THE CYTOTOXIC EFFECTS OF VESICULAR STOMATITIS VIRUS ON THP-1 MACROPHAGES

by

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ABSTRACT

Cancer cells do not act autonomously. The interaction between cancer cells and other 'normal' cell types creates a tumor microenvironment conducive for the development, growth, and progression of the disease. One example is the macrophage, an immune cell that phagocytizes pathogens during infection and removes unwanted cellular debris during injury, but which also accumulates in cancerous tissue as so-called tumor-associated macrophages (TAMs). M1-like TAMs are pro-inflammatory, immunostimulatory, and may directly target and kill cancer cells. M2-like TAMs, in contrast, generate the growth factors that support cellular growth, angiogenesis, and metastasis, and have thus been deemed a desirable target for therapeutics. Vesicular stomatitis virus (VSV) is known for its natural ability to target cancer cells, but its effect on TAMs is unclear. Here we used common agonists to pre-polarize model THP-1 monocytes into M0 (PMA), M1 (PMA, LPS, IFN-γ), or M2 (PMA, IL-4, IL-13) macrophages in order to measure their response to infection with a recombinant wild-type strain of VSV (rwt-GFP) as well as an isogenic mutant strain (rM51R-M-GFP) that is nonvirulent in normal tissues. The ability of VSV to infect and replicate in monocytes and macrophages was determined by live cell imaging of the green fluorescent protein (GFP). Results indicated that monocytes (73% GFP-positive), M0 macrophages (23% GFP-positive), and M2 macrophages (36% GFP-positive) supported the replication of both viral strains. Monocytes and M0 macrophages, however, were slightly more sensitive to the M protein mutant strain of VSV while M2 macrophages were slightly more sensitive to the wild type strain. M1 macrophages, in contrast, were completely resistant to both viral strains; no cells were GFP-positive under any experimental condition. The viability of macrophages following infection with VSV, as determined by an MTT assay,

showed similar results to the replication experiments. Most importantly, M1 and M2 macrophages were differentially susceptible to killing by rwt and rM51R-M viruses. Anti-tumor M1 macrophages were resistant to the cytotoxic properties of both viruses while tumor-promoting M2 macrophages were more sensitive, with greatest sensitivity to the rwt strain (31% viability) at an MOI of 10. The greater susceptibility of M2 macrophages to oncolytic VSV strains suggests newfound benefits for anti-cancer virotherapies targeting pro-tumor, M2-like TAM populations.

INTRODUCTION

Cancer Defined

Cancer is a collection of related diseases in which certain cells of the body divide without stopping and then spread to surrounding tissues. Cancer is caused by mutations in the oncogenes and tumor suppressor genes that control basic cellular behaviors like division, motility, and even death. These mutations may be genetically predisposed or may arise sporadically from exposure to carcinogenic stimuli such as tobacco smoke or UV radiation. Once the initial tumor is formed, cancers can spread locally to surrounding tissues or systemically throughout the body in a process called metastasis.

Cancer Treatment Modalities

Primary tumors (*e.g.* breast and prostate cancer) often have defined and successful treatment regiments when identified at early stages. This includes the traditional cancer treatments of chemotherapy, radiation, and surgery. Widely used against malignant or rapidly invading cancers, cytotoxic chemotherapy drugs kill cancer cells, but commonly have severe side effects such as alopecia, gastrointestinal irritation, and depletion of red blood cells (Gerber, 2008). Radiation, in contrast, uses high-energy x-rays or gamma rays to kill or shrink tumors and may be used in conjunction with chemotherapy and/or surgical intervention (Evans and Staffurth, 2017). Gaining in popularity are newer treatment modalities like hormonal therapy, immunotherapy, and targeted therapy. Hormone therapy is used prior to surgery or radiation in cancers of the breast or prostate tissue to reduce the size of the tumor or reduce the risk of recurrence (Abraham and Staffurth, 2016). Targeted therapy works to neutralize the signaling

pathways activated by oncogenes, including those involved in the uncontrolled proliferation of cancer cells (Scroff et al., 2018). Immune therapy refers to the stimulation of anti-tumor immune cells and is among one of the more exciting frontiers in cancer medicine (Khagi and Vlahovic, 2017). Regardless of the techniques in use today, metastatic cancers generally remain noncompliant, and there remains a constant need for novel treatment options along with clarification of the multiple and widespread factors that regulate this particular aspect cancer progression.

Oncolytic Virotherapies

Oncolytic virotherapies are also gaining in popularity. During a viral infection, host machinery is often manipulated in such a way as to promote the assembly of new viral particles. This compromise in host cell function may lead to irreparable harm and even death in the host cell. Thus, injection of an oncolytic virus into a tumor can directly kill infected cancer cells. Moreover, viral replication in infected cancer cells amplifies the viral load in tumor tissue, spreading the infection tumor wide (Biederer et al., 2001). Oncolytic virotherapies also induce an anti-viral response in cancer cells. These ‘danger signals' secreted as cytokines stimulate a host immune response, which also can promote destruction of the tumor (Biederer et al., 2001). Many viruses are being tested in clinical trials, including measles, herpes simplex, Newcastle disease virus, retrovirus, adenovirus, and vesicular stomatitis virus (Liu et al., 2013; Buijs et al., 2018).

Vesicular Stomatitis Virus

Vesicular stomatitis virus (VSV) is an RNA virus in the family Rhabdoviridae (Balachandran and Barber, 2000; Westcott et al., 2013). The Rhabdoviridae family is comprised

of enveloped viruses with a distinctive bullet-shape. Their genomes consist of single stranded negative-sense RNA. VSV is a common virus in livestock and causes a mild fever and blister-like lesions on the inside of the mouth, lips, nose, hooves, and udder (Buijs et al., 2015). Attachment to the host is initiated by viral glycoproteins and the virus enters the host cell via receptor-mediated endocytosis. Once inside the host, its genome is replicated, and progeny virus are assembled and released. One product is an RNA molecule that serves as a functional ligand for receptors that, when recognized, stimulates a type I interferon response in the host (Chávez-Galán et al., 2015). In the recombinant wild-type strain of VSV (rwt), inhibition of the host anti-viral response is achieved via the M protein. The M protein, encoded by one of 5 genes on the small VSV operon, blocks nuclear pores and thereby interferes with the ability of host mRNA to travel from the nucleus to the cytoplasm (Kopecky et al., 2001; Ahmed et al., 2010). It also inhibits all three host RNA polymerases (Kopecky et al., 2001). Thus, the M protein limits host gene expression, including interferon and interferon stimulated genes (ISGs) involved in host anti-viral defense (Westcott et al., 2013). It also has the effect of supporting viral replication by preferentially transcribing viral genes. A mutated VSV strain has been developed in which the M protein harbors a methionine to arginine substitution at the fifty-first amino acid (rM51R-M) (Ahmed et al., 2010; Redondo et al., 2015). Without the ability to inhibit host genome expression, this mutant strain is considered a safer, less virulent option for therapeutic use (Kopecky and Lyles, 2003). As an oncolytic therapy, VSV is used to induce autophagy in cancer cells. This leads to the presentation of tumor cell antigens and stimulation of an anti-tumor response by the immune system. Thus, the virus is able to directly kill cancer cells, but has the dual and equally important role of alerting the immune system to the presence of the tumor, and thereby inducing tumor cell death in an indirect manner (Bartlett et al., 2013). The virus can be

administered intravenously or intratumorally, alone or in conjunction with other cancer treatment modalities (Lun et al., 2006).

