

THERAPUETIC TARGETING OF MACROPHAGE POPULATIONS BY ONCOLYTIC
VESICULAR STOMATITIS VIRUS

A Thesis
by
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Abstract

THERAPUETIC TARGETING OF MACROPHAGE POPULATIONS BY ONCOLYTIC VESICULAR STOMATITIS VIRUS

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Vesicular stomatitis virus (VSV) is an oncolytic virus with preferential ability to infect and kill tumor cells over healthy cells. However, as tumor microenvironments contain other cell types that exert great influence on disease progression we wondered if and how VSV might target M2 macrophage populations given their natural ability to promote tumor growth and metastasis. Here we utilized model THP-1 monocytic leukemia cells that can be easily differentiated into various macrophage subsets and tested for viral susceptibility. First, naïve M0 and pro-tumorigenic M2 macrophages were susceptible to infection with both wild-type (rwt virus) and M protein mutant (rM51R-M virus) strains, thus supporting viral replication that led to decreased viability. Anti-tumorigenic M1 macrophages however were completely resistant to VSV infections. Second, the M1 polarization marker p-STAT1 was upregulated in monocytes, M0, and M2 cells upon infection with the rM51R-M virus suggesting that a phenotype switch to anti-tumor M1 macrophages may have occurred. And third, infection of M0 and M2 macrophages with VSV

also decreased podosome formation, a major driver of macrophage invasive behavior that in the context of cancer would be expected to diminish metastatic disease. This latter effect was most apparent in response to rwt virus. These results collectively indicate that macrophage populations are differentially susceptible to infection with VSV and that VSV has the capacity to modulate M2 macrophage subsets in such a way as to decrease the invasive phenotype of cancer cells.

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Dedication

This thesis is dedicated to my parents, Jeff and Joy Polzin, whose hard work and passion for learning has inspired my own. Their constant love, support, and guidance were the greatest comfort throughout my graduate career.

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Chapter I: Literature Review

Prologue

The human immune system is dedicated to ridding the body of foreign pathogens. However, in the context of cancer members of the innate and adaptive immune systems, M2d macrophages in particular, contribute to tumor growth and metastasis and are thus associated with poor patient prognosis. Metastasis is defined by the acquired ability of cancer cells to invade surrounding tissues and vasculature and is completed upon colonization of distant sites. As these patients respond poorly to traditional treatment options, the need for additional therapies has emerged. The therapies of interest in this thesis are oncolytic viruses that kill cancer cells while sparing healthy cells. This includes oncolytic vesicular stomatitis virus, which has not only demonstrated tumoricidal capabilities, but the ability to modulate immune cells to elicit anti-viral responses. This thesis seeks to investigate if different strains of this oncolytic virus, a recombinant wild-type (rwt) virus and a matrix (M) protein mutant (rM51R-M) virus, can infect and target pro-tumor M2d macrophages and inhibit their stimulatory role in cancer growth and metastasis. To explore this objective, monocytes, M0, M1, and M2 macrophages were infected with rwt virus or the M protein mutant virus whereupon viral replication, cell death, macrophage phenotypic switching, and the formation of invasive cytoskeletal structures called podosomes were analyzed. This review seeks to better clarify the context of this objective by elucidating how different types of macrophages of the innate immune system may assist in tumor growth and metastasis. This review will also seek to clarify oncolytic virotherapies, their current role in cancer treatment, and how several viruses utilized in this field impact macrophage populations.

Cancer Metastasis

Defined as a collection of approximately 200 related diseases, all cancers are characterized by the uncontrollable proliferation of cells that spread beyond the site of origin. Among cancer's many hallmarks is the process of tissue invasion and metastasis (Hanahan and Weinberg, 2011). Metastasis is defined by the acquired ability of cancer cells to invade surrounding tissues and vasculature. This can be facilitated by cytoskeletal structures called invadopodia, which create protrusions of the cancer cell surface where matrix-remodeling metalloproteinase (MMP) activity is focalized. Metastasis is completed if invasive cancer cells survive transport in the bloodstream or lymphatic system, extravasate, and the colonize distant organ tissues (Seyfried and Huysentruyt, 2013). The subsequent seeding of additional tumors throughout the body is the cause of ninety percent of cancer patient fatalities (Seyfried and Huysentruyt, 2013). Multiple factors are known to facilitate and enhance metastasis, including the presence of a unique M2 macrophage subtype that often resides in the invasive tumor microenvironment (TME) (Qian and Pollard, 2010).

Macrophages

Circulating mononuclear leukocytes *i.e.*, monocytes, are the main precursor cells to macrophages and comprise approximately ten percent of blood cells in humans (Chávez-Galán et al., 2015). Macrophages are large, specialized, professional phagocytic cells that arise from monocytes in response to chemical stimuli (Chávez-Galán et al., 2015). An immune cell found in all tissues, macrophages can distinguish self from non-self, enabling them to play a role in both the innate and adaptive immune responses. Most often found at the site of tissue damage or infection, macrophages function in the innate immune system via

the non-specific phagocytosis of particles or foreign pathogens. These foreign agents are then presented by macrophages at the cell surface to initiate an adaptive immune response against the specific pathogen. In addition to this, macrophages function in wound repair resulting from injury (Chávez-Galán et al., 2015).

Macrophages are heterogeneous in nature with a phenotype and function that is largely dependent upon location and chemical stimuli (Mantovani et al., 2007). Many macrophages are named according to their recruited site including alveolar macrophages in the lungs, microglia in the central nervous system, and Kupffer cells in the liver (Chávez-Galán et al., 2015). In addition to this, macrophages are able to change their phenotype and function quickly in response to chemicals released in their environment (Xue et al., 2014). Due to their different states, macrophages are organized into a nomenclature system that is further complicated by their versatile nature (Chávez-Galán et al., 2015). The names of the macrophage phenotypes are based on polarization-inducing stimuli and cytokine profiles and include naïve M0 macrophages, classically activated pro-inflammatory M1 macrophages, and alternatively activated reparative M2 macrophages. M2 macrophages can be further differentiated into four subtypes, including tumor-associated macrophages (Figure 1).

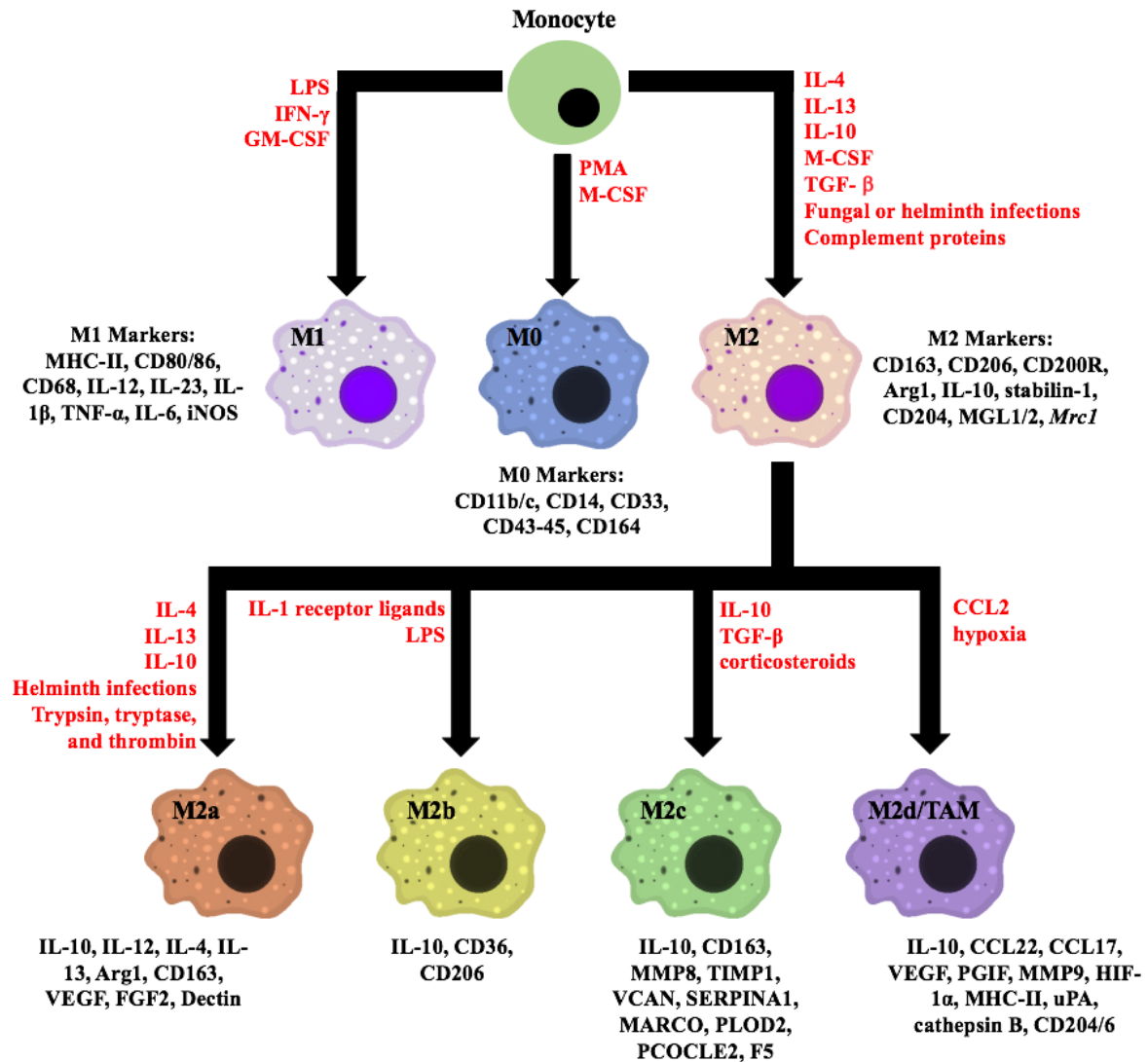


Figure 1. Schematic of M0, M1, and M2 macrophage polarization. Polarizing agents (red) and commonly expressed markers (black) are shown for each macrophage subtype.

M0 Macrophages

To stimulate M0 macrophage activation, monocytes from the human monocytic cell line, THP-1, are treated with the tumor promoter phorbol 12-myristate 13-acetate (PMA) (Figure 2) or M-CSF (Daigneault et al., 2010) (Figure 1). Stimulation by PMA activates protein kinase C resulting in the upregulation of specific markers like CD11b, CD11c, and

CD14 (Daigneault et al., 2010; Mittar et al., 2011; Schwende et al., 1996) (Figure 1). Specifically, a study published in 2011 examined marker differences between untreated THP-1 monocytes and PMA-treated THP-1 monocytes and identified 20 markers specific to the M0 macrophages and 21 markers found in both THP-1 monocytes and M0 macrophages. Some specific M0 markers identified in this study include CD33, CD43-45, and CD164 (Mittar et al., 2011). Another study identified an upregulation of TNF- α as another marker of the M0 phenotype (Schwende et al., 1996). Morphological changes that occur when polarizing a THP-1 monocyte to the M0 phenotype include increased cytoplasmic volume, enhanced adherence, and decreased proliferation, exhibiting what is largely considered to be general macrophage morphology (Daigneault et al., 2010; Schwende et al., 1996; Denholm and Stankus, 1995). Increased phagocytic abilities have also been observed upon polarizing to an M0 macrophage (Daigneault et al., 2010; Schwende et al., 1996). THP-1-derived M0 macrophages are also used to give rise to M1 and M2 macrophage subtypes. For polarization to the M1 macrophage phenotype, M0 macrophages are treated with LPS, IFN- γ , and PMA. To stimulate the M2 macrophage phenotype, M0 macrophages are treated with IL-4, IL-13, and PMA (Figure 2).

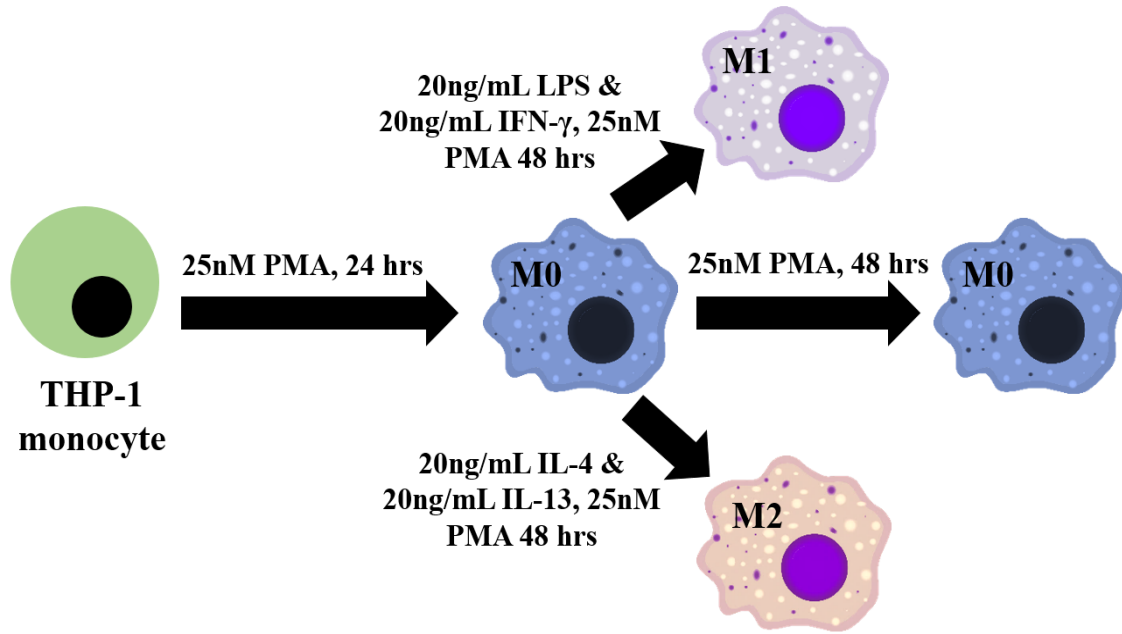


Figure 2. Schematic of THP-1 macrophage polarization. Monocytes are exposed to phorbol ester PMA for 24 hours, followed by treatments with either PMA alone (M0 macrophages); PMA, LPS, and IFN- γ (M1 macrophages); or PMA, IL-4, and IL-13 (M2 macrophages) for 48 hours.

M1 Macrophages

Classically activated M1 macrophages secrete pro-inflammatory cytokines. They are also involved in intracellular pathogen resistance, and exhibit microbicidal and tumoricidal properties (Chávez-Galán et al., 2015; Mantovani et al., 2007). M1 macrophages are characterized as being IL-12^{high}, IL-23^{high}, and IL-10^{low} and are known to readily produce effector molecules like inducible nitric oxide synthase (iNOS) or NOS2 and the pro-inflammatory cytokines IL-1 β , TNF- α , and IL-6. Several of these are known to possess tumoricidal effects when found within solid tumors (Mantovani et al., 2007). Additionally,

major histocompatibility complex (MHC)-II, surface molecule CD68, and the co-stimulatory molecules CD80 and CD86 are commonly expressed for antigen presentation (Chávez-Galán et al., 2015) (Figure 1).

Polarization to the M1 phenotype most often occurs via exposure to interferon (IFN)- γ secreted by Type 1 helper T cells (T_H1), cytotoxic T cells, or natural killer (NK) cells; lipopolysaccharide (LPS), a major component of gram negative bacterial cell walls; and granulocyte macrophage colony-stimulating factor (GM-CSF). However, signal transducer and activator of transcription (STAT) and suppressor of cytokine signaling (SOCS) proteins are also known to be involved in this polarization process (Shuai et al., 1993). STAT1 is crucial for M1 polarization through its phosphorylation and subsequent upregulation of toll-like receptor 4 (TLR4) and increased NF- κ B activity (Krausgruber et al, 2011). Additionally, SOCS3 is involved in the translocation of NF- κ B to the nucleus where phosphoinositide 3-kinase (PI3K) is activated and nitric oxide (NO), a known M1 marker for its microbicidal and tumoricidal capabilities, is produced (Chávez-Galán et al., 2015). In 2010, Fc γ receptors (Fc γ Rs) were shown to be involved in M1 polarization. Upon inactivation of Fc γ Rs in M2 macrophages, M1 markers were upregulated (Andreu et al., 2010) (Figure 1).

M2 Macrophages

M2 is the generic name for macrophages polarized beyond the classical activation pathway used by M1 macrophages and are thus given the name “alternatively activated” macrophages (Mantovani et al., 2007). M2 macrophages exhibit a wide variety of functions including the initiation of a Type 2 helper T cell (T_H2) immune response and tissue repair. M2 macrophages are also known to kill and encapsulate extracellular parasites in

collaboration with neutrophils and the complement system (Chávez-Galán et al., 2015).

There is some evidence of M2 macrophages' involvement in tumor growth, metastasis, and immune suppression. For example, the presence of M2 macrophages is associated with decreased survival in melanoma, breast, kidney, and bladder cancer patients (Edin et al., 2012).

M2 macrophage activation is induced by fungal or helminth infections, complement proteins, M-CSF, IL-4, IL-13, IL-10, and transforming growth factor (TGF)- β (Duluc et al., 2007; Röszer, 2015). IL-4 in particular upregulates STAT6 expression. Upon STAT6 phosphorylation, markers for the M2 phenotype are upregulated. IRF4 also acts in an antagonistic manner towards LPS-induced TLR signaling. This results in the downregulation of the M1 marker iNOS and the upregulation of the M2 marker STAT3. Another inducer of the M2 phenotype is PPAR γ , which was shown to induce an M2 phenotype from naive monocytes but remains less effective in differentiated macrophages (Chávez-Galán et al., 2015) (Figure 1).

Cytokine exposure remains a common and efficient polarization method of M2 macrophages *in vitro*. However, it has been previously noted that surrounding tissue structure and physical environmental cues can influence macrophage morphology and possibly function (Van Goethem et al., 2010). To test this idea, McWhorter and coworkers (2013) discovered that elongation of cell shape reduced inflammatory cytokine secretion and promoted an M2 phenotype. These changes also enhanced effects by IL-4 and IL-13 and provided protection against LPS and IFN- γ stimulation, thus creating an M2 macrophage less susceptible to M1 phenotypic switch. Upon inhibition of actin-myosin contraction by blebbistatin, cytochalasin D, Y27632, and ML-9 combined treatment, cell shape influence on

macrophage polarization was also prevented (McWhorter et al., 2013). This implies that changes to the ECM like injury or wound healing influences macrophage shape and therefore polarization status.

Like M1 macrophages, M2 macrophages exhibit their own set of markers. CD200R seems to be a distinct M2 marker (Jaguin et al., 2013). Additionally, stabilin-1, MGL1, MGL2, and CD163 in conjunction with CMAF are upregulated upon M2 polarization (Chávez-Galán et al., 2015; Mantovani et al., 2007). Recently identified markers also include MMR (*Mrc1*) and resistin-like molecule α (Chávez-Galán et al., 2015). Mannose receptor (CD206) was previously thought to be unique to M2, but a recent study showed no difference in CD206 expression between M1 and M2 macrophages (Jaguin et al., 2013) (Figure 1). Due to their eclectic polarization pathways and marker expression patterns, Mantovani et al., divided M2 macrophages into 4 subtypes: M2a, M2b, M2c, and M2d (Mantovani et al., 2004) (Figure 1).

M2a Macrophages

Due to their observed increase in number during wound healing processes, M2a macrophages are the M2 macrophage subset most associated with scar tissue formation whether in the wound healing process or during fibrosis (Ferrante and Leibovich, 2012; Sakai et al., 2006). In aging muscle, M2a macrophages are mostly responsible for an increased fibrotic state (Villalta et al., 2009). In fact, trypsin, tryptase, and thrombin encourage M2a polarization in monocytes, M1, and other M2 macrophage subtypes in order to promote a pro-fibrotic state to initiate scar tissue formation (White and Gomer, 2015). M2a macrophages are also associated with inflammatory inhibition and angiogenesis stabilization,

and under low glucose conditions are associated with increased fungal elimination in the gastrointestinal tract post-rosiglitazone treatment (Lefèvre et al., 2010; Lurier et al., 2017).

