Characterization and Development of Vesicular Stomatitis Virus For Use as an Oncolytic Vector

Joshua F. Heiber
University of Miami, jfheiber@gmail.com

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CHARACTERIZATION AND DEVELOPMENT OF VESICULAR STOMATITIS VIRUS FOR USE AS AN ONCOLYTIC VECTOR

By
Joshua F. Heiber

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CHARACTERIZATION AND DEVELOPMENT OF VESICULAR STOMATITIS VIRUS FOR USE AS AN ONCOLYTIC VECTOR

Joshua F. Heiber

Approved:

Glen N. Barber, Ph.D.
Professor of Medicine

Terri A. Scandura, Ph.D.
Dean of the Graduate School

Edward W. Harhaj, Ph.D.
Professor of Microbiology and Immunology

Eli Gilboa, Ph.D.
Professor of Microbiology and Immunology

Joseph D. Rosenblatt, M.D.
Professor of Medicine

Jaime Merchán, M.D.
Professor of Clinical Medicine

Stephen J. Russell, M.D., Ph.D.
Professor of Medicine
Mayo Clinic
Rochester, Minnesota
Abstract of a dissertation at the University of Miami.

Dissertation supervised by Professor Glen N. Barber.
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Oncolytic virotherapy is emerging as a new treatment option for cancer patients. At present, there are relatively few oncolytic virus clinical trials that are underway or have been conducted, however one virus that shows promise in pre-clinical models is Vesicular Stomatitis Virus (VSV). VSV is a naturally occurring oncolytic rhabdovirus that has the ability to preferentially replicate in and kill malignant versus normal cells. VSV also has a low seroprevalence, minimal associated morbidity and mortality in humans, and simple non-integrating genome that can be genetically manipulated, making it an optimal oncolytic vector. Currently, many labs are using a variety of different strategies including inserting trans genes that can modulate the innate and adaptive immune response. VSV can also be retargeted by altering its surface glycoprotein (G) or be made replication incompetent by deleting the G protein. Currently, our lab has engineered a series of new recombinant VSVs, incorporating either the
murine p53 (mp53), IPS-1, or TRIF transgene. mp53, IPS-1 and TRIF were incorporated into the normal VSV-XN2 genome and mp53 was also incorporated into the mutated VSV-ΔM vector generating VSV-mp53, VSV-IPS-1, VSV-TRIF and VSV-ΔM-mp53. Our data using these new viruses indicate that these viruses preferentially replicate in and kill transformed versus non-transformed cells and efficiently express the transgene. However, despite the ability for VSV-IPS-1 and VSV-TRIF to induce a robust type 1 IFN response, VSV-ΔM-mp53 was the only construct that had reduced toxicity and elicited an increased anti tumor response against a syngeneic metastatic mammary tumor model. VSV-ΔM-mp53 treatment lead to a reduction in IL-6 and IP-10 production, an increase in tumor specific CD8+ T cells, and immunologic memory against the tumor. Collectively these studies highlight the necessity for additional VSV construct development and the generation of new clinically relevant treatment schema.
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Chapter 1: Introduction to Cancer, Innate Immunity, and Oncolytic Virotherapy

The ancient Egyptians first documented Cancer over 3000 years ago. Throughout history there have been numerous theories of oncogenesis including Galen’s excess of black bile, Virchow’s chronic irritation theory, and Knudson's two hit hypothesis. However, in only the last 100 years there has been a more profound understanding of the molecular mechanisms that lead to cancer formation. One major finding that facilitated a dramatic change in the understanding of how tumorigenesis occurs was the discovery of deoxyribonucleic acid (DNA) by Watson and Crick in the early 1950’s. Although it was known that radiation, viruses, and chemicals could cause cancer the discovery of DNA proved the ability of these external forces to stimulate mutations in DNA. This also led to the discovery of critical genes, tumor suppressors and oncogenesis, which are the divers and roadblocks to cellular transformation(1, 2).

Out of necessity Homo sapiens evolved a complex, effective, and reasonably high fidelity DNA repair, base pairing mismatch correction, and mutation control system(3). DNA can be damaged by many internal and external factors, such as reactive oxygen species, chemical carcinogens, or retroviral insertion(4, 5). Minor DNA damage can be repaired, however mistakes made during the replication process can also lead to mutations(6). Furthermore, DNA that has been extensively damaged, beyond the capabilities of the repair system, can activate cellular senescence or apoptosis pathways(7, 8). This is significant since the human genome is comprised of approximately 3 billion base pairs (bp) and
has a mutation rate between $1 \times 10^{-8}$ to $1 \times 10^{-10}$ mutations/bp/replication yielding around one mutation per cell division(9). Thus, independent of external factors, genetic alterations are continuously occurring and accumulating throughout an individual’s life. Although these mutations occur randomly and are generally silent or selected against, occasionally tumor suppressors or oncogenes are “hit”, which begins the process of cellular transformation.

Over the last 150 years there have been significant advances in medical treatment and technology. As a result, the average life expectancy in the United States has increased from 72.6 in 1975 to 77.9 years in 2008. It is thus not surprising that National Cancer Institute (NCI) surveillance, epidemiology and end-results program (SEER) data indicates that the incidence of cancer has also risen over this period of time from 400 to 464 (per 100,000 people). Additionally SEER reported a nearly exponential increase in the likelihood of an individual developing cancer as age increases, due to the additional time for mutations to accumulate.

