THE TYPE I INTERFERON ANTI-VIRAL PATHWAY CONTRIBUTES TO MACROPHAGE POLARIZATION FOLLOWING INFECTION WITH ONCOLYTIC VESICULAR STOMATITIS VIRUS

A Thesis
by
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Abstract

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Vesicular stomatitis virus (VSV) is a promising oncolytic agent that directly kills cancer cells, but which also modulates immune elements within the tumor microenvironment. Here we were interested in how VSV affects tumor-associated macrophages (TAMs), a cell type that interconverts along a spectrum of polarizations from pro-cancer M2 to anti-cancer M1 subtypes. We hypothesized that VSV infections would switch M2 TAMs to an M1 phenotype via activation of the type I interferon anti-viral response. Such was the case with a mutant strain of VSV (rM51R-M) where the lack of a functional M protein led to activation of the anti-viral response and the upregulation of M1 markers (*i.e.* IFNα, STAT1, P-STAT1, MHC-II, and CD80, but not IRF5) in model M2 THP-1 macrophages. Our data suggest that rM51R-M virus has a previously unappreciated immunogenic potential based on modulation of TAM phenotypes.

Acknowledgments

This project has truly been a team effort. Firstly, I would like to thank my advisors Dr. Maryam Ahmed and Dr. Darren Seals, whose patient mentorship has guided and pushed my growth as a scientist. I would also like to thank Dr. Michael Opata for his input and guidance on this project. To Dalton Sizemore, Austin Simmons, Melissa Rowe, and the rest of my lab mates in the Ahmed and Seals labs, thank you for your collaborative efforts and support throughout my time in this program. Finally, thank you to Appalachian State University, the Department of Biology, and the Office of Student Research for providing the resources and funding to make this project possible.

Dedication

This thesis is dedicated to my fiancé Sierra Jackson, to our dog Gamgee, whose arrival helped me to finish this thesis, and to my family: Jeff, Chris, Cullen, and Morgan Owen. Sierra, your steady love and joyful spirit have been my most constant supports throughout my graduate career. Mama and Papa, you instilled in me the curiosity that led to my becoming a scientist. Cullen and Morgan, you both helped to keep me grounded when I needed it most. Thank you all.

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Chapter 1: Introduction

The Problem of Cancer

Cancer may be defined as the uncontrolled proliferation and spread of abnormal cells in the body. There are many different forms of cancer, depending on the tissue of origin and the specific mutations allowing the cancerous cells to overcome the natural barriers to over-proliferation (Iranzo et al., 2018). Cancer remains one of the leading causes of death worldwide (Torre et al., 2016). In the United States of America alone, the estimated number of cancer-related deaths will exceed 600,000 cases in 2020 (NIH National Cancer Institute, 2020). Some progress has been made against this disease. The number of cancer deaths in the U.S. fell 29% from 1991 to 2017 due to the reduced prevalence of smoking habits and the increased ability to detect and treat the disease (NIH National Cancer Institute, 2020). Nevertheless, the development of new cancer therapies to better fight the disease is still in high demand.

Cancer Therapies and Advantages of Oncolytic Virotherapy

The three main forms of anti-cancer therapy have historically been surgery, radiotherapy, and chemotherapy. While all three of these therapies are still widely in use today, each has its limits and drawbacks. Surgery and radiotherapy are only effective in treating localized tumors, and chemotherapy, while it is more systemically effective, often has harsh side effects. Due to these limitations, the development of new broadly effective and safe anti-cancer therapies is in high demand (Zhang and Chen, 2018).

One of the most promising new forms of cancer therapy is immunotherapy, in which the body's natural defenses are activated and armed to eliminate cancerous lesions. Increasing the presence of and/or effectiveness of tumoricidal immune cells may be accomplished with immune promoting drugs, adoptive cell therapies, tumor antigen specific antibodies, cancer vaccines, or oncolytic virotherapy. Immunotherapeutic drugs are generally aimed at promoting the proliferation and activation of the immune system in general. Adoptive cell therapies utilize the T lymphocytes within a tumor, as they are already specific for cancer antigens. These T cells are removed, expanded *in vitro*, and injected back into the patient where they can attack and destroy cancer cells. Tumor antigen specific antibodies bind to specific structures on the surface of cancer cells and make them easier to detect by the immune system. Cancer vaccines may be administered to induce an adaptive immune response against a specific tumor associated antigen (Zhang and Chen, 2018). Oncolytic virotherapy uses a non-pathogenic or modified pathogenic virus to selectively infect and kill cancer cells as well as elicit a strongly immunogenic response (Bastin et al., 2016). Whatever the mode of action, immunotherapies should help clear the cancer from the patient and then create lasting memory to cancer antigens.

The focus of my research is oncolytic virotherapy, which produces tumoricidal effects in several ways. The first, and originally intended effect of oncolytic virotherapy lies in their ability to selectively infect and kill cancer cells while leaving healthy cells intact (Bastin et al., 2016). Oncolytic viruses are unable to efficiently infect healthy cells due to several reasons. First of all, many oncolytic viruses are attenuated such that they may harbor genetic modifications that compromise their pathogenicity. Secondly, normal cells have intact anti-viral responses and are usually protected against assaults by viruses. Lastly, especially in the

case of viruses whose natural hosts are not humans, normal cells are usually not permissive to infection and killing by these viruses because they may lack the correct viral receptors or cellular environment. However, these same viruses may infect cancerous cells due to either a mutation present in the cancer genome or an engineered modification to the viral genome (Bastin et al., 2016).

The strategies in choosing and modifying oncolytic viruses aim to limit viral replication to specific cellular environments by controlling essential viral gene expression or making the virus reliant on tumor-specific properties. Preferential entry of an oncolytic virus in cancer cells can be engineered by modifying viral glycoproteins so that they bind to specific cell surface markers that are over-expressed in certain kinds of cancer (Au et al., 2005; Ochiai et al., 2006). Alternatively, oncolytic viruses may be directly delivered to tumors via carrier cells. Migratory cell types with tropism to the tumor microenvironment (TME), such as immune cells and mesenchymal stem cells, may be loaded with oncolytic viruses and presented like Trojan Horses to the tumor (Mahasa et al., 2020). Strategies relying instead on controlling efficiency of replication after initial transduction generally take advantage of aberrant gene expression within cancer cells. A common strategy utilized by viruses for efficient replication is to control host apoptosis. Removing anti-apoptotic genes from a viral genome causes the virus to only be able to efficiently replicate within cells with an anti-apoptotic program in place, as is present in cancer cells (Kasuya et al., 2007). Essential viral genes may be placed under tumor specific promoters, thereby limiting expression of those genes and thus efficient viral replication to infected cancer cells (Dorer and Nettelbeck, 2009). Environment specific gene expression related to the TME may also be used to control viral gene expression, as shown by Post et al. (Post and Van Meir, 2003) with

a hypoxia-inducible factor activated oncolytic adenovirus. Lastly, and most relevant to this project, are strategies taking advantage of mutations in cancers to key anti-viral proteins and pathways. The classic example of this strategy utilizes the debilitating mutations to the type I interferon (IFN) anti-viral pathway present in some forms of cancer. In these cases, viruses that are either naturally or engineered to be more susceptible to the type I IFN response will only be able to efficiently infect and replicate within cancer cells (Kim and Kang, 2007).

The Type I IFN Antiviral Response

The type I IFN response is best known for its potent anti-viral capabilities, though it has effective anti-bacterial and anti-cancer abilities as well. There are several type I IFN isoforms, the two most prominent of which are IFNα and IFNβ. Most cell types in the body produce IFNβ in response to viral infection. Activated cells of hematopoietic origin, including macrophages, are the primary producers of IFNα, though they can also produce IFNβ as well. Overall, the type I IFN response is designed to halt viral spread and stimulate various cells of the immune system to clear the infection (Ivashkiv and Donlin, 2014; Platanias, 2005; Zhou et al., 2010).

The type I IFN pathway (figure 1) is initiated following recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). PRRs then stimulate activation of several members of the interferon regulatory factor (IRF) family that induce expression of type I IFNs (Ivashkiv and Donlin, 2014; Wang et al., 2014). All forms of type I IFN then signal through the type I IFN receptor (IFNAR), which is made up of the two subunits, IFNAR1 and IFNAR2 (Hwang et al., 2006). Each subunit is bound to a

member of the janus kinase (JAK) family of receptor-associated tyrosine kinases. IFNAR1 is bound to tyrosine kinase 2 (TYK2) and IFNAR2 is bound to JAK1. When IFNα or IFNβ binds one of these receptors, it causes the receptor subunits to dimerize and JAK1 and TYK2 to become autophosphorylated. This leads to activation of several different signaling pathways leading to the expression of hundreds of different IFN stimulated genes (ISGs), many of which have anti-viral, anti-proliferative, and immunomodulatory properties (Platanias, 2005; Schreiber, 2017).

The best studied signaling pathway downstream of IFNAR activation is the JAK-STAT pathway. In this pathway, activated JAKs phosphorylate and activate signal transducer and activator of transcription (STAT) proteins. STATs are constitutively expressed and exist in the cytoplasm of cells. When activated, they form complexes with other STAT proteins and translocate to the nucleus where they function as transcription factors for many different ISGs. There are seven different STAT proteins, of which STAT1, STAT2, STAT3, and STAT5 are the most commonly activated by type I IFN signaling. STAT4 and STAT6 have roles in type I IFN signaling only in endothelial and lymphoid cells. The transcription factor complexes formed by these STAT proteins are diverse, as STAT proteins are able to form homodimer or heterodimer complexes. The classic JAK-STAT response to type I IFN stimulation is the activation of the IFN-stimulated gene factor-3 (ISGF3). This is a heterotrimeric complex of activated STAT1, STAT2, and IRF9. ISGF3 is the primary transcription factor responsible for binding IFN-stimulated response elements (ISREs) present in the promoter regions of many ISGs (Fu et al., 1990; Levy et al., 1989; Platanias, 2005).