Polarization to this phenotype occurs by IL-4 or IL-13 stimulation or helminth infections (Duluc et al., 2007; Villalta et al., 2011). However, during muscle inflammation, IL-10 is also required for M2a polarization (Villalta et al., 2011) (Figure 1). This subset is characterized by high CD163 and arginase-1, of which arginase is used in the production of collagen and the promotion of muscle fibrosis (Deng et al., 2012; Wehling-Henricks et al., 2010). Additionally, CD206 has been classified as an M2a marker as well as the secretion of IL-4, -10, -12, and -13, VEGF, and FGF2 (Mosser and Edwards, 2008; Loegl et al., 2016). Low levels of TNF- α have also been observed in this phenotype (Lefèvre et al., 2010) (Figure 1).

M2b Macrophages

Little information is known about M2b macrophages, but they are considered to be an intermediate phenotype between M1 and M2 macrophages. Recent research analyzing the function of M2b macrophages involved A549 lung adenocarcinoma cells where M1 and M2b macrophages promoted the greatest dispersal of A549 aggregates (Bai et al., 2015).

Polarization to this phenotype occurs in response to IL-1 receptor ligands and, like M1 macrophages, to LPS exposure (Duluc et al., 2007). Other similarities to M1 macrophages include elevated expression of CD36 and inflammatory cytokines. M2b macrophages also exhibit high CD206 and IL-10 and low IL-12 (Mantovani et al., 2004; Mosser and Edwards, 2008) (Figure 1).

M2c Macrophages

Shown to infiltrate wounded tissue during the early stages of injury, the M2c subtype of macrophages efficiently phagocytize apoptotic cells (Evans et al., 2013; Zizzo et al., 2012). They are also known to play a role in the recruitment of blood vessel stem cells as well as the prevention and reduction of lung fibrosis (Lolmede et al., 2009; Tang et al., 2016). According to a study published in 2016, M2c macrophages were shown to be more efficient than M2a macrophages at preventing lung injury and fibrosis and in reducing existing lung fibrosis. This effect is thought to be partially due to upregulated IL-10 production and activation of JAK1/STAT3/SOCS3 signaling pathway. Upon blocking IL-10, these superior effects were ablated (Tang et al., 2016).

Polarization to the M2c macrophage phenotype most often occurs by IL-10 or TGF- β stimulation, but can also occur via corticosteroid exposure (Duluc et al., 2007; Tang et al., 2016). In a study analyzing gene expression in 4 macrophage phenotypes, 17 genes in M2c macrophages were shown to be upregulated compared to M0, M1, and M2a macrophages. Many of these genes were associated with angiogenesis, matrix remodeling, and phagocytosis, including CD163, MMP8, TIMP1, VCAN, SERPINA1, MARCO, PLOD2, PCOLCE2, and F5. These genes were shown to be upregulated during early injury stages (Lurier et al., 2017). Further supporting evidence of their role in early wound repair, M2a and M2c macrophages also exhibit low pro-inflammatory cytokine secretion (Mantovani et al., 2004) (Figure 1).

M2d/Tumor-Associated Macrophages (TAM)

In 2002, Mantovani et al., determined TAM as a distinct M2 subset later referred to as M2d macrophages (Duluc et al., 2007). While TAM largely resemble M2 macrophages, they are an intermediate phenotype known to express M1 and M2 macrophage markers to varying degrees. As their name suggests, they are a dominant cell population in tumors, and when found in high numbers have been associated with poor patient prognosis (Chávez-Galán et al., 2015; Huang et al., 2011). However recent evidence has suggested that their diverse polarization pattern can enable them to take on more M1-like properties and improve patient prognosis.

The dualistic function of M2d macrophages observed within tumor patients is mimicked in their functions and marker expression. Monocytes are initially recruited to the TME via the secretion of CCL2, M-CSF, or VEGF by tumor cells (Condeelis and Pollard, 2006; Mantovani et al., 2004). Once recruited, CCL2 and hypoxic conditions within the tumor are thought to encourage M2d polarization (Huang et al., 2011; Mantovani et al., 2004) (Figure 1). M2d migration is inhibited by hypoxic conditions where they can then cooperate with tumor cells to promote tumor growth, migration, and metastasis (Grimshaw and Balkwill, 2001; Lewis et al., 2000).

To encourage tumor growth, M2d macrophages are influenced by the TME to take on an immunosuppressive role by recruiting and differentiating regulatory T-cells (Tregs) and secreting the immune suppressive cytokines and chemokines IL-10, CCL17, and CCL22 (Hoves et al., 2006; Wang et al., 2010). The production of VEGF, PGIF, MMP-9, hypoxia-inducible factor (HIF)-1 α , uPA, and cathepsin B promote angiogenesis, which contributes to

overall tumor growth and invasion (Bergers et al., 2000; Chávez-Galán et al., 2015; Huang et al., 2011; Talks et al., 2000) (Figure 1).

Though M2d macrophages are primarily M2-like, they simultaneously express M1 and M2 markers (Mantovani et al., 2006; Sica et al., 2006). M2d macrophages are known to express high levels of ILT3, CCL5, IL-10, as well as MGL-1, Dectin-1, CD68, CD206, NOS2, CD81, and scavenger receptor A (CD204) (Chávez-Galán et al., 2015; Duluc et al., 2007; Röszer, 2015). The recently identified markers FIZZ2 and Ym1 are also commonly expressed (Mantovani and Sica, 2010). Supporting the evidence that TAM are mostly M2-like and thereby classified as the M2d subtype, TAM possess poor antigen presentation capabilities and are defective at producing iNOS (Balkwill and Mantovani, 2001; Klimp et al., 2001). Interestingly, increased expression of IRF-3/STAT-1, molecules involved in the M1 polarization pathway, are characteristic of this phenotype (Figure 1). However, reduced NF- κ B expression likely inhibits further upregulation of M1 markers (Biswas et al., 2006).

Due to the versatile nature of these cells, much research has been dedicated to the alteration of M2d to a more anti-tumor M1 macrophage phenotype. Synthetic and natural immunotherapy-based drugs such as CDDO-Me and chlorogenic acid have demonstrated the ability to upregulate M1 marker expression and decrease M2/TAM marker expression in M2 macrophages, indicative of a phenotype switch (Ball et al., 2016; Xue et al., 2017). The upregulation of M1 markers in pre-polarized M1 macrophages was also observed in response to chlorogenic acid, suggesting an enhanced functional state (Xue et al., 2017). Additionally, oncolytic measles and mumps viruses have been successful at altering M2d phenotypes without inducing cell death (Tan et al., 2016). By altering M2d phenotypes, whether utilized

alone or in conjunction with traditional treatment options, the potential to inhibit tumor growth and metastasis is promising.

Concluding Remarks

Macrophages exhibit considerable plasticity, able to change their phenotype and function quickly in response to environmental cues (Mantovani et al., 2007; Xue et al., 2014). Divided into two main groups: M1 and M2 (including the many M2 subtypes), the macrophage polarization paradigm offers a convenient system for organizing macrophages by polarization stimulus and transcriptional profile, though it is complicated by their versatility (Chávez-Galán et al., 2015). Understanding the nature of these macrophages can provide valuable insights into their role in inflammation, wound healing, and diseases such as cancer where these cells are known to play a role in inflammation, angiogenesis, metastasis, and tumor immunity in ways that can encourage tumor growth and spread. The inhibition of an exacerbating macrophage phenotype and the encouragement of more beneficial ones is the basis for many novel cancer treatment options.

Macrophages and Inflammation

Inflammation, often the result of infection, is an innate immune response characterized by localized pain, heat, swelling, redness, and loss of function. Another key feature of inflammation is blood vessel dilation and infiltration of innate and adaptive immune cells, including macrophages (Grivennikov et al., 2010). This form of inflammation is called acute inflammation where the main goal is to eliminate the pathogen responsible. If

unchecked, these pathogens can undermine the host immune system and establish persistent infections resulting in chronic inflammation (Grivennikov et al., 2010).

Described as the seventh hallmark of cancer, there is much evidence connecting chronic inflammatory diseases and infections with cancer (Mantovani and Sica, 2010). In humans, chronic obstructive pulmonary disease (COPD) results in increased lung cancer risk (Moghaddam et al., 2011). Additionally, chronic colitis and subsequent colonic adenocarcinoma were found to be the result of inflammation caused by TNF- α and IL-6 secretion by macrophages (Deng et al., 2010). Inflammation from chronic irritation that causes increased cancer risk is termed “smoldering inflammation.” This form of inflammation is usually low grade, without immediate or overt consequences, and often the result of macrophage activity (Mantovani and Sica, 2010).

Due to these many forms of inflammation, a tumor can contain many immune cells from both the innate and adaptive immune systems (de Visser et al., 2006). These cells maintain constant communication with one another, either by direct contact or by cytokine and chemokine secretion, in order to control tumor growth. It is this communication as well as the abundance and activation status of these immune cells that can tip the balance of inflammation to become either pro-tumor or anti-tumor (Lin and Karin, 2007; Smyth et al., 2006). The immune system is in this constant balance until negative controls are compromised. This results in a microenvironment that is tumor-promoting, to which macrophages contribute (Qian and Pollard, 2010). Inflammation by macrophages relies heavily on the balance between NF- κ B and STAT3. NF- κ B is known to encourage inflammation by promoting an M1 macrophage phenotype. Upon TLR ligand binding, NF- κ B is activated and moves to the nucleus to upregulate pro-inflammatory M1 macrophage

markers IL-12, TNF- α , and iNOS. Research has shown NF- κ B signaling to be vital to a tumor-promoting environment (Karin and Greten, 2005).

Inflammation by macrophages is thought to play a role in tumor initiation by generating a mutagenic environment (Pang et al., 2007) (Figure 3A). M1 macrophages are known to produce reactive nitrogen intermediates (RNI) and reactive oxygen species (ROS). Particularly, NO is known to react with peroxidates to form pro-inflammatory compounds that cause mutations in the DNA of adjacent cells (Pang et al., 2007). Cytokines such as TNF- α produced by macrophages can upregulate RNI and ROS levels within malignant cells. This can result in mutations, or it can reduce the levels or activation status of mismatch repair enzymes, thus preventing DNA repair upon mutagenesis (Grivennikov et al., 2010).

Inflammation by macrophages is also known to promote genomic instability in developing tumor cells (Colotta et al., 2009) (Figure 3A). One protein known to be involved in this process is activation-induced cytidine deaminase (AID). AID which is overexpressed in many cancer types, is an enzyme that catalyzes the deamination of cytosines found in DNA. AID increases the probability of mutation during the joining of double stranded DNA breaks, a time during which mutations to tumor suppressors is known to occur. Expression of AID is induced upon NF- κ B upregulation by TGF- β and inflammatory cytokine production (Okazaki et al., 2007).

After tumor initiation, subsequent tumor promotion is encouraged by pro-inflammatory macrophages (Figure 3B). The pro-inflammatory cytokines IL-1, -6, and -23, along with TNF- α , are secreted by macrophages and in turn act on tumor cells via the transcription factors STAT3, NF- κ B, and/or AP-1. These transcription factors then upregulate genes in tumor cells involved in cell survival and proliferation, and the further

recruitment of more pro-inflammatory macrophages (Grivennikov et al., 2010). As replication progresses, mutations in the genome caused by macrophage-induced inflammation become permanent fixtures in the tumor cells.

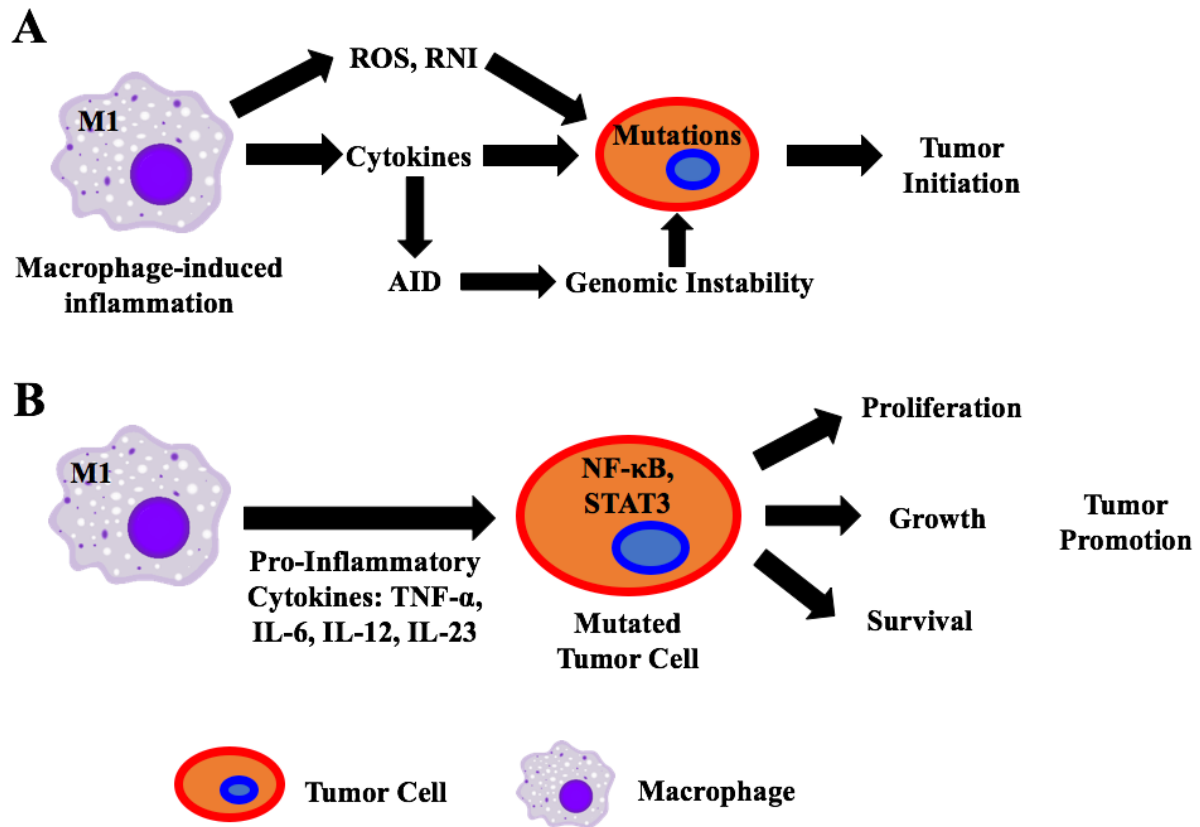


Figure 3. Role of M1 macrophage-mediated inflammation in tumor initiation and promotion. (A) M1 macrophages generate a tumor-promoting environment via secretion of pro-inflammatory cytokines, resulting in genomic instability and mutations. (B) Further cytokine secretion encourages tumor promotion via upregulation of pathways associated with proliferation, growth, and survival.

Macrophages and Tumor Angiogenesis

Oxygen and nutrients obtained from the vasculature are essential for cell function and survival, causing most cells to dwell within 100 μ m of a capillary. The process of forming

new blood vessels, called angiogenesis, is a heavily regulated process that occurs mostly during organ formation, wound healing, and menstruation. Due to the obligate dependency on blood vessels and the nutrients they provide, it would seem likely that dividing cells would possess the skills to control this process. However, cells with enhanced proliferative capabilities initially lack the ability to regulate angiogenesis, inhibiting their ability to proliferate uncontrollably. In order to subvert angiogenic regulations and acquire the ability to control this process, these abnormal cells utilize many techniques such as manipulating monocyte recruitment and their subsequent differentiation into macrophages (Bouck et al., 1996; Folkman, 1997; Hanahan and Folkman, 1996).

As a tumor moves from benign to malignant, there is occasionally a switch in macrophage phenotype from M1 to M2 within the TME. Because of this phenotype switch, there is a dramatic increase in tumor vascularization, known as the “angiogenic switch” (Hanahan et al., 1996). The role of M2 macrophages in this process was demonstrated in a 2007 study where upon the downregulation of CSF-1 by tumor cells, macrophage infiltration was diminished and there was a delay in angiogenesis. Upon restoration of CSF-1, blood vessel formation resumed (Lin and Pollard, 2007). Later, M2 polarized macrophages were shown to be associated with increased numbers of endothelial cells and tubular structures. This effect was the result of upregulated fibroblast growth factor (FGF-2), insulin-like growth factor-1 (IGF1), CCL2, and placental growth factor (PGF) by macrophages (Figure 4). A blocking antibody against PGF and FGF-2 reduced the formation of these tubular structures by 40% and 75%, respectively (Jetten et al., 2014).

Hypoxia is another inducer of macrophage-dependent angiogenesis. Monocytes are initially recruited to the TME via the secretion of CCL2, M-CSF, and/or VEGF by tumor

cells (Condeelis and Pollard, 2006). Once recruited, CCL2 and hypoxic conditions within the tumor are thought to encourage M2d polarization through NF- κ B (Mantovani et al., 2004; Huang et al., 2011). Once within the TME, M2d migration is inhibited by hypoxic conditions and pro-angiogenic genes, such as VEGF, are upregulated (Grimshaw et al., 2001; Murdoch et al., 2008). VEGF then acts by recruiting endothelial cells, the cell type that forms the internal lining of all blood vessels.

The secretion of MMP9 by macrophages has been shown to be another inducer of the angiogenic switch. M2d macrophages can produce VEGF, but can also free sequestered VEGF from the extracellular matrix (ECM) via the secretion of MMP9 (Qian and Pollard, 2010). A study performed in 2013 also found that M2 macrophages reduce levels of tissue inhibitor of metalloproteinase-1 (TIMP-1), thus relieving these cells of the negative regulation of MMP9 activity. This effect on angiogenesis was not observed in M0 or M1 polarized macrophages which maintain higher levels of TIMP-1. Either effect was lost upon re-establishing TIMP-1 expression in M2 macrophages or silencing TIMP-1 in M0 and M1 macrophages, respectively (Zajac et al., 2013).

Macrophages can exhibit dual functions within tumors concerning angiogenesis. M1 macrophages are known to stimulate coagulation and destroy blood vessel walls via damage to vascular cells. This results in hemorrhaging in the tumor (Mantovani et al., 1992). M1 macrophages are also known to upregulate TIMP-1 expression, thus diminishing the pro-angiogenic effects of MMP9 (Zajac et al., 2013).

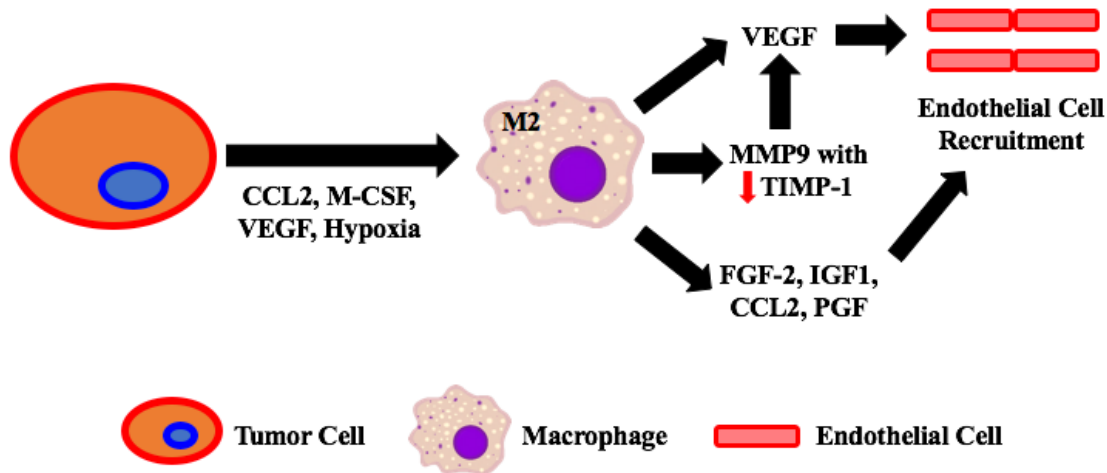


Figure 4. Role of M2 macrophages in tumor angiogenesis. Stimulated by tumor cells to the M2 phenotype, M2 macrophages secrete pro-angiogenic factors that encourage endothelial cell recruitment and blood vessel formation.