Podosomes

Actin-rich cell structures that aid in motility and invasion are known as podosomes. These ventral surface protrusions aid in invasion via motility and degradation of the extracellular membrane (Blouw et al., 2008). While found in other 'normal' cell types such as osteoclasts and vascular smooth muscle cells, podosomes are also found in both cancer cells and all polarized macrophages (Blouw et al., 2008; Polzin, 2017). Proteins associated with the actin filaments in the podosomes are involved in cancer cell invasion and subsequent metastasis, which is why podosome expression is examined in this study.

Macrophages

One of the cell types that can recognize pathogenic intruders, including viruses, are macrophages. Macrophages are part of the innate immune system and in a healthy organism are charged with ridding the body of harmful pathogens or repairing damaged cells and tissues (Italiani and Boraschi, 2014). As phagocytes, macrophages can present foreign antigens to other immune cells. They also help initiate an inflammatory response to danger signals and thus amplify the overall immune response to infection. The macrophages that reside in tissues have differentiated from circulating monocytes of the blood, bone marrow, or spleen in response to local cytokines like colony-stimulating factors (CSF) and interleukins (IL) (Italiani and Boraschi, 2014). Macrophages are assigned different names based on their resident tissue, including alveolar macrophages in the lung, osteoclasts in the bone, microglial cells in the central nervous

system, and Kupffer cells in the liver (Italiani and Boraschi, 2014). Resident macrophages are invasive. Their ability to move through tissues and to remodel the extracellular matrix is mediated by actin-rich protrusive structures along the ventral cell surface called podosomes (Burger et al., 2011). Podosomes are thus a well-known morphological marker of macrophages, though they are also made by other invasive cells (*e.g.* osteoclast and vascular smooth muscle cells), including the related invadopodia of cancer cells.

M1 and M2 Macrophages

Macrophages exhibit considerable plasticity and can exist among a wide range of phenotypes. At the two polar extremes are the classically activated M1 macrophages and the alternatively activated M2 macrophages (Italiani and Boraschi, 2014; Chávez-Galán et al., 2015).

M1 macrophages respond to infections. For example, M1 macrophage polarization is stimulated by lipopolysaccharide (LPS), a bacterial membrane component in gram-negative bacteria, and by pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α) or interferon-γ (IFN-γ) (Italiani and Boraschi, 2014). M1 macrophages are recognized by the cytokines they secrete, like IL-1β, TNF-α, IL-12, and IL-18, as well as by their expression of cell surface protein markers like major histocompatibility complex class II (MHC-II), CD68, and the costimulatory molecules CD80 and CD86 (Chávez-Galán et al., 2015). Infectious diseases with intracellular pathogens such as *M. tuberculosis* (tuberculosis) and *L. monocytogenes* (listeriosis) also activate pro-inflammatory M1 macrophages (Chávez-Galán et al., 2015). M1 macrophages also play roles in autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and inflammatory bowel disease. To the extent that mutations may create alterations in the surface of

cancerous cells, M1 macrophages may also recognize them as foreign, and hence M1 macrophages have an anti-tumor phenotype.

While they are responsive to parasites, alternatively-activated M2 macrophages are morphologically and phenotypically distinct from their M1 counterparts. M2 macrophages promote angiogenesis, tissue remodeling, and growth during wound healing. They are stimulated by cytokines like CSF-1, IL-4, IL-10, IL-13, and transforming growth factor-β (TGF-β). Their expression of the scavenger receptor A CD204 and the mannose receptor CD206 uniquely distinguishes M2 macrophages from other macrophage subtypes (Chávez-Galán et al., 2015). M2 macrophages are considered pro-tumor in the sense that they are able to support new vasculature and secrete growth-promoting cytokines. There is also evidence that M2 macrophages combine with endothelial and cancer cells to form a triad, which marks the sites of intravasation into blood vessels as an early step in metastasis (Robinson et al., 2009).

Tumor-Associated Macrophages

The tumor microenvironment (TME) is the tissue surrounding a tumor (Yang et al., 2015). It includes the blood vessels that supply oxygen and nutrients, as well as access to the circulation for tumor metastasis (Yang et al., 2015). It also includes neighboring stromal cells like fibroblasts, vascular endothelial cells, immune cells, adipocytes, and mesenchymal stem cells, as well as the cytokines they secrete. Macrophages are also present and functional within cancerous tissue where they sometimes can encompass as much as 50% of a tumor's mass (Vinogradov et al., 2014). As an internal wound site, the phenotype of these so-called tumor-associated macrophages (TAMs) often lies closest to the M2 end of the macrophage spectrum. High incidence of M2-like TAMs promotes tumor vascularization and secretes growth factors

that support tumor growth and metastasis. M2-like TAMs are thus associated with poor cancer patient prognosis (Lin et al., 2006; Bailey et al., 2007).

Hypothesis

In theory, inhibiton of the growth-promoting TME might reduce inflammation and/or aid the targeting of cancerous cells (Chávez-Galán et al., 2015). Thus, the goal of this study was to determine the effect of oncolytic VSV on macrophages in the tumor microenvironment. As a known oncolytic agent, the impact of VSV on pro-tumor M2 or anti-tumor M1 macrophages is unclear. Conflicting data regarding the effects of oncolytic virotherapy on macrophages in the tumor microenvironment poses further questions (Passaro et al., 2016; Tan et al., 2016; Liu et al., 2013). Is one macrophage phenotype preferentially killed over another? Do they mount different viral defenses? Does viral infection promote a phenotypic switch from M2 to M1 macrophages? Here the specific focus is on delineating techniques for the proper polarization of macrophages *in vitro* as well as an assessment of the susceptibility of M1 and M2 macrophages to replication by and cytotoxicity to VSV.

MATERIALS AND METHODS

Virus Stocks

Recombinant wild-type (rwt) and matrix protein mutant (rM51R-M) strains of vesicular stomatitis virus (VSV), both with or without insertions of the green fluorescent protein (GFP) gene, were a generous gift from Dr. Doug Lyles from the Wake Forest University School of Medicine (Winston Salem, NC). Viral stocks were grown in baby hamster kidney (BHK) fibroblasts using established procedures (Lyles et al., 1996). Briefly, BHK cells were infected using original recombinant viral stock. After a 24-hour incubation, supernatant media containing virus was harvested, serially diluted, and then applied to 80% confluent, non-infected BHK cells in a 6-well plate overlaid with 1% agar. After 48 hours, the cells were fixed with 3% formaldehyde/PBS for 3 hours. The agar overlay was then removed and crystal violet was added to identify plaques. The plaque assay was used to identify the number of plaque-forming units (pfu) in the viral samples. Titered virus in this study was kindly prepared by Ms. Megan Polzin.

Cell Culture

The nonadherent THP-1 leukemia cell line, used to model monocyte/macrophage behavior, was maintained at 37℃ and 5% CO_2 in RPMI-1640 media (Sigma-Aldrich). This media was formulated with 0.3g/L L-glutamine and sodium bicarbonate and supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich), 1% penicillin/streptomycin (Corning), 1% MEM vitamins (Sigma-Aldrich), 10mM HEPES (pH 7), and 0.05mM 2-mercaptoethanol (MP Biomedicals). Cells were subcultured every 2 to 3 days so that the density stayed between $2x10^5$ and $1x10^6$ cells/mL.