Not surprisingly, as a result of mutations cancer cells display critical differences from normal cells. Such differences give the cancer cells a selective advantage and include, the ability to replicate indefinitely, remain insensitive to apoptotic signals, self generated in growth and angiogenesis signals, and tissue invasion and metastatic potential(10). Although the mutations that cause disease are variable cancer can arise in nearly every cell type and organ system. However, pathway analysis has shown that mutations often cluster around critical cellular pathways that when deregulated can lead to cellular transformation(1, 2).
Recently, a variety of treatment options have been developed. Initially, surgery was the primary form of therapy; this was followed by the use of X-rays, which were discovered in the late 1800’s as a novel therapeutic approach. Until X-rays became a viable treatment option, a diagnosis of inoperable cancer was often considered a death sentence. Despite this significant advance, both surgery and X-rays were primarily effective against local malignancies and there was not treatment for hematological malignancies. However, over the last 100 years improvements in surgical and radiotherapy technologies have made the treatment of many more tumors possible. These, along with additional medical advancements and research to further our understanding of the molecular mechanisms that cause cancer, have significantly improved our ability to treat malignancies. However, many current cancer therapies such as chemotherapy, radiation, and immunotherapy, still rely on the differences between normal and tumor cells, as the discrepancies provide a therapeutic window for these treatments. Chemotherapy was discovered in the 1950’s when it was observed that nitrogen mustard inhibited leukemia cell growth; this changed the way people thought about treating cancer. Chemotherapeutic drugs come in many forms, including alkylating agents, anti-metabolites or topoisomerase inhibitors. Chemotherapy and radiation therapy work by damaging the DNA of rapidly dividing cells. Although cancer cells divide more rapidly than most normal cells, a number of normal cell types also have elevated replication rates, leading to negative side effects, as radiation and most chemotherapies are unable to differentiate between rapidly dividing tumor and normal cells. Side effects
include immunosuppression, debilitating nausea, hair loss, and diarrhea. Therefore, better methods to target the delivery of chemotherapy drugs specifically to tumor cells are currently being developed. Some tumors become “addicted” to specific signaling pathways and thus these tumors can be targeted by more specific therapies such as small molecules or antibodies. Due to these treatments specificity they have greatly reduced toxicity. Small molecule inhibitors and antibodies can be developed when the specific defect in a signaling pathway is identified and then targeted for therapeutic development. In a number of different malignancies a specific class of molecule, the tyrosine kinase, has been identified as the molecular driving force leading to tumorigenesis. Gain of function tyrosine kinases act by constitutively phosphorylating target molecules casing them to “turn on” or “turn off” leading too constitutive signaling. Tyrosine kinase inhibitors inactivate these pathways depriving the tumor cells of signals they need to survive and have been found to effective against some cancers(11, 12). Also, surgery and radiation therapy, are often combined with multiple chemotherapeutic drugs (a “multidrug cocktail”) to maximize efficacy. However, another potential negative consequence of chemotherapy is the selection of cancer cells that are refractory to treatment, due to elevated mutation rates in cancer cells(13). This can make treating cancer relapse more difficult and is the reason multidrug cocktails are used. It is important to note that chemotherapy, surgery, and radiation therapy are only effective against some cancers and therefore additional therapies must be developed.
Another form of cancer therapy being developed is tumor immunotherapy. Tumor immunotherapy activates the host immune system by various means against the tumor(14, 15). This treatment utilizes two strategies in order to elicit an immune response: 1) vaccination against tumor antigens or 2) treatment with therapeutic antibodies such as Rituximab or Trastuzimab, which bind to specific surface receptors and recruit host immune effector cells to kill these cells(16-19). One advantage of these therapies is that they are highly specific because the antibodies are generated against tumor antigens or highly over expressed surface receptors and have shown profound efficacy.

In addition to tumor vaccination and monoclonal antibody therapies other immunotherapeutic strategies are being developed that use cytokines or transgenic T cell receptors (TCR) are being developed(20-22). Cytokines like IFN-β or IL-2 have been proven effective against some malignancies by inducing apoptosis in tumor cells and further activating the adaptive immune response(23, 24). Although these immunotherapeutic methodologies can be very effective there use is limited, as there needs to be a receptor target, cytokine responsiveness, and also the immunosuppressive nature of the tumor microenvironment needs to be overcome. Through the use of these conventional therapies we are making progress in the fight against cancer. SEER indicates that 5-year survival rates for cancer at all sites have increased from 49.1% in 1975 to 67.4% in 2007, however improvements are still needed.
Oncolytic Virotherapy and Immunity

With the discovery of the viral particle in the 1800’s the idea that viruses could be used as cancer therapeutics began to take shape. The idea gained additional support in the early 20th century after clinical observations in which leukemia patients underwent a spontaneous, albeit incomplete remission upon infection by influenza virus(25). Oncolytic viruses are those that have the innate ability to preferentially replicate in and kill malignant cells. Many naturally occurring viruses, including Hepatitis B and C virus, Measles Virus, Mumps, Coxsakievirus, Herpes Simplex Virus, Vesicular Stomatitis Virus, Adenovirus, Vaccinia Virus, and others have oncolytic potential and have shown promise for use in this capacity(26-29). Research trials using Hepatitis B virus, West Nile virus, Adenovirus, and Mumps Virus began in the late 1940’s and continued through 1970’s(30). While these initial trials did lead to some tumor regression their success was limited due in part to the host immune responses and toxicity associated with using live viruses.