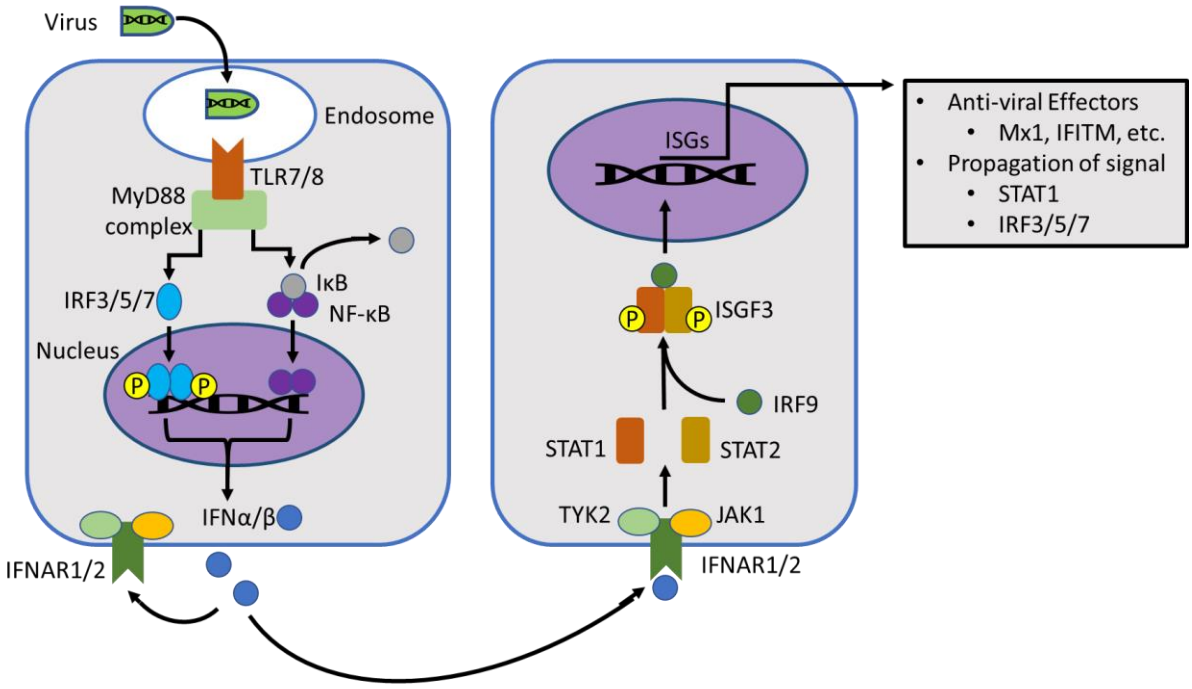

Figure 1. The type I IFN anti-viral signaling pathway. Viral RNA is recognized by TLR7 and 8, leading to activation of the IRFs 3, 5, and 7 as well as the release of IκB from NF-κB through the MyD88 complex, resulting in production of type I IFNs (IFNα and IFNβ), and other inflammatory markers. Type I IFNs signal in autocrine and paracrine fashion through the type I IFN receptors IFNAR1 and 2, which dimerize and cause JAK1 and TYK2 to autophosphorylate. Activated JAK1 and TYK2 phosphorylate STAT1 and STAT2, which form the transcription factor complex ISGF3 with IRF9. ISGF3 binds to ISRE promoters, upregulating transcription of hundreds of ISGs, including the anti-viral effector molecules Mx1 and IFITM and the transcription factors STAT1 and IRFs 3, 5, and 7, which induce further type I IFN production.

The wide array of ISGs being expressed due to type I IFN signaling act in concert to first limit viral spread and then to recruit and activate cells of the immune system to eliminate the infection. The variety of anti-viral effector ISGs inhibit key stages in the life cycle of viruses. Viral entry into cells may be inhibited by ISGs like Mx1 and IFITM (Y. P. Liu et al., 2013; Schneider et al., 2014), viral translation may be inhibited by ISGs like ISG15

(Rodriguez et al., 2014), and viral egress may be inhibited by ISGs like viperin (Vonderstein et al., 2017). Other ISGs limit viral spread by increasing pathogen sensing capability of the cell, limiting cell proliferation, and inducing apoptosis. These anti-proliferative and pro-apoptotic properties of type I IFN signaling cause the type I IFN response pathway to be commonly mutated in cancer cells. While these cancer cells have overcome a significant tumor suppressor, they have also made themselves vulnerable to viral infection as a side-effect. Oncolytic viruses engineered to induce a strong type I IFN response can take especially effective advantage of cancers with these mutations, as they specifically target cancer cells by inducing an anti-viral state in all but the cancer cells. A viral strain, rM51R-M virus, used in my research is an example of a virus that has been modified to selectively target cancer over normal cells due to its ability to stimulate a robust type I IFN response (Ahmed et al., 2004).

Oncolytic Virus Immunotherapy

The second, and arguably more important, effect of oncolytic viruses is the induction of an immune response that extends throughout the TME. The TME is a complex system of cancer cells, vasculature, immune cells, signaling molecules, and stromal tissue that is usually coerced by signals from the tumor to be immunosuppressive. This allows the cancer to avoid detection and destruction by tumoricidal components of the immune system. In contrast, infection of a tumor with an oncolytic virus releases danger signals and tumor-associated antigens that are responsible for immune stimulation (Achard et al., 2018). It is this immunogenic response that has been shown to be responsible for much of the efficacy of different oncolytic virus treatments. The importance of this effect was recently illustrated by

Selman et al. (Selman et al., 2018) in a study investigating a combination therapy of a type I IFN inducing mutant of vesicular stomatitis virus and an immune checkpoint inhibitor called vanadium. In this study, glioma tumors were injected bilaterally into the flanks of immunocompetent mice and the combination treatment was injected into just one tumor. Viral spread was found to only reach the initial tumor, but the anti-cancer immune response was able to reach and eliminate the distant tumor. Another study by Prestwich et al. (Prestwich et al., 2008) found that a single intravenous dose of oncolytic reovirus on its own was able to induce anti-tumor immunity and reduce metastatic melanoma lesions present in the lymph nodes of an immunocompetent mouse model.

The immunogenic cell death of cancer cells begins when viruses proliferate within and eventually lyse cancer cells (Donnelly et al., 2013). This results in the expression and release of damage-associated molecular patterns (DAMPs) and PAMPs. Both are 'danger signals' and serve to activate and recruit members of the innate immune system. DAMPs are components of the host's cells that are normally hidden from the immune system such as extracellular ATP, DNA, and heat shock proteins. PAMPs are common components of pathogens, such as double stranded RNA and lipopolysaccharide. These danger signals are recognized by PRRs in antigen presenting cells (APCs) like dendritic cells and macrophages, which bridge the gap between the innate and adaptive immune systems (Achard et al., 2018; Yongming Sang, 2015; Zhang and Mosser, 2008).

Virus-infected cancer cells and the debris resulting from lytic cancer cell death are phagocytosed and digested by APCs. These APCs then process and present foreign antigens from both the virus and cancer cells on major histocompatibility class II (MHC-II) molecules. After migrating to a lymph node, these APCs present the antigen-bound MHC-II

molecules and co-stimulatory molecules on their surface to naïve cells of the adaptive immune system (Guillerme et al., 2013). This stimulation leads to the activation and proliferation of cytotoxic and memory T cells specific to both viral and cancer antigens. The cytotoxic T cells are the primary cell type involved in cancer cell lysis and eventual tumor clearance (Tsung et al., 2002). Memory T cells specific to the tumor-associated antigens remain to help induce a quicker response to any future cancer containing the same antigens, such as what might occur during metastatic growth. In this way, oncolytic virotherapy behaves as a tumor specific *in situ* vaccine and presents a far wider range of tumor associated antigens than a man-made cancer vaccine could achieve (Achard et al., 2018).

VSV as an Oncolytic Agent

One of the most promising oncolytic viruses undergoing research is a non-segmented, negative-sense RNA virus in the family *Rhabdoviridae* called vesicular stomatitis virus (VSV). There are several aspects of VSV biology that make it a particularly good candidate for anti-cancer therapy. The natural hosts of VSV are horses and other livestock and it has been reported to travel between hosts by insect vectors (Bishnoi et al., 2018). Infection of a human host is generally asymptomatic as VSV is highly susceptible to human type I IFN defenses. Conversely, VSV will naturally infect and replicate within cancer cells lacking anti-viral defenses (Ahmed et al., 2004). Additionally, since pre-exiting immunity to VSV is generally limited to agricultural and laboratory workers, the majority of the human population has very low existing immunity to VSV (Hastie and Grdzelishvili, 2012). VSV is also effective in treating a wide variety of cancer types, as its method of entry into cells is non-specific. The glycoprotein (G) on the surface of the virion is thought to interact with

ubiquitous negatively charged membrane lipids, which leads to entry into the cell via clathrin-mediated endocytosis (Nikolic et al., 2018). The virus then replicates only within the cytoplasm of infected cells, averting any worry of virus-induced mutagenesis. Lastly, VSV has a relatively small genome that has been widely studied such that the genetic basis of replication and pathogenesis are well understood. Therefore, the virus can easily be manipulated and modified to specifically target cancer cells, have reduced pathogenicity, and/or include transgenes to aid in the oncolytic process (Hastie and Grdzelishvili, 2012).

Some VSV strains contain modifications in the G protein or matrix (M) protein. The G protein is responsible for initial binding and entry into cells, as well as fusion of the viral envelope with the endosomal membrane, thus releasing the virus into the cytoplasm of the cell (Nikolic et al., 2018). Mutation of the G protein results in an attenuated, though still actively oncolytic form of VSV, as shown by Janelle et al. (Janelle et al., 2011). Modifications to the M protein are aimed at increasing oncoselectivity and safety of VSV. Wild-type M proteins function by blocking host transcription and nuclear to cytosolic transport, thus inhibiting expression of new anti-viral genes, eventually leading to induction of virus-induced cytopathology. rM51R-M virus is a recombinant strain of VSV with a methionine to arginine substitution at position 51 of the M protein (Ahmed et al., 2010). This methionine is essential to the inhibitory function of the M protein, as it is responsible for binding Nup98 and other components of the nucleoporin complex as well as inhibiting the function of host RNA polymerases I, II, and III (Ahmed and Lyles, 1998). Disrupting the function of M protein at inhibiting host gene expression renders rM51R-M virus a selective and safer therapeutic option than wild-type VSV due to its inability to shut off the type I IFN anti-viral response.

The transgenes inserted into the genomes of oncolytic VSV include those engineered to have tumor suppressor and pro-apoptotic properties such as p53 (Heiber and Barber, 2011), and immunomodulatory properties like IFNβ. Other transgenic forms of VSV are used to increase specific binding and oncoselectivity by replacing the gene for the VSV G protein with another viral glycoprotein (Schreiber et al., 2019). The strain of VSV currently in clinical trials is one that includes an IFNβ transgene. VSV- IFNβ-NIS is in phase 1 clinical trials, determining safety and dosage in patients with a variety of resistant cancer types, including non-small cell lung carcinoma, endometrial cancer, and acute myeloid leukemia ("Clinical Trials Using VSV-hIFNbeta-NIS - National Cancer Institute," n.d.). The inclusion of an IFNβ transgene yields similar results to that of the rM51R-M virus. Both modifications cause the virus to induce a strong anti-viral response in infected cells, increasing oncoselectivity and activation of immune elements.

Many variants of VSV have been investigated for their potential as therapeutic agents in treating cancer. VSV-GP, in which the VSV G protein was replaced with the glycoprotein from lymphocytic choriomeningitis virus, was investigated in lung cancer mouse models and led to complete remission of the cancer, though not by induction of an immunogenic response (Schreiber et al., 2019). VSV-mp53, harboring the mouse p53 gene, was found to increase survival in mouse models with metastatic mammary adenocarcinoma while also being more attenuated to infection of healthy cells (Heiber and Barber, 2011). This effect was abrogated in athymic nude mice, illustrating the importance of a T cell compartment to the anti-cancer properties of VSV-mp53 (Heiber and Barber, 2011). Several studies have been performed investigating the efficacy of rM51R-M virus in prostate and breast cancer mouse models. rM51R-M virus was found to be successful in eliminating some forms of prostate

cancer (Ahmed et al., 2004), and treatment of breast cancers was found to be effective *in vitro* but may require a more vigorous combination therapy to be effective *in vivo* (Ahmed et al., 2010).