Macrophage Polarization

THP-1 monocytes were seeded into a 96-well plate at a concentration of 25,000 cells per well, and then differentiated into adherent macrophages with 25nM PMA (Sigma-Aldrich). After 24 hours, the media was replaced with fresh media containing polarization factors in conjunction with PMA for an additional 48 hours. M0 macrophages were made with a treatment of 25nM PMA alone; M1 macrophages were made with a treatment of 25nM PMA, 20ng/mL LPS (L5418; Sigma-Aldrich), and 20ng/mL IFN-γ (570202; BioLegend); and M2 macrophages were made with a treatment of 25nM PMA, 20ng/mL IL-4 (574002; BioLegend), and 20ng/mL IL-13 (571102; BioLegend). Macrophage polarization was verified by morphology and by the expression of subtype specific markers (Polzin, 2017).

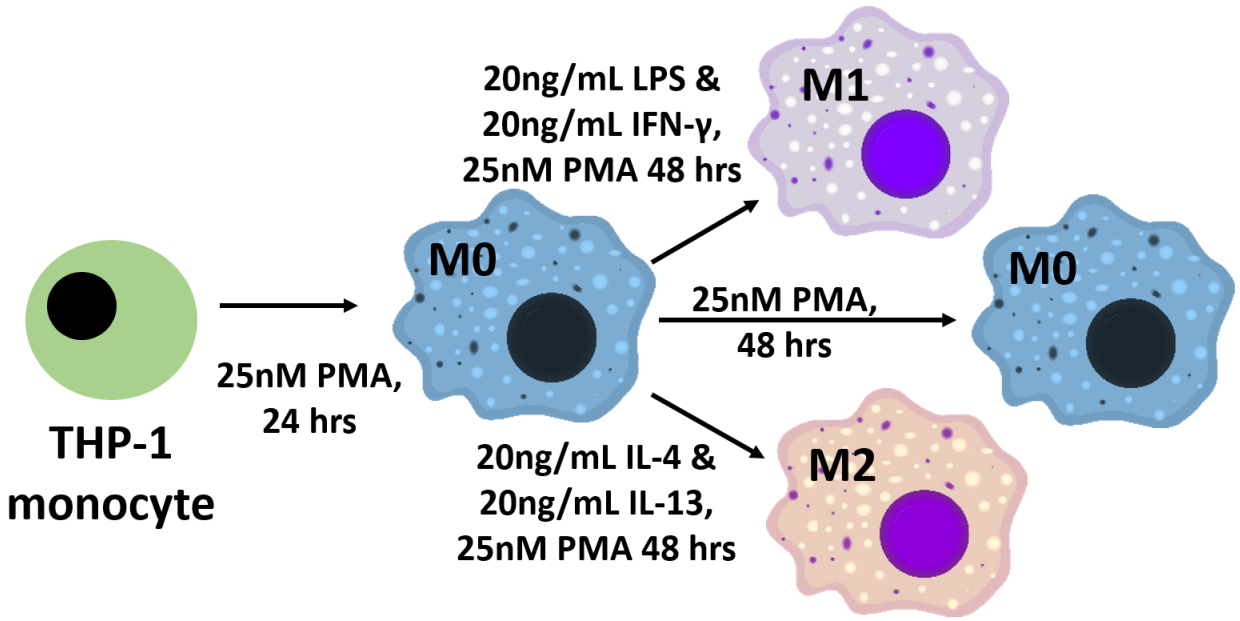

Figure 1. Polarization protocol of THP-1 monocytes. Model THP-1 monocytes were first differentiated into macrophages using the phorbol ester PMA for 24 hours. Continued PMA treatment for an additional 48 hours produced M0 macrophages or the PMA was supplemented with LPS and IFN-γ or IL-4 and IL-13 to produce polarized M1 or M2 macrophages, respectively.

Fluorescent Microscopy

THP-1 monocytes were seeded into a 6-well plate containing two sterile glass coverslips (Microscope Cover Glasses, 12mm; Carolina Biological Supply) at a concentration of 5 x 10^5 cells per well, and then polarized to macrophages using the protocol above. Macrophages were fixed on the slides using 0.3% formaldehyde (Electron Microscopy Sciences)/PBS for 10 minutes, permeabilized in 0.4% Triton-X-100/PBS for 10 minutes, and stained with Texas Red-conjugated phalloidin (1:200; Molecular Probes) in 5% donkey serum/PBS. After several washes in PBS, the cover glasses were mounted onto glass slides using a small droplet of ProLong® Gold Antifade with DAPI (8961S; Cell Signaling). Random representative images of each macrophage subtype were obtained using an Olympus BX51 inverted fluorescence and phase contrast microscope equipped with a Retiga EXi Fast1394 camera using the 40X and 100X objectives. Images were modified for contrast and brightness using Q-Imaging and Adobe Photoshop software.

Viral Replication Assay

To monitor VSV replication, THP-1 monocytes were seeded into 6-well plates at a concentration of 5 x 10^5 cells per well, and then polarized to macrophages using the protocol above. Both monocytes and M0, M1, and M2 macrophages were infected with the rwt-GFP or rM51R-GFP mutant strains of VSV at multiplicities of infection (MOIs) of 1 or 10 pfu/cell for 16 hours. Active replication of the virus was indicated by positive green fluorescent protein fluorescence in the cells. Live fluorescent microscopy images were obtained using an Olympus IX81 microscope at 20X magnification equipped with a DP71 color camera. Random images

were edited with MicroSuite B3 Biological Suite software, and the mean percentage of GFP-positive cells calculated from three independent experiments.

Cell Viability Assay

THP-1 monocytes were seeded in triplicate into a 96-well plate at a concentration of 25,000 cells per well, and then polarized to macrophages using the protocol above. Both monocytes and M0, M1, and M2 macrophage were infected with the rwt or rM51R-M mutant strains of VSV at MOIs of 1 or 10 pfu/cell for 16 or 32 hours. At endpoint the MTT Reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was applied to the cells according to manufacturer instructions (Cell Proliferation Kit, Roche Diagnostics; TACS MTT Cell Proliferation Assay, Trevigen) with viability based on the ability of metabolically active cells to produce purple-colored formazan. Cells were solubilized after 5 hours and the absorbance of each well was recorded with a VersaMax tunable microplate reader (89429-538) using SoftMax Pro 5.4.1 software at a wavelength of 570nm. Absorbance values from three independent experiments were normalized against mock-infected wells for each experimental condition.

RESULTS

Macrophage Polarization

THP-1 cells are widely used as a model of precursor monocyte differentiation into mature macrophages (Bosshard et al., 2016). When properly maintained, THP-1 monocytes are free-floating, spherically-shaped cells, but treatment with the phorbol ester PMA induces rapid cell adhesion and the formation of punctate actin structures known as podosomes (Burger et al., 2011). While this macrophage differentiation phenotype is reproducible, ambiguities remain as to a protocol that most successfully polarizes macrophages into M1 and M2 phenotypes (Debinski et al., 2014; Chanput et al., 2015). Classically activated anti-tumor M1 macrophages are noted for their production of pro-inflammatory cytokines and are induced by the bacterial outer membrane component LPS and the stress-inducing molecule IFN-γ. Alternatively activated pro-tumor M2 macrophages play roles in angiogenesis and tissue remodeling and are induced by IL-4 and IL-13 (Chávez-Galán et al., 2015). Based on previously published polarization methods, various techniques were tested to determine which provided the most robust phenotypic differences.

We first confirmed morphological differences by mounting monocytes and macrophage subtypes on glass coverslips for examination by phase contrast and fluorescent microscopy. As the THP-1 monocyte cell line is non-adherent, monocytes appear round and free-floating in phase contrast images (Figure 2A). When stained for F-actin to show the cytoskeletal architecture, monocytes did not have the defined, punctate structures known as podosomes that can be readily seen in the M0, M1, and M2 macrophage phenotypes (compare Figure 2B with Figures 2D, 2F, and 2H). M0 macrophages, as a non-polarized macrophage subtype, showed

remarkable morphological similarities to M2 macrophages (compare Figures 2C and 2G). Both were slightly larger and more flattened than THP-1 monocytes. M1 macrophages, in contrast, had more diverse, elongated morphologies based on both phase contrast and fluorescent microscopy images (Figures 2E and 2F).

