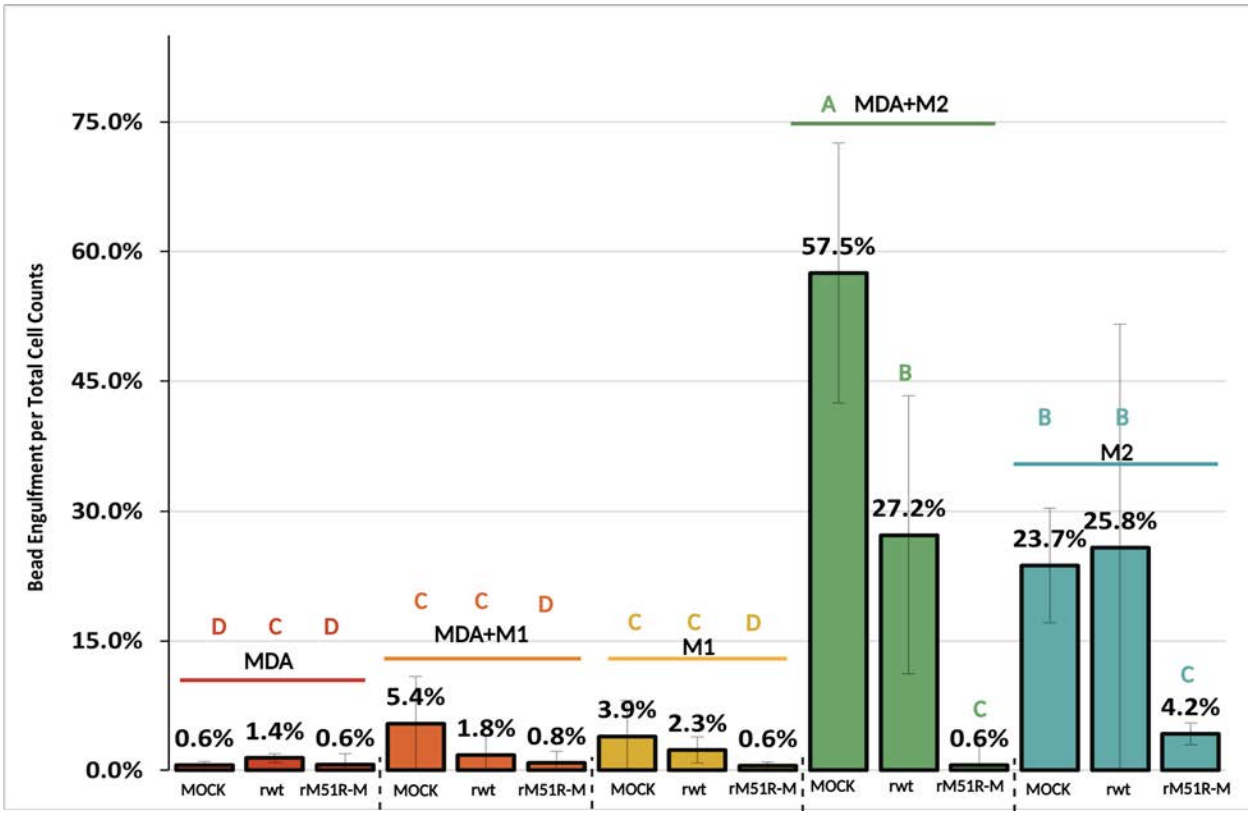



**Figure 4. Percentage of Phagocytic Cells in Pre-Polarized THP-1 Macrophage/MDA-MB-231 Breast Cancer Cell Mono- or Cocultures with or without VSV Infection.**
Quantitative analysis of the percentage of cells phagocytosing one or more fluorescent latex beads from composite, live cell images of M1 and M2 THP-1 macrophage/MDA-MB-231 (MDA) breast cancer cell mono- and cocultures (see Figures 1, 2, and 3). Cells were infected with (rwt or rM51R-M) or without (Mock) VSV virus (MOI 0.1 pfu/cell). Data are the mean and standard deviation of three independent experiments. Statistical significance between conditions was analyzed using a Two-Way ANOVA. Post-Hoc pairwise comparisons of results indicate differences by assigned letter (p-value $\leq$ 0.05). Means that do not share a letter are statistically different from one another. All statistical analyses were conducted using Minitab® statistics software.

phagocytose less because they were dead or dying from a viral infection. To rule out that possibility, we returned to our counts of the number of macrophages present within the images used in the phagocytosis assay, the very data used to determine the percentage of phagocytic cells. This is shown in Figure 5. First, the number of macrophages in coculture (Figure 5B and 5D) were always less than the number in monoculture (Figure 5A and 5C). This is because of the intended co-culture conditions that reduce the number of both THP-1 macrophages and MDA-MB-231 breast cancer cells in order to maintain the same overall confluency of cells. Second, neither rwt nor rM51R-M virus reduced the number of M1 macrophages in either mono- or coculture conditions (Figure 5A and 5B). This is consistent with previous data showing that M1 macrophages are resistant to VSV infection and cytopathicity (Polzin et al., 2020). Third, M2 macrophages did appear to be somewhat susceptible to VSV cytopathicity as macrophage numbers were statistically reduced following viral infection (Figure 5C and 5D). This has also been observed before (Polzin et al., 2020). However, the reduction in cocultured M2 macrophages by rwt virus was no different than that of rM51R-M virus, even though rM51R-M virus had a much more substantial effect on phagocytosis.

In sum, we observed the following from the phagocytic assay. (1) There was increased phagocytic activity in M2 macrophages compared to M1 macrophages in both mono- and coculture conditions. (2) Coculture with MDA-MB-231 breast cancer cells significantly increased the percentage of phagocytosing macrophages, particularly for the M2 macrophage subtype. (3) rM51R-M virus significantly decreased the phagocytic

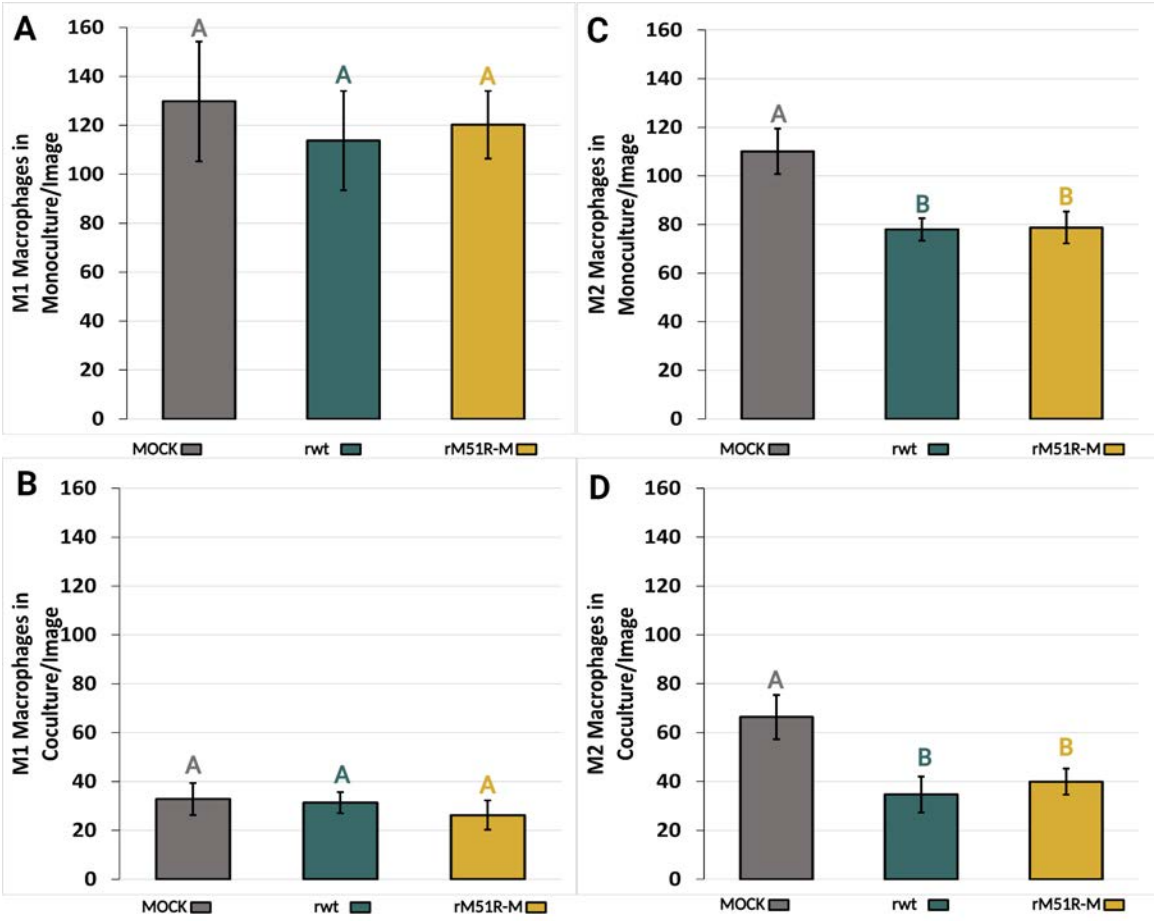



**Figure 5. THP-1 Macrophage Counts from the Phagocytosis Assays.** Quantitative analysis of the number of macrophages from composite, live cell images of M1 and M2 THP-1 macrophage/MDA-MB-231 (MDA) breast cancer cell mono- and cocultures (see Figures 1, 2, and 3). Cells were infected with (rwt or rM51R-M) or without (mock) VSV virus (MOI 0.1 pfu/cell). Data are the mean and standard deviation of three independent experiments. Statistical significance between conditions was analyzed using a Two-Way ANOVA. Tukey's Post-Hoc pairwise comparisons of results indicate differences by assigned letter (p-value $\leq 0.05$). Means that do not share a letter are statistically different from one another. All statistical analyses were conducted using Minitab® statistics software.

capacity of M1 and M2 macrophages in both mono- and coculture conditions. These decreases were such that rM51R-M-infected M2 macrophages were statistically indistinguishable from M1 macrophages under mock conditions. These data further suggest that the loss in M2 macrophage phagocytic function associated with rM51R-M virus infection is likely not due to cytopathicity, but to some other effect, such as the repolarization of M2 macrophages to an M1-like phenotype.