Oncolytic virotherapy is also possible due to the phenotypic difference between tumor and normal cells as a result of acquired mutations. Although the mechanism of oncolysis is not completely understood there are a number of theories that can explain why certain viruses have oncolytic ability. In some cases tumor cells overexpress a surface receptor, like CD46, which can then be used by a virus (in this case Measles) to enter the cell(31). This allows many more virus particles to infect a tumor cell causing its death. Also, viruses rely on
host translation machinery. Thus tumor cells, which continuously replicate due to a loss in their ability to regulate translation, are the perfect environments for viral replication(32). As a result many more viral progeny are produced and this leads to cell death and tumor antigen release. Finally, mutations can occur in malignant cells that inactivate the innate immune antiviral response. The antiviral response is typified by increased production of type 1 interferons (IFN-α/β). Defects in these pathways found in cancer cells include, alternate splice variants of IRF-3, mutations in STAT-1 or TRAF3, and deletion or methylation of STING, all of which reduce or ablate IFN production or effectiveness(33-36). Reduced IFN levels facilitate elevated levels of viral replication within tumor cells, thereby increasing cell death. Interestingly, some cancers when treated with IFN-α/β undergo apoptosis. Thus, if tumor cells are sensitive to IFN treatment, IFNs produced by virally infected cells, could also enhance tumor regression.

As mentioned the effectiveness of an oncolytic virus can be limited by endogenous circulating antibodies and the host immune response because viral infection potently activates the IFN system. Upon infection viruses display pathogen associated molecular patterns (PAMP) that are recognized by pattern recognition receptors (PRR). Viral PAMPs are generally positive or negative sense DNA or RNA that is single or double stranded. The RIG-I like receptors (RLR) RIG-I or MDA-5, and the Toll like receptor (TLR) pathway TLRs 3 and 7 both recognize different types of viral RNA whereas TLR-9 and the STING regulated pathway (SRP), for which the putative receptor is unknown, recognize viral DNAs(34, 37).
RLRs are located in most cell types and were discovered upon the observation that IFN-β was produced in response to RNA viral infection in a TLR independent manner(38) (Figure 1.1). After recognition of viral RNA, RIG-I or MDA-5 undergo a conformational change that exposes its caspase recruitment and activation domain (CARD)(39). Activated RIG-I or MDA-5 then interacts with the mitochondrially associated signaling adaptor IPS-1/VISA/MAVS/CARDIFF another CARD carrying protein(40-43). IPS-1 then assembles a complex consisting of TRAF2/3 and TANK which activates NAK and leads to the phosphorylation of TBK-1(40). TBK-1, a critical molecule in IFN signaling then activates the interferon regulatory factors (IRF-3 and 7), which dimerize and translocate to the nucleus(44). Dimerized IRF-3 or -7 can then initiate transcription of IFN-α/β(45, 46). Concomitantly, IPS-1 also recruits FADD and RIP-1, which lead to activation of the NF-κB pathway(47, 48). This is especially important because maximal IFN transcription occurs when all of four of IFN’s positive regulatory domains (PRD-I, II, III, IV) are activated by IRF-3 or -7, CBP300, NF-κB, and AP-1(49).

The TLR pathways can also initiate type 1 IFN production. There are currently 13 TLRs that can recognize a variety of PAMPS including bacterial lipopolysaccharide (LPS) by TLR 4 on the cell surface, double or single stranded RNA by TLR3 or TLR7 and CpG DNA by TLR9, which are in endosomes(50) (Figure 1.2). TLR7 and TLR9 are primarily expressed in plasmacytoid Dendritic cells (pDC) and signal through the MyD88 dependent pathway(51, 52). After stimulation MyD88 dissociates with IRAK4 facilitating an interaction with
Figure 1.1: Schematic representation of the RIG-I Like Helicase (RLH) signaling pathway that leads to IFN-β and NF-κB production.

TRAF6(53). TRAF6 is an E3 ubiquitin ligase that can target TAK1’s inhibitory complex of TAB1, 2, and 3 for K63 linked ubiquitination and allows TAK1
activation(54). TAK1 then drives the MAPK and NF-κB pathways by phosphorylating MAPK and IκBα facilitating an inflammatory response and IFN signaling(55). This pathway can also generate Type I IFN through the formation of a complex that includes MyD88, IRAK1, IRAK4, TRAF6 and IRF7 that results in IRF7 phosphorylation and subsequent IFN activation(54, 55). TLR3 activates IFN via the MyD88 independent pathway through the signaling adaptor molecule TRIF(56). TRIF can recruit TRAF3, TRAF6, and RIP1 and lead to the activation of NF-κB, MAPK, and IRF3 through a similar mechanism to that of TLR7 and TLR9(57, 58). Interestingly TLR4, which induced by LPS, can activate both MyD88 and TRIF dependent pathways through an additional adaptor molecule TIRAP or TRAM respectively(50, 59-61).