This growing body of evidence illustrates the versatility and efficacy of VSV as an oncolytic agent. The selectivity and cytotoxicity of VSV to a wide variety of cancer cells, combined with the ability to modify the virus for increased selectivity and immunogenicity, make VSV a "magic bullet" anti-cancer agent. However, VSV-based therapeutics do have their limitations. Not all cancers are susceptible to VSV infection, as shown by Ahmed et al. (Ahmed et al., 2004). Additionally, while VSV is relatively safe, over-dosing with the virus may cause hepatotoxicity or neurotoxicity (Zhang et al., 2016). Finally, in the case of strains modified to be immunogenic like rM51R-M virus, there may be a risk in over-stimulating the anti-viral response. Over-stimulation of the type I IFN response may induce adverse autoimmune and inflammatory symptoms and tissue toxicity (Trinchieri, 2010).

The Tumor Microenvironment

While many studies have investigated the oncolytic potential of VSV, one area that remains understudied is the capacity of VSV to manipulate the TME and the immune components within the TME. As mentioned before, the TME is a complex system, including proliferating cancer cells, stromal tissue, vasculature, and immune cells, supporting the tumor both physically and chemically. The TME is engineered by the tumor to provide a safe haven from tumoricidal components of the immune system. While inflammatory immune cells such as natural killer (NK) cells and cytotoxic T cells are recruited to the TME, the interactions

within the TME suppress their behavior (Whiteside, 2008). There are several mechanisms by which this immunosuppressive effect is induced in the TME. Alterations to cancer cell surface molecules to reduce presentation of self-antigens on MHC-I molecules or to increase presentation of negative costimulatory molecules reduces the ability of immune cells to detect and destroy cancer cells. Cancer cell secreted factors include those that are directly immunosuppressive like interleukin (IL)-10 and transforming growth factor β (TGF-β), and those that promote the expansion, activation, and recruitment of regulatory immune cells like regulatory T cells (Tregs) and tumor-associated macrophages (TAMs) (Croci et al., 2007).

Disrupting this immunosuppressive environment is crucial to the success of of anti-cancer immunotherapies. Many strategies directly target specific components of the TME involved in immunosuppression. For example, Hibino et al. (Hibino et al., 2018) found that treatment with inhibitors to the Nr4a receptor, a critical component of the cytotoxic T cell inhibiting pathway in Tregs, resulted in an increased anti-tumor immune response in model mice bearing colon adenocarcinoma tumors. Other strategies under investigation utilize the immunogenic effects of oncolytic virotherapy. A study investigating the efficacy and immunogenicity of an oncolytic type 2 herpes simplex virus determined that the virus was able to promote cancer clearance by induction of an anti-cancer inflammatory microenvironment in a mouse colon carcinoma model (Zhang et al., 2020).

The immunogenic capacity of VSV, and especially of its M protein mutant strains, potentially makes this virus a highly effective therapeutic for breaking the immunosuppressive profile of the TME. This capability is especially attractive in the potential to modulate the activity of TAMs, as they often are major contributors of immunosuppressive and tumor-promoting activity in the TME (Zhang et al., 2011).

Macrophages as Mediators of Oncolytic Activity

Macrophages, as phagocytes and APCs, trigger cellular immune responses and are thus vital component of oncolytic virotherapies. Their ability to secrete and respond to cytokines, chemokines, and growth factors means that they both influence and are influenced by the tissue in which they exist. For example, in helping to maintain tissue homeostasis, macrophages may be involved in either inducing or resolving inflammation. These diverse roles come with a range of different activation states. Macrophages are primarily derived from circulating blood monocytes and exhibit a range of phenotypes on a spectrum between two polarization extremes. These two extremes of the macrophage phenotypic spectrum are the classically activated M1 macrophages, with their pro-inflammatory, anti-bacterial, and anti-viral characteristics, and the alternatively activated M2 macrophages, with their anti-inflammatory, anti-parasitic, and wound healing characteristics. Macrophages are also phenotypically plastic and may convert between polarization states according to changing environmental signals (Sica and Mantovani, 2012; Yongming Sang, 2015).

Activation of PRRs by PAMPs leads to M1 macrophage polarization, as does pro-inflammatory signaling from other immune sources (Figure 2). This leads to the upregulation of pro-inflammatory signaling and antigen presentation by the macrophage, which recruits and activates other cells of the innate and adaptive immune system. M1 macrophages constitutively express low levels of pro-inflammatory cytokines, including the anti-viral IFNα and IFNβ (Yongming Sang, 2015). This inflammatory response is designed to quickly eliminate intracellular pathogens and foreign cells, but also includes cancer cells (Ohri et al., 2009; Stohlman et al., 1982).

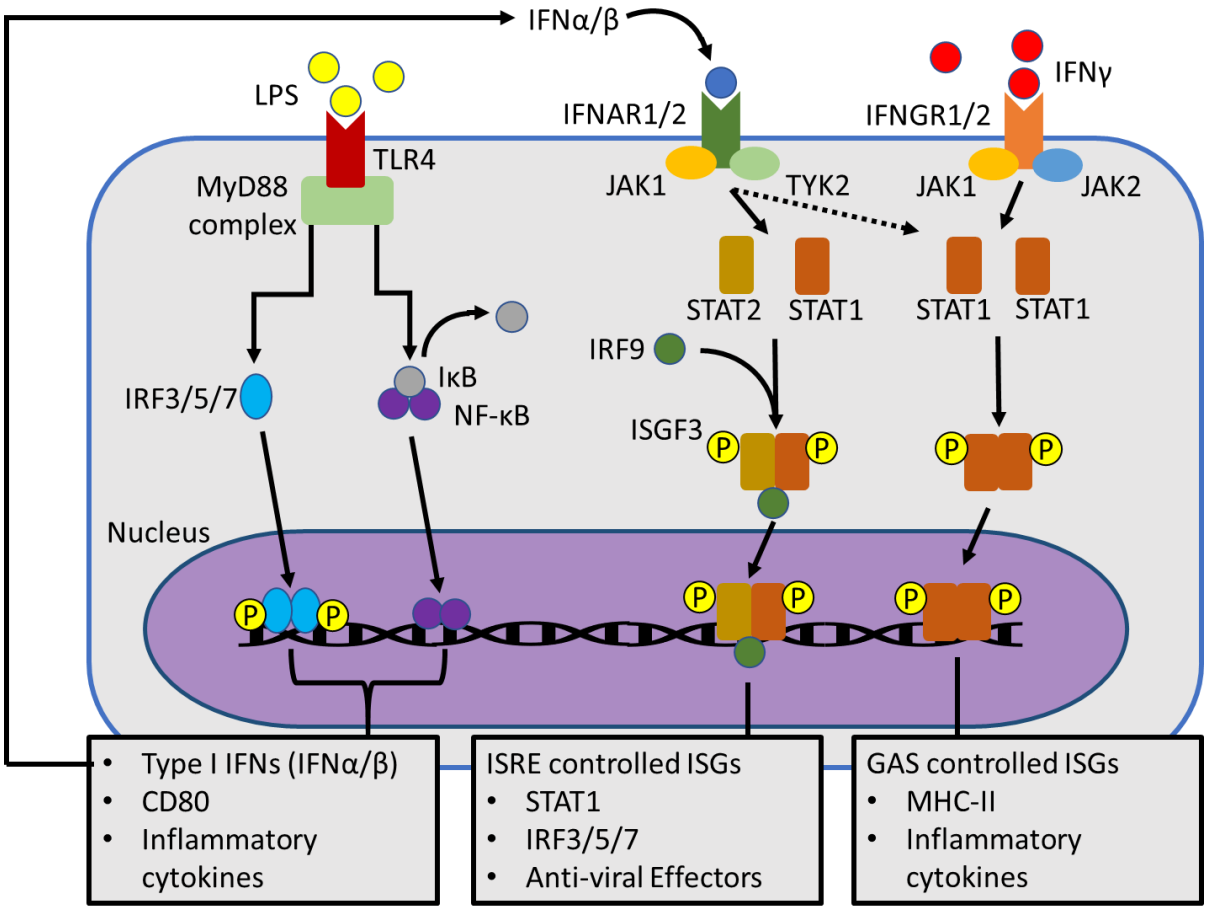

Figure 2. M1 polarization pathways in LPS and IFNγ stimulated macrophages. LPS is recognized by TLR4, leading to activation of the IRFs 3, 5, and 7 as well as the release of IκB from NF-κB through the MyD88 complex. Upregulated genes include CD80 and inflammatory cytokines including the type I IFNs which activate the type I IFN pathway described previously. IFNγ signals through the type II IFN receptor, IFNGR1 and 2, which dimerize and cause JAK1 and 2 to autophosphorylate. The STAT1 homodimer transcription factor complex is preferentially activated by IFNγ signaling, though it may be activated by type I IFN signaling as well. The STAT1 homodimer binds to GAS promoters and upregulate expression of MHC-II and other inflammatory factors.

Once a pathogen is cleared, macrophages switch to the M2 phenotype to aid in resolving inflammation. This switch is highly important, as uncontrolled inflammation can cause tissue damage and lead to a variety of different diseases, even tumorigenesis (Shi and Sun, 2018). Anti-inflammatory cytokines, like IL-10, are secreted to activate regulatory T cells and suppress the activity of inflammatory immune cells. M2 macrophages are also responsible for much of the tissue remodeling that comes with wound healing. Secretion of TGF-β causes an epithelial to mesenchymal transition (EMT) in surrounding epithelial cells, allowing the stationary epithelial cells to become migratory and fill the cleared out damaged tissue at the wound site (He et al., 2019). Unfortunately, cancers may utilize and take advantage of many aspects of M2 macrophage biology (Guo et al., 2016).

Macrophages in the TME

Macrophages are a key component of the TME, sometimes comprising up to 50% of the tumor volume (Guo et al., 2016). Macrophages in the TME are called TAMs and may have potent pro- or anti-cancer effects depending on their activation profile. TAMs with a pro-inflammatory M1 profile have been found to have anti-cancer properties, leading to increased survival time. This anti-cancer effect was illustrated in studies showing the increased survival heightened M1 TAM populations provided in patients with non-small cell lung cancer (Hou et al., 2009; Ohri et al., 2009) and ovarian cancer (Zhang et al., 2014). The anti-cancer effects of M1 polarized TAMs are related to their immunogenic ability, both by performing as an APC and via the production of pro-inflammatory cytokines (Ma et al., 2010; Yongming Sang, 2015). M1-type TAMs may also be directly tumoricidal, either by

secreting cytotoxic reactive oxygen and nitrogen species or by the induction of apoptosis via tumor necrosis factor-α (TNF-α) signaling (Ma et al., 2010).