## Part 2. Breast Cancer Cell Growth

The previous data show modulatory effects on the THP-1 macrophage phenotype following infection with rM51R-M virus. That is, M2 macrophage phagocytic capacity for latex beads, which is higher than that of M1 macrophages, was reduced to that of M1 macrophage levels under monoculture conditions and near to that of M1 macrophages when cocultured with MDA-MB-231 cells (Figure 4). Thus, alongside increases in M1 marker expression, the reduced function of M2 macrophages as phagocytes following infection with rM51R-M virus suggests a repolarization of macrophages to an M1 phenotype within the simulated conditions of the triple-negative breast TME used in this study. If so, then the therapeutic potential of VSV may extend well beyond its cytopathicity for cancer cells given that pro-tumor M2 TAMs may be prone to death, to loss of function, or to a possible repolarization to an antitumor phenotype (Polzin et al., 2020b). Seeing that VSV has already presented itself as a promising immunotherapy, even in the realm of triple-negative breast cancer (Niavarani et al., 2020 ), this study

aimed to detail further the impact that the rwt and rM51R-M strains of VSV have on MDA-MB-231 breast cancer cell/THP-1 macrophage coculture conditions *in vitro*. We hypothesized that classically-activated M1 macrophages would prohibit breast cancer growth while alternatively activated M2 macrophages would stimulate it. We further hypothesized that infection with VSV, and in particular with rM51R-M virus, would neutralize the stimulation of breast cancer cell growth even in the presence of M2 macrophage populations.

***M1 macrophages inhibit breast cancer cell growth in coculture; M2 macrophages have no effect.***

To study MDA-MB-231 cell growth, the breast cancer cells were first labeled with Cell Tracker™ Violet so that they could be differentiated from the macrophages when in coculture. Like the phagocytosis assays, it was the pre-labeled breast cancer cells that were lifted from a cell culture dish with trypsin-EDTA and placed onto new dishes containing pre-polarized macrophage populations. Growth was then monitored over time by counting the labeled cells under a fluorescent microscope (Figure 6A). Representative images of these cells at the 14-hour time point reveal the nature of the data, which was then quantitated as the fold-change in growth relative to that at 2 hours (Figure 6B-6E). The data suggest three conclusions. (1) The MDA-MB-231 cells grew over time by increasing their cell density by ~2.3 fold over the 38-hour growth period (Figure 6E). By the end of the experiment, there were a maximum of ~80

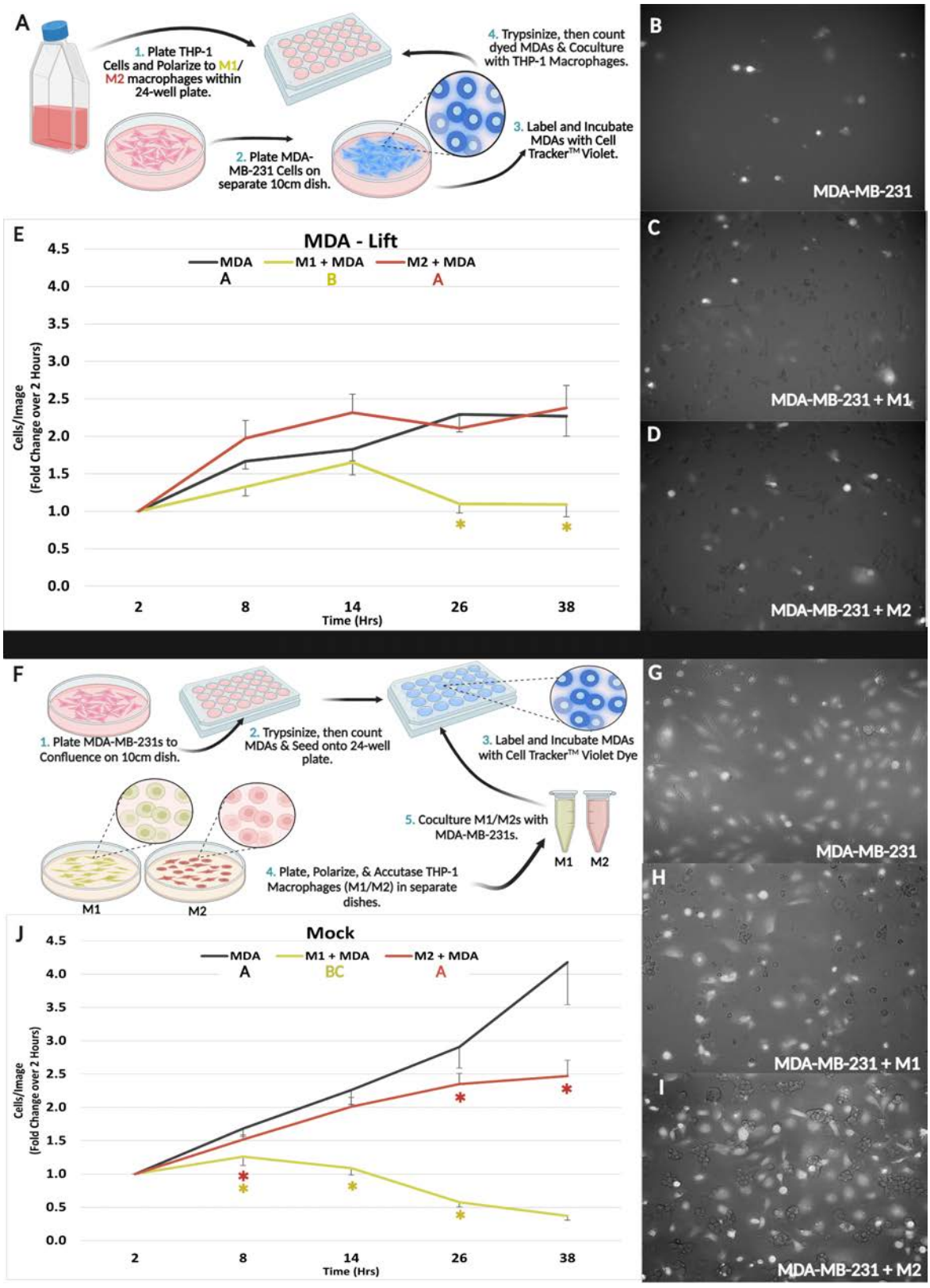

A
1. Plate THP-1 Cells and Polarize to M1/ M2 macrophages within 24-well plate.
2. Plate MDA-MB-231 Cells on separate 10cm dish.
3. Label and Incubate MDAs with Cell Tracker™ Violet.
4. Trypsinize, then count dyed MDAs & Coculture with THP-1 Macrophages.
B
MDA-MB-231
C
MDA-MB-231 + M1
D
MDA-MB-231 + M2
E
MDA - Lift
MDA
M1 + MDA
M2 + MDA
A
B
A
Cells/Image (Fold Change over 2 Hours)
Time (Hrs)
F
1. Plate MDA-MB-231s to Confluence on 10cm dish.
2. Trypsinize, then count MDAs & Seed onto 24-well plate.
3. Label and Incubate MDAs with Cell Tracker™ Violet Dye
4. Plate, Polarize, & Accutase THP-1 Macrophages (M1/M2) in separate dishes.
5. Coculture M1/M2s with MDA-MB-231s.
M1
M2
G
MDA-MB-231
H
MDA-MB-231 + M1
I
MDA-MB-231 + M2
J
Mock
MDA
M1 + MDA
M2 + MDA
A
BC
A
Cells/Image (Fold Change over 2 Hours)
Time (Hrs)

**Figure 6. Interval Plot of MDA-MB-231 Cell Counts with or without Cocultured THP-1 Macrophages – Comparison of Trypsin-lifted Breast Cancer Cells to Accutase®-lifted Macrophages.** Coculture method where Cell Tracker™ Violet-labeled MDA-MB-231 breast cancer cells were overlaid onto pre-polarized THP-1-derived macrophages (MDA-Lift, A) or where pre-polarized THP-1 macrophages were overlaid onto Cell Tracker™ Violet-labeled MDA-MB-231 breast cancer cells (Mac-Lift, F). Representative, composite, live cell images highlight fluorescent breast cancer cells (white) and macrophages (clear) in coculture at 10X magnification under MDA-Lift conditions (B-D) or Mac-Lift conditions (G-I). Quantitation of breast cancer cell counts/image were expressed as a fold-change relative to the 2-hour time point under MDA-Lift conditions (n=3, E) or Mac-Lift conditions (n=3, J). Cell counts across the complete time intervals were compared using a Repeating Measures Two-Way ANOVA. Tukey's Post-Hoc pairwise comparisons indicate significant differences by letter. Intervals that do not share a letter are significantly different from one another across the overall dataset. Individual time points were evaluated independently using a One-Way ANOVA. Dunnett's Post-Hoc analysis indicates statistical significance compared to the MDA-MB-231 monocultures under mock conditions. *, p-value $\leq$ 0.05). [Figure 6 is on the previous page.]

cells/image. (2) M1 THP-1 macrophages, as hypothesized, inhibited this growth of MDA-MB-231 cells. This was most noticeable by 26 hours and continued to the end of the experiment. This reduction in growth was lower than that of the breast cancer cells cocultured with M2 THP-1 macrophages too. (3) M2 THP1 macrophages neither reduced nor accentuated MDA-MB-231 cell growth in this experiment. This was an unexpected result as we anticipated a growth insurgence in response to M2 macrophages.