The stimulator of Interferon genes (STING) regulated pathway is responsible for recognizing and responding to DNA pathogens (Figure 1.3)(62). Although the putative DNA receptor that leads to IFN induction has not been identified, one molecule that is indispensable for an IFN response to foreign DNA is STING(34). It was found that STING knockout MEFs did not induce IFN in response to DNA analogues or DNA virus infection. Additionally, these knockout mice were highly susceptible to viral infection(63). Interestingly, STING associates with the translocon and interacts with TBK1, which phosphorylates IRF3 and 7 in order to induce IFN production(63, 64). Thus, multiple pathways converge on these few molecules that lead to production of type 1 IFNs. Surprisingly, STING may also play a role in the recognition of RNA viruses as it was found to interact with RIG-I and type 1 IFN production was blunted in STING
knockout mice infected with VSV(64). Although the definitive DNA receptor is unknown, two DNA binding molecules DAI and AIM2 have been discovered that

Figure 1.2: Schematic representation of TLR3, 7, and 9 signaling pathways that leads to IFN-β and NF-κB induction.
can activate type 1 IFN or IL-1β(65-67).

After IFN induction, IFNs are secreted from cells and act in an autocrine and paracrine manner by binding to the heterodimeric interferon alpha receptor (IFNAR1/2)(68) (Figure 1.4). IFN activates a JAK/STAT signaling cascade, with STATs, predominantly STAT1 and STAT2, homo or heterodimerizing in response to phosphorylation by JAK1(69). Phosphorylated STAT also interacts with IRF9 creating the ISGF3 complex, which translocates into the nucleus and activates transcription of IFN stimulated genes, of which there are over 100, by binding to IFN stimulated response elements (ISRE) or GAS sites in the DNA(39, 70). ISGs like PKR, RIG-I, 2-5' OAS, IFIT15, and Mx then create the antiviral environment within the cell(71, 72). Interestingly, IRF7 is also an ISG that can then initiate a positive regulatory loop to further bolster type 1 IFN production. Furthermore, IRF7 is postulated to be a master regulator of the IFN response as IRF7 knockout mice are incapable of producing high levels of IFN(45).

Type 1 IFN, in addition to activating the antiviral response, is also critically important for the maturation of dendritic cells (DC) and activation of natural killer (NK) cells(73). Activated DCs can mediate cross presentation of viral antigens to CD8+ cytolytic T cells (CTL) while NK cells can exert direct cytolytic effect creating viral antigens(74-76). IFN stimulation results in the production cytokines and chemokines that allow further immune cell activation and proliferation, as well as, facilitate increased trafficking to the site of infection. In particular, IFN leads to IP-10, an inflammatory chemokine that increases lymphocyte trafficking, and IL-15, which facilitates NK cell proliferation and activation in addition to
Figure 1.3: Schematic representation of IFN-β signaling. IFN-β activates a JAK/STAT cascade, which facilitates induction of antiviral ISGs like PKR.

Assisting the generation of CD8+ T cell memory (77-81). Studies in IRF7 or IFNAR deficient mice have highlighted the critical nature of IFN in these systems. IRF7 knockout mice, upon TLR9 stimulation with CpG DNA adjuvants, were unable to generate T cell responses to the accompanying antigen and T cells
lacking the IFNAR were unable to proliferate or differentiate into effector CTLs indicating that IFN provides a unique stimulus to these cell populations (45, 82).

Thus, the efficacy of oncolytic viruses can be limited as viral infection activates a complex network of innate signaling pathways that lead to activation of effector cells and the adaptive immune system. One advantage of oncolytic virotherapy is that this system can potentially be used against tumors. Our data, which has been supported by other research groups, suggest that tumor antigens generated during viral infection can be cross-presented and initiate an anti tumor T cell response (76, 83).

Figure 1.4: Schematic representation of IFN-β signaling. IFN-β activates a JAK/STAT cascade, which facilitates induction of antiviral ISGs like PKR.
Current Trends in Oncolytic Virotherapy

With the advancement of DNA and molecular cloning technologies much research has been focused on the manipulation of oncolytic viruses in order to better modulate the immune response leading with the hope of increasing efficacy and reducing toxicity. To achieve these goals, many different viruses and strategies are being combined to generate a greater number of viral vectors. Currently, there are a number of clinical trials; all in various stages, taking place using Herpes virus type 1 (HSV-1), Vaccinia virus, Seneca valley virus, Reovirus, Adenovirus, and others. Brief overviews of a number of these viruses and trials are described below.

Herpes virus type 1 (HSV-1) is an enveloped dsDNA virus that has a wide tissue tropism. HSV-1 has genes that are not required for replication or function in tumor cells that can be mutated and deleted including, thymidine kinase (TK), DNA polymerase, and ribonucleotide reductase (RR)(84). HSV1716 has most of the coding region of the $\gamma34.5$ gene deleted, whereas G207 has the lacZ gene inserted in $\gamma34.5$, in both cases effectively inactivating this protein which greatly reduces HSV-1 replication in normal cells and eliminates its toxicity(85). HSV1716 and G207 has been used in a number of clinical trials in which no toxicity was observed and there is evidence that viral replication is restricted to tumor cells. However, the anti tumor response was strongest in metastatic melanoma while HSV1716 and G207 had minimal effects against glioma and squamous cell carcinoma(86, 87). HSV can also have transgenes inserted, such
as OncoVEX GMCSF, in which the GMCSF gene has been inserted into the HSV genome(88). This additional expression of GMCSF from the virus augments and heightens the immune response, leading to enhanced efficacy and reduced toxicity. Currently, OncoVEX is scheduled to begin a phase III trial for metastatic melanoma and head and neck cancer(88).