TAMs with an anti-inflammatory M2 profile have been found to be associated with disease progression and lower survival rates in many forms of cancer due to their promotion of tumor growth, survival, angiogenesis, invasion, and metastasis. Additionally, the array of anti-inflammatory cytokines (*e.g.* IL-10) produced by M2 polarized TAMs assists in creating the immunosuppressive TME. Induction of IL-10 secretion in M2 polarized TAMs has also been found to directly contribute to promotion of the EMT program in pancreatic cancer cells (C. Y. Liu et al., 2013). Indeed, heightened levels of IL-10 have been found to be associated with a variety of cancers, including malignant melanomas and B cell and non-Hodgkin's lymphomas (Sato et al., 2011). Zhang et al. (Zhang et al., 2011) determined a negative correlation between heightened presence of M2-type TAMs and survival rates in patients with lung adenocarcinoma, which was found to be related to M2 TAM induced lymphangiogenesis, leading to lymph node metastasis. Unfortunately, the majority of TAMs have this M2 phenotype, as the signaling within tumors resemble an ever-present wound, recruiting macrophages to the tumor and promoting M2 polarization (Pathria et al., 2019).

This perfect storm of tumors promoting the pro-cancer phenotype in TAMs has led to the targeting of TAMs in anti-cancer therapies (Guo et al., 2016). These therapies utilize several different methods. Eliminating the presence of TAMs in the TME has been shown to be an effective strategy. Utilizing chemokine antagonists can block macrophage recruitment to the tumor (Argyle and Kitamura, 2018). Macrophage survival within the TME can be targeted with M2 TAM specific vaccines as shown by Luo et al. (Luo et al., 2006), who found that pro-tumor TAMs overexpress legumain. A vaccine against legumain abrogated

M2 polarized TAM presence and reduced pro-tumor growth factors and inhibited metastatic events. Alternatively, TAMs may be targeted for modulation, reprogramming them to a more M1-like phenotype with anti-cancer properties. Intra-tumoral injections of IL-21 alongside antibody therapy were found to be effective in modulating M2 polarized TAMs in a Her2/Neu+ breast cancer to an M1 phenotype (Xu et al., 2015). Oncolytic virotherapy with VSV has the potential to target TAMs in both fashions. The activation profile of M2 polarized macrophages does not include activation of the type I IFN response (Wang et al., 2014). This enables the infection and replication of VSV within M2 macrophages. Infection of M2 TAMs can lead to cell lysis, thus depleting their presence in the TME, but may also lead to their modulation to an M1-like profile. This would occur via activation of the type I IFN anti-viral response, which is intrinsically tied to M1 polarization in macrophages. The immunogenic profile of rM51R-M virus makes it especially attractive as an immunomodulatory agent in converting M2 polarized TAMs to an M1 phenotype. With the removal of the ability of the M protein to shut down the type I IFN response, rM51R-M virus becomes a potent inducer of that pathway.

This project seeks to determine the ability of rM51R-M virus to modulate M2 polarized TAMs to an anti-cancer M1 phenotype, and to determine whether the type I IFN pathway is responsible for the modulation. The pro-inflammatory pathways involved in inducing M1 polarization in macrophages share many factors with the type I IFN pathway. Both are initiated by activation of PRRs by PAMPs leading to activation of IRF and NF-κB transcription factors. Both rely heavily on IFN signaling, and many functional aspects of M1 polarized macrophages are downstream of type I IFN signaling. Indeed, previous data gathered by Vidyarthi et al. (Vidyarthi et al., 2018) indicated that stimulation of PRRs

leading to type I IFN signaling can alter the phenotype of M2 TAMs to an M1 phenotype. This data, along with the powerfully immunogenic capability of rM51R-M virus, led to the hypothesis that activation of the type I IFN pathway by rM51R-M virus infection will be sufficient to induce a phenotypic repolarization in M2-polarized TAMs towards the anti-cancer M1 phenotype. We tested this hypothesis using THP-1 monocytes, an immortalized cell line that may be activated and polarized to M1 and M2 macrophage phenotypes. Infection with rM51R-M virus, but not the recombinant wild type VSV, was able to induce activation and expression of several components of both type I IFN and M1 polarization pathways in M2 polarized THP-1 macrophages, including IFNα and the STAT1 component of the ISGF3 transcription factor. Infection with rM51R-M virus also proved to be capable of inducing some functional changes in M2 polarized macrophages including upregulation of the M1 associated costimulatory molecule cluster of differentiation (CD)80 and the antigen presenting MHC-II complex. These results support our hypothesis and provide evidence that the immunogenic rM51R-M virus is a potentially potent modulator of the TME.

Chapter 2: Materials and Methods

Cells and Viruses

THP-1 monocytic leukemia cells were propagated every 2-4 days at densities ranging from $2x10^5$ to $1x10^6$ cells/ml in RPMI media (Corning, 10-040-CV) supplemented with 10% fetal bovine serum (R&D Systems, S11150) and 0.05mM 2-mercaptoethanol (MP Biomedicals, 190242). Recombinant wild-type (rwt) virus and the M protein mutant (rM51R-M) virus were a gift from Dr. Douglas Lyles from Wake Forest University (Winston-Salem, NC) and have been previously described (Whitlow et al., 2006). Viral stocks were grown in baby hamster kidney (BHK) fibroblasts for 24 hours, spun down, and collected into cryovials, then stored at -80°C.

Macrophage Polarization and Procurement of Samples

In order to activate THP-1 monocytes into macrophages, cells were seeded into 6-well plates in THP-1 media containing 25nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, P1585) for 24 hours. Macrophages were then polarized by incubating for 48 more hours in THP-1 media containing 25nM PMA alone for M0 macrophages, or with 25nM PMA plus either 20ng/mL LPS (Sigma-Aldrich, L5148) and 20ng/mL IFNγ (Bio-Legend, 570202) for M1 macrophages or 20ng/mL IL-4 (Bio-Legend, 574002) and 20ng/mL IL-13 (Bio-Legend, 571102) for M2 macrophages. Stocks of PMA were made as a 25μM solution in DMSO. Stocks of all other polarization factors were made as 20μg/mL solutions in 0.05% BSA/phosphate buffered saline (PBS). Macrophages were then infected with rwt or rM51R-M virus at MOIs of 1 or 10 pfu/cell for 24 hours. To test the ability of IFNα to induce

the type I IFN response, M2 polarized macrophages were treated with Human IFN Alpha Hybrid (Universal Type I IFN) (PBL Assay Science, 11200-1) at 100U/mL for 24 hours. Supernatants were collected and stores at -80°C to be used for IFNα analysis by ELISA. Cell lysates were collected by scraping into RIPA buffer containing aprotonin (Fisher Scientific, ICN19455910) at 1:1000 following 2 washes with ice-cold PBS. The lysates were stored at -80°C. Cells were harvested for flow cytometry with Accutase (Innovative Cell Technologies, 7S0115A) for 1 hour and immediately prepared for flow cytometry analysis.

IFNα ELISA

Supernatants were analyzed for IFNα production by using a human IFNα (pan specific) ELISA development kit (MabTech, 3425-1H-6) per manufacturer's instructions. Data are the result of duplicate measurements from 3 independent experiments and are reported as the mean +/- standard deviation in units of pg/mL.

Western Blotting

Protein content in cell lysates was determined with Pierce® BCA Protein Assay Kit (Thermo Scientific, 23225) according to manufacturer's instructions against a series of bovine serum albumin (BSA) standards included in the kit. For western blot analysis, 20µg of lysate protein was denatured in SDS-containing sample buffer by boiling for 5 minutes before loading onto a 10% polyacrylamide gel. Following SDS-PAGE, proteins were transferred to a 0.45µm nitrocellulose membrane (Bio-Rad, 1620115) at 100V for 1 hour. After blocking in 5% milk/Tris-buffered saline containing 0.001% Tween 20 (TBS-T) for 1

hour, membranes were washed 3 times at 5 minutes each in TBS-T. Membranes were then incubated overnight at 4°C with primary antibody to STAT1 (Cell Signaling, 9172S), P-STAT1 (Y701) (Cell Signaling, 9167S), IRF5 (Cell Signaling, 20261), or β-actin (Cell Signaling, 4970S) at 1:1000 in 5% BSA/TBS-T. Membranes were washed as previously described before incubation with peroxidase-conjugated secondary antibody (GE Healthcare, NA9340V or NA931V) at 1:2000 in 5% milk/TBS-T for 1 hour at room temperature. Proteins were visualized using SuperSignal™ West Dura Extended Duration Substrate (ThermoFisher Scientific, 34075) or SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific, 34080) and Image Lab software (Bio-Rad). Data are the mean +/- standard deviation of pixel density normalized to β-actin in each lane from 3 independent experiments.

Flow Cytometry

Macrophages were washed with ice-cold 5% FBS/PBS before incubation with antibodies for CD80-PE (Beckman Coulter, MAB104) or FITC anti-human HLA-DR (BioLegend, 980402) at 1:20 in 5% FBS/PBS for 20 minutes at 4°C. Cells were washed twice more with 5% FBS/PBS before loading into the flow cytometer. Fluorescence was measured on the FL1 channel to determine HLA-DR expression and on the FL2 channel to determine CD80 expression. Flowjo software was used to analyze the data and determine percentage of cells expressing each marker. Data are the mean +/- standard deviation of the percentage of CD80 or HLA-DR positive cells from 2-5 independent experiments.

Chapter 3: Results

Activation of the type I IFN response

To appreciate whether VSV based oncolytic virotherapy activates the type I IFN response in an M2 polarized TAM population, we investigated IFNα production in M2 polarized THP-1 macrophages infected with recombinant wild type (rwt) VSV or rM51R-M virus *in vitro*. Cells were infected at either a multiplicity of infection (MOI) of 1 or 10 plaque-forming units/cell (pfu/cell) for 24 hours. An MOI of 1 is designed to mimic the natural infection process whereby one in ten cells are infected and the systemic antiviral response can be monitored as the virus spreads to neighboring cells. An MOI of 10 represents a synchronous infection in which approximately 90-100% of cells are infected. Therefore, any antiviral response detected represents the response of individual cells to virus infection. Levels of IFNα released into the supernatants of infected cells were monitored by an enzyme-linked immunosorbent assay (ELISA). Mock-infected M2 macrophages express low levels of IFNα (Figure 3). Infection with rwt virus at either an MOI of 1 or 10 yielded similar results to the mock infection, which is consistent with the ability of a functioning M protein to prevent activation of the type I IFN response. As hypothesized, infection with rM51R-M virus induced heightened production of IFNα in M2 macrophages. At an MOI of 1, IFNα levels rose to ~20 pg/ml, and this increased to ~70pg/ml at an MOI of 10. While infection with neither virus strain yielded a statistically significant increase of IFNα relative to a mock infection, there was a consistent trend towards increased levels of type I IFN in response to infection with the mutant virus. In addition to virus infection, we tested the ability of type I