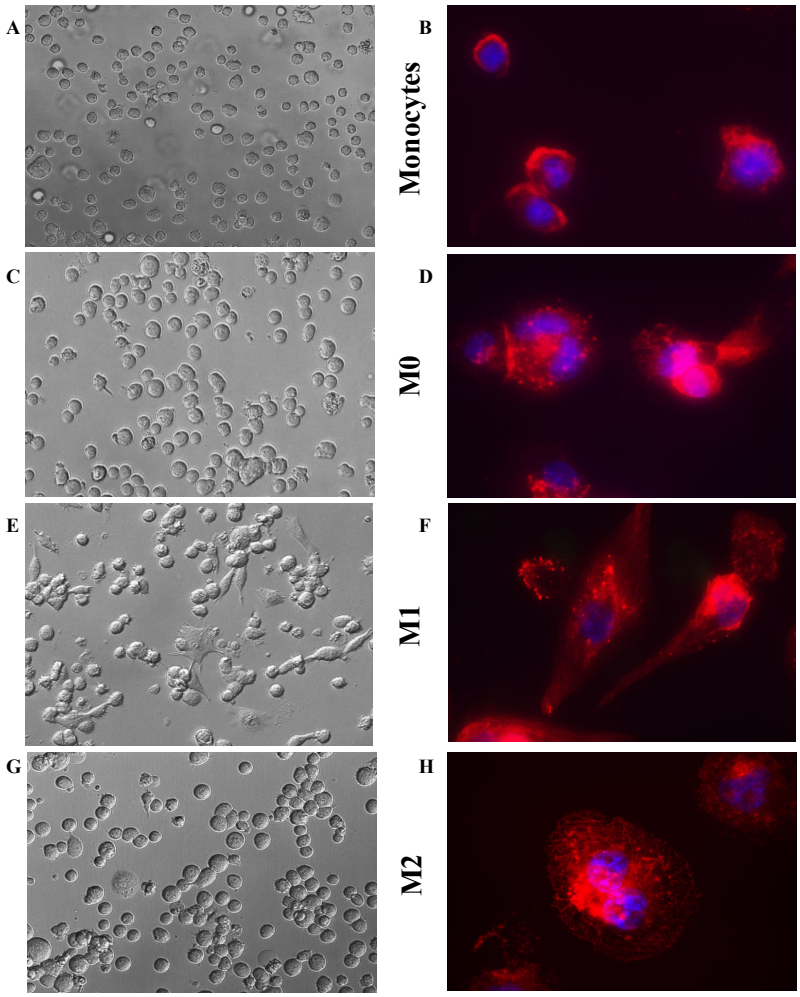

Figure 2. Phase contrast and fluorescent microscopy images denote morphology. THP-1 monocytes were pre-polarized into M0, M1, and M2 macrophages. Phase contrast images appear on the left while fluorescent microscopy images appear on the right (red, F-actin; blue, nuclei). (A,B) Monocytes, (C,D) M0 macrophages, (E,F) M1 macrophages, (G,H) M2 macrophages. Images A, C, G, and G were provided by Dalton Sizemore. Image E was provided by Megan Polzin.

As indicated earlier, all macrophage subtypes made punctate F-actin podosome structures (Figures 2D, 2F, and 2H). While podosome multiplicity was the focus of another study (Polzin, 2017), their presence here noted by the punctate staining pattern of F-actin helped confirm the desired differentiation of monocytes to macrophages in the THP-1 cell line. This, along with the morphologies of the macrophage subtypes, provided a distinct visual cue that helped verify their proper polarization. Based on the robust responses of THP-1 cells seen here, the polarization protocol involving the addition of PMA, its removal from the media after 24 hours, and then the re-addition of PMA in conjunction with polarization factors for an additional 48 hours was chosen for all future experiments in this study.

VSV Replication in Monocytes and Polarized Macrophage Populations

Oncolytic virotherapies like VSV are known for their selective cytotoxicity towards cancer cells. However, as the tumor microenvironment is home to many other cell types, we sought to determine whether VSV might have a broader cytotoxic profile, focusing this study on tumor associated macrophage populations. Here we used the THP-1 monocytic leukemia cell line as a surrogate for macrophages as they are easily cultured and can be polarized to specific phenotypic profiles, including putative anti-tumor M1 and pro-tumor M2 macrophages (Figure 2). Monocytes and pre-polarized macrophages were each assayed for their ability to become infected with two forms of VSV: a recombinant wild-type strain (rwt) and an M protein mutant strain (rM51R-M) that does not diminish the host response to VSV infection. Both strains contained a GFP insertion such that any cell that appeared green by fluorescent microscopy was supporting VSV replication.