Vaccinia virus, another dsDNA virus that is closely related to the Small Pox virus, is also being developed as an oncolytic vector. Naturally occurring vaccinia virus has been used over the last 60 years in a number of trials against malignant melanoma, chronic lymphocytic leukemia and bladder cancer and has shown to be effective in some patients(89, 90). JX-594, a vaccinia virus in which the TK gene was deleted and the GMCSF gene was inserted, was developed to try and improve vaccinia’s ability to modulate the immune response and increase the anti tumor efficacy. JX-594 infection increased white blood cells (WBC), monocytes, and eosinophils and evidence of antitumor effect was observed in most patients(91, 92). JX-594 is being used in a number of phase II trials against hepatocellular carcinoma and metastatic colorectal cancer.

Seneca valley virus (SVV), a naked ssRNA picornavirus, recently discovered in 2002, was found to have potent oncolytic potential in a variety of model systems including small cell lung carcinoma cell lines and pediatric neuroendocrine tumors(93). Additionally, SVV is not known to cause morbidity in humans and its seroprevalence is very low. Thus, a phase 1 trial using SVV-001 was performed. Surprisingly, a maximum tolerable dose (MTD) was not reached as infections with the highest doses had minimal morbidity that included, low
grade fever with chills, arthralgias, and myalgias, which were all resolved by day 7 and no mortality (94). They also showed evidence of viral replication within tumors by recovering increased post fusion viral titers and all patients developed antibodies and cleared the virus within approximately 7 days (95).

Discovered in the 1950’s, Reovirus (RV) a naked dsRNA virus, was also observed to have oncolytic capabilities (30). Preclinical studies using RV indicated that many different tumors and cell lines including pancreatic cancer, ovarian cancer, lymphoma, breast cancer, non-small cell lung cancer, malignant glioma, and a number of pediatric tumors were susceptible to virus infection (96, 97). Unmodified RV serotype 3 had been developed under the commercial name Reolysin and has undergone a number of phase I studies in which the virus was administered both intratumorally (IT) and intravenously (IV), both alone and in combination with other therapies. RV displayed minimal toxicity during dose escalation and lead to a reduction in tumor specific markers and a stabilization of disease in some patients (98). As a result of the success observed in phase I testing, Reolysin is now in phase II trials where it is being combined with various chemotherapy regimens and is planned for a randomized phase III trial in which RV will be combined with paclitaxel/carboplatin for patients with head and neck cancer that are not responsive to platinum based therapy (99, 100).

Onyx Pharmaceuticals initially developed Oncolytic Adenovirus (AV) in the 1990s. Their vector ONYX-015 has the E1b-55k protein deleted (101). This deletion attenuated the replication capacity of the resulting virus in normal cells due to normal p53 function. P53 mutation is one of the most common genetic
Lesions in cancer and tumors harboring this mutation become permissive for adenovirus oncolysis. Unfortunately, ONYX-015 has displayed limited clinical efficacy (102). Despite this setback, research has continued with new adenoviral vectors such as adenovirus H-101, based on the ONYX-015 virus, which is currently approved for clinical use in combination with chemotherapy for a variety of malignancies (103).

As oncolytic viruses continue to be proven as effective cancer therapeutics it is likely that additional oncolytic viral vectors, shown to be effective in preclinical models, will begin phase 1 trials. These new viruses attempt to utilize and expand upon current methods in order to increase viral efficacy and safety. In addition to transgene insertions and genome manipulation, oncolytic viruses can be fully retargeted to tumor cells by altering their surface receptors using single chain antibodies. MV have been generated that express a single chain antibody to EGFR or CD38 and have SLAM deleted in order to prevent binding to its normal CD46 receptor (104). These viruses were tumor specific in cases where tumors highly overexpress the EGFR or CD38 receptors. Oncolytic viruses can also be retargeted by incorporating miRNA binding sites into the genome. This strategy has been used to fully retarget VSV expressing miRNA 125 and completely abolishes its normal neurotropism (105). VSV has also been retargeted to CD4+ T cells by fusing the gp120 domain of HIV gp160 to the cytoplasmic tail of the VSV-G protein (106). VSV is on the frontline of oncolytic research and is described in detail next.
Chapter 2: Vesicular Stomatitis Virus as an Oncolytic Vector

Background

Cancer is a leading cause of mortality worldwide accounting for more than 7 million deaths in 2007(107). Cancer can occur in nearly all cell types resulting in various types of malignancies. While a number of treatment options exist including surgery, chemotherapy, radiotherapy, gene therapy, and bone marrow transplant, the side effects of these treatments can often be debilitating. Additionally, current treatment regimens can lead to the selection of resistant cancer cells, which may produce refractory relapses that are more difficult to treat or even untreatable using current available methods(108). Therefore, it remains important to try and develop safe treatment strategies and therapeutics that are cancer specific, lack the negative side effects often associated with established therapies, and that can be used in conjunction with established treatment strategies to improve efficacy.

Mechanisms of Viral Oncolysis: VSV

Viral oncolysis, the use of a virus to kill cancer cells, is emerging as a potential new therapy in the fight against cancer, in part due to the ability of oncolytic viruses to preferentially kill transformed cells compared to normal cells(109). For example, VSV and other viruses are extremely sensitive to type 1
IFN and can generally only replicate in cells defective in innate immunity(110). The innate immune system has evolved to control and inhibit the replication and subsequent release of viral progeny. This system relies on PRR that detect specific PAMPs and subsequently activate an antiviral response characterized by the production of IFN-α/β(59, 111).