Macrophages and Metastasis

Many studies point toward M2 macrophages as being required for tumor cell migration and invasion. In one proposed model, this migration of tumor cells is a direct result of a paracrine loop between tumor cells and M2 macrophages that encourages migration of both cell types (Goswami et al., 2005). Specifically, tumor cells secrete CSF-1, which binds the CSF-1 receptor (CSF-1R) on macrophages. This binding event triggers the production of epidermal growth factor (EGF) by macrophages, which binds to the EGF receptor (EGF-R) on tumor cells. As both CSF-1 and EGF are effective motogens, this positive feedback paracrine loop promotes tumor cell motility and invasion in the absence of any other cell types or signals. Inhibition of CSF-1, EGF, or their corresponding receptors inhibits the migration of both the tumor cell and the macrophage in this model (Goswami et al., 2005).

Tumor cells are known to migrate by attaching to protein fibers in the extracellular matrix (ECM). Some of these fibers are collagen and fibrillar collagen I. Macrophages are known producers of osteonectin, which deposits collagen in the extracellular matrix. This provides surface area for tumor cell adherence and a substratum on which tumor cells can travel (Qian and Pollard, 2010). Fibrillar collagen I also provide structures with which tumor cells can adhere and use to travel. Because fibrillar collagen I anchors itself to blood vessels, tumor cells now have a direct route to the circulatory system (Condeelis and Segall, 2003). Macrophages also tend to cluster outside of blood vessels where the release of EGF can entice the tumor cells towards them. Travelling towards macrophages along collagen fibrils anchored to the blood vessels better enables tumor cells to enter the blood stream and metastasize (Gligorijevic et al., 2009; Wyckoff et al., 2007).

Macrophages may also be enhancing tumor cell movement through basement membrane and ECM breakdown. This contribution is thought to occur through the use of actin-rich extensions from the ventral surface of the cell called podosomes, which serve both motility and degradation functions for the macrophage (Gawden-Bone et al., 2010). Macrophages are known producers of many proteases including cathepsins, MMPs, and serine proteases, which are secreted from these podosomes. As macrophages can make up a large percentage of solid tumors, their contributions to the collective proteolytic activity within the TME is substantial, highly utilized, and perhaps even encouraged by the tumor cells to promote local tumor cell invasion and metastasis. Macrophage secretion has been shown to be important for tumor cell movement as the deletion of cathepsin B and S in macrophages resulted in reduced tumor cell invasion and the inhibition of metastasis (Gocheva et al., 2010). Another study in 2015 found that in SKAP2-null mice, macrophages

produced fewer podosome structures and the percentage of macrophages with podosomes dropped to below 10%. This resulted in a significant reduction of coordinated invasion by macrophages and MDA-MB-231 breast cancer cells *in vitro*. *In vivo*, the number of lung metastases was significantly reduced, an effect thought to be due to reduced macrophage infiltration in the metastatic tumors (Tanaka et al., 2015). Overall, the reduction in tumor cell invasion and metastasis could partially be due to reduced proteolytic activity by podosomes within the TME.

Once a tumor cell reaches a blood vessel, their direct entrance into the blood stream (intravasation), subsequent survival, and exit from the blood stream at a secondary location (extravasation) can be supported by macrophages. Macrophages have been observed forming triads with tumor and endothelial cells, a grouping referred to as “tumor microenvironment of metastasis” (TMEM). In breast cancer patients, an increase in TMEM density correlates with an increase in metastasis risk. This suggests that the three cell types cooperate to facilitate tumor cell intravasation (Robinson et al., 2009). Tumor cells will travel along collagen fibrils attached to blood vessels, following signals secreted by macrophages congregated outside the blood vessel wall. Once the tumor cell reaches a blood vessel, intravasation has been shown to occur upon physical contact with the macrophages present. A study in 2014 found that direct contact between tumor cells and macrophages induced RhoA activation in tumor cells. RhoA activation then triggered the formation of proteolytic extensions on the tumor cell called invadopodia. This provided the tumor cells with the invasive machinery necessary to degrade the basement membrane lining of blood vessels and the endothelium for entry into the blood stream (Roh-Johnson et al., 2014). Podosomes formed by macrophages have also been noted to have a proteolytic function, enhancing cell movement (Gawden-Bone et al.,

2010). It is possible that macrophages are utilizing these structures and the secretion of MMP to degrade through the blood vessel wall and allow for tumor cell entry in to the blood stream.

Intravasation and subsequent survival in the blood stream have also been shown to occur via fusion between tumor cells and macrophages. In 2011, Powell and coworkers found that macrophages naturally fuse with carcinoma cells during the intravasation process. This fusion produces a cell that retains the transcriptomes of both parental cells while also expressing its own unique profile. This fusion event provides tumor cells with some of the behavioral characteristics of macrophages including navigation while in circulation, evasion of the immune system, and migration. The ability to migrate might also encompass the proteolytic activity that macrophages possess. This would allow the fusion cell to degrade the endothelium and enter the blood stream. This enhanced migratory ability might also play a role in the epithelial to mesenchymal transition (EMT), a process of de-differentiation and increased motility by tumor cells during migration and invasion. Additionally, macrophages might also be transferring their identity to the tumor cell and allowing them to evade immune surveillance once in the circulatory system (Powell et al., 2011).

Upon extravasation, macrophages are recruited to exiting tumor cells. It is not known exactly how macrophages contribute to this process, but the degradative function of podosomes has been implicated. The presence of macrophages has been shown to influence extravasation as shown by a study performed in 2009 where elimination of macrophages decreased tumor cell extravasation efficiency and survival (Qian et al., 2009).

More data support the idea that macrophages promote malignancy. However, there are exceptions. In bone marrow, M1 macrophages phagocytize cells that do not express anti-

death receptor CD47 and Kupffer cells phagocytose circulating tumor cells in the liver (Jaiswal et al., 2009; Heuff et al., 1993).

Macrophages and Tumor Immunity

The M1 macrophage phenotype is most associated with cancer initiation. However, as tumor cells move towards malignancy, this phenotype changes from M1 to M2, an effect mostly due to secreted cytokines and the hypoxic conditions of the TME. Once this occurs, M2 macrophages take on an immunosuppressive role. This role involves the inhibition of cytotoxic T cell responses (Figure 5A). This inhibition can occur via the secretion of IL-10, which then triggers the expression of PD-L1 by monocytes. PD-L1 binds to its receptor PD-1, inhibiting IL-2 production and subsequent T cell activation and proliferation (Kuang et al., 2009). Additionally, M2 macrophages inhibit cytotoxic T cell response and recruit regulatory T cells (Tregs) into the tumor via CCL22 secretion (Curiel et al., 2004). To enhance Treg differentiation and recruitment, macrophages also secrete CCL18 and IL-10 (Duluc et al., 2009). M2 macrophages also express low levels of the co-stimulatory molecules CD80 and CD86 and express high levels of the inhibitory molecule ILT3, thus preventing antigen presentation (Duluc et al., 2009).

In order to combat tumor growth, M1 macrophages possess enhanced antigen presentation capabilities, upregulating MHC-II and the co-stimulatory molecules CD80 and CD86. This enables them to participate in the initiation of an adaptive immune response against the tumor cells (Fidler and Schroit, 1988). M2 macrophages do not possess this enhanced ability. M1 macrophages can also directly kill tumor cells by the production of RNI

and ROS. These can cause additional DNA mutations with tumor cells, triggering apoptosis (Allavena et al., 2008).

The role of M1 macrophages in inhibiting tumor growth and promoting immune surveillance can be demonstrated by studies that convert M2 macrophages to the M1 phenotype. Tumor growth has been shown to be inhibited by treating tumor cells with GM-CSF (Eubank et al., 2009) or anti-IL10. This activates TLR activity, upregulating the M1 phenotype, thus increasing suppression of the tumor (Guiducci et al., 2005). Another study performed in 2009 supports this idea. Among 32 tested cytokines, IFN- γ was the sole cytokine able to switch M2 macrophages to M1 macrophages. Upregulation of IL-12 and CD86 and downregulation of immunosuppressive IL-10 and ILT3 was observed upon IFN- γ treatment. IFN- γ -exposed macrophages also enhanced T lymphocyte proliferation, induced cytotoxic abilities in CD8⁺ T cell populations (Figure 5B), and reduced secreted levels of CCL18, VEGF, and MMP9 (Duluc et al., 2009). These results demonstrate the importance of the immunostimulatory capabilities of M1 macrophages. It is the suppression of M1 macrophage phenotypes and/or the activation of M2 macrophage phenotypes that ultimately enhances tumor cell immune evasion and subsequent tumor growth and spread.

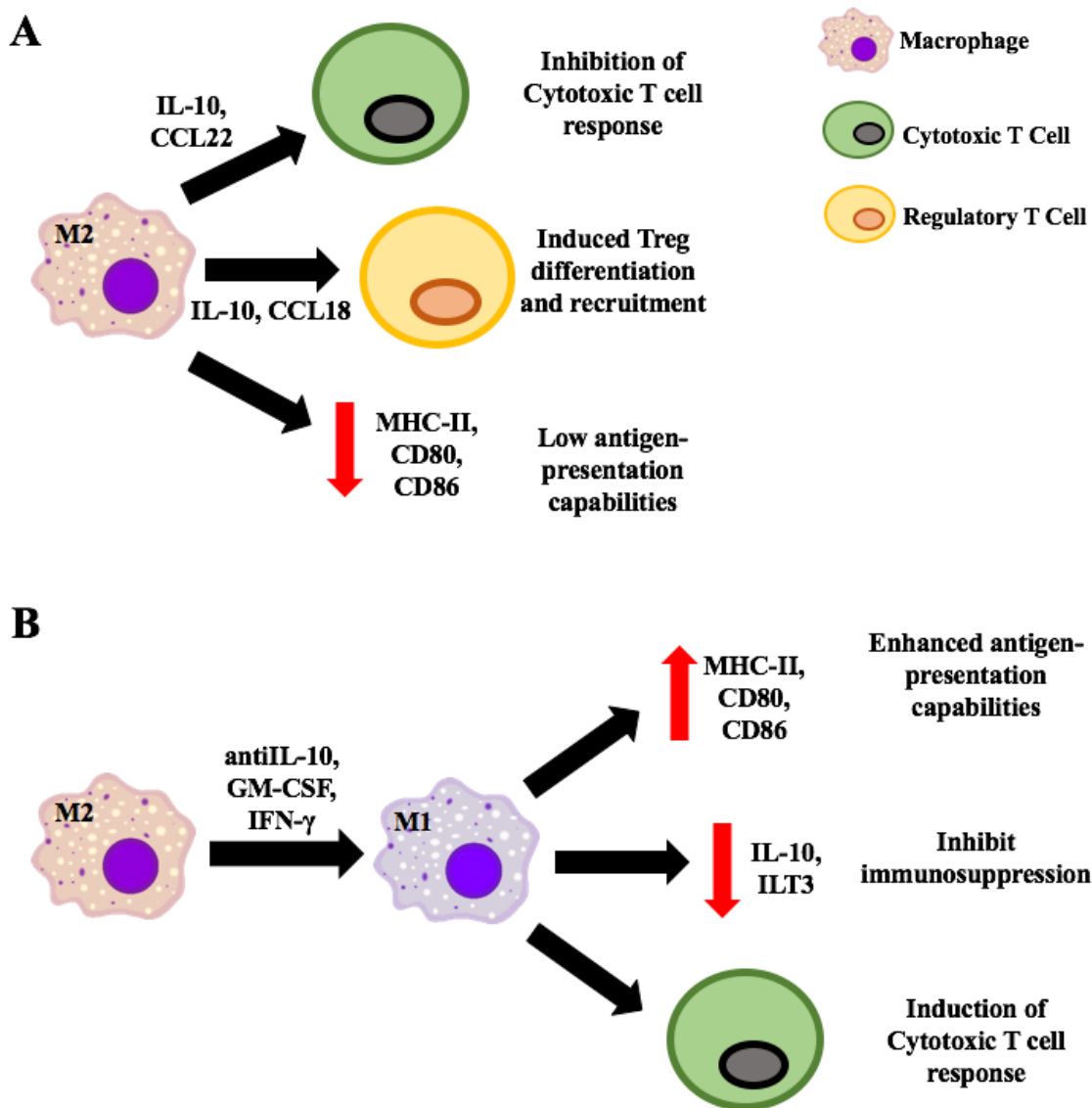


Figure 5. Role of macrophages in suppressing or stimulating immune responses to tumors cells. (A) M2 macrophage-mediated immunosuppression by inhibition of cytotoxic T-cell response, induction and recruitment of Tregs, and low antigen presentation capabilities. (B) Macrophage conversion from M2 to M1 enhances immune surveillance by enhanced antigen presentation, inhibition of immunosuppression, and induction of cytotoxic T-cell response.

Traditional Macrophage Targeting Therapies

M2d macrophages are one of the main components of solid tumors and have been indicated in tumor angiogenesis, metastasis, and immune suppression with their abundance

and activity associated with poor patient prognosis. Conversely, there is strong evidence for specifically targeting these macrophages in order to improve patient outcome. This includes inhibiting monocyte/M2d recruitment to the TME, decreasing M2d survival, and promoting a phenotype switch to the M1 macrophage phenotype (Panni et al., 2013).

To block the recruitment of monocytes to the TME, several research groups have attempted to block CCL2 or its receptor CCR2 (Panni et al., 2013). CCL2 is secreted by tumor cells to recruit monocytes and stimulate their differentiation to pro-tumor M2 macrophages (Roca et al., 2009; Zhang et al., 2010). Upon treatment with pharmacological inhibitors such as MLN1202, an anti-CCR2 antibody, or PF-04136309, a CCR2 antagonist, these migratory and polarization effects were inhibited (Panni et al., 2013; Sanford et al., 2013).

Another potential targeting strategy is to decrease M2d macrophage survival in the TME. Specifically, the bisphosphonates clodronate and zoledronic acid have been shown to kill TAM populations (Rogers and Holen, 2011; Zeisberger et al., 2006). Targeting surface marker CD204 using immunotoxin-conjugated mAbs has also been studied in ovarian cancer and yielded promising results concerning M2d depletion (Bak et al., 2007). Additionally, bacteria that target macrophages have been used to induce apoptosis including *Shigella flexneri*. A study published in 2010 found that a single injection of attenuated *Shigella* bacteria induced M2d apoptosis resulting in a >70% reduction in tumor size (Galmbacher et al., 2010). *Listeria monocytogenes*, *Chlamydia psittaci*, and *Lionella pneumophila* are other bacteria being considered for targeted M2d apoptosis (Weigert et al., 2009).

Due to the versatile nature of these cells which enables them to change their phenotype in response to environmental cues, much research has been dedicated to the

alteration of M2d to a more anti-tumor M1 macrophage phenotype. Synthetic and natural immunotherapy-based drugs such as CDDO-Me and chlorogenic acid have a demonstrated ability to upregulate M1 marker expression and decrease M2/TAM marker expression in M2 macrophages, thus indicative of a phenotype switch (Ball et al., 2016; Xue et al., 2017). The upregulation of M1 markers in pre-polarized M1 macrophages was also observed in response to chlorogenic acid, suggesting an enhanced functional state (Xue et al., 2017). Additionally, drugs against cell surface marker CD40, which inhibits cytotoxic effects, have been shown to upregulate expression of the M1 markers MHC-II and CD86 (Beatty et al., 2011). Treatment with cytokines like GM-CSF are known to polarize M2d macrophages towards an M1 phenotype and is currently used as an immunotherapy in neuroblastoma patients (Yu et al., 2010).

Oncolytic Virotherapies

Oncolytic virotherapy, the use of viruses to treat cancer, is a relatively recent and controversial concept in the field of cancer treatments designed for patients with aggressive forms of the disease or distant metastases (Woller et al., 2014). Oncolytic viruses are novel anti-tumor agents that possess the ability to selectively replicate within and lyse cancer cells while sparing healthy cells (Kelly and Russell, 2007). Some viruses inherently possess this unique ability, while other oncolytic viruses are made to do so via genetic alteration (Woller et al., 2014). These viruses are often introduced to the tumor by direct injection where the infection is then allowed to spread to neighboring cells, or through intravenous administration when metastasis has already occurred. Once the tumor has been infected, the

expanding viral infection is often impeded by the body's natural IFN response or the use of antiviral drugs (Woller et al., 2014).

The first successful clinical trial utilizing oncolytic virotherapies was based on the development of the Adenovirus (Ad) Onyx-015, a genetically altered form of the virus responsible for the common cold. This confirmed the safe administration of certain oncolytic viruses in human patients while additionally establishing synergism with other cancer treatments, including radiation therapy and chemotherapy (Heise et al., 1997). Alongside its tumoricidal effects, infection by various oncolytic viruses is known to impact the tumor vasculature by selective replication in tumor-associated endothelial cells. These viruses also impact the secretion of cytokines by tumor-infiltrating immune cells. This influx of immune cells to the TME is known to increase the efficacy of the viral treatment (Breitbach et al., 2013). Alongside Ad-Onyx-015, Herpes simplex virus, the measles virus, poliovirus, and vesicular stomatitis virus are other viruses being utilized to treat cancer (Woller et al., 2014).

The ability of certain viruses to directly kill tumor cells has long been appreciated, however this ability goes beyond direct lysis. These viruses are also capable of stimulating innate and adaptive immune responses that may augment their overall therapeutic potential. A case in point is the macrophage. Macrophages often accumulate in the TME, where they are known to exhibit dual functions for oncolytic virotherapies. To enhance the therapeutic benefit of viruses, macrophages of the M1 subtype secrete pro-inflammatory cytokines and undergo antigen presentation to promote immune responses at the tumor site. However, these very responses may also inhibit viral replication, and thus subsequent tumor cell lysis by the virus. In contrast, M2 macrophage subtypes can take on immunosuppressive functions that are more permissive of viral replication but prevent the beneficial immune responses directed

against tumor cells following oncolytic virus treatment (Denton et al., 2016). These complications broaden the implications for therapies designed to reduce tumor associated macrophage numbers or alter macrophage phenotypes.

Oncolytic viruses can increase macrophage infiltration

Viruses such as oncolytic HSV (oHSV), adenovirus, and the Chinese vaccine strain of vaccinia virus, Tian Tian (VTT), have been shown to display anti-tumor effects through direct tumor cell lysis, but their effects on the immune system were unknown. Talimogene laherparepvec (T-VEC) is an oHSV type 1 strain designed to replicate within tumor cells. T-VEC is modified by the deletion of two viral genes: ICP34.5 and ICP47. ICP34.5 is the herpes virus neurovirulence factor gene. Upon its deletion, viral pathogenicity is decreased and selectivity for tumor cells is increased. The ICP47 gene was deleted to inhibit antigen presentation by incoming phagocytic cells including that by macrophages. Additionally, T-VEC has been modified to express GM-CSF, a protein known to recruit and activate M1 macrophages, to induce a tumor-specific immune response (Andtbacka et al., 2017). In addition to recruiting macrophages, GM-CSF serves to enhance dendritic cell function and cytotoxic T cell response to tumor associated antigens. Although the above alterations to the virus encourage immune cell infiltration and function, the deletion of ICP47 limits antigen presentation to prevent an immune response from occurring against the virus. Melanoma patients that received T-VEC exhibited increased overall survival at 23.3 months compared to the 18.9 months exhibited by patients that received GM-CSF alone. (Andtbacka et al., 2017). Other forms of oHSV have been modified to express IL-12 or CCL2. These have also

been shown to increase macrophage infiltration without reducing viral titer in the tumor (Parker et al., 2000; Parker et al., 2005).

Oncolytic adenovirus (Delta24-RGD) was shown to have similar effects on macrophage infiltration in mouse models of cancer. For example, a 50% increase in survival of mice with glioma was observed after Delta24-RGD treatment. The immune changes that occurred in response to viral treatment included increased macrophage infiltration and increased pro-inflammatory cytokine and chemokine secretion. These effects were lost upon treatment with dexamethasone, an immunosuppressive drug (Kleijn et al., 2014). This demonstrates that immune cell infiltration post-adenovirus treatment was imperative to its anti-tumor capabilities against glioma.