Another result that was unexpected, but readily apparent, was that the MDA-MB-231 breast cancer cells did not appear as healthy as desired nor was there consistent fluorescence omitted from the Cell Tracker™ Violet dye. In many cases the fluorescence from the cells was barely detectible at all. To remedy this, the cancer cell growth experiment was repeated, but this time with the macrophages overlaid onto the breast

cancer cells. In short, pre-polarized macrophage populations were lifted from a cell culture dish with Accutase® and then placed onto new dishes containing the pre-labeled breast cancer cells (Figure 6F). Growth was again monitored over time by counting the labeled cells under a fluorescent microscope (Figure 6J). The most notable difference between the two methodologies could be seen in the total cell counts at 38 hours, which under these new conditions increased to as many as 180 cells/image (Figure 6G-6I). Moreover, the MDA-MB-231 cells were visibly healthy, and with more cells retaining Cell Tracker™ Violet dye, the overall trends in growth at each time point were far more apparent.

Generally, the trends in breast cancer growth were the same regardless of which cell type was lifted and plated into the cocultures. First, when MDA-MB-231 cells were grown in monoculture, the cells expectedly showed a steady, nearly linear increase in growth up to the final time point of 38 hours (Figure 6J). The breast cancer cells also demonstrated a healthy morphology with little cell death outside what can be expected for a proliferating cell culture.

In contrast, cocultured M1 THP-1 macrophages significantly inhibited MDA-MB-231 cell growth across the entire growth interval, resulting in a nearly 75% loss in cell number by 38 hours (Figure 6J). This inhibition was first detected at 8 hours of coculture, a much earlier time in the Mac-Lift method (Figures 6H and 6J) than the 26 hours of coculture seen in the MDA-Lift method (Figures 6C and 6E). The images at 14 hours show the

reduced breast cancer cell numbers (Figures 6G and 6H), which were nearly 6-fold lower than MDA-MB-231 monocultures by the end of the experiment at 38 hours. Aside from the detached cells that had died already, we also noticed an unhealthy morphology in the attached breast cancer cells, some of which represented classical interpretations of necrotic or apoptotic cells with ruptured/fragmented shapes and blebbing/ disintegration, respectively (Yan et al., 2020; Ziegler & Groscurth, 2004). As observed in the MDA-Lift experiment, the M2 macrophages did not stimulate MDA-MB-231 breast cancer cell growth in coculture (Figures 6E and 6J). Indeed, across the entire growth interval, the M2 THP-1 macrophages showed some inhibition of breast cancer growth, but this was not statistically significant (Figure 6J). Thus, despite other studies showing that the presence of M2-polarized macrophages increases the growth rate/number of cancer cells, we did not detect that here.

The best coculture procedure to use may depend on the nature of the assay and the cell type being analyzed. It was relatively easy to discern the phagocytic capacity of macrophages when it was the breast cancer cells that were lifted and replated in the assay. Conversely, when studying breast cancer growth, imaging worked best when it was the macrophages that were lifted and replated. Regardless, it bodes well that the breast cancer growth assay gave similar results using both methods as it allowed us to proceed with confidence into the next round of breast cancer cell growth experiments involving both coculture with macrophages and VSV infection.

***VSV infection neutralizes breast cancer cell growth regardless of macrophage presence.***

The penultimate experiment in this study was to investigate the growth of breast cancer cells in coculture with pre-polarized macrophage populations following infection with the rwt and rM51R-M strains of VSV. This experiment was carried out as described in Figure 6F, except that virus was added to the cocultures after an initial 2-hour pre-incubation and was kept in the cultures for rest of the experiment. Figure 7 shows representative images from the 14-hour time point where the impact of the virus was beginning to become apparent (Kopecky et al., 2001; Kopecky & Lyles, 2003). As before, we can see a visible difference in the number of MDA-MB-231 cells in monoculture relative to the cells cultured with M1 THP-1 macrophages under mock conditions (Figures 7A and 7D). More apparent, however, is the reduction in breast cancer cells seen under this coculture condition in the presence of either viral strain at an MOI of 0.1 pfu/cell (Figure 7D-7F). The breast cancer cells also appear more rounded following virus infection, particularly with rM51R-M virus (Figure 7C, 7F, and 7I). This indicates poor health, though these cells were not so damaged that they were left out of the quantification. Figure 8 shows the breast cancer cell counts from all assay conditions presented as a fold- change over the 2-hour time point. Figure 8A is the same data as in Figure 6J and reminds of the prevailing trends in breast cancer growth in the absence of VSV. We hypothesized that both viral strains would inhibit breast cancer growth in monoculture because VSV is an oncolytic virus. We further reasoned that this inhibition

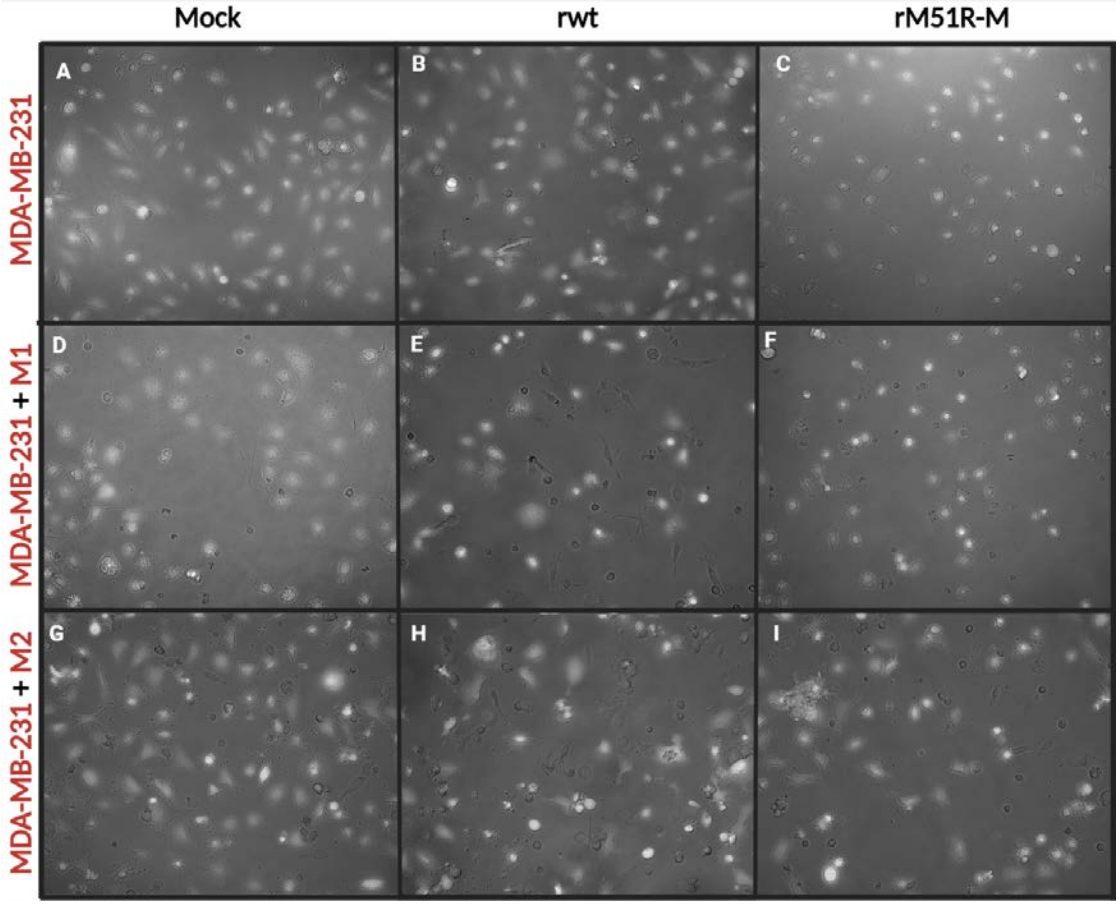



**Figure 7. Representative Images of MDA-MB-231/THP-1 Macrophage Cocultures Following Infection with VSV Strains at an MOI of 0.1.** Cell Tracker™ Violet-labeled MDA-MB-231 breast cancer cells (white) in mono- or 1:1 coculture with pre-polarized THP-1 macrophages (Mac-Lift conditions – see Figure 6F) were pre-incubated for 2 hours before infection with or without (Mock) rwt or rM51R-M virus at an MOI of 0.1 pfu/cell. Representative, composite, live cell images were taken at 14 hours and highlight fluorescent breast cancer cells (white) and macrophages (clear) at 10X magnification.