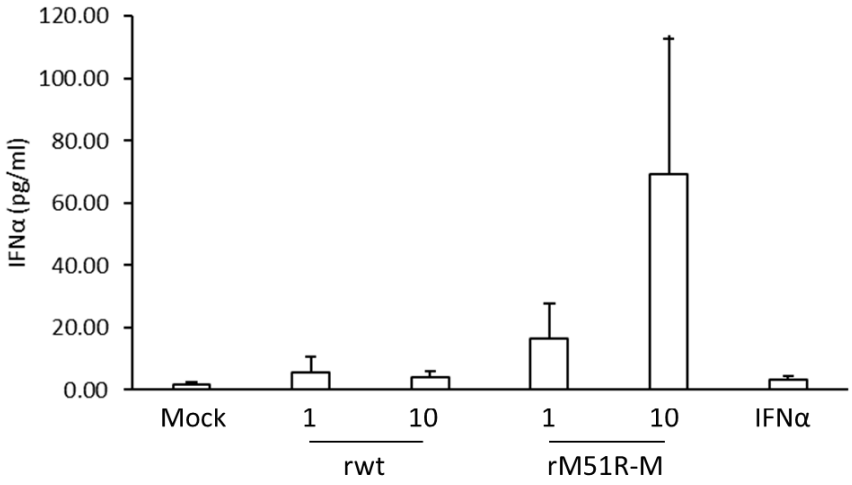

Figure 3. IFNα production in infected M2 macrophages. In these and subsequent experiments, THP-1 monocytes were polarized to an M2 phenotype with a 72-hour treatment with 25nM PMA and 20ng/mL IL-4 and IL-13. Shown are the IFNα concentrations (pg/mL) in cell supernatants following a 24-hour infection with rwt and rM51R-M viruses (MOIs of 1 or 10 pfu/cell) or treatment with IFNα (100U/mL). Data are the mean ± standard deviation of three (n = 2 for IFNα treatment) independent experiments. Statistical analysis between mock and viral infections was conducted using paired one-tailed Student's t-test and between mock and IFNα treatment using an unpaired one-tailed Student's t-test.

IFN (100 units/mL IFNα) to induce further production of IFNα in M2 THP-1 macrophages, but interestingly, levels did not increase above those observed in mock-infected cells. These data provide evidence that some additional stimulus (such as stimulation of PRRs by rM51R-M virus) is required to activate the type I IFN response in these cells.

Activation of antiviral transcription factors

In order to more fully determine the level of activation of the type I IFN response in VSV infected M2 macrophages, we investigated the expression of two key transcription factors in the antiviral response. STAT1 is a component of the ISGF3 complex, which is activated by IFNα and IFNβ binding to the IFNAR1/2 receptor and leads to expression of hundreds of ISGs, including the type I IFNs themselves. IRF5 is a transcription factor activated downstream of pathogen sensing toll-like receptor (TLR) signaling and induces expression of type I IFNs and other proinflammatory cytokines. Both transcription factors have been found to be directly tied to M1 polarization in macrophages (Krausgruber et al., 2011; Tugal et al., 2013). We used western blot analysis to investigate STAT1 and IRF5 expression as well as STAT1 activation in response to infection of M2-polarized THP-1 macrophages with rwt and rM51R-M viruses. Representative images are shown in Figure 4A, while densitometry was used to quantitate the differences in expression relative to β-actin (Figures 4B-4D). As before, cells were infected at either an MOI of 1 or 10 pfu/cell for 24 hours, but this time cell lysates were collected and subjected to western blot analysis with antibodies specific to each of the factors. IRF5 expression was not altered significantly following infection with either virus. However, cells infected with rwt virus at increasing MOIs trended towards lower levels of IRF5. STAT1 expression, in contrast, was significantly increased by rM51R-M virus at both MOIs. STAT1 activation also increased in response to rM51R-M virus, with a near significant increase at an MOI of 1 and a significant increase at an MOI of 10. Infection with rwt virus did not significantly alter STAT1 expression or activation.

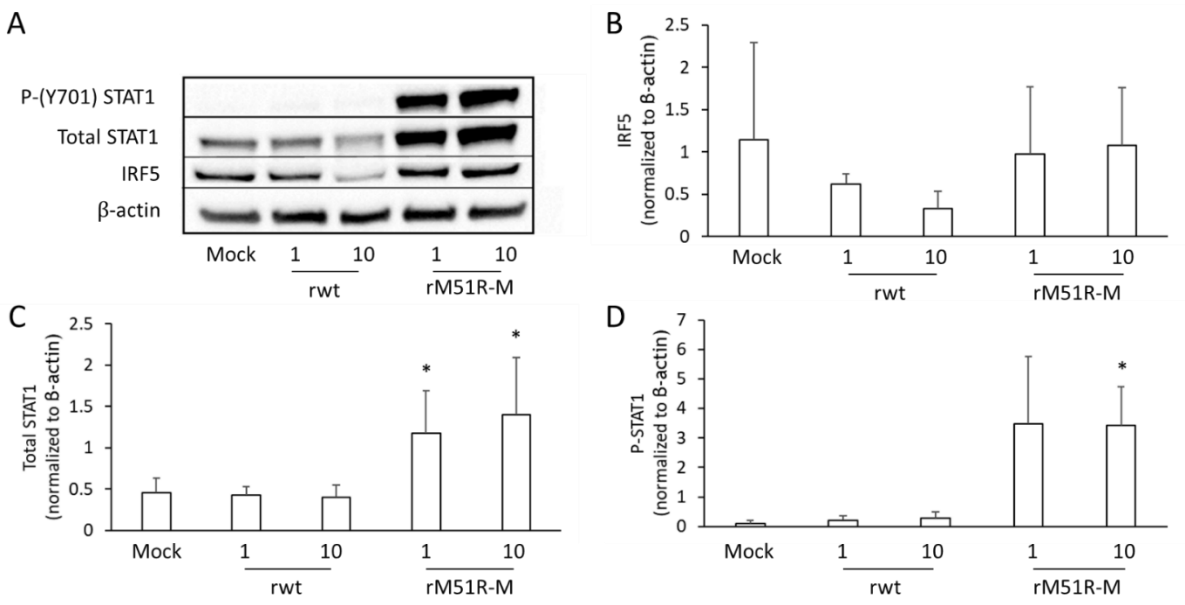

Figure 4. Transcription factor expression and activation in infected M2 macrophages. (A) Representative western blot images depicting changes in P-STAT1, total STAT1, IRF5, and β-actin expression in M2 polarized THP-1 macrophages following a 24-hour infection with rwt and rM51R-M viruses (MOIs of 1 or 10 pfu/cell). IRF5 (B), Total STAT1 (C), and P-STAT1 (D) levels were normalized to β-actin levels in each lane and are the mean ± standard deviation of 3 independent experiments. Statistical analysis was conducted using paired one-tailed Student's t-tests.

Expression of M1 macrophage surface markers

The results so far indicate that rM51R-M virus has the ability to induce the type I IFN response in infected M2 macrophages to potentially induce a switch in phenotype. However, to further determine if rM51R-M virus-infected M2 macrophages are behaving like M1 pro-inflammatory macrophages, it is important to determine their functional capabilities. To that end, we investigated whether rM51R-M virus can promote the expression of M1-related cell surface markers that contribute to M1 macrophage functions. MHC-II is found on the surface

of professional APCs and is responsible for presenting foreign antigens to the adaptive immune system to initiate an antigen specific immune response. CD80 is a co-stimulatory surface molecule responsible for promoting full adaptive immune cellular expansion and cytokine production. Both of these surface markers are important aspects of M1 macrophage functionality.

We first used flow cytometry to determine the expression of HLA-DR, an isotype of the MHC-II complex, on M1, M2, and M0 THP-1 macrophages (Figure 5). Cells were infected with rwt or rM51RM viruses at an MOI of 1 or 10 for 24 hours, and then collected and stained with antibodies to MHC-II prior to flow cytometry analysis. Uninfected M2 polarized macrophages expressed MHC-II on approximately 37% of cells. This was in contrast to M1 macrophages, which exhibited higher MHC-II expression on about 70% of cells following mock infection. Infection with rwt virus at both MOIs significantly reduced MHC-II expression on M2 macrophages while infection with rM51R-M virus at both MOIs significantly increased MHC-II expression on M2 macrophages.

Next, we used flow cytometry to address CD80 expression in M1 and M2 polarized macrophages infected with rwt and rM51R-M viruses at an MOI of 10 (Figure 6). Mock-infected M1 polarized macrophages displayed CD80 expression on about 30% of cells. In contrast, less than 1% of M2 polarized macrophages expressed CD80 under these conditions. While neither virus significantly altered CD80 expression on M1 macrophages, changes were apparent in M2 macrophages. When infected with rM51R-M virus, CD80 expression on M2 polarized macrophages increased and was significantly higher than levels observed under mock-infection conditions. Furthermore, levels of CD80 in rM51R-M virus infected M2

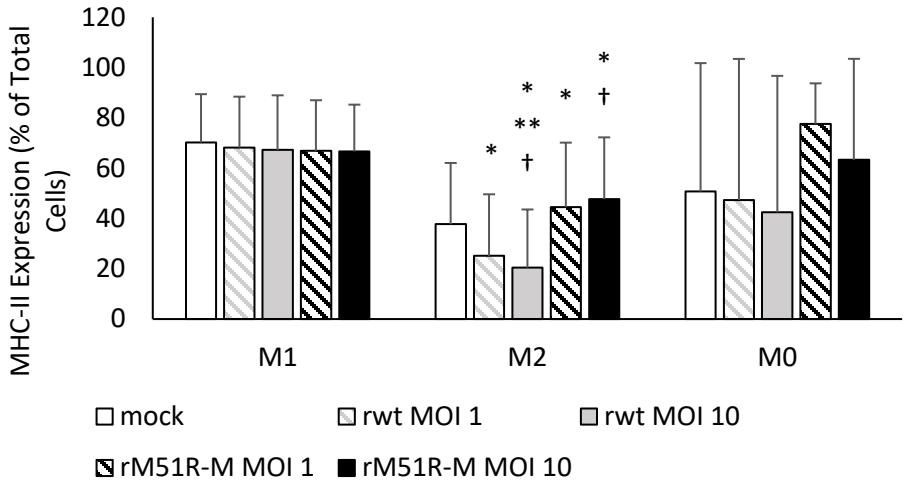

Figure 5. MHC-II expression in infected M0, M1, and M2 macrophages. THP-1 monocytes were polarized with a 72-hour treatment with 25nM PMA with or without (M0) 20ng/mL IL-4 and IL-13 (M2) or 20ng/mL LPS and IFNγ (M1). The percentage of cells expressing MHC-II on the surface of M0, M1, and M2 polarized macrophages following 24-hour infection with rwt and rM51R-M viruses (MOIs of 1 or 10 pfu/cell) was determined by flow cytometry. Data are mean ± standard deviation of 2 (M0 and M1) or 4 (M2) independent experiments. Statistical analysis was conducted within cell types with paired one-tailed Student's t-tests and between cell types with unpaired one-tailed Student's t-tests. * indicates statistical differences between mock and viral infections. ** indicates statistical differences between MOI of 1 and 10. † indicates statistical differences between rwt and rM51R-M virus infections.

macrophages were not significantly different from those in M1 macrophages. Infection with rwt virus did not significantly alter CD80 expression on M2 macrophages.