Viruses may encourage phenotype switching from M2 to M1

M2d macrophages are known to modulate tumor cell response to oncolytic virotherapies (Tan et al., 2016). Due to the versatile nature of these cells, it is possible for these macrophages to convert between phenotypes in response to environmental conditions (Mantovani and Sica, 2010; Tan et al., 2016). By altering M2d to an M1 phenotype, an innate and adaptive immune response could be initiated against the tumor cells. Many oncolytic viruses are being investigated for their potential capacity to induce a switch in macrophages to the M1 phenotype and therefore promote anti-tumor immunity.

A study in 2016 found that the presence of monocyte-derived macrophages, irrespective of their initial polarization state, enhanced tumor cell death by oncolytic measles (MeV) and mumps viruses (MuV). These enhanced effects were not due to increased viral titer, but rather the switch from M2-like macrophages to an M1 anti-tumor phenotype. This

was demonstrated by upregulation of the M1 markers TNF- α , TRAIL, NO, CD80, CD64, IL-1 β , CXCL9, CXCL10, and IL-6 (Tan et al., 2016). Another study in 2013 found that reovirus significantly increased expression of the M1 antigen presentation markers MHC-I, β 2-microglobulin (β M2), TAP-1, and TAP-2 (Gujar et al., 2013).

Another virus shown to encourage macrophage phenotype switch is adenovirus. Adenovirus encoding CCL16 demonstrated increased macrophage infiltration in mice injected with breast and colon cancer cells (Guidicci et al., 2005). The direct injection of TLR-9 ligand CpG, used to suppress T-cell activation and proliferation, and anti-IL-10 receptor antibody, an adjuvant, into the tumor switched infiltrating macrophages from the M2 to the M1 phenotype (Balkwill and Mantovani, 2001; Guidicci et al., 2005; Klinman, 2004). This was demonstrated by elevated levels of M1 markers TNF- α , IL-12, and NO. This resulted in decreased tumor volume as early as 16 hours post-treatment (Guidicci et al., 2005). The adenovirus strain dl922-947 exhibited similar effects in anaplastic thyroid carcinomas *in vivo*. Treatment with dl922-947 induced a phenotype switch in infiltrating macrophages from M2d to M1 as demonstrated by the upregulation of M1 marker Nos2. This effect occurred without the upregulation of M2 markers Ym1 or Arg1 (Passaro et al., 2015).

Other oncolytic viruses known to enhance pro-inflammatory cytokine secretion by tumor infiltrating macrophages, indicative of an M1 polarization status, include Newcastle Disease Virus (NDV) and vaccinia virus. NDV had long been known to possess immunostimulatory capabilities, but by an unknown mechanism. In 2003, Washburn et al discovered that NDV-treated monocytes differentiated into macrophages that killed multiple breast and colon cancer cell lines. This occurred by the upregulation of TRAIL by the macrophages. These effects did not change upon treatment with UV-inactivated NDV,

indicating that the induction of TRAIL and subsequent killing of tumor cells was independent of NDV replication. Instead, this effect was triggered by IFN- α and IFN- β secretion by surrounding macrophages, resulting in the upregulation of TRAIL at the mRNA and protein level (Washburn et al., 2003). Another strain of NDV, 73-T, yielded similar results (Zorn et al., 1994). Treatment of co-cultured peripheral blood mononuclear cells (PBMCs) and kidney, chronic myelogenous leukemia, or fibroblast tumor cells with 73-T demonstrated enhanced cytotoxic effects by PBMCs. This effect was not observed in viral treatment of tumor cells alone or PBMCs alone, indicating that the cytotoxicity depended on communication between both cell types. In this case, the cytotoxic effects by PBMCs correlated with an increase in IFN- γ and TNF- α expression. Additionally, the lack of cytotoxic effects observed in PBMCs alone demonstrated that they were not initiating an immune response against the virus, thus facilitating continued increases in viral titer and direct tumor cell lysis by the virus (Zorn et al., 1994).

Treatment with oncolytic vaccinia virus (GLV-1h68) was also shown to increase pro-inflammatory cytokine secretion. Upon treatment of colon cancer xenograft models, a greater number of macrophages infiltrated into the tumors and upregulated the secretion of cytokines involved with either an anti-viral or anti-inflammatory immune response, including IFN- γ , CXCL10, IL-3, IL-6, M-CSF1, MCP-1, and RANTES. Increased colon cancer cell death, inhibited tumor growth, and an increased survival in mice were observed, effects that were most likely due to the upregulation of these cytokines (Ehrig et al., 2013).

Macrophages encourage viral spread

The ECM and its density is a barrier for oncolytic virus spread within a tumor. Macrophages, however, produce proteases that, upon their expression or upregulation, could encourage viral spread in a tumor. Lavilla-Alonso and co-workers (2012) tested matrix metalloproteinase 12 (MMP12) alone and in conjunction with oncolytic adenovirus (Lavilla-Alonso et al., 2012). MMP12 exhibited proteolytic activity against most components of the ECM, decreasing much of the ECM surrounding the tumor cells (Zucker and Vacirca, 2004). This resulted in increased anti-tumor activity by the oncolytic adenovirus in HCT116 primary tumor xenografts and in a liver metastasis model. Both decreased tumor growth and invasiveness (Lavilla-Alonso et al., 2012).

Macrophages can inhibit viral titer, replication, and direct tumor lysis

To enhance virotherapeutic effects against tumor cells, macrophages secrete pro-inflammatory cytokines and undergo antigen presentation. However, these processes are also known to inhibit viral replication and subsequent tumor cell lysis by the virus (Denton et al., 2016). Immune responses by macrophages have been shown to limit oHSV efficacy in glioblastoma patients. In 2015, Han et al., attempted to determine if tumor exposure to an immunosuppressive cytokine, TGF- β , before viral treatment would reverse these effects. The authors found that TGF- β pre-treatment to oHSV injection decreased immune cell infiltration into the tumor and reduced the M1 macrophage activation status. As a result, viral titers increased in glioblastoma cells (Han et al., 2015). Similar effects have been observed using adenovirus to treat glioma. Like oHSV, adenovirus can have immunosuppressive transgenes incorporated into their genome for expression upon replication. In 2006, adenovirus was

modified to express the immunosuppressive compound cyclophosphamide (CPA). Without CPA, mice with glioma exhibited decreased viral replication after virus injection. The addition of CPA pro-longed viral gene expression (Lamfers et al., 2006).

Another group in 2015 attempted to clarify the role of macrophages in oHSV treatment of glioblastoma and found that oHSV increased macrophage infiltration by 7.9-fold. Furthermore, these infiltrating macrophages exhibited an M1 phenotype, expressing high levels of CD86, MHC-II, Ly6C, and TNF- α . Using TNF- α blocking antibodies and studying macrophages from TNF- α knockout mice, the authors found that TNF- α was mainly responsible for the increased glioblastoma cell apoptosis in infected cells, but also decreased viral replication and earlier viral clearance. Blocking TNF- α resulted in increased viral replication and prolonged survival of the mice (Meisen et al., 2015).

Another strain of oHSV called oHSV1 also demonstrated limited viral replication in glioblastoma cells. This effect was due to a protein called cysteine-rich 61 protein (CCN1), which activated the IFN response in glioblastoma cells. CCN1 also increased macrophage infiltration by directly stimulating their migration through binding to integrin $\alpha_M\beta_2$. Upon macrophage entrance into the tumor, CCN1 then upregulated pro-inflammatory M1 macrophages, leading to increased viral clearance. Adding neutralizing antibodies against integrin $\alpha_M\beta_2$ reduced CCN1-induced macrophage infiltration and activation, increased oHSV1 titer, and increased glioblastoma cell death (Thorne et al., 2014).

Modification of macrophage phenotype and cytokine secretion are known to enhance the efficacy of certain oncolytic viruses. However, complete ablation of macrophages is also known to promote this same effect. hrR3 is an oncolytic virus derived from HSV-1 and contains a *LacZ* gene from *Escherichia coli*. Generally, the HSV1-derived hrR3 virus is

rapidly cleared in rat models of glioma, and this effect correlates with increased CD68 and CD163 marker expression in macrophages. When macrophages expressing these markers were depleted via clodronate liposomes, viral titers increased more than 10-fold, thus demonstrating that viral clearance is dependent upon CD68 and CD163 expressing macrophages (Fulci et al., 2007). Viral modification by immunosuppressive cytokine/protein incorporation is beneficial for its ability to alter macrophages to a state that encourages viral titer, but in some cases the complete prevention of macrophage infiltration has the greatest impact for the patient.

Macrophages can be utilized to deliver viruses to hypoxic regions in tumors

As monocytes are recruited from the blood by tumor cells, they are stimulated to become macrophages. These M2-like macrophages tend to accumulate in areas where the tumor cells rapidly outgrew the established blood supply leaving pockets with reduced oxygen concentrations, called hypoxic regions. When found within these areas, macrophage movement can be inhibited. These hypoxic regions tend to be resistant to many treatment options due to their low blood supply, which prevents many treatment options from naturally exiting the blood stream in these locations and impacting the tumor cells. Despite the negative impacts macrophages are known to have on oncolytic virotherapeutic efficacy, macrophages are now being utilized as vehicles for the administration of tumor therapies to these hypoxic regions (Muthana et al., 2011). In one case, macrophages were transduced with hypoxia-dependent E1A/E1B and an E1A-dependent oncolytic adenovirus. Once these macrophages arrived in hypoxic regions, they expressed E1A/E1B, which then induced adenovirus replication. The macrophages then released the virus, which infected nearby

tumor cells. A study in 2011 using subcutaneous or orthotopic prostate tumors, utilized these modified macrophages and found that the virus only proliferated in and killed tumor cells after initial replication in the macrophages. This subsequently reduced tumor growth and decreased tumor metastases to the lungs (Muthana et al., 2011). Using these macrophages and their ability to migrate into and survive hypoxic regions, enhanced oncolytic virotherapeutic effects by providing a vehicle to sites notoriously difficult to impact.

Vesicular Stomatitis Virus

Vesicular stomatitis virus (VSV), a member of the *Rhabdoviridae* family, is a helical, enveloped, negative sense RNA virus with a monopartite genome. VSV is a natural pathogen of livestock animals including horses, cattle, pigs, alpaca, and llamas, where infection often results in mucous membrane lesions of the mouth and nose, as well as sores above the hooves in horses (Ahmed and Lyles, 1998). While humans are not the natural host for VSV, a less virulent infection can cause mild flu-like symptoms or, given the strength of the antiviral response, be entirely asymptomatic (Ahmed and Lyles, 1998). The cause of this decreased virulence comes from the IFN response exhibited by healthy cells, which inhibits viral replication (Ahmed and Lyles, 1998). Cancer cells, with their mutated genomes, do not initiate a strong antiviral response and are thus more likely to be killed by these viruses, hence the exploration of VSV as an oncolytic agent.

The matrix protein of VSV, one of five encoded proteins, is most responsible for its pathogenicity. The matrix protein prevents nuclear to cytoplasmic transport of mRNAs, and thus inhibits the expression of host genes that might prevent or inhibit viral infection. The matrix protein is the basis for the two strains of VSV used in this study. The wild-type (rwt)

form of the virus contains the naturally occurring matrix protein, while the second form, rM51R-M virus, possesses a methionine to arginine substitution at the fifty-first amino acid of the matrix protein. This results in a less pathogenic virus with and even greater selectivity for cancer cells over normal cells. With a defective matrix protein, this mutant form of VSV could also potentially be a safer option as an oncolytic agent (Ahmed et al., 2015). VSV has demonstrated cancer cell killing capabilities in breast, pancreatic, colorectal, glioblastoma, and prostate cancer cell lines (Ahmed et al., 2010; Cary et al., 2011; Fehl and Ahmed, 2017; Felt et al., 2015; Stewart et al., 2011), but less is known about how this virus impacts other cells within the TME, particularly pro-tumor M2d macrophages.

Objectives

There is convincing evidence linking M2d macrophages with malignancy including tumor angiogenesis, immune suppression, and metastasis. Additionally, the TME is known to play a role in monocyte recruitment and subsequent M2 macrophage activation through the secretion of various cytokines such as GM-CSF, CCL2, and VEGF. Due to their role in malignancy and their ability to switch phenotypes in response to environmental cues, these cell types have become a target for emerging cancer treatments including that by viruses. The virus of interest, VSV, is known to preferentially infect and kill cancer cells, but certain VSV forms have been shown to modulate immune cells to induce antiviral responses. This leads to the speculation that VSV might have an impact on other cells within the TME, specifically pro-tumor M2d macrophages.

We hypothesize that VSV will inhibit the pro-tumor function of M2d macrophages within the TME by directly targeting these macrophages in ways that will reduce their role in

tumor growth and metastasis. We address this hypothesis through the following specific aims. First, we will address whether M2d macrophages are susceptible to VSV replication and subsequent cell death. Second, we will determine if there is evidence of a potential phenotype switch from a pro-tumor M2d macrophage to an anti-tumor M1 macrophage. Third, we will address whether infection by VSV inhibits the formation of potential metastasis-contributing structures called podosomes by the M2d macrophages. Should VSV be able to kill, convert, or disarm M2d macrophages, their stimulatory role in tumor growth and metastasis might be inhibited.

Chapter II: Introduction, Materials and Methods, Results, and Discussion

Introduction

Metastasis is the acquired ability of cancer cells to invade local surroundings and colonize distant organ tissues (Seyfried and Huysentruyt, 2013). Multiple factors facilitate the metastatic disease process, including the presence of a unique M2 macrophage subtype that often resides in the tumor microenvironment (TME) (Qian and Pollard, 2010).

Macrophages are a heterogeneous and highly plastic cell type of the innate immune system, but whose functions may vary based on location and local chemical stimuli (Mantovani et al., 2007). Macrophages are broadly categorized into classically activated inflammatory M1 macrophages and alternatively activated M2 macrophages. M1 macrophages are characterized by the expression of pSTAT1, MHC-II, and co-stimulatory molecules CD80/86 as well as secretion of pro-inflammatory cytokines IL-6, IL-1 β , TNF- α , and iNOS, which assist in the innate immune response and the initiation of an adaptive immune response. M2 macrophages, in contrast, have been characterized by surface expression of CD204 and secretion of VEGF, IL-10, and matrix metalloproteinase 9 (MMP9) which assist in wound healing and reducing inflammation.

There is much evidence to support the contribution of M2 macrophages to cancer cell metastasis. Macrophages themselves are professional invasive, and form actin-rich extensions at the ventral cell surface called podosomes (Gawden-Bone et al., 2010). These podosomes recruit cathepsins, MMPs, and serine proteases that degrade extracellular matrix (ECM) proteins and thus facilitate movement through tissues. This behavior may also support the invasive movement of nearby tumor cells (Gocheva et al., 2010). In fact, M2 macrophages and cancer cells have been shown to be mutually dependent upon one another

for migration/invasion and collagen deposits by macrophages are known to be utilized by cancer cells for movement towards blood vessels (Goswami et al., 2005; Qian and Pollard, 2010). Macrophages also tend to cluster outside of blood vessels where the release of EGF can entice tumor cell chemotaxis (Gligorijevic et al., 2009; Wyckoff et al., 2007). It is the plasticity of macrophages and the influence of M2 macrophages on cancer cell invasion that has facilitated the search for therapeutics that modulate M2 macrophage viability, subtype, and/or function within the TME.

As metastasis patients tend to respond poorly to traditional treatment options, the need for additional therapies has emerged. Oncolytic virotherapies utilize viruses to kill cancer cells while sparing normal, healthy cells (Woller et al., 2014). An example is oncolytic vesicular stomatitis virus (VSV), which has not only demonstrated tumoricidal capabilities, but has the ability to modulate innate immunity (Ahmed et al., 2006). Much research has been devoted to VSV and its abilities to kill cancer cells, but less is known about how this virus impacts other cells within the TME, particularly pro-tumor M2 macrophages. We hypothesized that VSV will preferentially target and infect M2 macrophages, thereby inhibiting their stimulatory effects on cancer invasion. For these studies, we utilized two strains of VSV, a recombinant wild-type strain, rwt virus, and a matrix (M) protein mutant strain, rM51R-M virus (Black et al., 1993; Kopecky et al., 2001; Lawson et al., 1995; Whelan et al., 1995;). M protein mutants such as the rM51R-M virus have previously shown efficacy in both the killing of cancer cells as well as the promotion of anti-tumor immunity through stimulation of genes involved in host innate immune responses (Ben et al., 2013; Bondreau et al., 2009; Diallo et al., 2010; Diaz et al., 2007; Fernandez et al., 2002; Peng et al., 2013; Wongthida et al., 2010). We believe that the rwt and rM51R-M viruses are capable

of modulating M2 macrophages in ways that could increase their anti-tumor capabilities and reduce the metastatic potential of tumor cells.

Utilizing the ability of the THP-1 monocytic leukemia cell line to differentiate into various macrophage subsets, we observed that pro-tumor M2 macrophages were susceptible to infection by both rwt and rM51R-M viruses leading to decreased viability. Anti-tumor M1 macrophages, however, were resistant to VSV infection by both viruses. We further observed an upregulation of the M1 polarization marker pSTAT1 in monocytes, and M0 and M2 macrophages upon infection with the rM51R-M virus suggesting a phenotype switch to anti-tumor M1 macrophages. Infection of M0 and M2 macrophages with VSV also decreased the formation of podosomes, a major regulator of macrophage invasion. This effect on podosomes was most apparent upon infection of cells with the rwt virus. These results indicate that macrophage populations are differentially susceptible to infection by VSV, and that both the rwt and rM51R-M viruses uniquely modulate M2 macrophage subsets in ways that decrease their invasive behavior.

Materials and Methods

Cell Culture and Viruses

The human monocytic leukemia cell line THP-1 was cultured at 37°C and 5% CO₂ in RPMI-1640 (Sigma-Aldrich) formulated with 0.3g/L L-glutamine and sodium bicarbonate and supplemented to a final concentration of 10% fetal bovine serum (Sigma-Aldrich), 1% penicillin/streptomycin (Corning), 1% vitamins (GE Healthcare, Hyclone), 10mM HEPES, and 0.05mM 2-mercaptoethanol (MP Biomedicals). Recombinant wild-type (rwt) virus, the matrix (M) protein mutant (M51R-M) virus, and rwt and rM51R-M viruses expressing green fluorescent protein (rwt-GFP and rM51R-GFP) were a generous gift from Dr. Douglas Lyles from Wake Forest University School of Medicine (Winston-Salem, NC) and have been described previously (Whitlow et al., 2006). Virus stocks were prepared in BHK cells using methods described previously (Kopecky et al., 2001).

Macrophage Polarization

To initiate macrophage differentiation, THP-1 monocytes were seeded into 6-well plates at 5.0×10^5 cells/well for 24 hours in media containing 25nM PMA (Sigma-Aldrich). The cells were then further polarized to different macrophage subtypes by washing with sterile PBS and treating for 48 hours with 25nM PMA for naïve M0 macrophages, with 20ng/mL LPS (L5418; Sigma-Aldrich), 20ng/mL IFN- γ (570202; BioLegend), and 25nM PMA for M1 macrophages, or 20ng/mL IL-4 (574002; BioLegend), 20ng/mL IL-13 (571102; BioLegend), and 25nM PMA for M2 macrophages (Figure 6A).

Virus Replication in Infected Cells

To examine the ability of VSV to replicate in THP-1 cells, THP-1 monocytes were polarized to M0, M1, and M2 macrophages according to the protocol above. After macrophage polarization, monocytes were seeded into another 6-well plate at 5.0×10^5 cells/well. All cells were infected with rwt-GFP or rM51R-GFP viruses at multiplicity of infections (MOI) of 1 or 10 pfu/cell (plaque forming units/cell) for 16 hours. The GFP-expressing viruses (rwt-GFP and rM51R-GFP) were engineered to express GFP from the viral genomes and thus, serve as a means to measure active virus replication in infected cells (Whitlow et al., 2006). Images of the cells were taken on an Olympus IX81 under 20X magnification using MicroSuite B3 Biological Suite software. The percentage of GFP-positive cells was determined using the ImageJ 1.51a software cell counting tool. Data are the mean \pm standard deviation of three, independent experiments.