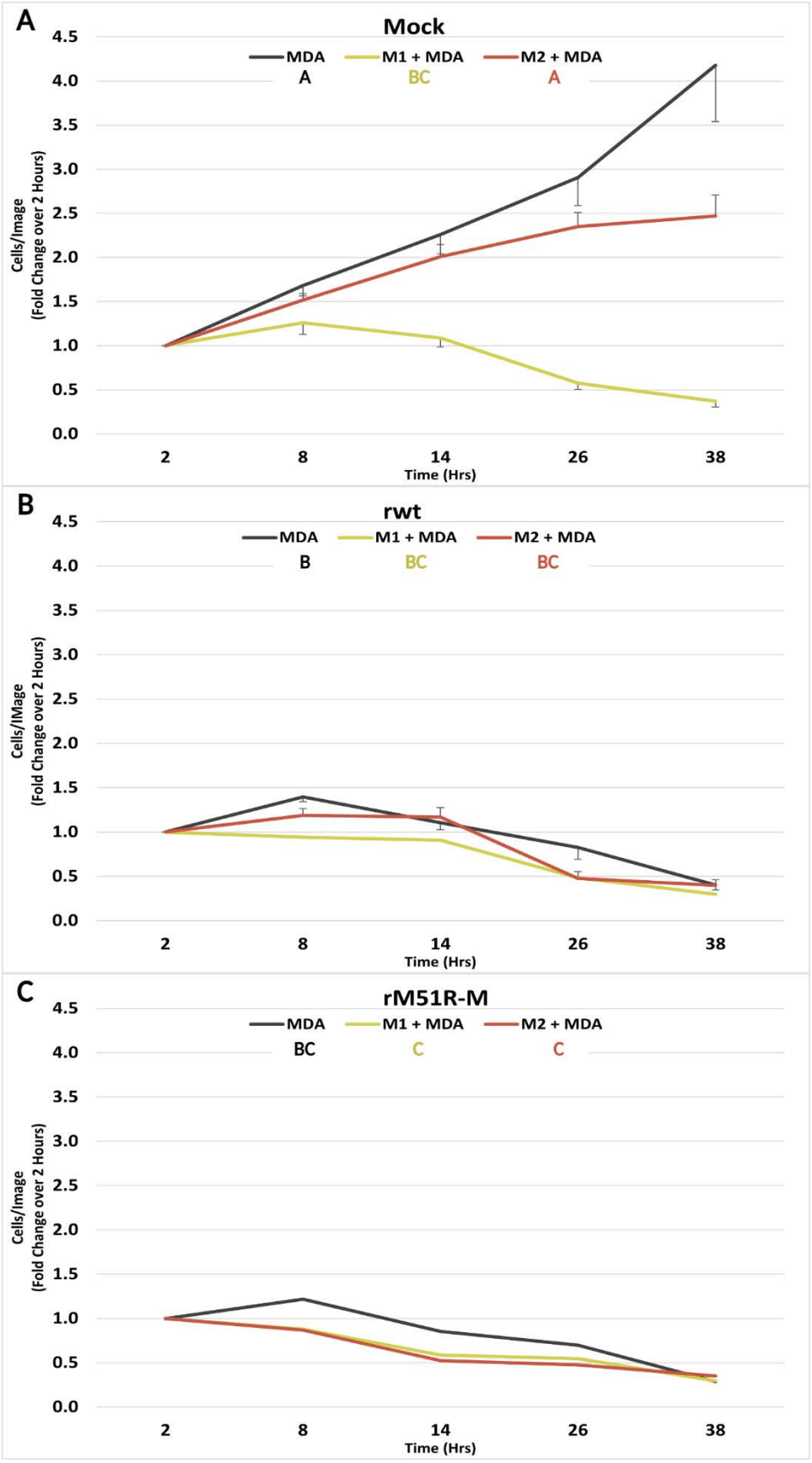

A
Mock
MDA
M1 + MDA
M2 + MDA
A
BC
A
Cells/Image
(Fold Change over 2 Hours)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2
8
14
26
38
Time (Hrs)
B
rwt
MDA
M1 + MDA
M2 + MDA
B
BC
BC
Cells/IMage
(Fold Change over 2 Hours)
Time (Hrs)
C
rM51R-M
MDA
M1 + MDA
M2 + MDA
BC
C
C
Cells/Image
(Fold Change over 2 Hours)
Time (Hrs)

**Figure 8. Interval Plot of MDA-MB-231 Cell Counts in the Presence of M1 or M2 THP-1 Macrophages with or without VSV Infection at an MOI of 0.1.** Experimental conditions involving MDA-MB-231 breast cancer cell mono- or 1:1 cocultures with THP-1 macrophages following infection with or without the indicated VSV strains (MOI of 0.1 pfu/cell) are described in Figure 6F. Breast cancer cell growth was monitored over 38 hours. Quantitation of breast cancer cell counts/images was expressed as a fold-change relative to MDA-MB-231 cells at 2 hours under mock conditions. Only MDA-MB-231 cells with evident, notable fluorescence were counted. Data are the mean $\pm$ SE of three independent experiments. Statistical significance was determined by Repeating Measures Two-Way ANOVA. Here, the overall average trend in the data is expressed by letter. Conditions that do not share a letter differ significantly (p-value $\leq$ 0.05). [Figure 8 is on the previous page.]

of breast cancer growth might even be more pronounced in the presence of M1 macrophages as there would then be two entities with growth inhibitory effects present in culture. What we observed, however, was that VSV exhibited such an unexpected potency as a cancer cell growth inhibitor in the assay that it overshadowed all the experimental conditions being studied. Whether alone in monoculture (Figure 8A) or in coculture with macrophages (Figures 8B and 8C), breast cancer cells did not grow in the presence of either viral strain across the time interval of the assay. Even the M2 macrophages were unable to protect the breast cancer cells from the cytopathic effects of VSV on breast cancer cells.

We also conducted a statistical analysis at each time point in isolation to see if any subtle effects in the prior trend data would become more apparent when analyzed across all conditions (Figure 9). What can be gleaned by these independent evaluations