Specifically, three pathways, namely the RIG-I like helicase (RLH), Toll TLR, and SRP, are responsible for detecting viral infection and activating an antiviral response(37, 112) (Figure 2.1). These pathways are activated by a variety of different PAMPs. The RLH pathway is comprised of RIG-I, which recognizes viral double stranded RNA and Melanoma Differentiating Factor 5 (MDA-5) that is activated by positive stranded RNAs(113, 114). There are 13 TLRs that are stimulated by an array of PAMPs including single or double stranded RNA, LPS, CpG methylated DNA, and others(50). Interestingly, the SRP is essential for generating a response to DNA pathogens and is also involved in the RIG-I response to VSV(63, 115). Each of these pathways induces an antiviral state characterized by the production of type 1 IFN α/β(73). It is known that tumor cells can have defects in innate immune signaling pathways, which make them permissive to viral infection. These defects include alternative spliced isoforms of IRF-3, CpG methylation of IRF-7, mutations of CYLD, reduced ability to phosphorylate STAT-1 and activate transcription of IFN stimulated genes (ISGs), mutations in JAKs, and TRAF-3 mutations(110). Also, cancer cells have the ability to continuously replicate due to acquired mutations
that disrupt translation inhibition, which also facilitates high levels of viral replication within malignant cells (116, 117).

**VSV Infection Activates the RLH Pathway**

The importance and necessity of both the innate and adaptive immune systems have been demonstrated through the use of mice deficient in critical components of these systems (118-122). The antiviral response is activated by recognition of double stranded RNA intermediates produced during viral replication by the RLH pathway which leads to production of IFN-α and -β (59). Activation of this cascade relies on two DExD/H helicases, RIG-I and MDA-5. RIG-I recognizes 5'-triphosphates on viral RNA, such as those encoded by negative-stranded viruses like VSV or Ebola Virus (EV) (123). In contrast, MDA-5 recognizes longer RNA (> 1kb), which are encoded by positive-stranded viruses such as the picornavirus encephalomyocarditis virus (EMCV) (124). Binding of viral RNA leads to a conformational change in these helicases. This change exposes the N-terminal CARD, which facilitates a CARD-CARD interaction with a second CARD carrying mitochondrial associated protein referred to as Interferon-β Promoter Stimulator 1 (IPS-1) (125). IPS-1 then mediates the phosphorylation of TRAF3, which leads to activation of TBK1 and I KKı. TBK-1 can then phosphorylate IRF-3 and 7, which are needed for activation of type I IFNs (126). Concomitantly, RIG-I/MDA-5 also activates the NF-κB pathway through the Fas Associated Death Domain (FADD) and Receptor Interacting Protein 1 (RIP-1) (48,
This is important since IRF-3, NF-κB, and AP-1 are required for maximal production of IFN-β. IFN-α/β can be secreted from most cell types, and TLR7-dependent pDC’s are able to produce type I IFN in very high quantities (51).

Type I IFN can act in both an autocrine and paracrine manner and binds to species specific IFN-α/β receptor (IFNAR), which is composed of two subunits IFNAR1 and IFNAR2 (128). Activation of the IFNARs triggers the JAK-STAT signaling cascade. After IFN binds to IFNARs, JAK1 and TYK2 interact with the intracellular domain of IFNARs (129). Additionally, JAK1 has tyrosine kinase activity, which allows it to phosphorylate the phosphotyrosine-binding domain (SH2) on SH2 containing STATs. Phosphorylated STATs then homo or hetero dimerize. Although STAT-1 and STAT-2 are commonly activated by type I IFNs additional STAT molecules including STAT-3, -4, -5, and -6 can also be activated by this pathway (129-131). Predominantly, hetero or homo dimerized STAT-1/2 associate with an additional molecule, IRF9, completing the IFN-stimulated gene factor 3 (ISGF3) complex, which then translocates into the nucleus (71). After translocation, ISGF3 binds to IFN-stimulated response elements (ISRE) or IFN-activated sites (GAS) in the promoters of more than 100 IFN stimulated genes (ISGs) including PKR, Mx, OAS, IFI16, ISG15 and others (131, 132).

Our lab, as well as other research groups, have demonstrated that mouse embryonic fibroblasts (MEFs) and mice deficient in components of signaling networks that lead to IFN production, become highly susceptible to viral infection. Cells lacking RIG-I allowed high levels of viral replication in response to infection by several classes of viruses including VSV, influenza, Sendai Virus, and
Newcastle Disease Virus (124, 133). Additionally, TRADD and IPS-1 deficient cells were unable to produce interferon in response to infection. Also, TBK-1 or IRF 3/7 knockouts cells highlight the absolute necessity of these molecules for efficient activation of the interferon response and showed IRF-7 to be a master regulator of IFNα production (45). Furthermore, knockouts have been generated to components of the IFN responsive system including IFNAR, STAT-1, or PKR and these deletions also render cells permissive to viral infection (68, 116, 117, 121, 134).