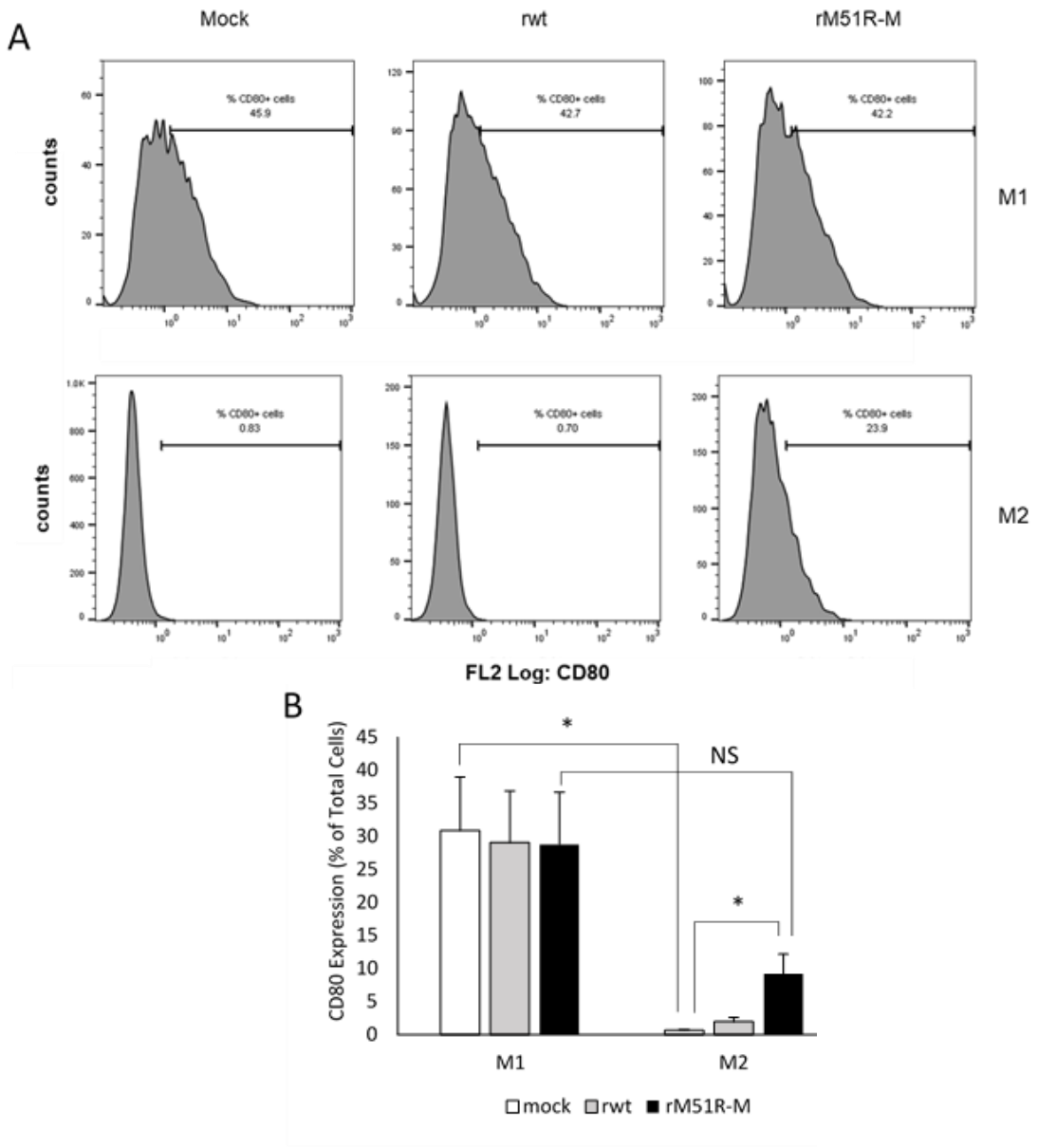

Figure 6. CD80 expression in infected M1 and M2 macrophages. THP-1 monocytes were polarized with a 72-hour treatment with 25nM PMA and 20ng/mL IL-4 and IL-13 (M2) or 20ng/mL LPS and IFNγ (M1). Representative histograms (A) and percentage of cells (B) expressing CD80 on the surface of M1 and M2 polarized macrophages following 24-hour infection with rwt and rM51R-M viruses at an MOI of 10, as determined by flow cytometry. Data are mean ± standard deviation of 4-5 independent experiments. Statistical analysis within cell types was conducted with paired one-tailed Student's t-tests and between cell types with unpaired one-tailed Student's t-tests. * indicates statistical difference

Chapter 4: Discussion

VSV is a promising oncolytic virus by many measures. Being vulnerable to human type I IFN anti-viral responses, it is relatively safe for humans (Stojdl et al., 2003). Because of the common presence of debilitating mutations in the type I IFN response, many cancers are vulnerable to VSV infection and cytopathogenicity (Stojdl et al., 2003; Wollmann et al., 2010). Some studies have also shown, by a variety of mechanisms, that VSV induces apoptosis in infected cancer cells (Ahmed et al., 2010, 2004; Kopecky et al., 2001; Stojdl et al., 2003; Wollmann et al., 2010). A consequence of virus-mediated cell death is the release of DAMPs, VSV-related PAMPs, and tumor antigens (Achard et al., 2018; Guo et al., 2014). The combination of these signals induces a more powerful, cancer-clearing immune response against both the virus and the cancer. The M protein mutant strain of VSV, rM51R-M virus, displays greater immunogenicity than the wild-type strain of VSV, as the former is a potent inducer of the type I IFN response (Ahmed et al., 2006, 2003). This immunogenic capability makes rM51R-M virus a promising therapeutic agent for targeting immunologically ‘cold’ tumors, which exist in a highly immunosuppressed TME. This study focused on the ability of rM51R-M virus to modulate the phenotype of TAMs, as they are some of the primary drivers of tumor immunosuppression and disease progression.

TAMs can be potent positive or negative effectors within the TME. This phenotypically plastic population of cells can be activated to a variety of polarization states between the two extremes of M1 and M2 (Chanmee et al., 2014). TAMs with the M2 phenotype are pro-cancer by promoting cancer survival and growth, as well as by contributing to an immunosuppressive TME (C. Y. Liu et al., 2013; Zhang et al., 2011). In

contrast, M1 macrophages secrete pro-inflammatory cytokines and chemokines, present antigens, and thus constitute the first line of defense against pathogens (Tarique et al., 2015; Yongming Sang, 2015). Promoting the inflammatory and anti-cancer M1-like phenotype in this population can be an effective strategy in immunotherapies targeting cancer (Zhang et al., 2014).

Our results indicate that rM51R-M virus has the ability to activate pro-inflammatory pathways in M2 polarized TAMs, and partially modulate their phenotype to that of M1 macrophages. IFNα and STAT1, key components of the type I IFN pathway (Hervas-Stubbs et al., 2011), were highly expressed following infection with rM51R-M virus. Furthermore rM51R-M virus induced STAT1 activity in M2 macrophages, which is indicative of an active antiviral response as well as coercion to an M1 phenotype (Müller et al., 2018). IFNα is one of the main forms of type I IFN (Platanias, 2005), and is produced as part of the amplification phase of the type I IFN response (Marié et al., 1998). Activation of anti-viral signaling by IFNα then relies on STAT1, a component of ISGF3, which is the main driver of type I IFN stimulated gene expression (Au-Yeung et al., 2013). Both IFNα and STAT1 are upregulated downstream of type I IFN signaling, thus forming a positive feedback loop that amplifies the antiviral response (Cheon et al., 2011; Hu et al., 2008; Lehtonen et al., 1997). While the increase in IFNα production was not statistically different from the mock treatment, the increasing trend in response to rM51R-M virus infection combined with the increased expression and activation of STAT1 indicate that the type I IFN response was activated in these experiments.

A further indication of the type I IFN response following infection with rM51R-M virus was the upregulation of the surface markers CD80 and MHC-II, both of which relate to M1 macrophage functionality (Vidyarthi et al., 2018). MHC-II is an antigen-presenting complex and CD80 is a costimulatory molecule. These molecules work in concert on the surface of professional APCs like macrophages to activate the adaptive immune response to internalized foreign antigens (Gujar and Lee, 2014; Subauste et al., 1998). The antigen-presenting MHC-II molecules are recognized by T cells, thus producing an antigen specific adaptive immune response. Moreover, co-stimulation with CD80 is required for effective expansion and cytokine production of the adaptive immune system (Gujar and Lee, 2014; Subauste et al., 1998). The ability of rM51R-M virus to induce expression of CD80 and MHC-II on M2 macrophages indicates its ability to promote an adaptive immune response against both viral and tumor antigens (Guo et al., 2014). Such immunogenicity is a crux of oncolytic virotherapies. Indeed, increasing adaptive immune system activating factors in TAMs is not a novel concept in the utilization of oncolytic virotherapy. Other oncolytic viruses, including oncolytic measles, mumps, reovirus, and murine cytomegalovirus, studied for their abilities to induce M1-like functions in TAMs, have been found to upregulate CD80 and/or MHC-II in M2 polarized model macrophages or in TAMs collected directly from a tumor (Gujar et al., 2011; Tan et al., 2016; Wilski et al., 2019).

An active type I IFN response is therefore both a marker for and driver of M1 polarization in macrophages. Indeed, many of the factors involved in the type I IFN response are shared with LPS- and IFNγ-induced M1 polarizing pathways (compare Figures 1 and 2) (Hervas-Stubbs et al., 2011; Xie et al., 2016). Both pathways involve stimulation of PRRs. LPS stimulates TLR4, and viruses may stimulate a variety of PRRs depending on their

method of entry (Achard et al., 2017; Schneider et al., 2014). Downstream of PRR signaling in both pathways is activation of the interferon regulatory factors IRF3, IRF5, and IRF7, as well as NF-κB, which collectively lead to production of pro-inflammatory cytokines, including the type I IFNs, as well as the expression of CD80 (Liu et al., 2010; Platanias, 2005). In our M1 polarizing protocol, IFNγ binds to the type II IFN receptor IFNGR1/2. This leads to activation of a STAT1 homodimer, and thus expression of M1 macrophage-related pro-inflammatory factors controlled by gamma activated sequences (GAS), including MHC-II (Schroder et al., 2004; Ting and Trowsdale, 2002). The type I IFNs produced in both responses signal through the type I IFN receptor IFNAR1/2, activating the ISGF3 transcription factor complex. The transcriptional targets of ISGF3 include IRF7, which in turn upregulates production of the type I IFNs themselves (Au-Yeung et al., 2013; Hu et al., 2008), the STAT1 transcription factor (Cheon et al., 2011; Lehtonen et al., 1997), and IRF5, as well as a host of other anti-viral and pro-inflammatory factors (Au-Yeung et al., 2013; Xie et al., 2016). While rM51R-M virus does not induce production of IFNγ, IFNAR1/2 stimulation may make up for activation of this important pathway in macrophage polarization by also activating the STAT1 homodimer, leading to expression of MHC-II and the other GAS controlled M1 genes (Au-Yeung et al., 2013; Tugal et al., 2013).