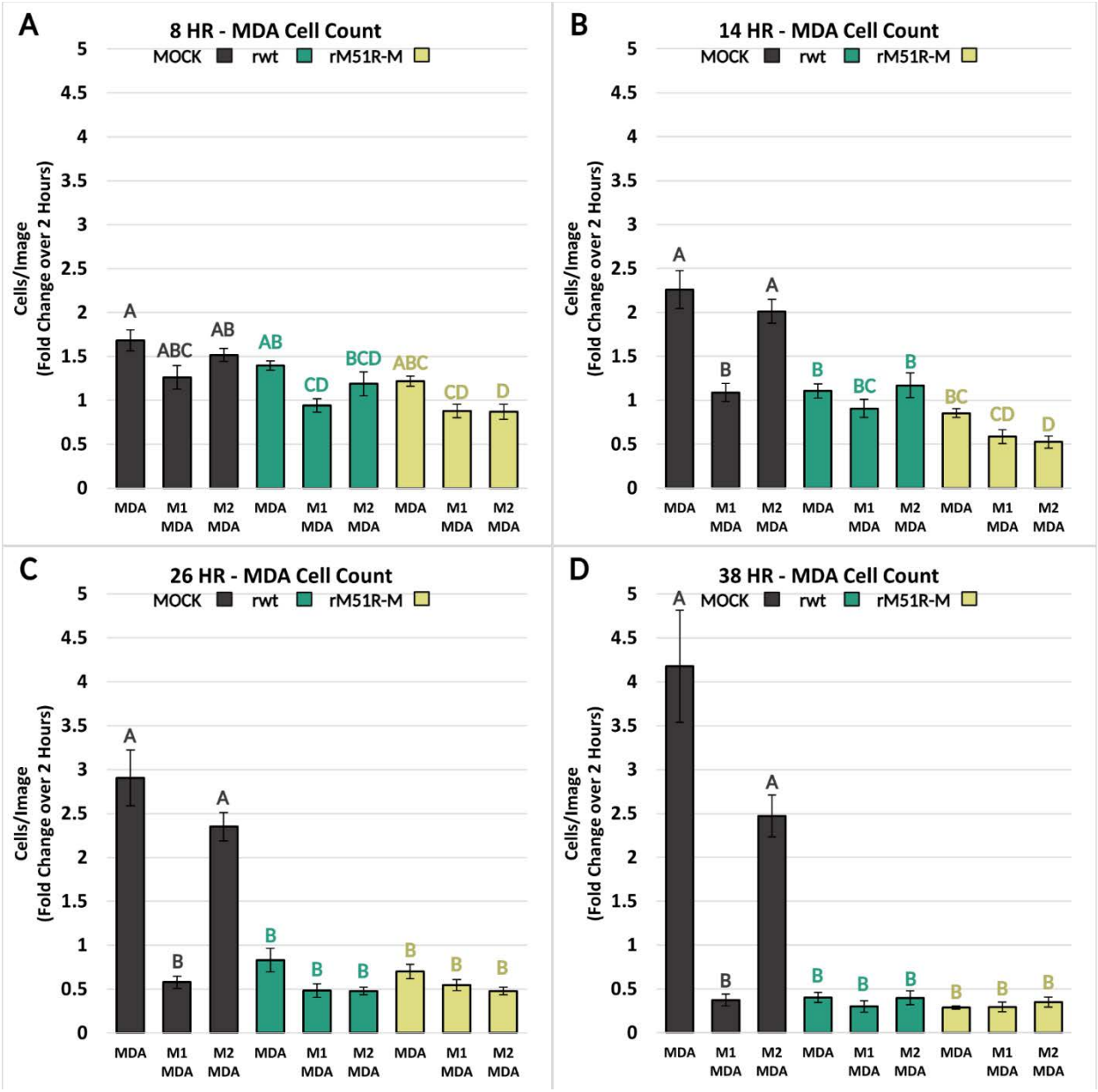



**Figure 9. MDA-MB-231 Cell Counts in the Presence of M1 or M2 THP-1 Macrophages with or without VSV Infection at an MOI of 0.1 – Analysis of Independent Time Points.** This figure provides additional statistical analysis of the data in Figure 8. Experimental conditions involving MDA-MB-231 breast cancer cell mono- or 1:1 cocultures with THP-1 macrophages following infection with or without the indicated VSV strains (MOI of 0.1 pfu/cell) are described in Figure 6F. Quantitation of breast cancer cell counts/image were expressed as a fold-change relative to MDA-MB-231 cells at 2 hours under mock conditions. Data are the mean $\pm$ SE of three independent experiments. Significant differences were analyzed using Two-Way ANOVA. Each time interval was analyzed independently. Tukey's Post-Hoc pairwise comparisons indicate differences in means by letter. Means that do not share a letter differs significantly (p-value $\leq$ 0.05).

is that starting at 14 hours (Figure 9B), but more apparent by 26 and 38 hours (Figures 9C and 9D), the M1- and M2-breast cancer cell cocultures under both virus conditions were showing identical growth inhibitory trends that were statistically significant from mock conditions. Evident in both Figures 8 and 9 is the reduction in fold-change by the 38-hour time point, indicating that there were hardly any living MDA-MB-231 cells left in culture under any virus condition.

The effect of VSV on MDA-MB-231 breast cancer cell growth was seen at a low MOI of 0.1 pfu/cell. This represents an asynchronous infection. An asynchronous infection means that not all cells are subject to infection and that antiviral responses can be communicated across the culture. Clearly the breast cancer cells were obliterated under this infection regime making it difficult to parse out any mechanistic effects exerted by each macrophage subtype or viral strain. That said, much data supports using different MOIs to examine the ultimate impact of VSV infection as a cancer therapeutic(Ludwig-Begall et al., 2021; Tang et al., 2023; Tang et al., 2022) To be thorough in our investigations, we also did breast cancer cell growth assays in mono- and coculture and in the presence of either VSV strain at a higher MOI of 10 pfu/cell. This represents a synchronous infection where the presence of excess viral particles ensures that nearly every cell in culture may be infected. We were curious as to whether the cocultured M2 macrophages might be protective of the breast cancer cells under this infection regime.

Figure 10 shows the visual landscape of MDA-MB-231 breast cancer cell mono- and

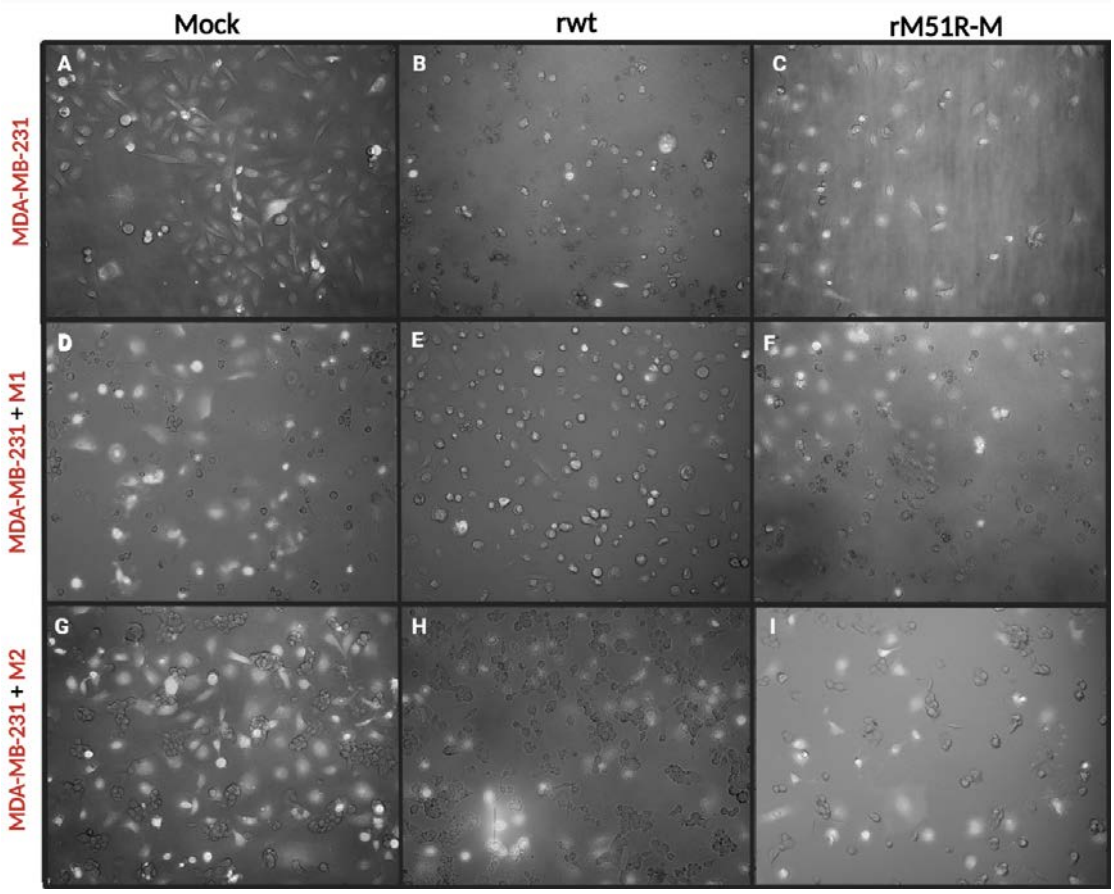
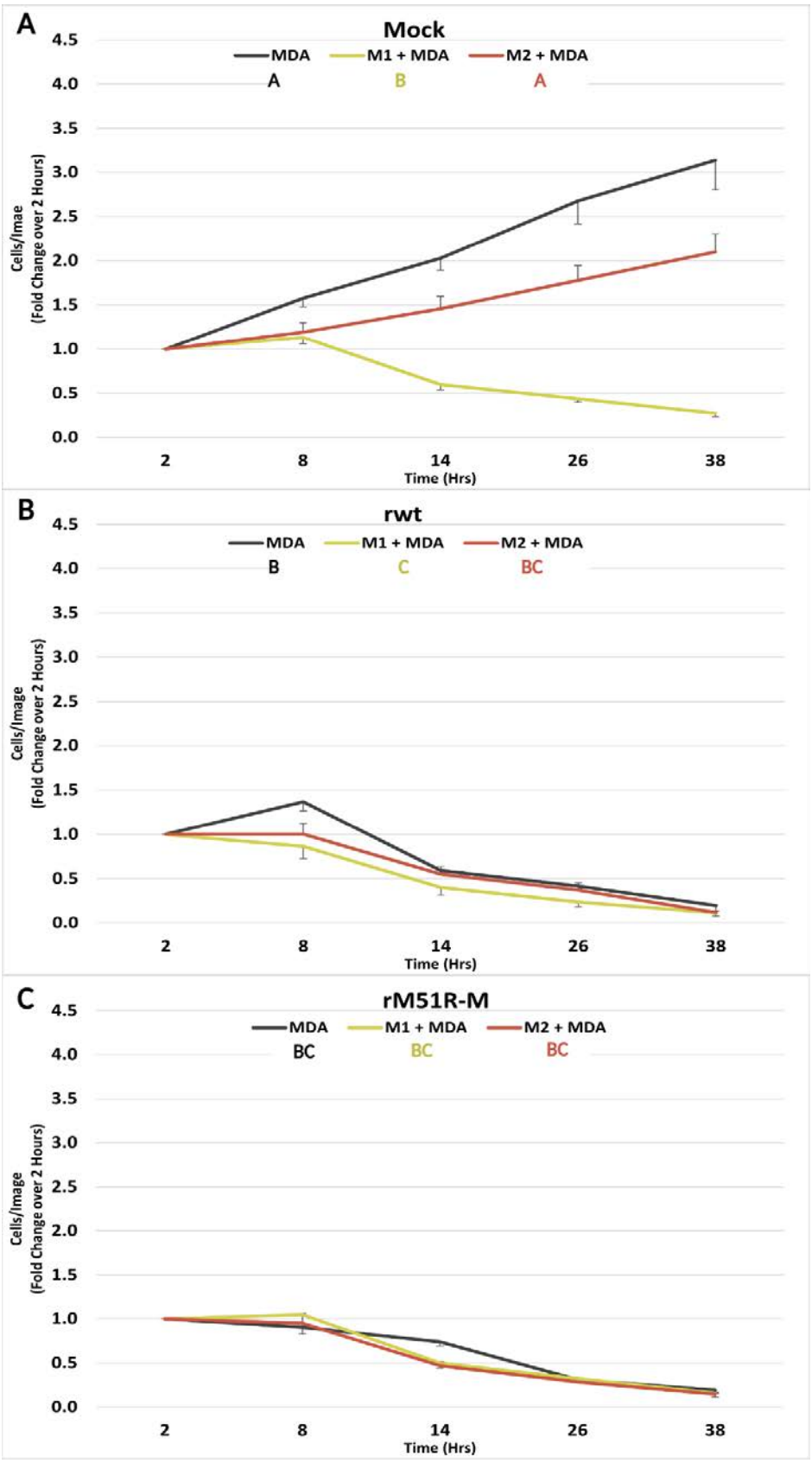