Measurement of Cell Viability

The viability of THP-1 monocytes and macrophages in response to VSV infection was carried out by an MTT Assay (Cell Proliferation Kit I (MTT), Roche Diagnostics, Indianapolis, IN; TACS MTT Cell Proliferation Assay, Trevigen). To examine viability, THP-1 monocytes were seeded into 96-well dishes at 2.5×10^4 cells/well and polarized according to the protocol above. After macrophage polarization, monocytes were seeded into 96-well plates at 2.5×10^4 cells/well. Cells were infected with rwt and rM51R-M viruses at MOIs of 1 or 10 pfu/cell for 16 and 32 hours before being assayed for viability according to the manufacturer's directions. Data are the mean \pm standard deviation from three,

independent experiments with each sample performed in triplicate. All data were normalized to mock-infected cells.

Cell Lysates

THP-1 monocytes were polarized to M0, M1, and M2 macrophages according to the protocol above, then along with monocytes were infected with rwt-GFP or rM51R-GFP viruses at MOIs of 1 or 10pfu/cell for 12 hours. During the cell lysis procedure, all plates and buffers were kept on ice. Cultured cells were washed twice with 1mL of ice-cold 1 mM sodium orthovanadate in PBS, then lysed using 75 μ L of lysis buffer composed of 20mM HEPES (pH = 7.0), 110 mM sodium chloride, 40 mM sodium fluoride, 1% NP40, 1 mM sodium orthovanadate, 10 μ g/mL aprotinin, 10 μ g/mL benzamidine, 10 μ g/mL leupeptin, 10 μ g/mL pepstatin, 2mM DTT, and 1 mM PMSF. The cells were scraped from the plates on a bed of ice, incubated on ice for 10 minutes, and the cellular debris removed by centrifugation at 10,000g for 10 minutes at 4°C. Protein concentrations in the supernatants were determined using a detergent-compatible protein assay kit according to manufacturer's instructions (Bio-Rad) against a series of known BSA standards.

SDS-PAGE/Immunoblotting

For immunoblotting analysis, 8 μ g of whole cell lysate protein were denatured in SDS-containing sample buffer and heated at 95°C before loading on a 10% polyacrylamide gel (Bio-Rad). Proteins were separated at 90V for approximately 90 minutes, and then transferred to a 0.45 μ m nitrocellulose membrane (Bio-Rad) at 100V for 1 hour. After blocking in 5% milk/0.1% PBST, the membrane was incubated overnight at 4°C with a

primary antibody specific to CD204 (1:500; #sc-166184; Santa Cruz), pSTAT1 (Y701) (1:1000; #9167S; Cell Signaling), STAT1 (1:1000; #9172S; Cell Signaling), or β -actin (1:2000; #4970S; Cell Signaling), in 5% BSA/0.1% PBST. This was followed by incubation in a species-specific peroxidase-conjugated secondary antibody (1:2500; NA9340V; NA931V; GE Healthcare) in 5% milk/0.1% PBST for 1 hour at room temperature. Proteins were visualized using SuperSignalTM West Dura Extended Duration Substrate (ThermoFisher Scientific), Premium X-Ray Film (Phenix Research Products), Developer & Replenisher Concentrate XLR (#XLD1032; Co-exist & Prosperity Enterprises Co., Ltd) at a 1:3 dilution, and Fixer & Replenisher Concentrate XLR (#XLF1032; Co-exist & Prosperity Enterprises Co., Ltd) at a 1:3 dilution. Relative band intensity was quantified using Quantity One Software 4.6.6 (Basic). Data are the results of three, independent experiments, normalized to the levels of β -actin in each lane and expressed relative to the mock samples. Data are the mean \pm standard deviation.

Podosome Formation

To analyze podosome formation, THP-1 monocytes were polarized to M0, M1, and M2 macrophages according to the protocol above, except that each well contained a sterile glass coverslip (Microscope Cover Glasses, 12mm; Assistent). Cells were infected with rwt-GFP or rM51R-GFP viruses at MOIs of 1 or 10pfu/cell for 12 hours. The macrophages on the coverslips were fixed in 0.3% formaldehyde (Electron Microscopy Sciences)/PBS for 10 minutes, permeabilized in 0.4% Triton X-100/PBS, and stained with Texas Red-X phalloidin (1:200; Molecular Probes) in 5% donkey serum/PBS. Coverslips were mounted onto glass slides using small aliquots of ProLong[®] Gold Antifade with DAPI (8961S; Cell Signaling).

Using an Olympus BX51 microscope (OPELCO) equipped with a Retiga EXi Fast 1394 camera (QImaging), ten random images were collected for each experimental condition. Image processing was conducted using a 100X objective, Type F Immersion Oil (Olympus), and Q-Capture 64 Suite software. The number of podosomes per GFP-positive and GFP-negative macrophage in each image was quantified using ImageJ 1.51a software and then normalized to the mock-infected cells. Podosome incidence, multiplicity, and distribution was determined for total, GFP-positive, and GFP-negative cells for each sample group. Data are the mean \pm standard deviation of three, independent experiments.

Results

M1 and M2 THP-1 Macrophages Have Unique Polarization Profiles

Macrophages have long been conveniently divided into classically activated M1 and alternatively activated M2 subtypes based on polarization stimulus and transcriptional profile (Chávez-Galán et al., 2015). However, the heterogeneous and highly plastic nature of macrophages is well appreciated and necessitates a precise definition of any macrophage polarization procedure and their resulting phenotypes. In this study, an established macrophage polarization protocol using model THP-1 monocytes was followed with minor modifications (Genin et al., 2015) (Figure 6A). Successful polarization was based on phenotypic marker expression and observation of morphological changes.

Two markers, pSTAT1(Y701) and CD204, were analyzed by immunoblotting to determine the polarization status of THP-1 macrophages. STAT1 is a transcription factor associated with IFN- γ -induced M1 polarization. Upon STAT1 phosphorylation, it dimerizes and moves into the nucleus to induce the expression of additional M1 macrophage markers (Ihle et al., 1994). Scavenger receptor-A (SR-A/CD204), an M2 marker, is known to scavenge the ligands of TLR4, thus inhibiting LPS-induced M1 macrophage phenotype (Huanfa et al., 2009). We observed an increase in the M2 macrophage marker CD204 in naïve M0 and M2 polarized macrophages while remaining absent in monocytes and M1 macrophages, consistent with previous data utilizing a similar polarization protocol (Debinski et al., 2014) (Figure 6B). A slight increase in β -actin was also observed with M2 polarization. In contrast, STAT1 was activated (phosphorylated) upon treatment of cells with LPS and IFN- γ , thus confirming the phenotype of the M1 macrophages, also consistent with previous data (Debinski et al., 2014) (Figure 6B).

The morphological changes in the four cell types were also consistent with unique polarization statuses (Figure 6C). THP-1 monocytes are small, rounded, and uniformly shaped non-adherent cells. M0 macrophages, in contrast, stopped proliferating, became adherent, and exhibited a common enlarged, rounded morphology. M2 polarized macrophages induced a phenotype similar to M0 macrophages, but were slightly larger in size and had a tendency to cluster. M1 polarized macrophages were also adherent, but had a much more variable morphology, including a significant number of cells that were large, flat, and spindle-shaped.

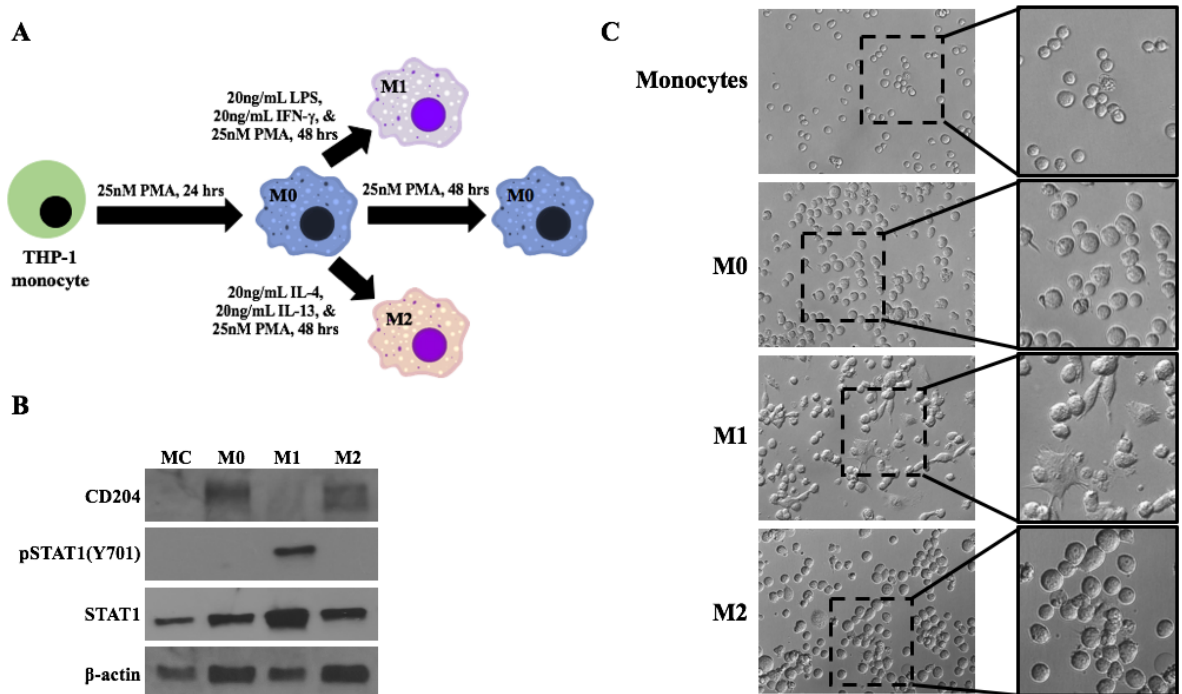


Figure 6. THP-1 Macrophage Polarization Protocol and Profiling. (A) Macrophage polarization protocol as described in the methods. (B) Protein marker expression following macrophage polarization (MC, monocyte) (C) Representative differential interference contrast images taken at 20X magnification showing the different morphologies of THP-1 monocytes and macrophage subtypes.

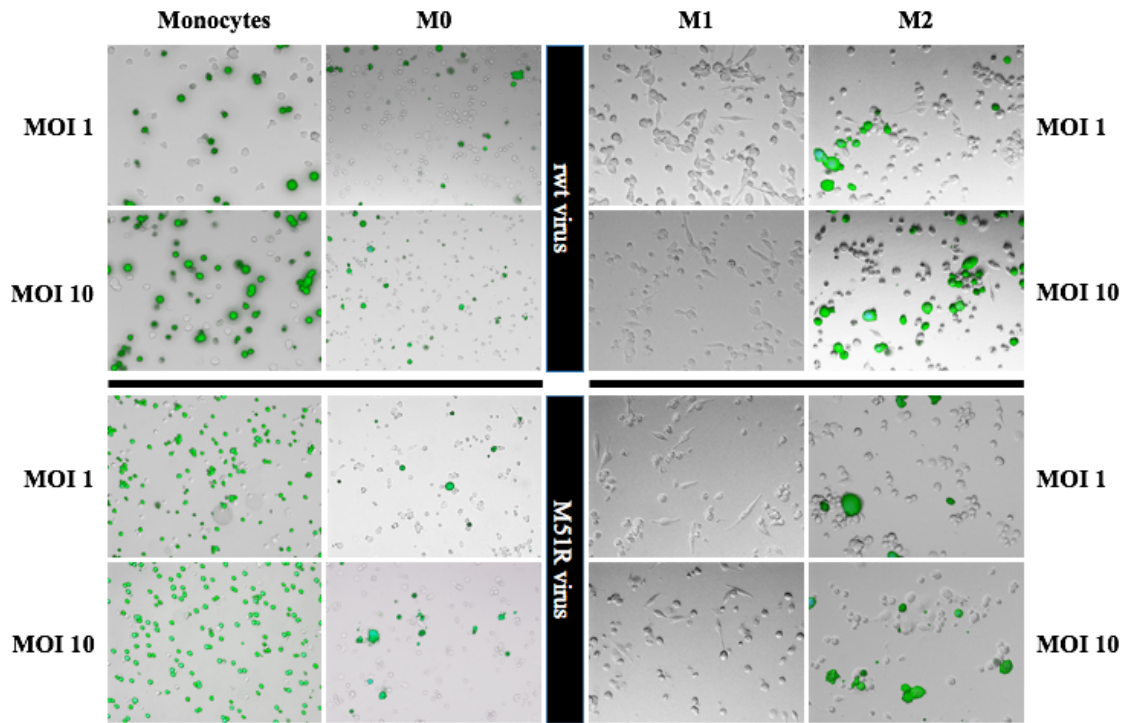
M1 Macrophages are Resistant and M2 Macrophages Sensitive to Replication by rwt and rM51R-M viruses

Polarization to the M1 phenotype most often occurs via exposure to IFN- γ secreted by T_H1 cells, cytotoxic T cells, or NK cells (Shuai et al., 1993). IFN- γ then binds to its associated receptor composed of 2 ligand-binding subunits (IFNGR1) and 2 signal transducing subunits (IFNGR2). Two Jak tyrosine kinases, Jak1 and Jak2, bind to IFNGR1 and IFNGR2, respectively, and IFNGR1 gets phosphorylated in order to provide a binding site for STAT1. STAT1 is then phosphorylated, leaves the binding site on IFNGR1, and dimerizes with another phosphorylated STAT1. Acting as a transcription factor, pSTAT1:pSTAT1 homodimers move into the nucleus to induce upregulation of other M1 polarization markers as well as antiviral genes (Matsumoto et al., 1999). Antiviral genes include the GTPase, Mx, which binds and sequesters nucleocapsid proteins making them unavailable for the next generation of viruses (Haller et al., 2007), and 2'-5' Oligoadenylate synthase 1 (OAS-1), which leads to the degradation of viral RNA following activation of RNase L (Sadler and Williams, 2008). This signaling pathway primes macrophages for increased responsiveness to extracellular stimuli including that by viruses (Taniguchi and Takaoka, 2001). This is an effect not observed upon M0 or M2 macrophage polarization. As a result, there are no constitutive levels of antiviral proteins found in these macrophages before viral infection. Thus, we hypothesized that M1 macrophages would be resistant to infection and killing by VSV, while monocytes and M0 and M2 macrophages would exhibit varying degrees of sensitivity.

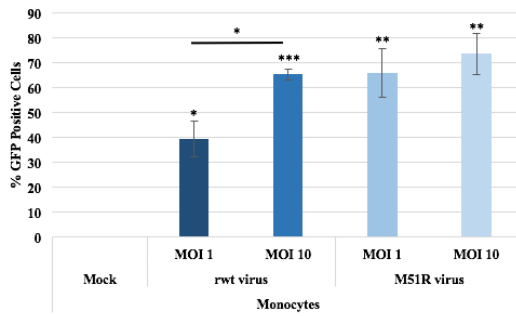
In order to test this hypothesis, THP-1 monocytes and M0, M1, and M2 macrophages were infected with rwt-GFP or rM51R-GFP viruses at MOIs of 1 or 10pfu/cell. After 16

hours, the cells were imaged and the percentage of GFP-positive cells was analyzed as an indication of the percentage of cells with active virus replication. Figure 7A shows representative images with GFP-positive cells indicated in green. Upon quantitation, monocytes exhibited the highest percentage of GFP-positive cells ranging from 40% to almost 75% (Figure 7B). This effect occurred in a dose-dependent manner and was greatest upon infection with the M protein mutant virus (Figure 7B). The percentage of GFP-positive cells decreased following macrophage polarization. Supporting our hypothesis, M1 macrophages exhibited no evidence of viral replication, consistent with their constitutive anti-viral state (Haller et al., 2007; Matsumoto et al., 1999; Sadler and Williams, 2008) (Figure 7C). M0 and M2 macrophages were susceptible to infection with both rwt and rM51R-M viruses with the percentage of GFP-positive cells ranging from 7% to 23% for rwt virus and 13% to 36% for rM51R-M virus (Figure 7B, D). M2 macrophages were more susceptible to infection with rwt virus infection as compared to rM51R-M virus (Figure 7D). This evidence suggests that monocytes demonstrate the greatest susceptibility to viral infection followed by M2, M0, and M1 macrophages. The ability for M2 macrophages to support viral replication may imply that VSV strains have the ability to modulate this cell type through various means.

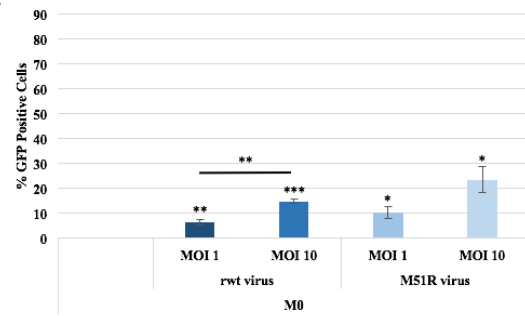
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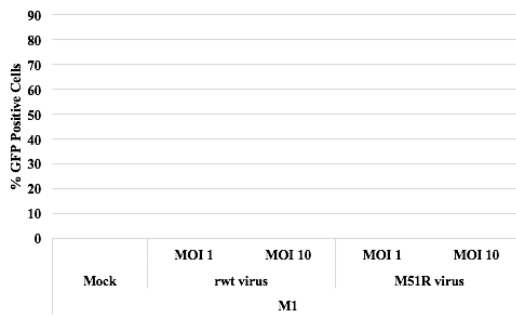
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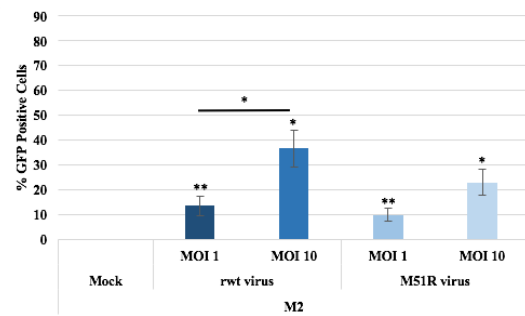


Figure 7. Replicative Ability of rwt and rM51R-M Viruses in THP-1 Monocytes and Macrophages. (A) Representative images of GFP-positive cells taken by live phase contrast and fluorescent microscopy 16 hours post-infection by rwt and rM51R-M viruses at MOIs of 1 or 10 pfu/cell. Percentage of GFP-positive (B) monocytes, (C) M0, (D) M1, and (E) M2 macrophages. Data are the means \pm standard deviations of three, independent experiments. Statistical analyses between mock and viral treatments and between MOIs were conducted using a Student's t-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

The Cytopathicity of VSV Towards Macrophage Subtypes is Consistent with the Sensitivity of Macrophages to Replication by VSV

Previous studies have targeted M2 macrophage survival as a potential anti-cancer therapy. This has been accomplished in ovarian cancer with pro-apoptotic bisphosphonates like clodronate and zoledronic acid, which accumulate in highly phagocytic TAM populations, by targeting the M2d macrophage surface marker CD204 with immunotoxin-conjugated antibodies, and by infecting macrophages with *Shigella flexneri* (Bak et al., 2007; Galmbacher et al., 2010; Rogers and Holen, 2011; Zeisberger et al., 2006). To determine whether macrophage populations that support virus replication were also susceptible to killing by the virus, the viability of macrophages following virus infection was determined by an MTT assay (Figure 8).