In addition to defects in innate signaling pathways, mutations in key ISGs or the translation regulatory machinery can also render cells permissive for viral infection. For example, PKR is an IFN inducible serine/threonine protein kinase that becomes auto phosphorylated in response to double stranded RNA species leading to inhibition in translation (116). Therefore, in normal cells as IFN and ISGS are being produced, active PKR phosphorylates eukaryotic translation initiating factor 2α (eIF2α), inhibiting translation and facilitating initial control of the infection (32). In another series of experiments it was established that both the α and ε subunits of the eIF2B translation initiation complex play critical roles in the control of viral infection. Defects in either of these molecules render cells susceptible to infection. More recently, an additional RNA binding molecule has shown to be involved in the response to viral infection. Nuclear factor associated with dsRNA (NFAR1/2) is a novel regulator of host translation in response to viral infection. Recognition of viral RNA by PKR enabled phosphorylation of NFAR on
critical threonines, allowing it to associate with viral RNAs and inhibit protein translation (134).

**Vesicular Stomatitis Virus (VSV) Background**

The optimal oncolytic virus for the treatment of various cancers must possess a number of specific characteristics including: i) low incidence and seroprevalence in the population, ii) inability to integrate into host genomes causing transformation, iii) low morbidity and mortality, iv) genetic malleability, and v) relative specificity (i.e. preferentially replicating and killing tumor cells compared to normal cells). While a number of candidate viruses are currently being tested, such as Measles Virus (MV), Herpes Simplex Virus (HSV), Adenovirus (AV), Coxsakie Virus (CV) and others, Vesicular Stomatitis Virus (VSV) is one of the few viruses that meets all of these criteria (104, 135, 136).

VSV is a negative stranded RNA virus of the rhabdoviridae family. Rhabdoviruses, of which there are more than 100, can infect vertebrates, invertebrates and plants. This family is comprised of 5 genera: Vesiculoviruses, Lyssaviruses, and Ephemeroviruses, which can infect animal cells and Cytorhabdoviruses and Nucleorhabdoviruses, which infect plant cells. Rhabdoviruses are generally 100-400 nm long and 45-100 nm in diameter (Figure 2.1 B)(137). The wild type virus is comprised of an 11 kilobase negative sense, single-stranded RNA genome coding for 5 proteins: nucleocapsid (N), polymerase (P) and (L), matrix (M), and surface glycoprotein (G) (Figure 2.1
A)(138). VSV is an enveloped virus tightly encapsulated by the N protein and coated with trimers of the typical type I membrane G protein that facilitates binding and fusion(139). G is also the major antigen responsible for serotype specificity and is the primary target for neutralizing antibody(140). This virus is packaged with the P and L polymerase proteins as well as some M protein(138). M is a multifunctional protein, involved in viral assembly and defense against innate immune responses(141, 142). The G protein binds to cells through an unknown receptor that is predicted to be fairly ubiquitous given VSV’s capability to infect nearly all cell types. After binding, cellular entry occurs through an actin dependent clathrin mediated endocytosis in vesicles incompletely coated with clathrin(143). Fusion occurs following acidification of the vesicle, now considered an endosome, which initiates a conformational change in the G protein, leading to viral entry into the cytoplasm(144). Then, the packaged P and L polymerase proteins rapidly produce VSV protein mRNA’s. This entire process occurs within about 30 minute of infection(143). VSV produces each of its gene transcripts as a sub-genomic RNA, that is capped and polyadenylated. After polyadenylation, the P and L polymerase proteins are free to reinitiate transcription of the next VSV gene(145). The viral mRNAs then use the host translation machinery, Golgi apparatus and endoplasmic reticulum, to translate its proteins(146). Although the mechanism is not completely understood, the switch from generating sub genomic mRNAs, to the production of full length genomes, occurs after the production of sufficient VSV proteins. It is known that the original viral genome is tightly associated with the N protein, forming a bead on a string helical structure.
In order for viral replication to occur, N must dissociate from the RNA, as this enables transcription by the P and L proteins. It was also shown that phosphorylated N can interact with the P protein, which may regulate the transcription of viral genes versus genomes\(^\text{(147, 148)}\). The VSV N and M proteins then form ribonucleic particles (RNP) with the complete genome shuttling to rafts of G protein bound to the membrane of infected cells. The RNP complex is then further encapsulated in N protein and buds off from the cell taking with it G protein containing cellular membrane producing functional new virions\(^\text{(141, 149)}\). Since infection occurs through a ubiquitous undetermined

Figure 2.1: A, Schematic representation of the VSV genome and virion. B, Electron micrograph of VSV.
receptor, VSV has the unique ability to target many different cell types and malignancies. This benefit of VSV is also a limiting factor, as the virus lacks true cellular specificity, despite its ability to preferentially replicate in and kill malignant cells.

**VSV Pathology and Immune Response**

Another benefit of using VSV is its low population incidence and minimal morbidity caused in humans. It can, however, cause a persistent non-lethal infection characterized by sores on the hooves or mucus membranes of the mouth and nose in animals such as cattle, horses, sheep, and pigs(150). VSV can also have neuropathic effects in mice infected intranasally (IN) or intravenously (IV). Interestingly, upon IV administration the virus disseminates throughout the host, infecting most organ systems, and is able to transverse the blood brain barrier, through the olfactory nerve. In contrast, IN administration does not result in dissemination of virus to other organ systems, and infection remains restricted to the brain. The lack of systemic virus dissemination upon IN administration is likely due to activation of an innate immune response characterized by the production of IFN-β(151-155). CNS infection by VSV can cause lethal encephalitis or hind limb paralysis; however, the infection can be controlled and cleared in immuno-competent mice through the activation of both the innate and adaptive immune systems(156).
Initially, the immune response against VSV is characterized by the activation of the innate immune system and production of interferon beta (IFN-β). IFN-β protein acts in an autocrine or paracrine manner, which leads to the activation of interferon-stimulated genes (ISGs), upregulation of antigen processing machinery and activation/maturation of antigen presenting cells (i.e., dendritic cells, NK cells, macrophages)(72, 132). VSV is highly susceptible to the effects of ISGs, like PKR, which upon activation inhibits translation initiation by phosphorylating eIF2α. Despite this, activation of PKR alone is not sufficient to completely control infection by VSV(119).