**Figure 10. Representative Images of MDA-MB-231/THP-1 Macrophage Cocultures Following Infection with VSV Strains at an MOI of 10.** Cell Tracker™ Violet-labeled MDA-MB-231 breast cancer cells (white) in mono- or 1:1 coculture with pre-polarized THP-1 macrophages (Mac-Lift conditions – see Figure 6F) were pre-incubated for 2 hours before infection with or without (Mock) rwt or rM51R-M virus at an MOI of 10 pfu/cell). Representative, composite, live cell images were taken at 14 hours and highlight fluorescent breast cancer cells (white) and macrophages (clear) at 10X magnification.

cocultures containing M1 and M2 THP-1 macrophages subjected to rwt and rM51R-M infection. Much like before, the health of these cells at 14 hours was declining in the presence of VSV. The breast cancer cells appeared rounded and fragmented, suggesting a possible induction of apoptotic pathways. Meanwhile, the macrophages were also being impacted. Most noticeable was the reduction of cocultured M2 macrophages when subjected to rM51R-M virus infection (Figure 10I). These results were not as pronounced as with rwt virus infection (Figure 10H). M1 macrophages, in contrast, appeared to be unaffected by both viruses in coculture (Figures 10E and 10F).

In this synchronous infection condition at an MOI of 10 pfu/cell, the overall MDA-MB-231 cell counts reduced in nearly an identical way to the asynchronous condition at an MOI of 0.1 pfu/cell (compare Figures 8 and 11). Moreover, M2 macrophage cocultures still failed to shield the breast cancer cells from the cytopathicity of either VSV strain. In an independent time analysis, the most notable feature of this data was the drastic drop

**Figure 11. Interval Plot of MDA-MB-231 Cell Counts in the Presence of M1 or M2 Macrophages with or without VSV Infection at an MOI of 10.** Cell Tracker™ Violet-labeled MDA-MB-231 breast cancer cells in mono- or 1:1 coculture with pre-polarized THP-1 macrophages (Mac-Lift conditions – see Figure 6F) were pre-incubated for 2 hours before infection with or without (Mock) rwt or rM51R-M virus at an MOI of 10 pfu/cell. Breast cancer cell growth was monitored over 38 hours. Only MDA-MB-231 cells with evident, notable fluorescence were counted. Quantitation of breast cancer cell counts/image were expressed as a fold-change relative to MDA-MB-231 cells at 2 hours under mock conditions. Data are the mean $\pm$ SE of three independent experiments. Cell counts across the complete time intervals were compared using a Repeating Measures Two-Way ANOVA. Tukey's Post-Hoc pairwise comparisons indicate significant differences by letter. Intervals that do not share a letter are significantly different from one another across the overall dataset (p-value $\leq$ 0.05). [Figure 11 is on the next page.]


A
Mock
MDA
M1 + MDA
M2 + MDA
A
B
A
Cells/Imae
(Fold Change over 2 Hours)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2
8
14
26
38
Time (Hrs)
B
rwt
MDA
M1 + MDA
M2 + MDA
B
C
BC
Cells/Image
(Fold Change over 2 Hours)
Time (Hrs)
C
rM51R-M
MDA
M1 + MDA
M2 + MDA
BC
BC
BC
Cells/Image
(Fold Change over 2 Hours)
Time (Hrs)

in MDA-MB-231 cell counts at 14 hours and persisting to 38 hours (Figures 12B-12D). This differs slightly from the experiment done at the lower MOI of 0.1 pfu/cell, as it took until 26 hours to note the drastic drop in MDA-MB-231 cell counts (Figures 9B-9D). Nevertheless, both viruses at both MOIs neutralize the breast cancer cells under the conditions assayed in this study.

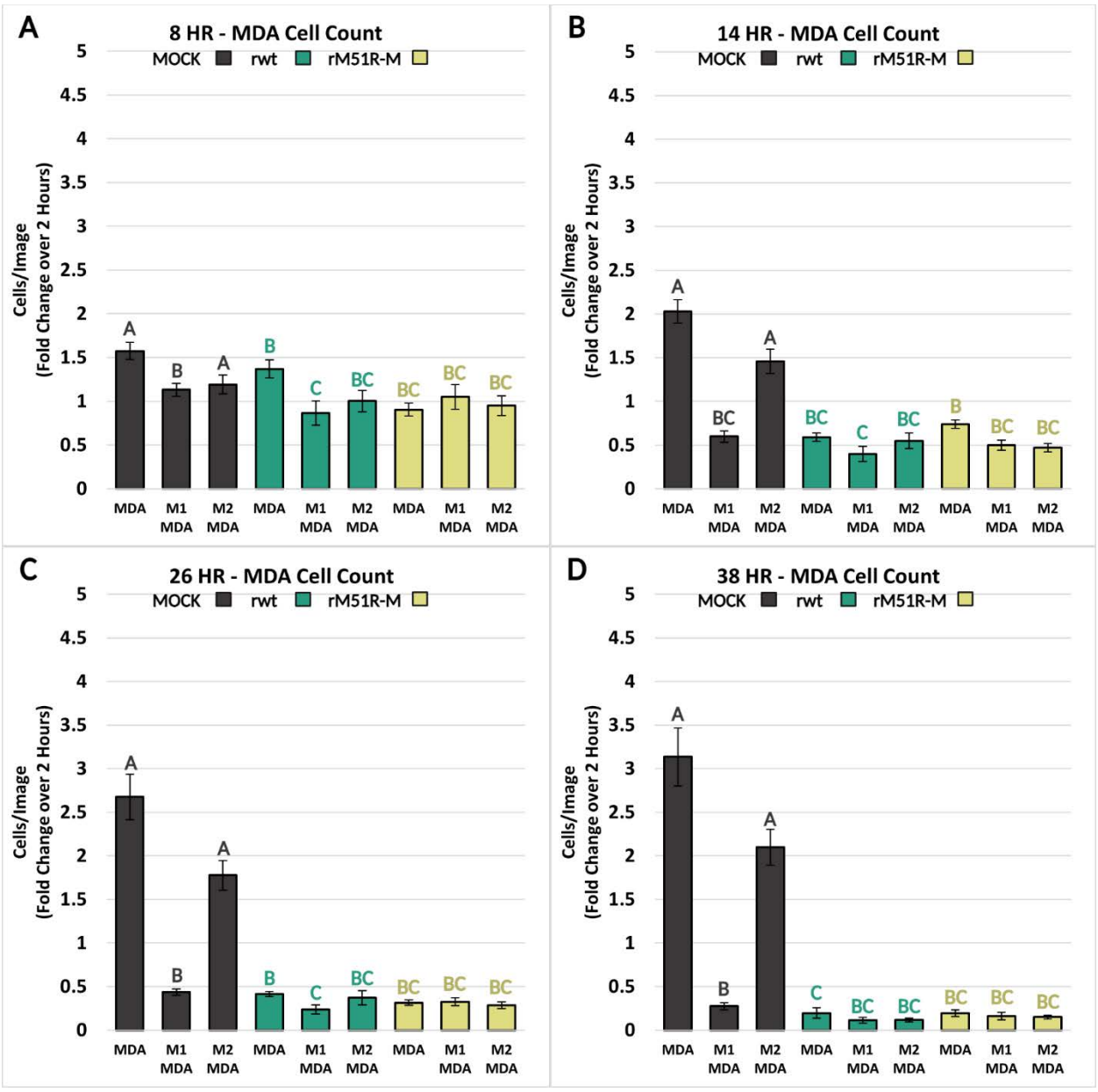



**Figure 12. MDA-MB-231 Cell Counts in the Presence of M1 or M2 THP-1 Macrophages with or without VSV Infection at an MOI of 10 – Analysis of Independent Time Points.** This figure provides additional statistical analysis of the data in Figure 11. Experimental conditions involving MDA-MB-231 breast cancer cell mono- or 1:1 cocultures with THP-1 macrophages following infection with or without the indicated VSV strains (MOI of 0.1 pfu/cell) are described in Figure 6F. Quantitation of breast cancer cell counts/image were expressed as a fold-change relative to MDA-MB-231 cells at 2 hours under mock conditions. Data are the mean $\pm$ SE of three independent experiments. Significant differences were analyzed using Two-Way ANOVA. Each time interval was analyzed independently. Tukey's Post-Hoc pairwise comparisons indicate differences in means by letter. Means that do not share a letter differs significantly (p-value $\leq$ 0.05).