Monocytes demonstrated the greatest susceptibility to viral-induced cell death (Figure 8A). This effect occurred in both a dose- and time-dependent manner and was most apparent in response to rwt virus, with a close to 80% reduction in viability when infected for 32 hours at an MOI of 10 pfu/cell. M0 and M2 macrophages were also sensitive to the cytopathic effects associated with both viral strains but to a lesser degree as compared to monocytes. Cell death was also dose- and time-dependent in these subtypes and was most apparent in response to the rwt virus (Figures 8B, D). At most, M2 macrophages showed a 70%

reduction in viability while M0 macrophages showed a 65% reduction in viability when infected for 32 hours at an MOI of 10 pfu/cell. In contrast, M1 macrophages showed no statistical differences in viability relative to mock-infected cells, indicating that they are resistant to virus-induced cell death (Figure 8C). The differential ability of VSV to kill these macrophage subtypes is thus consistent with the extent to which these cells support viral replication (Figure 7).

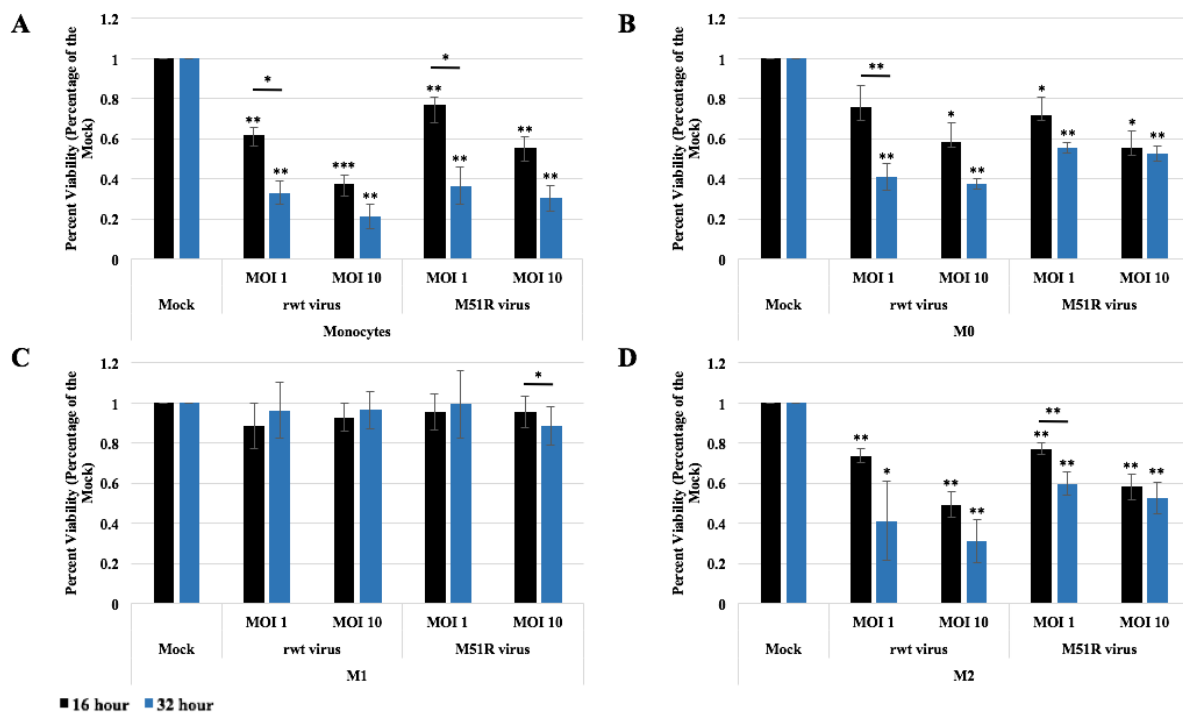


Figure 8. The Effects of VSV on THP-1 Monocyte and Macrophage Viability. Percentage of viable cells in (A) monocytes, (B) M0, (C) M1, and (D) M2 macrophages in response to rwt or rM51R-M viruses at MOIs of 1 or 10 pfu/cell at 16 and 32 hours post-infection. Data are the means \pm standard deviation of three, independent experiments. Statistical analyses between mock and viral treatment and between 16 and 32 hours post-infection were conducted using a Student's t-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Monocytes, M0, and M2 macrophages upregulate M1 marker pSTAT1(Y701) in response to rM51R-M virus.

M2 macrophages are known to modulate tumor cell response to oncolytic virotherapies (Tan et al., 2016). Due to the versatile nature of these cells, it is possible for these macrophages to convert between different subtypes in response to environmental conditions (Mantovani and Sica, 2010; Xue et al., 2014). By altering M2 macrophages to an M1 phenotype, a newfound innate and adaptive immune response could be initiated against the tumor cells. Many oncolytic viruses, including adenovirus, measles and mumps viruses, and reovirus have successfully induced a switch to the M1 phenotype and promote anti-tumor immunity *in vitro* (Guidicci et al., 2005; Gujar et al., 2013; Tan et al., 2016).

In order to determine if VSV was effective at modifying the phenotype of macrophage subtypes, expression of the M1 marker pSTAT1(Y701) and the M2 marker CD204 was determined by immunoblotting. Representative images are shown in Figure 9A. Most noteworthy, infection by the mutant virus led to an upregulation of pSTAT1(Y701) in monocytes (Figures 9B), M0 (Figures 9C), and M2 macrophages (Figures 9E). This effect was not observed in response to rwt virus. The upregulation of the M1 marker provides evidence that rM51R-M virus has the potential to induce a switch from M0/M2 to an M1 phenotype. This may be due to the M protein mutation in the mutant virus allowing for expression of new host genes, including those involved in host innate immunity and antiviral responses. In M1 macrophages, constitutive STAT1 activation (pSTAT1) was not altered following virus infection, further indicating that M1 macrophages are resistant to virus infection and any effects the virus may have in altering the M1 phenotype (Figures 9D). Interestingly, CD204 expression significantly increased in response to rwt virus in the M0

(Figures 9B) and M2 macrophages (Figures 9E). This effect was not observed upon infection with rM51R-M virus nor in M1 macrophages.

Despite the upregulation of pSTAT1, further evidence is needed to support a model of VSV-induced macrophage phenotype switch. pSTAT1 is essential for responsiveness to IFN- γ and IFN- α , the former of which is involved in M1 macrophage polarization and both of which are involved in the anti-viral immune response (Durbin et al., 1996; Meraz et al., 1996). Therefore, it is difficult to determine if the observed effect is due to a true phenotype switch or due to the initiation of the anti-viral immune response. Cytokine secretion has been noted as one of the best ways to indicate macrophage polarization state. Further studies clarifying these results could include enzyme-linked immunosorbent assays (ELISAs) to determine alterations in pro-inflammatory (M1) and anti-inflammatory (M2) cytokine secretion.

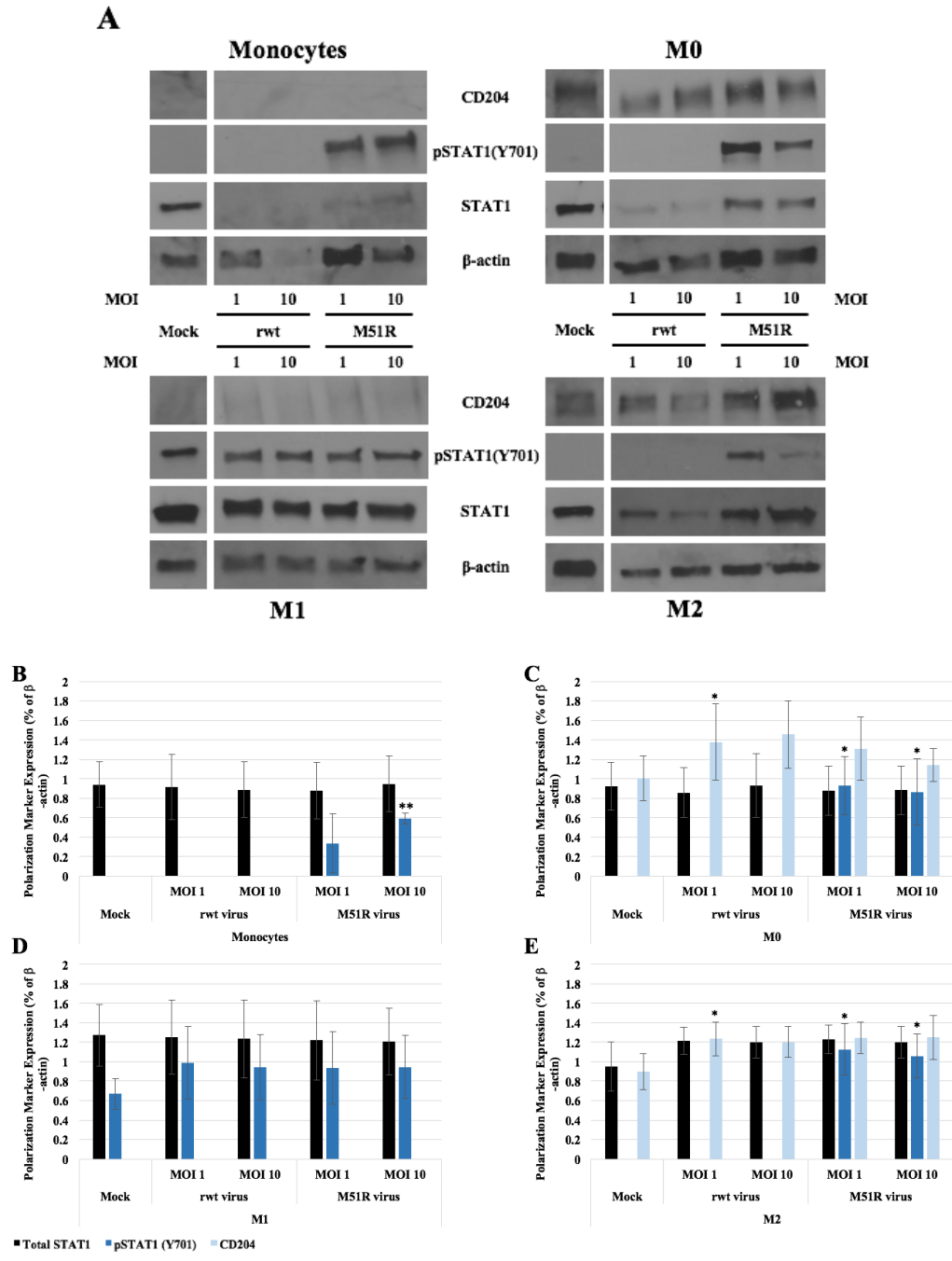


Figure 9. Effects of Viral Infection on the Polarization Markers pSTAT1 and CD204. (A) Representative images depicting changes in polarization markers in monocytes, and M0, M1, and M2 macrophages following infection by rwt or rM51R-M virus at MOIs of 1 or 10 pfu/cell. Quantification of polarization marker data for (B) monocytes, (C) M0, (D) M1, and (E) M2 macrophages relative to mock treatments. Data are the means \pm standard deviation of three, independent experiments. Statistical analyses between mock and viral infections were conducted using a Student's t-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

M1 Macrophages Produce Significantly Fewer Podosomes than M0 and M2 Macrophages

Previous evidence indicates that a high density of M2 macrophages in the tumor microenvironment correlates with a poor patient prognosis, an effect thought to be partially due to M2 macrophage involvement in cancer cell invasion and metastasis (Mantovani and Sica, 2010). One study found that macrophages and tumor cells are involved in a paracrine loop that drives forward the invasion of both cell types (Goswami et al., 2005). This effect may not be true of M1 macrophages as IL-4-polarized M2 macrophages were more invasive in a 3D culture environment than TNF- α -polarized M1 macrophages (Cougoule et al., 2012). As podosomes serve a motility function for the macrophage, it is possible that this effect on tumor cell and macrophage invasion could be partially due to podosome formation by these cells (Gawden-Bone et al., 2010). However, no studies have assessed differences in podosome formation between these cell types and correlated podosome formation to individual macrophage function.

Here we compared M0, M1, and M2 macrophages for differences in podosome incidence, multiplicity, and distribution. Figure 10A shows representative images of each of the macrophage subtype with podosomes based on positive staining of punctate F-actin structures (red) with phalloidin. Quantitation of podosome incidence, the percentage of cells with at least one podosome, revealed that podosomes are found in every M0, M1, and M2 macrophage analyzed (Figure 10B). While there were no differences associated with podosome incidence, podosome multiplicity, the average number of podosomes per cell, differed statistically between macrophage subtypes (Figure 10C). M0 macrophages formed approximately 120 podosomes/cell. This significantly dropped to 50 podosomes/cell upon M1 polarization. M2 macrophages formed approximately 90 podosomes/cell, a significantly

greater amount compared to M1 macrophages, but not significantly different from M0 macrophages (Figure 10C). In analyzing podosome distribution, the range in the number of podosomes, few M0 macrophages exhibited less than 50 podosomes with most forming between 50-150 podosomes/cell (Figure 10D). Upon M1 polarization, the number of cells forming fewer than 50 podosomes increased and very few of these cells formed greater than 100 podosomes. M2 macrophages exhibited an intermediate distribution between the M0 and M1 macrophages, with several cells forming fewer than 50 podosomes and several cells forming greater than 100 podosomes (Figure 10D). These results indicated that despite no differences in podosome incidence, M1 macrophages formed significantly fewer podosomes/cell based on a podosome distribution significantly lower than that of the M0 and M2 macrophages. This could provide evidence for the decreased invasion exhibited by M1 macrophages compared to M2 macrophages (Cougoule et al., 2012).

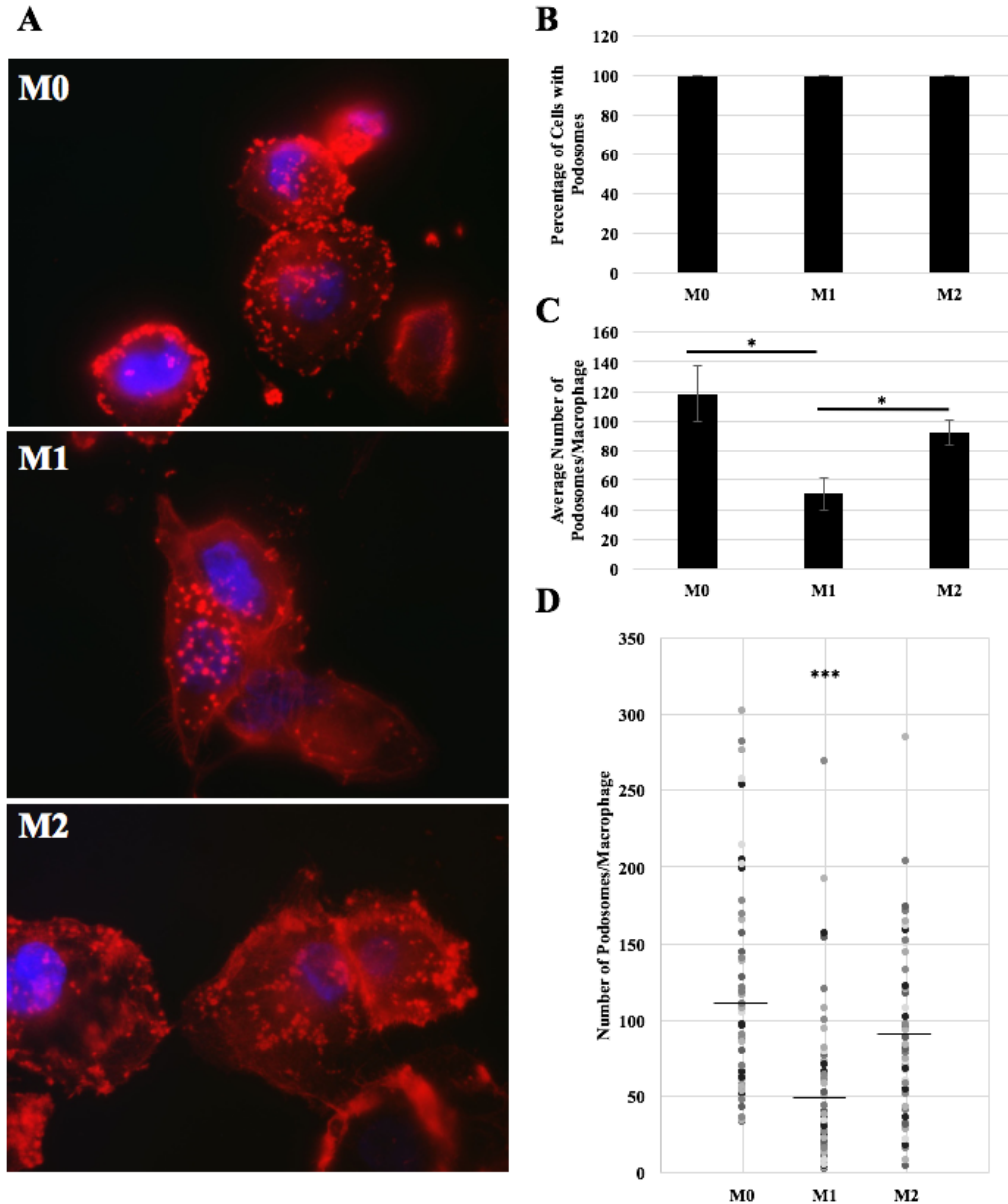


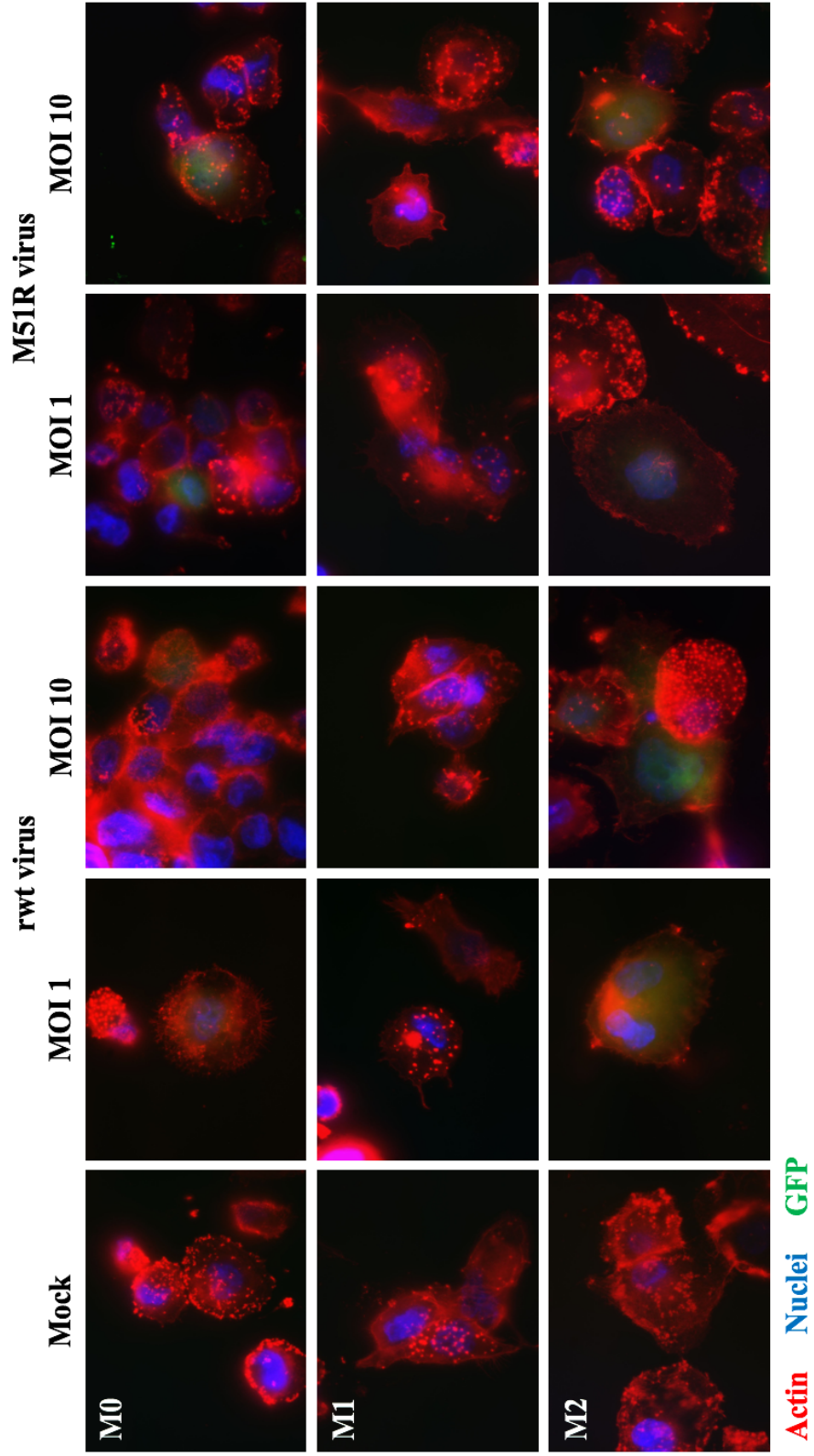
Figure 10. Analysis of Podosome Formation in THP-1 Macrophage Subtypes. (A) Representative images of podosomes in M0, M1, and M2 macrophages based on the punctate distribution of F-actin (red) (nuclei, blue). Images were taken at 100X magnification. (B) Podosome incidence (percentage of cells with podosomes), (C) multiplicity (number of podosomes/cell), and (D) distribution (range in the number of podosomes/cell) are indicated for mock-infected M0 (n=54), M1 (n=55), and M2 (n=49) macrophages. Podosomes were counted using ImageJ 1.51a. Data are the means \pm standard deviation of three, independent experiments. Statistical analyses were conducted using a Student's t-test or one-way ANOVA. *, $p < 0.05$; **, $p < 0.01$; p < 0.001.