Activating these pro-inflammatory properties of the antiviral response in M2-type TAMs through infection with rM51R-M virus could break their anti-inflammatory and pro-tumor properties and replace them with the pro-inflammatory and anti-cancer properties of M1 macrophages. Evidence gathered in several studies lend support to this hypothesis. Müller et al. (Müller et al., 2018) and Xie et al. (Xie et al., 2016) both found that a combination of type I IFN response activation and either TLR stimulation or IRF1 activity in

mouse bone marrow-derived macrophages (BMDMs) and U937 model mouse macrophages respectively activated them to an M1-like state. Furthermore, Vidyarthi et al. (Vidyarthi et al., 2018) found that activation of the type I IFN response via TLR3 stimulation was capable of re-polarizing M2-type BMDMs to an M1-like state. The combination of an activated type I IFN response and upregulated M1-related surface markers suggests that rM51R-M virus induces a phenotype switch in infected M2 macrophages.

In contrast to the other marker proteins investigated, the expression of IRF5, an important transcription factor in M1 polarization (Krausgruber et al., 2011), was not upregulated in these experiments. This lack of rM51R-M virus-induced change in IRF5 expression calls into question the ability of the virus to induce a complete phenotypic switch in M2 THP-1 macrophages. One of the primary determinants of macrophage polarization is the expression and activity of the transcription factor IRF5. Expression of one of the isoforms of IRF5 is controlled by an ISRE promoter, which may be bound by the ISGF3 transcription complex (Xie et al., 2016). IRF5 is a transcription factor activated downstream of PRR stimulation that functions to upregulate several pro-inflammatory cytokines, including type I IFNs. It also acts as an antagonist of the IRF4 transcription factor by competing for activation by the MyD88 complex. Through these mechanisms, IRF5 promotes M1 macrophages associated signaling and suppresses M2 macrophage associated signaling (Krausgruber et al., 2011; Negishi et al., 2005). Heightened expression of IRF5 is one of the most important determinants of the M1 phenotype (Krausgruber et al., 2011). It is possible that, rather than through changes in expression, it is the activation state (*i.e.* phosphorylation) of IRF5 that controls its activity. This was not investigated. Additionally, the balance between IRF5 and IRF4 expression might be a better indicator of macrophage phenotype (Negishi et al., 2005;

Van Den Bossche et al., 2018). Determining the expression of both IRF4 and IRF5 in response to rwt and rM51R-M virus infections would help to clarify our results.

The immunogenic effects seen in M2 macrophages in response to VSV infection can be attributed to the mutation engineered into the rM51R-M virus genome. Viral infection, and the release of viral genetic material into the endosome and cytoplasm of the cell, stimulates PRRs, leading to activation of the anti-viral response (Au-Yeung and Horvath, 2018). Viruses like VSV have developed means to control this response (Alto and Orth, 2012; Lyles and McKenzie, 1997). The wild-type viral M protein is central to the ability of VSV to control host gene expression and its strategy in ensuring survival long enough to proliferate in typical infections (Kopecky et al., 2001). One function of the M protein is to disable the anti-viral response by blocking host transcription and nuclear to cytosolic transport, thus allowing the virus to replicate unchecked (Lyles and McKenzie, 1997). rM51R-M virus, in contrast, has a point mutation at position 51 of the M protein amino acid sequence that disables this function (Kopecky et al., 2001). The resulting inability to block the anti-viral response makes rM51R-M virus a safer and more immunogenic option for oncolytic virotherapy than its wild-type counterpart (Chirayu M, 2015; Polzin et al., 2020). In cases where the targeted cancer has deficiencies in the anti-viral response, rM51R-M virus preferentially infects and kills cancer cells over healthy somatic cells (Ahmed et al., 2010, 2004). In cells that do have an intact type I IFN pathway, rM51R-M virus can induce some immunogenic change. This effect was seen in the type I IFN receptor dependent maturation of dendritic cells in response to infection with rM51R-M virus (Ahmed et al., 2006). Each of these effects are centered on the ability of rM51R-M virus to induce the type I IFN response.

The focus of these studies was based on the ability of rM51R-M virus to induce the type I IFN anti-viral response in order to re-polarize macrophages from an M2 to M1 phenotype. The wild-type (rwt) strain of VSV differs from this ability by preventing the activation of the type I IFN response in infected cells. Neither does it induce any of the other re-polarizing effects that we saw in response to rM51R-M virus. Infection with rwt virus did not alter expression of IFNα, STAT1, or CD80, nor did it alter activation of STAT1 in M2 macrophages. Of note, these factors are already downregulated in M2 macrophages, therefore virus infection does not seem to reduce expression significantly further. Expression of both MHC-II and IRF5 in M2 macrophages was decreased in response to rwt virus infection, with MHC-II expression being decreased significantly. This suppression of pro-inflammatory and anti-viral signaling can be attributed to the functional M protein expressed by rwt virus, either for its ability to shut off host gene expression or for the increased cytotoxicity to infected M2 macrophages shown by Polzin et al. (Polzin et al., 2020). rwt virus also failed to induce significant changes in the factors we investigated in M1 polarized THP-1 macrophages. Rather than an ability exhibited by rwt virus, this effect is most likely due to the anti-virally primed nature of M1 macrophages (Yongming Sang, 2015).

The lack of any effect by IFNα on type I IFN signaling in this study suggests that macrophage repolarization requires more than just stimulation of the type I IFN receptor. The mechanism by which rM51R-M virus modulates M2 macrophages must then be at least partially reliant on the process of viral entry and stimulation of PRRs. The induction of pro-inflammatory and anti-viral pathways in response to intracellular PRR stimulation is more powerful and diverse than the signal transduction pathway following IFNAR1/2 stimulation, especially in an anti-inflammatory cell type (Müller et al., 2018). Many oncolytic viruses

modulate immunity in the TME in similar ways. The immunogenicity of oncolytic reovirus stems from activation of these host anti-viral defenses in both immune and cancer cells (Gujar et al., 2011). Reovirus preferentially infects cancer cells by taking advantage of upregulated Ras signaling and overrides tumor-associated immune evasion mechanisms. This is achieved by stimulating pro-inflammatory cytokine signaling in lymphoid cells and by re-inducing self-antigen presentation on infected cancer cells by upregulation of MHC-I expression (Gong et al., 2016; Gujar et al., 2011). Others have taken advantage of virus-directed gene expression to modulate immunity in the TME by engineering a strain of oncolytic Newcastle Disease Virus (NDV) that expresses the inducible co-stimulator ligand (ICOSL). NDV-ICOSL, in turn, activates adaptive immunity within infected tumors by activating co-stimulatory pathways in infected cells (Zamarin et al., 2017).

Oncolytic virus induced modulation of phenotypically plastic immune cells like macrophages does not always result in a complete polarization switch. There are many more factors that govern macrophage polarization. There is also a spectrum of phenotypes between the two extremes of M1 and M2. The classification of these intermediate macrophage phenotypes, as well as the M1/M2 paradigm itself, is currently a topic of debate. However, the currently agreed upon macrophage phenotypes are the classically activated M1 phenotype, M0, an activated but unpolarized phenotype, and 4 subtypes of M2 macrophage (M2a, M2b, M2c, and M2d) (Martinez and Gordon, 2014). The different M2 subtypes are divided based on the stimuli driving their differentiation. Our experimental model falls into the M2a category, driven by stimulation with IL-4 and IL-13. M2a is known as an alternatively activated macrophage and is the furthest phenotypically from the M1 state. The M2b phenotype is driven by TLR and IL1 receptor stimulation and immune complexes (e.*g.*

secreted antibodies bound with antigens) and is considered to be the most intermediate phenotype between M1 and M2a. The M2c, or 'deactivated' phenotype is driven by IL-10 and can originate from IL-10 deactivated M1 macrophages. The M2d phenotype is the typical phenotype of pro-cancer TAMs and is stimulated by tumor-derived factors (Chanmee et al., 2014).

Our data indicate that rM51R-M virus induces an incomplete polarization switch in THP-1 macrophages from M2a to an intermediate phenotype. rM51R-M virus did induce activation of the antiviral response in M2 macrophages, which was tied to the M1 phenotype, and it also induced adaptive immune response stimulating factors like MHC-II and CD80. But rM51R-M virus did not upregulate IRF5, which is one of the primary indicators of M1 polarization. The best we can claim at this point is that rM51R-M virus induces an "M1-like" phenotype in infected M2 macrophages, though the intermediate M2b phenotype is likely the closest classification. In order to more accurately determine the phenotype of rM51R-M virus infected macrophages, we plan to follow the example of other studies examining oncolytic virus-induced re-polarization of TAMs. Tan et al. (Tan et al., 2016) examined an array of macrophage markers including CD80, IL-1β, TNF-α, CXCL9, CXCL10, and IL-6 to support their findings that oncolytic measles and mumps viruses re-polarize M2 macrophages to an M1-like state. In order to support the idea that rM51R-M virus does the same, the next steps for this project must include examination of a similarly wide array of M1 and M2 markers. We plan to do so by investigating M1 and M2 polarization markers at the mRNA level by real time PCR. Our experimental plan is to investigate mRNA expression in M1 and M2 polarized THP-1 macrophages infected with rwt virus or rM51R-M virus at MOIs of 1 or 10. We will investigate M1 macrophage-related mRNAs that are representative of many M1-

related functions. Some of the factors we will investigate include expression of the type I IFNs, IFNα and IFNβ, and IL-12, all of which are pro-inflammatory cytokines. We will also determine expression of IRF5 and IRF9 as M1 phenotype-promoting transcription factors (Günthner and Anders, 2013), as well as TLR7 and retinoic acid inducible gene I (RIGI), which are PRRs involved in pathogen sensing ability of M1 macrophages (Malmgaard et al., 2004; Sabbah and Bose, 2009). The final M1 marker we will look at is expression of myxovirus resistance 1 (Mx1), which is an anti-viral effector molecule involved in inhibition of viral entry (Schneider et al., 2014). The M2 macrophage related mRNAs we are interested in are IRF4 as an M2 phenotype promoting transcription factor (Wang et al., 2014), transforming growth factor-β (TGF-β) as an immunosuppressive M2 signaling molecule (Zhang et al., 2011), and the scavenger receptor CD204, which antagonizes inflammatory M1 signaling by scavenging TLR4 ligands (Yi et al., 2009). So far, we have confirmed accuracy and efficacy of the RNA primers to be used in this experiment in samples derived from an M0 THP-1 model (Dalton Sizemore unpublished data) (Figure 7). Samples amplified from primers were run on an agarose gel, were detectable in the gel, and were found at the expected molecular weights.