# CHAPTER 4: DISCUSSION

Triple-negative breast cancer (TNBC) remains one of the most challenging and aggressive subtypes of breast cancer, primarily due to its lack of hormone receptors and HER2 amplification, which limits the available targeted therapies. The TME plays a pivotal role in TNBC progression, with infiltrating immune cells, particularly macrophages, contributing to tumor growth and evasion of the immune system. Macrophages exist in two main phenotypes: M1, which is associated with antitumor activity, and M2, which is associated with pro-tumoral activity. The balance between these two macrophage phenotypes within the TME plays a crucial role in cancer progression. Understanding the complex interplay between TNBC, macrophage subtypes, and innovative therapeutic approaches is of paramount importance in the quest for more effective treatment strategies.

This study investigated the potential of vesicular stomatitis virus (VSV) as a therapeutic option for TNBC. VSV is well-known for its selective oncolytic properties. However, its influence on the intricate crosstalk between cancer cells and TAMs, specifically in the context of TNBC, remains an underexplored area of research. To remedy that, we cocultured MDA-MB-232 TNBC cells with pre-polarized THP-1 macrophages to simulate a TME containing TAM-like populations. We then infected the cocultures with either the wild-type (rwt) or mutant (rM51R-M) strains of VSV as a therapy. One aim included the measurement of macrophage phagocytic activity, as this enabled dissection of the highly

malleable macrophage phenotype. Indeed, previous research from our lab has shown that the rM51R-M strain of VSV can repolarize M2 THP-1 macrophages in monoculture towards an M1-like phenotype, thereby suggesting still another benefit to VSV's antitumor arsenal, *i.e.*, the ability to turn a 'friend' of cancer into a tumor 'foe' (Polzin et al., 2020). This led us to the primary aim of this study, to compare VSV's inhibition of breast cancer cell growth in both mono- and coculture conditions. We hypothesized that VSV infection would not only exhibit oncolytic effects on TNBC cells, but would also shift the cocultured macrophages to the M1 phenotype, thereby taking advantage of the antitumor properties of these macrophages to exert an even more robust inhibition of breast cancer development.

The potential findings from this study were considered to have profound implications for the development of more targeted and efficacious therapeutic strategies against TNBC. If VSV could effectively disrupt the TME by reprogramming TAMs towards an antitumoral state, it may represent a novel approach for TNBC treatment. This approach also aligns with the broader goals of immunotherapy, where harnessing the power of the immune system to combat cancer is an evolving paradigm in oncology.

***Discussion of Phagocytosis Data***

Previous research in our lab discovered several important effects of VSV on THP-1 macrophages (Polzin et al., 2020). First, the rwt strain of VSV induced significant cell

death on M2 macrophages, possibly by inhibiting host gene expression and triggering rapid apoptosis. The rwt virus also disarmed podosome formation in the M2 macrophages. On the other hand, the rM51R-M strain of VSV activated the antiviral STAT1 pathway in M2 macrophages, a pathway that produces M1-polarizing cytokines that, in turn, promoted certain aspects of M1 programming like production of the pro-inflammatory factor TNF-α. Meanwhile, M1 macrophages, polarized by LPS and IFN-γ, remained resistant to infection, further emphasizing the potential of VSV to completely reprogram TAM populations. More recently, Austin Simmons, in his graduate thesis work, explored the phagocytic abilities of THP-1 macrophages, focusing on how that activity responds to VSV infection (Simmons, 2021). He solidified, as was true of other studies, that M2 macrophages were more robust phagocytes than their M1 counterparts (A-Gonzalez et al., 2017; Aderem & Underhill, 1999a; Aderem & Underhill, 1999b; Schulz et al., 2019; Uribe-Querol & Rosales, 2020). He also found that infection with rM51R-M virus lowered the phagocytic capacity of M2 macrophages towards *E.coli* bioparticles to that of M1 levels, validating the possible change in phenotype suggested by the previous marker analysis (Owen, 2020; Polzin et al., 2020a). Simmons also examined VSV replication within MDA-MB-231 breast cancer/THP-1 macrophage cocultures, finding that VSV primarily infects cancer cells over macrophages and remains effective in decreasing cancer cell proliferation despite diminished replication in coculture conditions (Simmons, 2021).

We first aimed to expand upon the phagocytic assay utilized in the Simmons thesis by (1) focusing on the ingestion of fluorescent latex beads, (2) using both M1 and M2 THP-1 macrophage populations, and (3) studying the effects of both rwt and rM51R-M strains. The focus on a phagocytic assay to identify macrophage polarization states was partly due to previous failed attempts to detect reactive oxygen/nitrogen species in THP-1 cells (Owen, 2020). In contrast, Simmons showed that phagocytosis was both measurable and distinguishable in THP-1 macrophage subtypes (Simmons, 2021). We now sought to determine whether that was also true within coculture systems and whether the effect of rM51R-M virus was retained.

First, we confirmed that more M2 THP-1 macrophages phagocytose latex beads than M1 THP-macrophages, both in monoculture as well as in coculture with MDA-MB-231 breast cancer cells (Figure 4). The reduced phagocytic capability of the M1 macrophages aligns with the alternative mechanisms (*e.g.*, ROS production) these cells use to aid the immune response (Acharya et al., 2020; Aderem & Underhill, 1999a; Gonzalez et al., 2023; Mendoza-Coronel & Ortega, 2017; Scott et al., 2001). Second, we were somewhat surprised to see that coculturing macrophages with MDA-MB-231 breast cancer cells increased phagocytosis so significantly, particularly in M2 macrophages.

What would be the benefit for breast cancer cells to increase the percentage of actively phagocytosing macrophages? Before addressing this, it might first be important to mention that our assay measures the phagocytosis of non-opsonized latex beads, not

infectious agents (like the *E.coli* bioparticles studied by Simmons) and not cancer cells (studied by other groups) (Cao et al., 2022; Fraser et al., 2009; Garcia-Aguilar et al., 2016; Hess et al., 2009; Lingnau et al., 2007; Simmons, 2021; Swanson & Hoppe, 2004; Wright & Silverstein, 1982). Currently, there is a lot of interest in stimulating phagocytosis to target and eliminate opsonized cancer cells. This not only kills the cancer cells, but also enables antigen presentation to the adaptive immune system (Benoit et al., 2012; Benoit et al., 2012; Jaiswal et al., 2009; Pio et al., 2014; Tseng et al., 2013; Vinay et al., 2015; Wang et al., 2017; Zhang et al., 2016; Zhang et al., 2017). However, this is not what we were studying in this assay. Another key function of macrophages is to phagocytose extracellular matrix material and apoptotic cells as part of wound healing. The phagocytosis of apoptotic cells, including dying cancer cells, is known as efferocytosis, which is very similar to phagocytosis, and is critical to maintaining tissue homeostasis (A-Gonzalez et al., 2017; Mukundan et al., 2009; Rőszer et al., 2011; Scott et al., 2001; Uribe-Querol & Rosales, 2020). Efferocytosis is thought to mediate a tolerance for cancer cells. This is based on the consistent exposure to antigens from dying cancer cells (Hochreiter-Hufford & Ravichandran, 2013). Efferocytosis can also lead to the release of immunosuppressive factors like TGF-β and IL-10, which are both known to inhibit the activity of cytotoxic T cells and natural killer cells (Szondy et al., 2017; Zhou et al., 2020). Whether efferocytosis is going on in an actively growing MDA-MB-231 breast cancer culture is unknown, but it might explain the observed increases in bead uptake here. Alternatively, stimulating a phagocytic mechanism that can be directed against other particles in the tumor microenvironment that need clearing (*i.e.*, the

extracellular matrix), might also be beneficial to the kind of space-clearance needed to support continued cancer growth, if not invasion and metastasis as well.

We hypothesized that rM51R-M virus would reduce phagocytosis of latex beads by M2 THP-1 macrophages because we had seen that before with *E.coli* bioparticles (Simmons, 2021). Not only did we see that reduction, but it also occurred in coculture with the breast cancer cells, and the results were indistinguishable from M1 macrophage mono- and cocultures. This suggests a repolarization of M2 macrophages to M1 macrophages based on a functional assay. rwt virus, meanwhile, had a comparatively less effect on M2 macrophage phagocytosis in coculture and no effect at all in monoculture. Since we had also previously seen rM51R-M virus-mediated decreases in viability in M2 macrophages, it was prudent to evaluate whether this decrease in phagocytic activity was simply due to M2 macrophage cell death (Polzin et al., 2020a). It was not. When comparing the M2 macrophage counts in response to rM51R-M virus infection versus rwt virus infections, both decreased to comparable levels. Seeing that both infection conditions had similar numbers of macrophages, the effects we were witnessing can be attributed to virus modulation of the macrophages, not simply oncolysis.

Why does the rM51R-M virus reduce M2 macrophage phagocytosis more substantially than the rwt virus? One answer may lie in what happens in macrophages infected by these two viral strains. rM51R-M virus has the potential to be a potent cancer therapeutic because deactivation of the M protein enables the host antiviral response.