Macrophage Podosome Incidence Is Not Significantly Affected by VSV Infection

As indicated previously, M2 macrophages are thought to enhance ECM breakdown and tumor cell movement through the basement membrane via the formation of podosomes (Gawden-Bone et al., 2010). We wanted to determine whether viral infection would modulate podosome formation among the macrophage subtypes. As M0 and M2 macrophages (but not M1 macrophages) are susceptible to viral infection and replication, we hypothesized that viral infection of M0 and M2 macrophages would reduce podosome incidence, perhaps through direct modulation of the factors necessary for podosome formation.

To test the ability of VSV to modulate podosome formation in macrophages, M0, M1, and M2 macrophages were infected for 12 hours with rwt-GFP or rM51R-GFP viruses at MOIs of 1 or 10 pfu/cell, after which the cells were stained with phalloidin for visualization of F-actin. Figure 11A shows representative images of the stained cells. The punctate F-actin structures were counted in total cells and in GFP-positive (cells in which virus was actively replicating) and GFP-negative cells (cells with no virus replication) and analyzed for podosome incidence. No differences were observed in podosome incidence between mock and virus-infected M0 (Figure 11B), M1 (Figure 11C), and M2 (Figure 11D) macrophages. Podosome incidence in GFP-positive and GFP-negative cells did not differ from that of mock-infected cells either.

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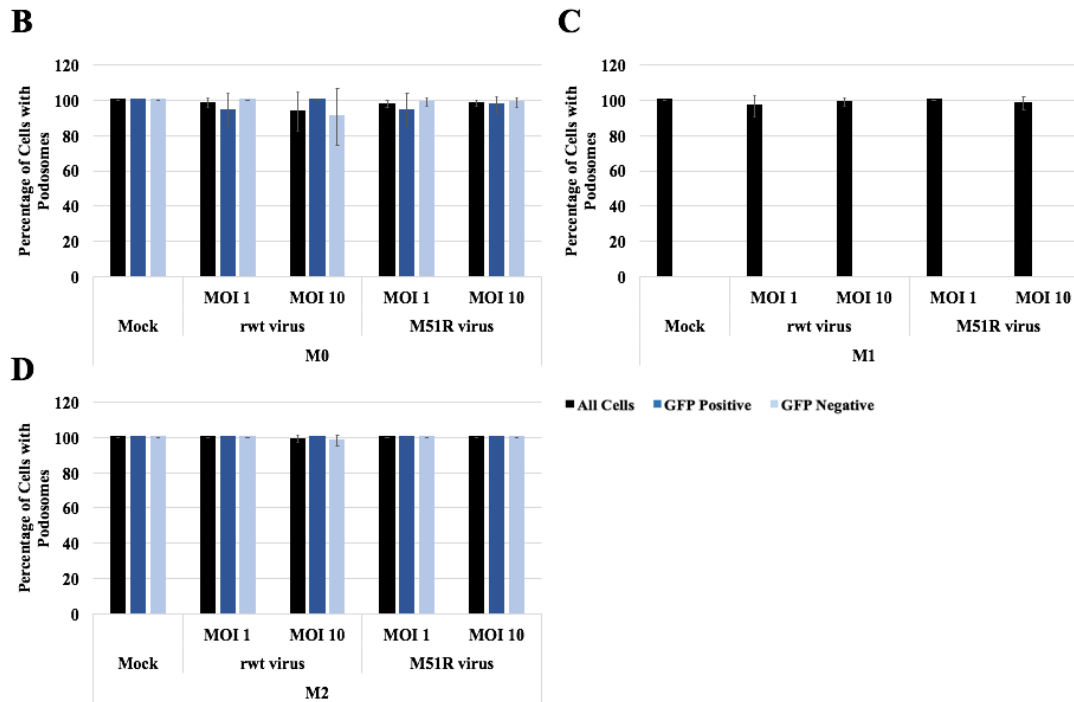


Figure 11. The Effect of VSV on Podosome Incidence. (A) Representative images of podosomes (punctate F-actin structures, red) in M0, M1, and M2 macrophages following infection for 12 hours with rwt-GFP or rM51R-GFP viruses at MOIs of 1 or 10 pfu/cell. GFP-positive cells (green), are actively replicating virus. (nuclei, blue), Images were taken at 100X magnification. Podosome incidence (percentage of cells with podosomes) was determined in all cells, GFP-positive cells, and GFP-negative cells for (B) M0, (C) M1, and (D) M2 macrophages. Podosomes were counted using ImageJ 1.51a. Data are the means \pm standard deviation of three, independent experiments. Statistical analyses between mock and viral infections were conducted using a Student's t-test. *, $p < 0.05$.

M0 and M2 Macrophages Exhibit Greater Susceptibility to Changes in Podosome Multiplicity in Response to VSV Infection than M1 Macrophages

While no differences were observed with podosome incidence, it is possible that podosome multiplicity could be affected upon infection with VSV. A study published in 2003 found that cortactin, an actin-associated protein and podosome marker, was utilized in clathrin-mediated endocytosis, the process by which VSV enters host cells (Cao et al., 2003). It is therefore possible as a consequence of VSV infection that cortactin is recruited by the

virus to facilitate virus entry, thus debilitating podosome machinery. While this effect may not be impacting podosome incidence, it could still be reducing podosome multiplicity, or the average number of podosomes within each cell. As M0 and M2 macrophages (but not M1 macrophages) are susceptible to viral infection and replication, we hypothesized that podosome multiplicity in M0 and M2 macrophages would be decreased by VSV.

To test this hypothesis, the images of M0, M1, and M2 macrophages analyzed for podosome incidence were also analyzed for podosome multiplicity. Punctate F-actin structures were counted in total cells and in GFP-positive and GFP-negative cells and normalized to the mock. Results showed that M0 and M2 macrophages experienced a significant decrease in podosome multiplicity relative to mock. This decrease was observed in cells infected with both rwt-GFP and rM51R-GFP viruses, but differed depending on the MOI used to infect the cells. The reduction in podosome multiplicity was also observed when analyzing the GFP-positive and GFP-negative M0 and M2 macrophages. That GFP-negative macrophages also exhibited reductions in podosome numbers suggests that the GFP-positive macrophages actively exert a ‘field effect’ across the culture that leads to phenotypic changes in non-infected neighboring cells. In contrast to M0 and M2 macrophages, no significant changes were observed in the podosome numbers of M1 macrophages, supporting our hypothesis that these cells remain resistant to the various effects of virus infection (Figure 12B).

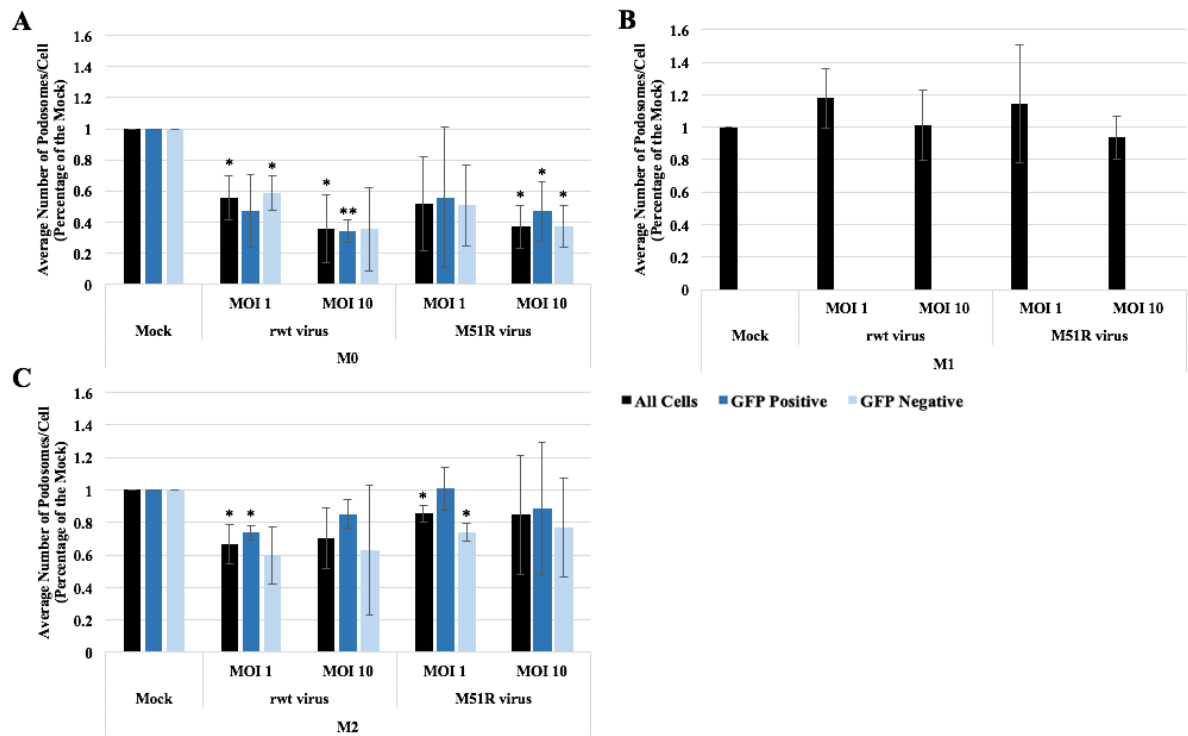


Figure 12. The Effect of VSV on Podosome Multiplicity. Podosome multiplicity (numbers of podosomes/cell) was determined in all cells, GFP-positive cells, and GFP-negative cells for (A) M0, (B) M1, and (C) M2 macrophages following infection for 12 hours with rwt-GFP or M51R-GFP viruses at MOIs of 1 or 10 pfu/cell. GFP-positive cells are actively replicating virus. Podosomes were counted using ImageJ 1.51a. Data are the means \pm standard deviation of three, independent experiments. Statistical analyses between mock and viral infections were conducted using a Student's t-test. *, $p < 0.05$; **, $p < 0.01$.

Treatment with rwt Virus Results in More Cells with Fewer Podosomes in M0 and M2

Macrophages

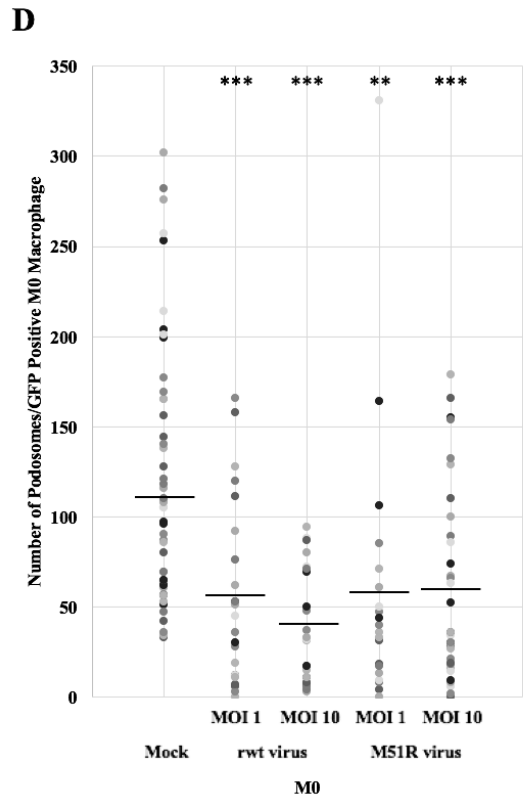
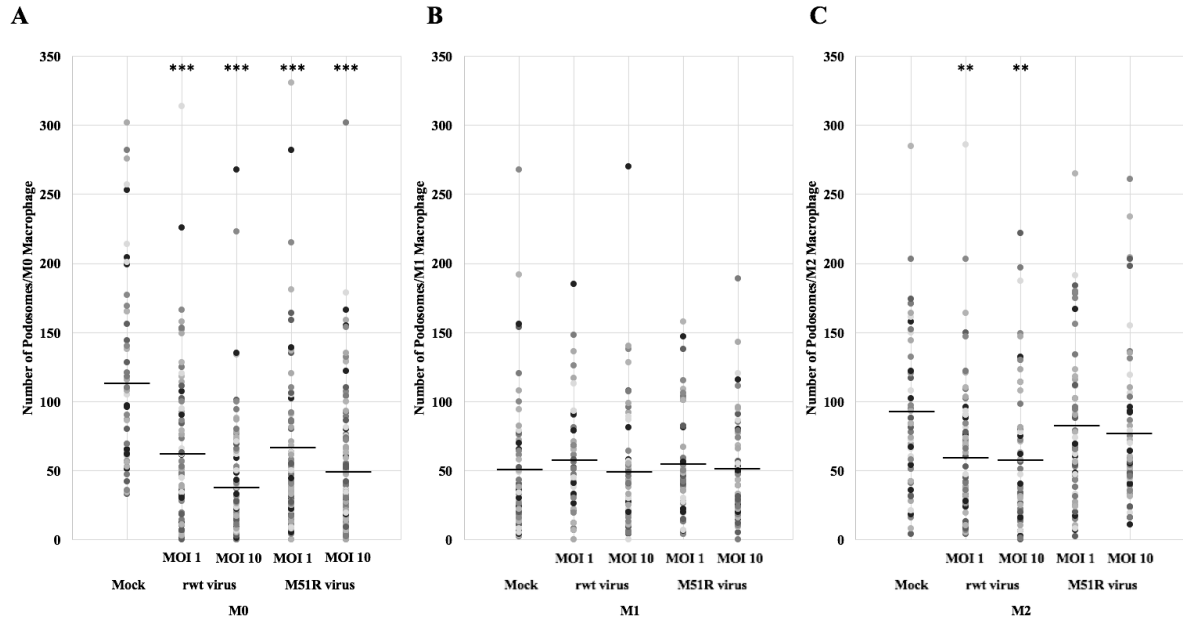
To further tease out differences in podosome formation among macrophage subtypes following viral infection, we also analyzed the range in podosome number per cell. Doing so might enable detection of significant reductions in podosome development following an infection that were not seen in the analysis of podosome multiplicity. Thus, the images of M0, M1, and M2 macrophages analyzed for podosome incidence and multiplicity were also

graphed for podosome distribution. Punctate F-actin structures were counted for total cells and for GFP-positive and GFP-negative, then normalized to the mock. First in corroboration with previous data, there was a significant decrease in podosome distribution in M0 macrophages infected with either rwt-GFP or rM51R-GFP viruses (Figure 13A). Mock-infected M0 macrophages exhibited an average of 116 podosomes per cell and this dropped to 63 (1 pfu/cell) and 38 (10 pfu/cell) podosomes per cell when infected with rwt-GFP virus and to 67 (1 pfu/cell) and 49 (10 pfu/cell) podosomes/cell when infected with rM51R-GFP virus (Figure 13A). Furthermore, the percentage of cells forming fewer than 50 podosomes increased from 9.3% to 46.6% (1 pfu/cell) and 70.23% (10 pfu/cell) when infected with rwt virus and 59% (1 pfu/cell) and 61.6% (10 pfu/cell) when infected with rM51R-M virus (Figure 13A). Similar to M0 macrophages, there was also a significant decrease in podosome distribution in M2 macrophages (Figure 13C). This too corroborates with the data on podosome multiplicity (Figure 12C). Mock-infected M2 macrophages exhibited an average of 91 podosomes per cell and this dropped to 59 (1 pfu/cell) and 49 (10 pfu/cell) podosomes per cell when infected with rwt-GFP virus and to 78 (1 pfu/cell) and 76 (10 pfu/cell) podosomes per cell with rM51R-GFP virus. Additionally, upon rwt virus infection, the percentage of M2 macrophages forming fewer than 50 podosomes increased from 24.5% to 50% (1 pfu/cell) and 52.5% (10 pfu/cell) in response to rwt virus infection and 34.8% (1 pfu/cell) and 44.3% (10 pfu/cell) in response to rM51R-M virus infection (Figure 13C). Consistent with our prediction, M1 macrophages experienced no changes concerning podosome distribution across all groups (Figure 13B).

Analyzing the podosomes of GFP-positive and GFP-negative macrophages enabled further refinement of the data. GFP-positive M0 macrophages demonstrated similar trends in

distribution to that observed in total cells with more cells forming fewer than 50 podosomes (Figure 13D). These results indicate that active virus replication may be altering the podosome machinery in these cells. However, GFP-negative cells also exhibited a similar distribution suggesting that secreted factors following virus infection alter podosome formation in neighboring M0 macrophages (Figure 13F). This may even be more relevant in cells infected with rM51R-GFP virus where a greater effect was observed in the GFP-negative cells (Figures 13D, F). In terms of M2 macrophages, significant changes in podosome distribution upon virus infection were only observed in GFP-negative macrophages, indicating that the indirect effects of the virus supersede those effects seen in cells directly infected with the virus (Figures 13E, G).

Evidence suggests that M2 macrophages are susceptible to viral infection and support viral replication, an effect also observed in monocytes and M0 macrophages but not M1 macrophages. Cell types susceptible to viral infection are also more susceptible to cell death. Further evidence indicates that the cells remaining are potentially switching over to an anti-tumor M1 phenotype in response to rM51R-M virus as demonstrated by the upregulation of pSTAT1. Infection by the rwt virus also has implication concerning formation of podosomes, a cellular structure thought to be involved in various stages of metastasis.