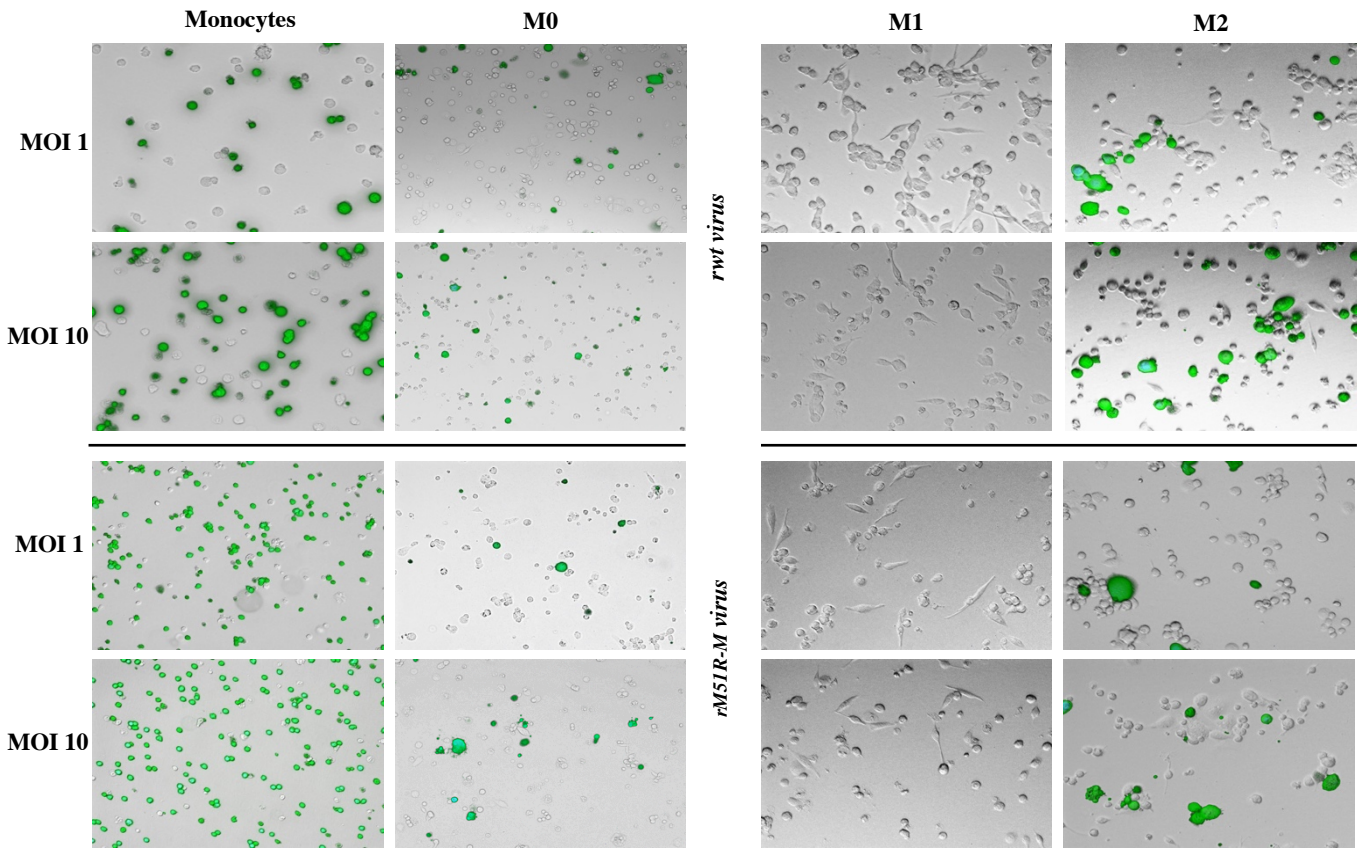

Figure 3. Differential susceptibility of THP-1 monocyte and macrophage populations to VSV replication. THP-1 monocytes and M0, M1, and M2 macrophages were infected for 16 hours with either wild-type (rwt-GFP) or M protein mutant (rM51R-M-GFP) strains of VSV at multiplicities of infection of 1 and 10 for 16 hours. Replication was based on live cell imaging by fluorescent microscopy. Cells containing actively replicating virus appear green in the images. Images provided by Megan Polzin (Polzin, 2017).

Monocytes showed very little resistance to infection and replication of VSV at both the low and high MOIs tested. The percentage of GFP-positive THP-1 monocytes varied from 39% to 73%, with maximal VSV replication occurring with the rM51R-M mutant strain of the virus at an MOI of 10 (Figure 3). On the other end of the spectrum were the M1 macrophages, which showed no sensitivity to VSV infection and replication. Regardless of viral strain or MOI, no M1 macrophage showed signs of VSV replication after a 16-hour incubation with VSV (Figure 3).

M0 and M2 macrophages exhibited results that were somewhere in between the sensitive monocytes and resistant M1 macrophages. Focusing on the M2 macrophages, replication by the rwt virus occurred in up to 36% of cells at an MOI of 10, dropping to 22% of cells when infected with rM51R-M at an MOI of 10 (Figure 3). The data suggest that monocyte and M2 macrophage populations may be sensitive to the cytotoxic properties of VSV, while M1 macrophages may not be.

Monocyte and Macrophage Viability in Response to VSV Infection

To determine the relative cytotoxicity of VSV towards monocyte/macrophage populations, THP-1 monocytes and M0, M1, and M2 THP-1 macrophages were infected with either the rwt or rM51R-M mutant strains of VSV and tested for their viability by MTT assay 16 and 32 hours later. A dose-dependent decrease in cell viability was noted for monocytes, M0 macrophages, and M2 macrophages, with the monocytes being the most affected. For example, the rwt strain of VSV decreased monocyte viability to 61.9% (MOI 1) and 37.8% (MOI 10) of mock infection levels, while the rM51R-M mutant strain decreased viability to 77.1% (MOI 1) and 55.4% (MOI 10) (Figure 4A). This can be compared favorably to non-polarized M0 macrophages whose viability in response to the rwt VSV strain decreased to 75.6% (MO1 1) and 58.2% (MOI 10) of mock infection levels, whereas the mutant strain decreased M0 macrophage viability to a slightly lesser degree of 71.9% (MOI 1) and 55.3% (MOI 10) (Figure 4B).

Interestingly, pro-tumor M2 macrophages also saw significantly reduced cell viability relative to mock infections, and to levels comparable to the M0 macrophages (Figure 4D).

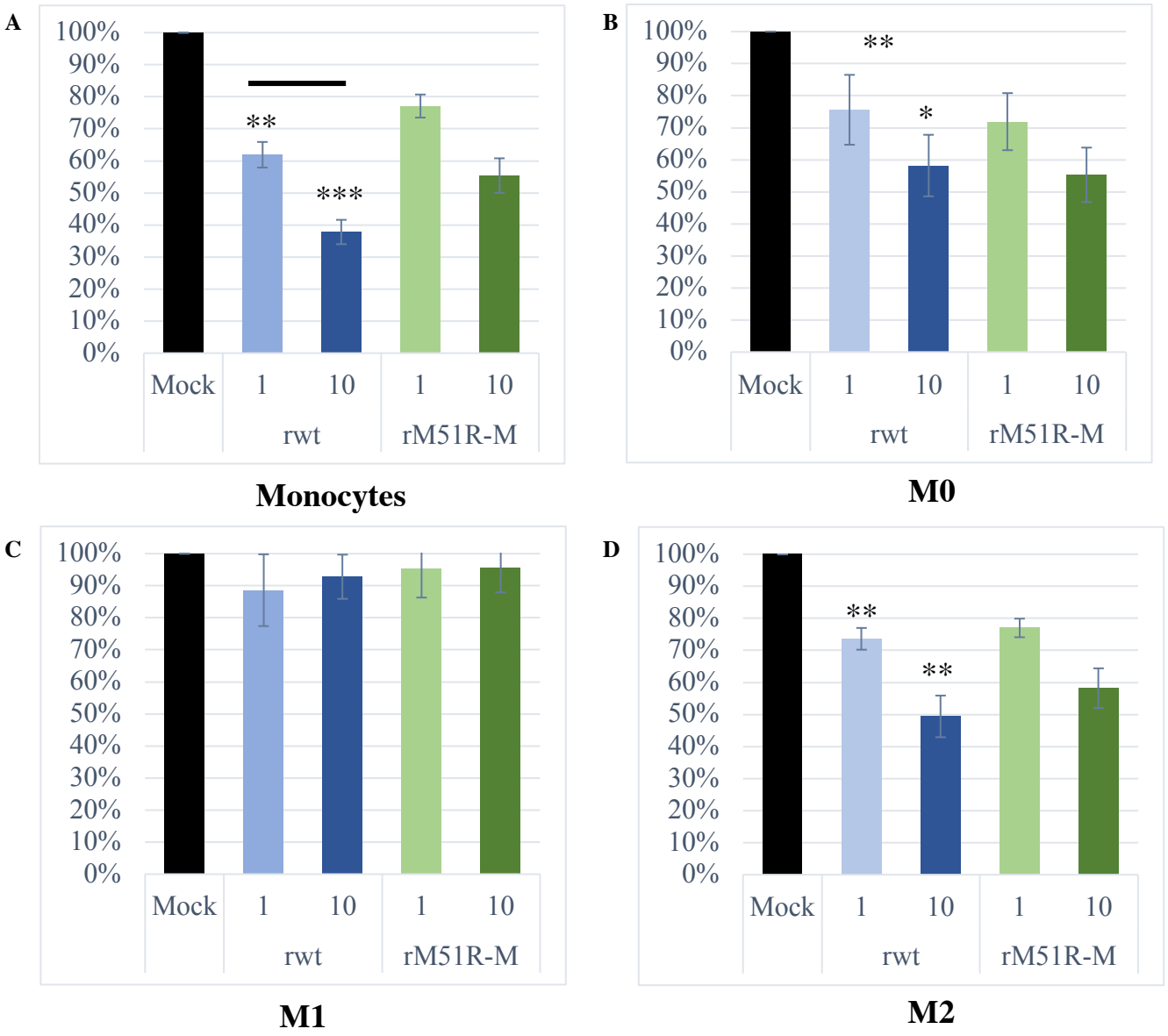

Figure 4. Percent viability of monocytes and macrophages following a 16-hour VSV infection. THP-1 monocytes (A) and pre-polarized M0 (B), M1 (C), and M2 (D) macrophages were infected for 16 hours with either wild type (rwt) or M protein mutant (rM51R-M) strains of VSV at MOIs of 1 and 10. Percent viability was based on an MTT assay and is recorded relative to mock infections. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (n=3).