Our results to this point indicate that rM51R-M virus induces activation of the type I IFN response and modulates the phenotype of M2 macrophages. Logically, the former (type I IFN) would be responsible for the latter (M1 polarization). We wish to validate this conclusion by examining the ability of rM51R-M virus to modulate the phenotype of M2 polarized THP-1 macrophages in the presence of an inhibitor to STAT1, as a key component of the type I IFN pathway. We will use fludarabine, an inhibitor of STAT1 phosphorylation (Ye et al., 2016), to determine the ability of rM51R-M virus to modulate M2 macrophages in

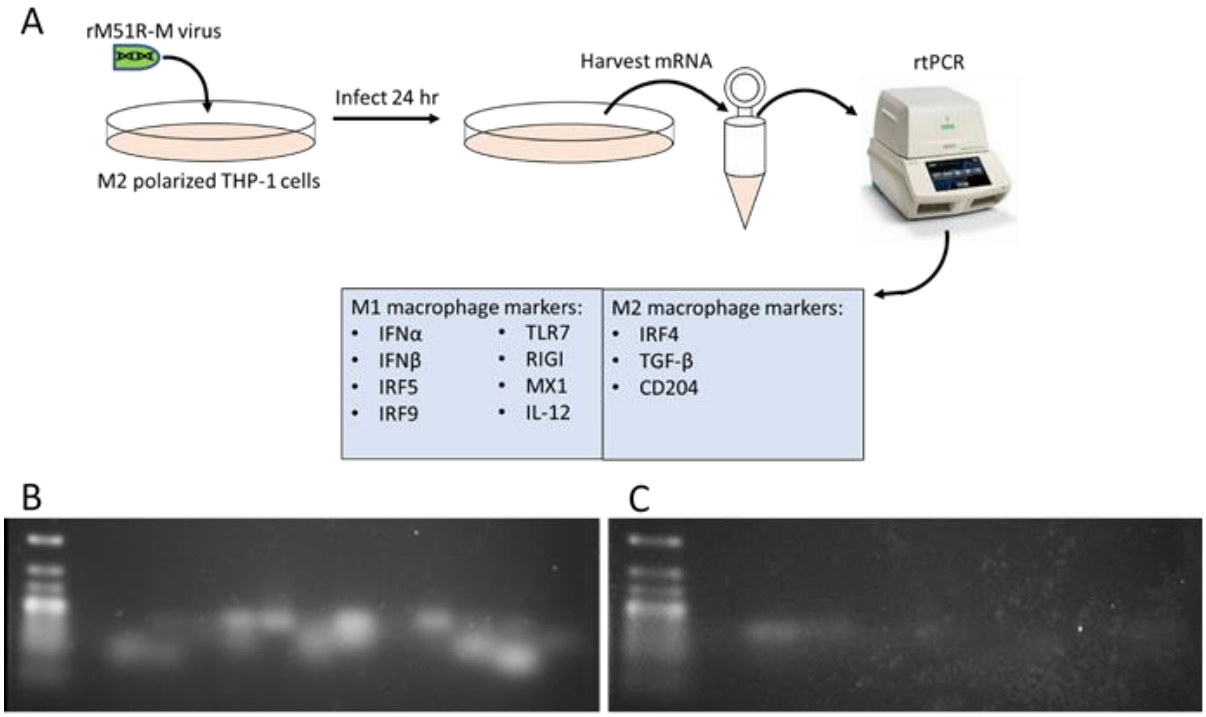

Figure 7. Qualitative analysis of macrophage polarization transcript expression in response to rM51R-M infection. (A) Experimental plan for determining mRNA expression of M1 and M2 markers in rM51R-M virus infected M2 macrophages by real time PCE (rtPCR). (B,C) THP-1 monocytes were differentiated into M0 macrophages by treatment with 25nM PMA for 72 hours. mRNA was harvested with TRIzol according to manufacturer's instructions and quantified on a NanoDrop™. After conversion to cDNA, rtPCR was completed with gene-specific primers using a SYBR Green assay kit. Samples were run on a 2% agarose gel at 150V for 1 hour. DNAs were stained with ethidium bromide. (B) From left to right: Molecular Weight Ladder, LSP1, MMP-14, HCK, vinculin, Arp2/3, N-WASp, VSV N Protein, Dynamin 2, cortactin, cofilin, STAT1, STAT6, ARG-1, and Tks5. (C) Molecular Weight Ladder, IRF5, IRF4, IRF9, RIG-1, MX1, IL-12, TGF-β, IFNα, and IFNβ.

the absence of the type I IFN response. We used western blot analysis to determine STAT1 expression and activation in fludarabine pre-treated M2 macrophages infected with rM51R-M virus at MOIs of 1 and 10pfu/cell (Figure 8). As seen before, rM51R-M virus infection

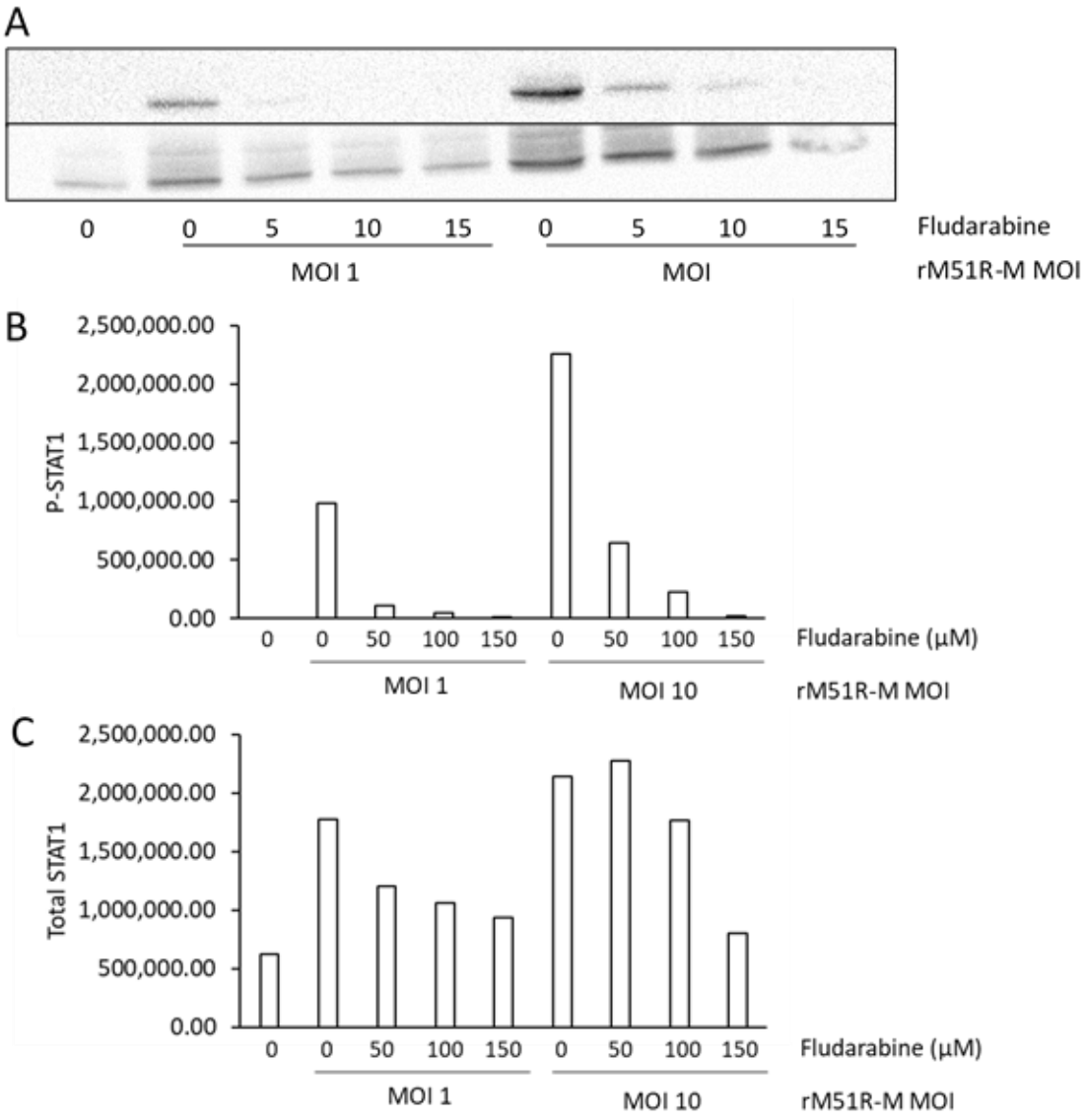

Figure 8. STAT1 inhibition by fludarabine in rM51R-M virus infected M2 macrophages. THP-1 monocytes were polarized to an M2 phenotype with a 72-hour treatment with 25nM PMA and 20ng/ml IL-4 and IL-13. M2 polarized macrophages were then treated with fludarabine (Selleckchem,; S1491) at 50, 100, or 150μM for 1 hour before infection with rM51R-M virus at an MOI of 1 or 10 pfu/cell. Lysates were then collected as before for western blot analysis following 24-hour infections. Representative western blot images (A) and quantified data depicting changes in P-STAT1 (B) and total STAT1 (C) in M2 polarized THP-1 macrophages following 24-hour infection with rM51R-M virus (MOI of 1 or 10 pfu/cell) with or without fludarabine treatment at 50, 100, or 150 μM (n=1).

increased both expression and activation of STAT1 in M2 macrophages. Pre-treatment with fludarabine, however, reduced this response down to mock infected M2 macrophage levels in a dose-dependent manner.

Our plan is to examine M1 macrophage-related functional activity in rM51R-M infected M2 polarized THP-1 macrophages in the presence or absence of fludarabine treatment. We have shown the ability of rM51R-M virus to modulate expression of the M1 macrophage surface markers CD80 and MHC-II, which both function in activating the adaptive immune system. We will repeat the flow cytometry experiments with fludarabine treatments to determine the activity of the type I IFN pathway in this response. Another functional difference between M1 and M2 polarized macrophages lies in their phagocytic ability (Tarique et al., 2015). Our lab has found that M2 polarized THP-1 macrophages have higher phagocytic ability than their M1 polarized counterparts, and infection of M2 macrophages with rM51R-M virus reduces their phagocytic ability to that of M1 macrophage levels (Austin Simmons, unpublished data). Reversal of the modulatory ability that rM51R-M virus exhibits on M2 macrophages with the addition of fludarabine would provide evidence that the mechanism of action is through activation of the type I IFN pathway.

Both wild-type VSV and rM51R-M virus have previously been shown to be effectively cytopathic to cancers without an intact type I IFN response (Ahmed et al., 2010, 2004). Direct cancer cell lysis and the resulting immunogenic cell death already make rM51R-M virus a promising cancer therapeutic. The added ability to repolarize TAMs would also have an immunogenic effect within the TME. rM51R-M virus may even have the edge over other TAM-modulating oncolytic virotherapies due to the low existing immunity against VSV and the low risk of a high-grade infection from VSV in human populations (Hastie and

Grdzelishvili, 2012). However, there are limitations to an rM51R-M virus-based therapy as well. For example, efficient infection and viral spread, which would appear to be crucial to the ability of rM51R-M virus to modulate TAMs may be inhibited in tumors with an intact anti-viral response (Carey et al., 2008). Investigation into the full phenotype of rM51R-M virus infected TAMs along with the replication of this study *in vivo* would better clarify the efficacy and relevance of rM51R-M virus as an immune activating anti-cancer therapeutic.

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Vita

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