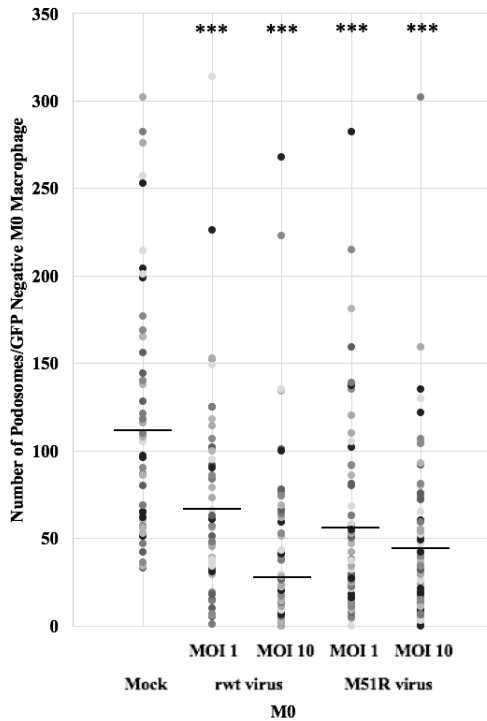
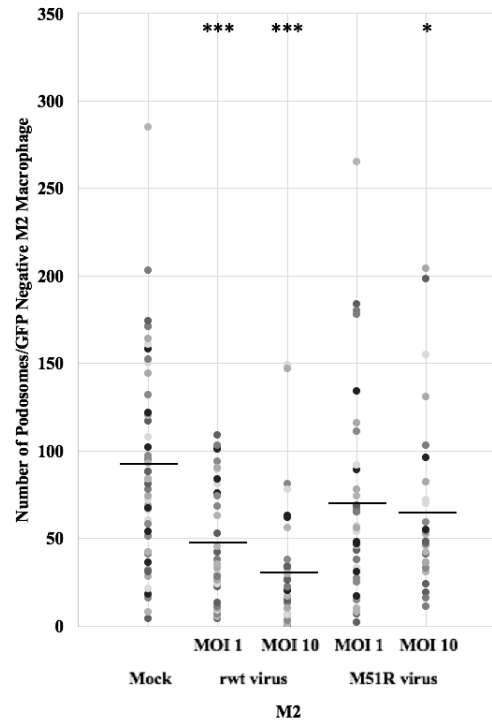
F**G**

Figure 13. The Effect of VSV on Podosome Distribution. Podosome distribution (range in the number of podosomes/cell) was determined in all (A) M0, (B) M1, and (C) M2 macrophages, in GFP-positive (D) M0 and (E) M2 macrophages, and in GFP-negative (F) M0 and (G) M2 macrophages following infection for 12 hours with rwt-GFP or rM51R-GFP at MOIs of 1 or 10 pfu/cell. GFP positive cells are actively replicating virus. Podosomes were counted using ImageJ 1.51a. Data are the means of three, independent experiments. Statistical analyses differences between mock and viral infections were conducted using a one-way ANOVA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Discussion

The data presented here indicate that oncolytic vesicular stomatitis virus has the capacity to modulate M2 macrophage viability, phenotype, and function in ways that could have inhibitory effects on cancer cell invasion and metastasis. Both the rwt and M protein mutant viruses replicated effectively within monocytes, M0, and M2 macrophages, resulting in substantial cell death. The remaining viable cells within those populations demonstrated the potential to switch to an M1 pro-inflammatory phenotype especially in response to the rM51R-M virus. We believe that since rM51R-M virus is defective at inhibiting host gene expression, these cells responded by upregulating the expression of antiviral genes, including the M1 macrophage marker pSTAT1. Finally, M0 and M2 macrophages experience a decrease in podosome multiplicity and distribution mostly in response to rwt infection, while M1 macrophages remain unaffected. It is therefore possible that treatment with different strains of oncolytic VSV provides benefits for cancer patients beyond oncolysis alone, including by decreasing M2 macrophage number, converting M2 macrophages to an anti-tumor M1 phenotype, and reducing the number of metastasis-contributing macrophage podosomes.

Utilizing a previously established protocol with minor modifications (Genin et al., 2015), we have been able to polarize M0, M1, and M2 macrophages from the monocytic leukemia THP-1 cell line. Successful polarization was demonstrated through the induction of CD204 in M0 and M2 macrophages and of STAT1 phosphorylation in M1 macrophages (Figure 6). Additionally, we observed distinct morphological changes in M0, M1, and M2 macrophages upon treatment with polarizing cytokines (Figure 6). Activated M1 macrophages are known to be the macrophage phenotype that responds to most bacterial and

viral infections. The use of IFN- γ to polarize to the M1 phenotype activates the JAK/STAT pathway and STAT1, and upregulates M1 macrophage markers and antiviral genes including Mx and OAS-1 (Haller et al., 2007; Matsumoto et al., 1999; Sadler and Williams, 2008). This can also result in priming, where the activated macrophages exhibit increased sensitivity to environmental stimuli, including viruses (Taniguchi and Takaoka, 2001). This creates a highly sensitive and activated M1 macrophage prepped to handle a viral infection before it ever occurs. As evidence of this phenotype, M1 macrophages were resistant to infection and killing by both strains of VSV. This does not occur in monocytes or in M0 or M2 macrophages following polarization, thus leaving these other cell types vulnerable to viral infection, subsequent replication, and potentially cell death.

Our results show that rwt virus and, to a lesser extent, rM51R-M virus are effective at killing M2 macrophages (Figure 8). In fact, increasing reports are demonstrating that targeting and/or killing M2 macrophages in the TME for killing is a potential therapeutic strategy. Bisphosphonates like clodronate and zoledronic acid have been shown to kill TAM populations (Rogers and Holen, 2011; Zeisberger et al., 2006). Targeting surface marker CD204 using immunotoxin-conjugated mAbs has also been studied in ovarian cancer and yielded promising results depletion of M2 macrophages (Bak et al., 2007). Additionally, bacteria that target macrophages have been used to induce apoptosis including *Shigella flexneri*. In one study, a single injection of attenuated *Shigella* bacteria induced M2 apoptosis resulting in a >70% reduction in tumor size (Galmbacher et al., 2010). *Listeria monocytogenes*, *Chlamydia psittaci*, and *Lionella pneumophila* are other bacteria being considered (Weigert et al., 2009). Previous studies have shown that the matrix M protein of VSV inhibits host RNA and protein synthesis and as a result induces apoptosis through the

mitochondria-associated (intrinsic) pathway (Kopecky and Lyles, 2003; Kopecky et al., 2001). In contrast, the rM51R-M virus induces apoptosis via the extrinsic apoptotic pathway, in a manner that is independent of M protein (Gaddy and Lyles, 2005). Although both viruses induce cell death in infected cells, the mechanisms by which they do so are distinct and may explain differences we observed in our results. In response to infection with rM51R-M virus, host gene expression fails to be shut off thus allowing the M0 and M2 macrophages to induce anti-viral genes like Mx and OAS-1. This in turn, leads to a delay in cell death. We also believe that activation of the antiviral pathway may be inducing these cells to switch to the M1 macrophage phenotype. rwt virus, with its ability to inhibit host gene expression, prevents expression of antiviral genes and rapidly induces apoptosis in infected cells. Taken together, this information provides a rationale for the differential cell death and phenotypic changes observed in macrophage subtypes following infection with either rwt or rM51R-M viruses.

Despite the ability of VSV to induce cell death in monocytes and M0 and M2 macrophages, a significant proportion of cells remained viable, especially in the case of infection with rM51R-M virus. As indicated above we have evidence that rM51R-M virus effectively induces an anti-viral response in these cells and may be inducing the conversion of M2 polarized macrophages to the M1 phenotype. There is some evidence to support promotion of macrophage phenotype switching by oncolytic agents as a therapeutic option for cancer patients. Oncolytic measles (MeV) and mumps viruses (MuV) have been shown to modulate the TME by inducing a switch from M2-like macrophages to an M1 anti-tumor phenotype that could enhance tumor cell death. This change as demonstrated by the upregulation of M1 markers TNF- α , TRAIL, NO, CD80, CD64, IL-1 β , CXCL9, CXCL10,

and IL-6 (Tan et al., 2016). Reovirus significantly also enhanced the expression of the M1 antigen presentation markers MHC-I, β 2-microglobulin (β M2), TAP-1, and TAP-2 (Gujar et al., 2013). Finally, the adenovirus strain d1922-947 exhibited similar effects in anaplastic thyroid carcinomas *in vivo*. Treatment with d1922-947 induced a phenotype switch in infiltrating macrophages from M2d to M1 as demonstrated by the upregulation of M1 marker NOS2. This effect occurred without the upregulation of M2 markers Ym1 or Arg1 (Passaro et al., 2015). Therefore, there is some precedence for the use of oncolytic viruses to manipulate macrophages in the TME. However, much work is necessary to understand the underlying mechanisms by which viruses may induce macrophage phenotype switching and to what effect on tumor growth and progression.

In addition to the use of viruses to target M2 macrophages in the TME, several groups have explored other strategies for altering the phenotype of this pro-tumorigenic macrophage population. For example, the addition of the TLR-9 ligand CpG, used to suppress T-cell activation and proliferation, and an anti-IL-10 receptor antibody, an adjuvant, switched tumor infiltrating macrophages from the M2 to the M1 phenotype (Balkwill and Mantovani, 2001; Guidicci et al., 2005; Klinman, 2004). This was demonstrated by the elevated levels of the M1 markers TNF- α , IL-12, and NO. This approach also resulted in decreased tumor volume as early as 16 hours post-treatment (Guiducci et al., 2005).

One method to analyze the ability of macrophages to undergo phenotype switching is to evaluate marker protein levels for each population. In our study, we used immunoblot analysis to measure the levels of the M1 marker pSTAT1 and the M2 marker CD204 in monocytes, M0, M1, and M2 macrophages. The rwt virus did not induce changes in the activation of STAT1 in any of the cell types. However, upon infection with rM51R-M virus,

STAT1 became phosphorylated in monocytes, M0, and M2 macrophages, and was maintained in M1 macrophages (Figure 9). In contrast to the rwt virus, this M protein mutant virus has been known to modulate immune cells to secrete pro-inflammatory cytokines and express surface markers associated with antigen presentation. Specifically, myeloid dendritic cells have been shown to undergo maturation in response to rM51R-M virus as characterized by the expression of the cell surface markers CD40, CD80, and CD86 and the secretion of IL-12, IL-6 and type I IFN (Ahmed et al., 2006). As M1 macrophages and activated dendritic cells share overlapping functions and surface marker expression, this research provides promising evidence that rM51R-M is stimulating monocytes, M0, and M2 macrophages to induce an antiviral response and produce cytokines that promote the M1 macrophage phenotype. On the other hand, sustained activation of this antiviral transcription factor in M1 macrophages illustrates that M1 macrophages maintain their antiviral and pro-inflammatory properties following virus infection.

The idea of phenotype switching is complicated by the upregulation of the M2 macrophage marker CD204 in rwt virus-infected M0 and M2 macrophages (Figure 9). While CD204 upregulation may be interpreted as further M2 polarization, this protein has been implicated in the phagocytosis and degradation of adenovirus, and in the detection of human cytomegalovirus in THP-1 cells *in vitro* (Haisma et al., 2009; Todt et al., 2008). This leads to the idea that CD204 upregulation may be indicative of an M1 macrophage in some cases, and not a characteristic of an M2 macrophage.

While activation of STAT1 is evidence of a phenotype switch to M1, additional factors must be analyzed to conclusively determine the polarization status of macrophages. Previous studies indicated that Newcastle disease virus (NDV)-treated monocytes

differentiated into macrophages that went on to kill multiple breast and colon cancer cell lines. In this study, NDV-differentiated macrophages underwent a type I IFN response and upregulated the expression of the pro-apoptotic protein TRAIL (Washburn et al., 2003). Treatment of colon cancer xenografts in mice with oncolytic vaccinia virus (GLV-1h68) was also shown to increase pro-inflammatory cytokine secretion resulting in the infiltration of macrophages at the tumor site. Infiltrating macrophages induced the secretion of cytokines involved with either an anti-viral or anti-inflammatory immune response, including IFN- γ , CXCL10, IL-3, IL-6, M-CSF1, MCP-1, and RANTES. This all resulted in colon cancer cell death and increased survival rates (Ehrig et al., 2013). Our observation that the rM51R-M strain of VSV induced STAT1 phosphorylation as part of the M1 polarization of M2 macrophages suggests a novel benefit to this oncolytic virotherapy, but further evidence of a phenotype switch must be acquired through analysis of secretory cytokines.

While the ability of VSV to kill M2 macrophages and/or induce a phenotype switch is highly encouraging, we also observed that VSV reduces the ability of M2 macrophages to form podosomes. Utilizing our polarization protocol, M1 macrophages produced significantly fewer podosomes than M0 or M2 macrophages (Figure 10). As podosomes are considered to be invasive structures, this supports previous data that M2 macrophages polarized by IL-4 were more invasive when placed in a 3D culture environment relative to M1 macrophages polarized by TNF- α (Cougoule et al., 2012). Upon infection with rwt and rM51R-M viruses, podosome incidence was not affected in any of the macrophage subtypes, but podosome multiplicity and distribution were reduced (Figures 11-13). Macrophages recruit cathepsins, MMPs, and serine proteases to podosomes as a means to degrade the ECM and migrate through tissues. In the TME, the proteolytic activity of macrophages has been

shown to contribute to tumor cell invasion and metastasis. Previous studies have demonstrated that deletion of macrophage cathepsin B and S resulted in reduced tumor cell invasion and inhibition of metastasis (Gocheva et al., 2010). Furthermore, in SKAP2-null mice, macrophages produced fewer podosome structures resulting in a significant reduction of coordinated invasion by macrophages and MDA-MB-231 breast cancer cells *in vitro* as well as a decrease in lung metastases *in vivo* (Tanaka et al., 2015). Therefore, it is possible that a reduction in the number of podosome structures in M2 macrophages could have implications concerning the ability of macrophages in the TME to contribute to cancer cell invasion and metastasis.

Surprisingly, the reduction in podosome formation that we observed was found in both macrophages with active virus replication (GFP-positive) as well as in macrophages with no detectable virus replication (GFP-negative). To explain this observation, we have several hypotheses. First of all, it is possible that VSV is altering membrane structures during viral entry leading to an alteration of podosome structures. Clathrin-mediated endocytosis, the process by which VSV enters into host cells, involves the recruitment of cortactin, an F-actin associated protein, and other factors involved in podosome formation (Cao et al., 2003; Sun et al., 2005). Therefore, it is possible that upon attachment of VSV at the plasma membrane, the virus is sequestering cortactin and F-actin for viral entry mechanisms at the expense of podosome formation. This would explain the observed decrease in podosomes not only in cells supporting viral replication (GFP-positive) but also in those in which the virus may have attached but not yet gone through the process of virus replication (GFP-negative).

It is also possible that the reduction in podosome formation that we observed in GFP-negative cells is an indicator of a switch from M0 and M2 macrophages to an M1

macrophage phenotype, especially in the case of infection with the rM51R-M virus. M1 macrophages consistently produced less podosomes per cell as compared to M0 and M2 macrophages. There is some evidence to support that manipulation of the cytoskeleton can influence macrophage phenotype. McWhorter and coworkers discovered that manipulation of actin-myosin contraction in macrophages altered cell shape, reduced inflammatory cytokine secretion, and promoted an M2 phenotype. Upon drug-induced inhibition of actin-myosin contraction, M2 polarization was prevented as demonstrated by decreased arginase-1 expression (McWhorter et al., 2013). Therefore, it is possible that as portions of the cytoskeleton, including F-actin and cortactin, are utilized for viral entry, actin-myosin contraction may be inhibited. These changes could alter the phenotype of cells in the form of reduced podosome formation and downregulated M2 macrophage markers. However, our results also indicate that rwt virus effectively kills specific macrophage populations, including M0 and M2 macrophages. Therefore, the decreased podosome numbers that we observed in rwt virus-infected cells may be due to the initiation of virus-induced cytopathology, where podosome machinery is utilized for viral entry and then disposed of as the cell undergoes apoptosis. Future work will attempt to dissect these possibilities and determine the mechanisms by which VSV influences the development of podosome structures in macrophages.

The data presented here indicate that oncolytic vesicular stomatitis virus has the capacity to modulate macrophages in ways that could have inhibitory effects on cancer cell invasion and metastasis. It is increasingly being appreciated that cancer therapies that recruit or stimulate components of the immune system are more efficacious. This has been demonstrated in several VSV strains. In a melanoma/bone marrow cell co-culture model,

VSV induced expression of Type III IFN IL-28 by bone marrow cells and allowed for the enhanced recognition of tumor cells by NK cells (Wongthida et al., 2010) and VSV expressing melanoma tumor associated antigens induced IFN- γ secretion by immune cells and activated naïve T cells (Diaz et al., 2007). This resulted in a more effective cancer treatment. Therefore, it is imperative that optimal treatment by viruses including VSV not only involves a direct cancer-cell killing mechanism, but also involves modulation of the immune system to build anti-tumor immunity.

Chapter III: Future Directions

Validating VSV-Dependent M1 Polarization of Macrophages

Several oncolytic viruses, such as NDV and vaccinia virus, have been known to encourage a macrophage polarization switch by modulating cytokine secretion (Ehrig et al., 2013; Washburn et al., 2003; Zorn et al., 1994). Treatment with oncolytic measles virus, mumps virus, and adenovirus were also shown to increase secretion of pro-inflammatory cytokines indicative of an M1 polarization switch (Ehrig et al., 2013; Guidicci et al., 2005; Tan et al., 2016). In this study, we analyzed a single M1 macrophage marker pSTAT1. However, pSTAT1 is not a cytokine, but a transcription factor known to be involved in processes beyond M1 polarization. Without analysis of cytokine secretion, we have limited understanding of the macrophage phenotypes being identified. However, other evidence, such as viability and podosome formation, found throughout this project provide further evidence that a switch is occurring towards the M1 phenotype.

TNF- α is a known M1 macrophage marker (Mantovani et al., 2007) and upon infection by oHSV or NDV, is induced in macrophages. This induction resulted tumor cell death of glioblastoma, kidney, fibroblast, and leukemia cancer cells (Meisen et al., 2015; Zorn et al., 1994), making TNF- α a good candidate for M1 macrophage phenotype confirmation. A proposed method for analyzing TNF- α secretion includes utilizing our macrophage polarization model whereupon monocytes and M0, M1, and M2 macrophages will be infected by rwt and rM51R-M viruses at MOIs of 1 or 10 pfu/cell. ELISAs of collected supernatants will be used to measure TNF- α levels.

Defining the Nature of Macrophage Podosomes Following VSV Infection

Macrophages make up a large percentage of solid tumors. The accumulation of cathepsins, MMPs, and serine proteases at macrophage podosomes generates substantial matrix remodeling potential across the TME that supports the local invasion and metastasis of tumor cells. For example, deletion of several cathepsins in macrophages resulted in reduced tumor cell invasion and metastasis (Gocheva et al., 2010). Reducing the number of podosome structures also resulted in a significant reduction in coordinated invasion by macrophages and MDA-MB-231 breast cancer cells *in vitro*. *In vivo*, the number of lung metastases was also significantly reduced, an effect thought to be due to reduced podosome formation and macrophage infiltration in the metastatic tumors (Tanaka et al., 2015). In this study, we showed a reduction in podosome number in M2 macrophages in response to rwt virus infection. We speculate that matrix degradation by macrophages and/or macrophage motility/invasion might also be inhibited by VSV. One caveat to our investigation is the assumption that the F-actin puncta that were counted truly represent podosome structures. Without an analysis of other podosomes markers co-localized with F-actin puncta, cortactin for example, it is possible that other actin structures are being affected by the virus. Fluorescent microscopy could be performed to assess these possibilities. Moreover, it may be the case that podosome-associated matrix degradation will be unaffected or even enhanced in response to viral infection despite observed decreases in podosome formation. Degradation assays using fluorescently labelled gelatin and Boyden chamber assays could clarify the effects of VSV on macrophage podosome-mediated degradation and motility. It will be prudent to consider more refined criteria for podosome identification as well as determination of podosome functionality in future studies of VSV-infected macrophages.

Addressing How Macrophage Podosome Marker Proteins response to VSV Infections

Previous studies have linked podosome machinery with the same machinery that facilitates VSV entry into host cells (Cao et al., 2003). F-actin, cortactin, and dynamin have been shown to assist in the scission of clathrin-coated pits from the plasma membrane (Cao et al., 2003). This is a crucial stage in clathrin-mediated endocytosis, the process by which VSV enters host cells (Cao et al., 2003). Due to this, it is possible that VSV particles are infecting host cells, resulting in the recruitment of podosome markers to sites of viral entry. Analysis of protein localization by fluorescent microscopy would help determine if these podosome markers are leaving podosomes, and if they are moving to sites of VSV entry. This would provide a mechanism by which podosome number is reduced upon viral infection in both GFP-positive and GFP-negative cells. Analysis of protein levels by immunoblot could further validate these results, demonstrating that if protein levels remain the same upon viral infection, that they could be re-localized and recycled by the cell. Should there be a reduction in podosome machinery, it could imply that these proteins are discarded by the cell after being used for VSV entry.

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