In response to the rwt strain of VSV, viability dropped to 73.6% (MOI 1) and 49.4% (MOI 10) of mock infections, while the mutant rM51R-M strain decreased viability to 77.9%

(MOI 1) and 58.2% (MOI 10). As with the monocytes and M0 macrophages, there was always a statistical significance increase in cytotoxicity by both viral strains between MOIs of 1 and 10, indicating an ability to decrease viability when virus is applied at higher concentrations. In addition, the wild type strain of VSV tended to reduce cell viability more than the M protein mutant strain.

M1 macrophages were uniquely different in their response to viral infection. Regardless of strain or multiplicity of infection, M1 THP-1 macrophages were resistant to viral infections and did not to succumb to its cytotoxicity in the way that monocytes, M0 macrophages, and M2 macrophages did (Figure 4C). Indeed, no significant cell death was recorded under any of these standard experimental conditions. This was consistent to the extent of replication observed in M1 macrophages after a 16-hour infection (Figure 3). Thus, putative anti-tumor M1 macrophages appear less susceptible to the cytotoxic properties of VSV.

Viability after a 32-Hour VSV Infection

In order to further explore the sensitivity of M2 macrophages to VSV as well as the ability of M1 macrophages to remain resistant to viral infection, the viability of monocytes and M0, M1, and M2 macrophages was also investigated as before, but after a 32-hour infection. The resulting trends appeared similar to those from the 16-hour VSV infection, but with more extensive cytotoxicities. For THP-1 monocytes viability decreased to 33.8% (MOI 1) and 23.2% (MOI 10) of mock infections with the rwt strain, and to 36.8% (MOI 1) and 30.7% (MOI 10) with the rM51R-M strain (Figure 5A).

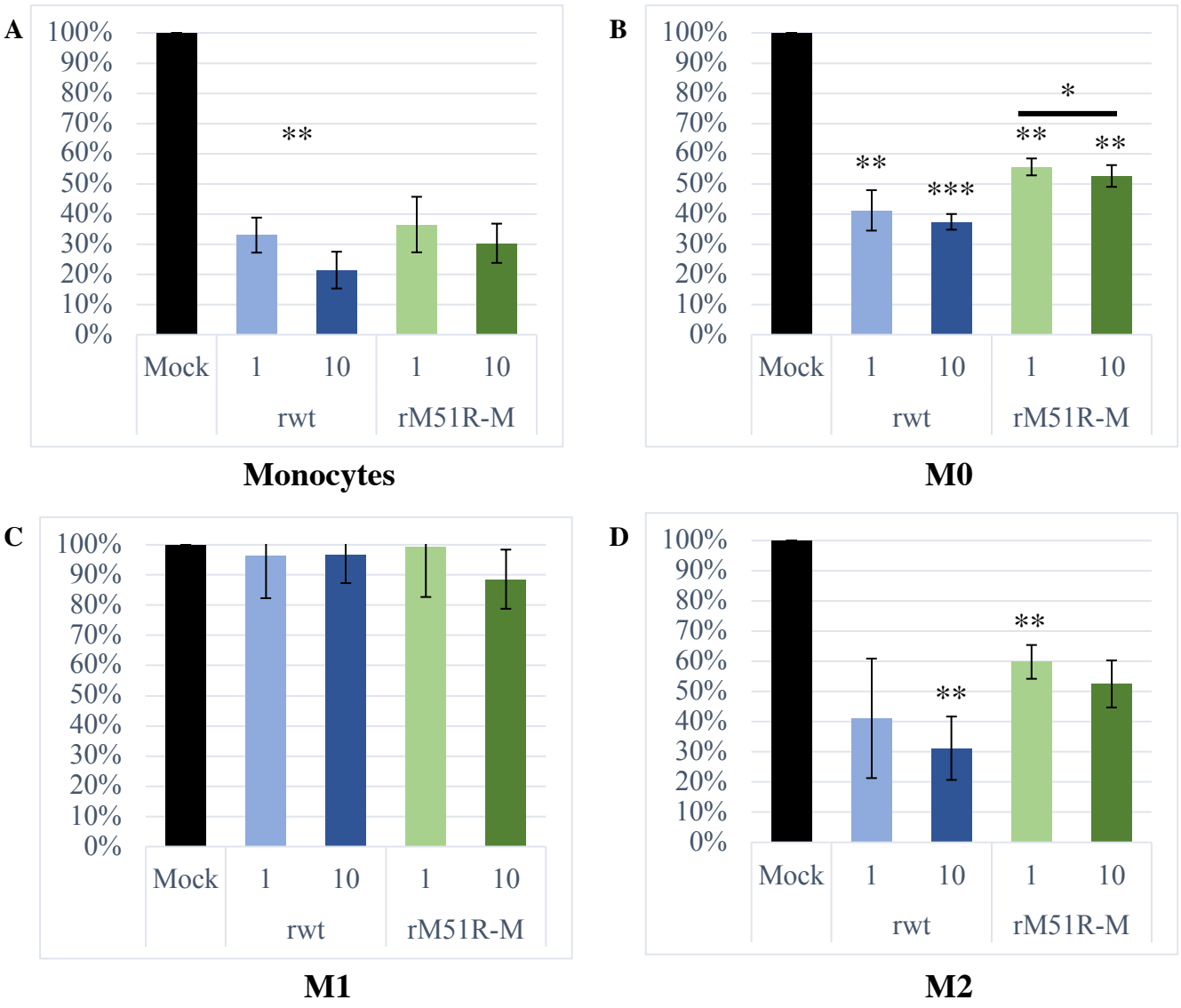

Figure 5. Percent viability of monocytes and macrophages following VSV infection. THP-1 monocytes (A) and pre-polarized M0 (B), M1 (C), and M2 (D) macrophages were infected for 32 hours with either wild type (rwt) or M protein mutant (rM51R-M) strains of VSV at MOIs of 1 and 10. Percent viability was based on an MTT assay and is recorded relative to mock infections. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (n=3).

Thus, precursor monocytes remained highly susceptible to killing by VSV as seen with the decreased viability after a longer viral infection period. Significant cell death was similarly recorded for M0 and M2 macrophages. Most death occurred at an MOI of 10 and in response to

the rwt strain (M0, 42.9% of mock infections; M2, 31.3% of mock infection) (Figures 5B and 5D). In support of previous data from the 16-hour VSV infection, M1 THP-1 macrophages remained resistant to both strains and at both dosages, even after 32 hours. The lowest viability was in response to the M protein mutant virus at an MOI of 10 (89% of mock), but this was not statistically significant. Thus, putative anti-tumor M1 macrophages maintained their antiviral activity despite longer exposure to the virus, while monocytes and M0 and M2 macrophages remained highly susceptible to cell death.