This response naturally comes in the form of the type I IFNs (IFN-α and IFN-β). These IFNs are secreted by infected cells and induce antiviral activity in neighboring cells (McNab et al., 2015; Murira & Lamarre, 2016). Strikingly, such antiviral signaling is similar to the response of macrophages to LPS and the type II IFN-γ during M1 polarization. IFN-γ is produced by many immune cells, including cytotoxic T-cells and natural killer cells, and stimulates macrophages to clear infectious agents (Green et al., 2017; Lee & Ashkar, 2018). By virtue of the overlap between antiviral and M1 polarization signaling, rM51R-M virus infections essentially promote M1-polarization, replete with M1 markers and M1 functions, which in the case of phagocytosis of non-opsonized latex beads is far lower than that of M2 macrophages. This suppression in phagocytosis in M1 macrophages has been previously attributed to the downregulation of macrophage receptors with collagenous structure (MARCO), which is a major binding receptor on the surface of macrophages for nonopsonized particles (Wang et al., 2015). To this end, IFN-γ stimulates M1-like behavior and activity as a means of confronting infection, which translates to a decrease in phagocytosis in favor of other mechanisms of defense (Schütze et al., 2021). Infection with rwt virus does not follow this paradigm as its intact M protein does shut down host gene expression, no antiviral response is elicited, and thereby no M1 polarization factors are released into the media.

***Discussion of Breast Cancer Cell Growth Data***

The ultimate goal of this study was to investigate breast cancer cell growth *in vitro*, but to do so under conditions in which the cells were intermixed with pre-polarized M1 and M2 macrophage populations. The macrophages would thereby serve as a surrogate for TAMs when in coculture. Under mock conditions MDA-MB-231 monocultures showed a trending increase in growth. This was sensible given the aggressively proliferative nature of this TNBC cell line. A similar expectation was met with regards to the breast cancer cell/M1 macrophage cocultures in that the macrophages exerted a significant inhibitory effect on breast cancer cell growth. This result aligns with data supporting the anti-proliferative nature of M1 macrophages on cancer cell populations both *in vitro* and *in vivo* (Gavalas et al., 2010b; Cao et al., 2022; Edin et al., 2012; Finn, 2012; Jochems & Schlom, 2011; Lv et al., 2022; Pan, 2012). What deviated from our initial hypothesis was the behavior of breast cancer cell/M2 macrophage cocultures. While there was a trending increase in growth under these conditions, it was not significantly different from MDA-MB-231 monocultures. M2 macrophages are typically shown to support cancer cell growth (Biswas et al., 2012; Genin et al., 2015a; Liu et al., 2021; Mantovani et al., 2002; Salmaninejad et al., 2019; Sousa et al., 2015; Yuan et al., 2015; Zhou et al., 2022). However, there are some key differences in how other groups conduct their *in vitro* studies when showing the growth-supporting qualities of M2 macrophage populations on various forms of cancer. Many researchers stray from the utilization of THP-1 cells in favor of primary blood monocytes (Chanput et al., 2014; Genin et al., 2015; Hoppenbrouwers et al., 2022). For example, one assay showed statistically significant increases in pancreatic cancer cell growth when the conditioned media from

M2 peritoneal blood macrophages was applied. Perhaps, the use of THP-1-derived macrophages, a monocytic leukemia cell line, fails to match the nature of primary cells when in coculture. The robustness of interleukin expression in primary monocytes as compared to THP-1-derived cells may also explain our dampened effect (Hoppenbrouwers et al., 2022).

Perhaps no other result in this study was more pronounced than the reductions in MDA-MB-231 cell growth following infection with rwt or rM51R-M virus. No cell growth was detected beyond the initial counts at 2 hours, and there were significant decreases as early as 14 hours. These decreases were observed with both asynchronous (MOI of 0.1 pfu/cell) and synchronous (MOI of 10 pfu/cell) infection conditions, and with or without cocultured macrophages of either polarization state. All growth intervals showed the same statistical reductions. Even M2 macrophages failed to block the inhibitory impact of these viral strains on this breast cancer cell line. There is data to support the loss in breast cancer cell growth. It is based on MTT assay results from a former lab member Jessica McCanless, who was the first to identify the harmful effects of VSV infection on MDA-MB-231 cell viability (McCanless, 2019). Other data support the susceptibility of breast cancer cells to rM51R-M infection as well (Ahmed et al., 2010). However, no previous study has, to our knowledge, seen the kind of decimation seen here, though it is possible that cell count data reveals a more substantial effect on the cells than the metabolic activity measured in MTT assays.

As mentioned prior, one of the most crucial findings in this study was how the M2 macrophages failed to protect MDA-MB-231 cells from the oncolytic activity of VSV. This is in direct opposition to similar studies regarding the protective qualities of M2s in response to chemotherapies (Shree et al., 2011). Although macrophages were not directly counted in the growth assays as they were in phagocytosis assays, we have evidence from those prior counts to suggest that this effect is not due to a similar effect on macrophages. Macrophages are still present and visible in the growth assay images as well. In the case of rM51R-M virus, these data suggest that the lack of protective qualities from the M2 macrophages might simply be due to their newfound modulation into M1 macrophages following virus infection. However, a different hypothesis must be formulated for rwt virus, which unlike rM51R-M virus, does not have the same macrophage repolarization effects in culture.

***Future Directions***

Several oncolytic viruses have been known to encourage a macrophage polarization switch by modulating cytokine secretions (Cao et al., 2021; Cripe et al., 2015; Delwar et al., 2018; Heo et al., 2011; Kwan et al., 2021; Kwan et al., 2021; Packiriswamy et al., 2020; Piersma et al., 2007; Shalhout et al., 2023). In this study, through analysis of macrophage phagocytic function and breast cancer cell growth, we add commentary to the hypothesis that rM51R-M virus induces an M1-like phenotype change in M2 macrophages. We have seen that the phagocytosis activity of M2 macrophages dropped

to that of M1 macrophages, but we have not exhausted our means of identifying the totality of this possible polarization change. One clear objective following this study would be to use flow cytometry to identify surface markers associated with M1 (CD80, CD86, TLR2, TLR4) and M2 macrophages (CD204, CD163, and CD206) to further substantiate changes in macrophage subtype (Liu et al., 2022). We then, could evaluate the levels of these markers as a function of mono- and coculture conditions and in the presence or absence of VSV infections.

Another direction of analysis would be to establish a more direct phagocytosis assay that pertains to the engulfment of cancer cells themselves. This can be accomplished using a mixture of flow cytometry and fluorescent microscopy. Flow cytometry can be used to identify tumor antigen-positive macrophages undergoing presentation following phagocytosis of cancer cells. Complimenting this, fluorescent microscopy imaging could identify if labeled cancer cells are being internalized by macrophage populations. However, it is important to note that most of these approaches are based in flow cytometry given the efficient means of collecting larger data sets and the many parameters that can be measured using this technique (Alvey & Discher, 2017; Martinez-Marin et al., 2017; Munn et al., 1990; Xu et al., 2023).

In our experiments, we did not see a stimulatory effect by M2 THP-1 macrophages on MDA-MB-231 breast cancer cell growth. It is possible that such effects were not seen because of the nature of this cancer cell line. MDA-MB-231 cells are considered among

the more aggressive of breast cancer cell lines, so proliferative that it may be difficult for a supporting cell to stimulate it any further. Future studies of cancer cell/macrophage cross-talk in coculture might be advised to employ less aggressive breast cancer cell lines, such as MCF7 or T47D. These alternate cell line models may help tease out whether M2-to-M1 macrophage repolarization by rM51R-M virus is a part of its antitumor arsenal. Relatedly, another potential avenue could be found in testing primary macrophages in coculture. Previous work by graduate student Dalton Sizemore has established the use of human peripheral blood monocyte-derived macrophage, which behave in monoculture in ways similar to THP-1 macrophages (Sizemore, 2021). We could reassess the current coculture assays using these primary cells to see if our data relating to THP-1-derived macrophages is significantly different. This approach could potentially elevate the experiments conducted in this lab to be more functionally relevant to data in the cancer research field.

Regardless of the possible issues with this coculture model system, the conducted experiments do carry profound implications for the treatment of TNBC. Successful outcomes such as the decline in MDA-MB-231 cell counts by M1 THP-1 macrophages alone or by either infection with rwt or rM51R-M virus suggest a potentially powerful 2-pronged therapeutic approach. It is uncertain whether TNBCs might be uniformly sensitive to M1 macrophages and/or VSV, but, if so, then employment of such a mechanism against a type of cancer that is recalcitrant to current therapies is a promising consideration in oncology research.

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## Vita

Eliza Grace Watson was born in Bryson City, North Carolina, to Paula A. Watson. She graduated from Swain County High School in June 2017. Congruent with her high-school diploma she received a dual-enrollment associate in science degree, cum laude, from Southwestern Community College. In the fall of 2017, she entered the University of North Carolina at Asheville, and in December 2020 was awarded the Bachelor Science degree. In the fall of 2021, she began the Master of Science in Biology program at Appalachian State University in the laboratories of Dr. Darren Seals and Dr. Maryam Ahmed. Upon completion of her Master of Science in Biology in December 2023, she aims to work in the cancer research industry.