DISCUSSION

Summary of Results

VSV is known for its oncolytic properties against cancer cell lines (Balachandran et al., 2000). However, the effects of VSV on polarized macrophage populations remains less clear. As effectors of an immunogenic response and potential drivers of tumor progression (Lichty et al., 2004; Fernandez et al., 2002; Berkey et al., 2017), we believed that the effects of oncolytic viruses on tumor-associated macrophages should be further studied to better elucidate the potential benefits or drawbacks of virotherapies. To that end, we developed an *in vitro* model system to evaluate the effects of VSV on putative TAM populations. First, THP-1 monocytes could be successfully polarized into distinct M1 and M2 macrophage phenotypes. This was superficially confirmed by the variable morphologies exhibited by monocytes and macrophages under various polarization regiments as well as podosome production in the macrophage subtypes (Figure 2). Validation came later by determining the expression profiles of known M1 (pSTAT1) and M2 (CD204) markers (Polzin, 2017). Only M1 macrophages expressed the activated (phosphorylated) form of the transcriptional activator protein STAT1. Only M0 and M2 macrophages expressed the cell surface scavenger receptor CD204. Second, different THP-1 macrophages subtypes exhibited differential susceptibilities to VSV infection as measured by the ability of the virus to replicate in and kill the cells (Figures 3, 4, and 5). Most noteworthy was the remarkably complete resistance of anti-tumor M1 macrophages to VSV infection, and the much greater sensitivity of the pro-tumor M2 macrophages in which over a third of the cells supported replication of VSV and over two-thirds of the cells eventually died. Such data suggest that the

positive benefits of VSV as an oncolytic virotherapy might include the ability to target and kill tumor promoting M2 macrophage populations within the tumor microenvironment.

Validation of THP-1 Macrophage Polarization

Since much depended on a valid macrophage polarization procedure in this study, the earliest work on this project involved testing various protocols from the literature (Debinski et al., 2014; Chanput et al., 2015; Genin et al., 2015; Voloshyna et al., 2014). While there are other published procedures, we chose to adapt a protocol from the Debinski laboratory group at Wake Forest University. Obvious morphological distinctions demonstrated phenotypic differences between the macrophage subtypes. M1 THP-1 macrophages appeared elongated and spread out, while M2 THP-1 macrophages appeared rounder, and larger, and had a tendency to clump together (Figure 2). Images of M0, M1, and M2 macrophages all contained punctate actin structures known as podosomes while monocytes lacked these structures, all of which is consistent with previous data (Polzin, 2017). M0 and M2 macrophages also appeared to contain more podosomes per cell in comparison to M1 macrophages, which further validates previous observations about THP-1 polarization into divergent macrophage phenotypes (Polzin, 2017).

A more definitive indication of phenotype differentiation was determined by the expression profiles of classic macrophage markers. THP-1 macrophages exposed to PMA, IFN-γ, and LPS saw increased expression of the M1 marker pSTAT1, a transcription factor in the interferon signaling pathway (Polzin, 2017). When exposed to IFN-γ from other immune cells like helper and cytotoxic T cells, or natural killer cells, a signaling cascade is activated in macrophages that transcribes M1 polarization and antiviral genes (Shuai et al., 1993; Matsumoto et al., 1999). Our preliminary analysis of M1 macrophages has also shown an upregulation of the

costimulatory molecule CD80 (Chávez-Galán et al., 2015). In contrast, THP-1 macrophages exposed to PMA, IL-4, and IL-13 saw increased expression of the M2 marker CD204 (Polzin, 2017), which is consistent with a previous study (Debinski et al., 2014). CD204, or scavenger receptor A, is a common surface marker protein on M2 macrophages, and in the context of the tumor microenvironment is associated with angiogenesis and immunosuppression and hence a poor cancer patient prognosis (Miyasoto et al., 2017; Kelley et al., 2014). Future studies aim to confirm previously reported cytokine secretions by M1 and M2 macrophages as measured by ELISA (Chávez-Galán et al., 2015). In particular, the M1 markers TNFα, IL-6, and IFN-α are all being studied, both before and after infection with VSV. Aside from these continued investigations, the collective data obtained to date reasonably confirms polarization of THP-1 cells into M1 and M2-like phenotypes, thus making this cell line a reasonable model for assessing the effects of VSV on macrophage viability.

The Differential Susceptibility of THP-1 Monocytes and Macrophages to VSV Infection

THP-1 monocytes, M0 macrophages, and M2 macrophages were all susceptible to infection and replication by recombinant VSV, while M1 macrophages maintained resistance to the virus. These results were generally true for both the wild-type (rwt) and M protein mutant (rM51R-M) strains of VSV and at both of the studied concentrations. Monocytes were the most sensitive to VSV replication as 65% (rwt) and 73% (rM51R-M) of monocytes were GFP-positive after 16 hours at an MOI of 10 (Figure 3). Based on an MTT assay, VSV infections also led to high THP-1 monocyte cytotoxicities as the viability of infected monocytes ranged from 21% (rwt) to 30% (rM51R-M) of mock infections after 32 hours at an MOI of 10 (Figure 5). On the opposite end of the spectrum were the M1 macrophages. Live fluorescent microscopy imaging of

GFP as a measure of viral replication indicated complete resistance at 16 hours post-infection (Figure 3). That is, no GFP-positive cells were observed. Moreover, the minimal cytotoxic properties of VSV, based on the 81% viability relative to mock infected cells, were not statistically significant thus making M1 macrophages one of the more resistant cell types to VSV infection studied to date (Figures 4 and 5). M0 and M2 macrophages were, in contrast, somewhere in between the responses of monocytes and M1 macrophages. Susceptibility to VSV replication in the M0 macrophages was greatest in response to the rM51R-M mutant strain of VSV (23% GFP-positive) while cytotoxicity was greatest in response to the rwt strain at 32 hours post-infection (34% of mock viability) (Figures 3 and 5). For M2 macrophages, both replication (36% GFP-positive) and cytotoxicity (29% of mock viability) were greatest in response to the rwt strain (Figures 3 and 5).

Extending the Value of VSV-based Therapies to Susceptible M2 THP-1 Macrophage Populations

M2 macrophages are polarized using the phorbol ester PMA. This activates protein kinase C signaling, including two activators of JAK/STAT signaling called IL-4 and IL-13 (Daigneault et al., 2010; Chávez-Galán et al., 2015; Gandhi et al., 2016). While these cytokines stimulate polarization to M2 macrophages, they do not initiate any kind of antiviral response, leaving M2 macrophages (as well as monocytes and M0 macrophages) susceptible to VSV infection. M2 macrophages secrete growth factors that promote cancer cell proliferation and angiogenesis, and their podosome-associated invasive behavior is known to stimulate tumor metastasis (Alblazi et al., 2015). Their high susceptibility to VSV infection thus poses potential benefits for the cancer patient by broadening the targets VSV can hit within the tumor microenvironment.

The general susceptibility of M0 and M2 macrophages to VSV infection and cytotoxicity relates, in part, to the strain being studied. For example, M2 macrophages were most susceptible to the rwt strain of VSV in this study. The rwt strain of VSV has an intact M protein, which aids in the shutdown of host genome expression so that the virus is able to more effectively infect cells and replicate its viral genome. This contrasts with the rM51R-M mutant strain of VSV, which renders the M protein defective and disables the shutdown of host genome expression by the virus. IFN and interferon-stimulated genes (ISGs), which are thus still expressed, protect the cell against VSV infection (Ahmed et al., 2010). The M protein mutant strain of VSV was developed as a less virulent and thereby safer virus as it still kills cancer cells but has a somewhat diminished ability to target normal cell populations. As a nonvirulent form of VSV, a similar potency by the M protein mutant virus to the wild-type virus would prove beneficial for oncolytic virotherapies, which is why both strains were included in this study. Results indicate a more pronounced drop in viability when M2 macrophages were exposed to rwt (29% of mock viability), but there was still significant cell death in response to r-M51R-M (47% of mock viability). Thus, there is utility in the ability of both viral strains to target tumor-promoting M2 macrophage populations while anti-tumor M1 macrophage populations retained resistance to both strains (Figures 4 and 5). This suggests a further benefit of using the M protein mutant VSV strain in that it maintains some efficacy against M2 macrophages despite the lack of ability to shut down host genome expression.

In addition to studies of cytotoxicity, the Ahmed-Seals labs are also exploring ways by which VSV can target M2 macrophage populations. One possibility lies in the ability of VSV to disarm the tumor-promoting functions of M2 macrophages. This refers to the podosome machinery of M1 and M2 macrophages. The Ahmed-Seals lab is currently investigating the

functions of macrophage podosomes within the tumor microenvironment, as macrophages are known to associate with tumor cells during intravasation and thus the metastatic spread of cancer to distant anatomic sites (Robinson et al., 2009). Based on recent data, all macrophages (M0, M1, and M2) are found to possess podosomes but at varying multiplicities. M1 macrophages produce the least with approximately 50 podosomes per cell while M2 (90 podosomes per cell) and M0 (120 podosomes per cell) macrophages produce much more (Polzin, 2017). When infected with VSV, surviving M2 macrophages experience a marked and statistically significant reduction in podosome multiplicity, even in the cells that were not directly replicating the virus (Polzin, 2017). Beyond podosome development, we hope to elucidate podosome function in TAMs by examining the matrix degradation ability of both M1 and M2 macrophages with and without exposure to VSV. Those experiments are currently in progress. If M2 macrophages can be selectively disarmed of their podosome structures, then the tumor-promoting invasive function of M2 macrophages may be another benefit of a VSV-based oncolytic virotherapy. A second possible implication of VSV infection is a phenotypic switch from an M2-like to M1-like phenotype in surviving macrophages. After infection with the M protein mutant strain of VSV, we have observed an upregulation of the M1 marker pSTAT1 in M2 macrophages (Polzin, 2017). This suggests a possible phenotypic switch that would reduce the functional numbers of pro-tumor M2 macrophages and increase the functional number of anti-tumor M1 macrophages, both of which could provide a benefit to the cancer patient.

Primed Antiviral Resistance in M1 THP-1 Macrophages

The M1 macrophages demonstrated remarkable resistance to VSV infection. We believe that this is likely the result of pre-activated type I IFN signaling in these cells. By polarizing

THP-1 cells with the bacterial outer membrane component LPS and the stress-inducing molecule IFN-γ, the resulting M1 macrophages are naturally primed for infections through stimulation of an antiviral interferon response (Sadler and Williams, 2008). These cells become alerted via the binding of type I IFN ligands to the IFNAR1 and IFNAR2 receptor complex at the cell surface (Sadler and Williams, 2008). This begins a signaling cascade leading to the activation of transcription factors like phosphorylated signal transducers and activators of transcription (STATs; *e.g.* pSTAT1) that in turn upregulate antiviral genes. These effector genes include Mx GTPase, ribonuclease L, and protein kinase R, and serve to protect cells like M1 macrophages against viral replication. In mouse knockout studies, these effector proteins are known to block transcription, degrade viral RNA, inhibit translation, and modify proteins to downregulate all steps of viral replication (Sadler and Williams, 2008). Thus, when M1 macrophages are infected with VSV, they are already primed to resist an infection. In the context of the tumor microenvironment, this would mean that anti-tumor M1 macrophages might remain viable while M2 macrophages succumb to replication of VSV with subsequent cell death.

Considerations on the Susceptibility of THP-1 Monocytes to VSV Infection

As monocytes were the most sensitive to viral replication and death by VSV, concerns could be raised as to the impact of VSV as an oncolytic virotherapy on precursor monocytes. If injected intratumorally, rwt or rM51R-M virus might not be expected to have a wide-scale impact on the primary monocytic population, as they circulate in the blood throughout the body. However, if applied systemically (*e.g.* intravenously), VSV may significantly impact the larger monocyte population. High monocytic susceptibility lends itself to a high death rate when exposed to VSV, in which case the monocytes would be eradicated before getting a chance to

differentiate into either anti-tumor M1 or pro-tumor M2 macrophage subtypes. Such a therapy might also diminish the number of monocytes and macrophages systemically, and quite possibility compromise host immunity in general. Clearly, future studies, particularly those being done *in vivo,* need to consider whether these *in vitro* observations still prevail and whether there are broad impacts of such a therapy across the host immune system.

Considerations on the THP-1 Model of TAM Activities

Another important consideration in this study is the use of THP-1 monocytes as the model system. THP-1 is actually a cell line derived from a one-year-old monocytic leukemia patient (Tsychiya et al., 1980). They are neither primary monocytes derived directly from the peripheral blood or a macrophage derived from the peritoneum or bone marrow, which are two common techniques for acquiring primary monocyte/macrophage populations (Chanput et al., 2014). Moreover, these cells are artificially stimulated into macrophages, treated directly with polarizing agonists instead of exposed to infection, wounding, or cancerous cells. They may therefore produce different results from experiments using primary cells when co-cultured with cancer cell lines or exposed to virus. In fact, THP-1 monocytes are known to be less reactive to polarization factors such as LPS than peripheral blood-derived monocytes (Bosshart and Heinzelmann, 2016). Results conducted with the THP-1 cell line should ideally be confirmed with studies using THP-1/cancer cell co-cultures, primary human peripheral blood monocytes, or TAMs studied *in vivo.* Indeed, ongoing experiments are now being conducted in the Ahmed-Seals labs on a simulated tumor microenvironment created by the co-culture of THP-1 monocytes and macrophages with breast cancer cell lines. Cytokine secretion by co-cultured THP-1 monocytes and macrophages is being measured to detect changes in the immunogenic

response of the cells within this simulated tumor microenvironment with and without infection by VSV. While the THP-1 monocytic cell line is not a perfect model, the relative ease of cell line maintenance and low cost more than makes up for any undesirable properties (Tedesco et al., 2018). Indeed, monocytic cell lines (*e.g.* THP-1, U937) are commonly employed for the study of many macrophage-associated activities (Chanput et al., 2015).

VSV-based Oncolytic Virotherapies

VSV is an advantageous and convenient type of cancer therapy. The small genome is easily manipulated and produces high viral titers in a wide range of cell types. Intracytoplasmic replication presents low risk of integration into the host genome, and there is no pre-existing immunity found in humans against this virus (Buijs et al., 2015). *In vivo* studies of VSV using dogs and monkeys have shown promising results as anti-cancer agents (Buijs et al., 2015). Phase I clinical trials are ongoing (Buijs et al., 2015). VSV is also not the only virus being studied in clinical trials for its oncolytic properties; others include the Newcastle disease virus (NDV), measles virus, herpes simplex virus, and many others (Buijs et al., 2015). Intratumoral application of oncolytic viruses are currently being tested, while promising results have also been seen following the systemic application of VSV (Ding et al., 2018).

An intratumoral or systemic treatment with VSV would be expected to lead to infection of cancer cells with considerable cytotoxic effects. The death of cancer cells would presumably lead to the expression of tumor antigens on the surface of phagocytic cell types and the stimulation of a long-term immune-based response to the cancer as well. Here we suggest that pro-tumor M2 macrophages may also be a part of the casualties of virus treatment, and thus offers another potential benefit for the cancer patient. However, much work remains as to

whether these initial observations seen *in vitro* yield any efficacy in more realistic co-culture experiments with cancer cells or in mouse models of the disease.

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