The importance of both arms of the immune response has been demonstrated using knock out mice that lack key components of host defense, such as the IFN receptor (IFNAR), PKR, or STAT1. These knockout mice show lethality around 4-5 days after infection due to the virus’ ability to efficiently replicate throughout the mouse(118, 121, 146). B-cell deficient mice that maintain an otherwise intact innate response often show toxicity around 9 or 10 days post infection, due to the lack of endogenous circulating antibodies and the production of neutralizing antibodies against the N and G proteins by plasma cells. Further, T-cell deficient mice succumb after 30 days due to their inability to provide B cell help and generate memory against the virus(119). These studies illustrate that the initial control of infection by the innate response is essential to provide enough time for the activation of the adaptive immune response. This leads to activation of VSV specific T cells and activation of B cells, which
differentiate into plasma cells and produce high levels of neutralizing antibody, primarily to the surface G and N proteins, facilitating clearance of the infection.

*Wild Type VSV’s as Oncolytic Agents*

It is now well established that VSV has oncolytic ability and is able to kill numerous types of solid and hematological transformed and malignant cell lines(109, 157, 158). VSV selectively replicates with high efficiency in these cells and rapidly induces apoptosis(159-162). Generally, normal cells treated with IFN are protected from VSV infection, as IFN initiates an antiviral response characterized by the induction of specific ISGs such as PKR(109, 116). However, tumor cells, which appear deficient in either innate signaling pathways or translational control mechanisms, are highly susceptible to VSV infection(116). The specific defects can vary from tumor to tumor. For example, bladder cancer, prostate cancer and mesothelioma, are resistant to VSV mediated oncolysis because these cancer types can produce or respond to IFN-β production(163-165). However these cells can be rendered permissive by targeting and inactivating critical molecules in innate signaling pathways like the IFN receptor (IFNAR)(164). Additionally, data from our lab indicates that, although many VSV permissive cell lines have a functional PKR response, viral translation rates are not diminished(32, 116, 166). Therefore, translation deregulation in cancer is hypothesized to play a major role in facilitating VSV mediated oncolysis, as VSV and all other oncolytic viruses require host translation machinery to replicate(32,
In addition to determining the mechanisms that facilitate VSV’s ability to kill malignant cells, it has been established that VSV mediates cell death by activating the Bax, Bcl-2, and caspase cascade (168, 169). More recently, it was shown that VSV causes cell death by binding to the mitotic spindle complex, thereby inhibiting mitotic progression (170). The inhibition of mitotic progression accounts for the characteristic rounding of cells observed during VSV induced cell death (170). It has also been demonstrated that VSV oncolysis of T lymphocytes, in chronic lymphocytic leukemia (CLL), requires cell cycle entry and translation initiation and this is likely true for other tumors as well (171).

Our research has demonstrated that VSV can exert its oncolytic ability against tumors when administered intra-tumorally in a variety of models including CT-26, a murine colorectal carcinoma cell line, in athymic nude mice and B16(F10), a murine melanoma cell line, in immuno-competent mice (109, 172). Additionally, systemic intra-venous (IV) treatment was also found to mediate the regression and clearance of TS/A, a metastasizing mammary adenocarcinoma, in wild type Balb/c mice (173, 174). The clearance of TS/A involved the generation of tumor specific T cells and appears to induce immunologic memory against tumor antigens (173). Furthermore, it was determined that VSV infection was able to modulate the tumor microenvironment by increasing tumor vascular permeability, enabling better drug and lymphocyte infiltration into glioma cells implanted in rat brains (175, 176).

Upon IV delivery, VSV infects both normal and tumor cells. When normal cells, including plasmacytoid dendritic cells and macrophages, are infected,
production of IFN-β and inflammatory cytokines is triggered, inducing an antiviral response in surrounding healthy cells. In contrast, infected tumor cells permit VSV replication and the released progeny virus can then infect surrounding malignant tissue. Additionally, as tumor cell lysis occurs tumor antigens are released which are recognized and processed by immune effector cells. Ultimately these tumor cell antigens are cross presented to naïve T cells, in the draining lymph nodes, which can facilitate the generation of a tumor specific T cell response and potentially induce immunologic memory against the tumor (172, 177, 178). Interestingly, findings suggest that using a replication competent VSV is not required to activate the adaptive immune system against tumors. In fact, it appears that replication incompetent VSV (VSV-ΔG) were sufficient to drive both an anti-tumor and antiviral immune response (179). Specifically, infection with replication competent VSV induced high titers of class switched neutralizing antibody, predominantly to N and G proteins, produced by activated plasma cells and the viral infection is controlled in approximately 10 days (180, 181).

**Recombinant Vesicular Stomatitis Virus (rVSV)**

One significant advantage of the oncolytic VSV system is the ability to produce infectious viral particles from cDNA plasmids (138, 139). Initially, cells are infected with a vaccinia virus that expresses T7 polymerase (VTF7-3). T7 polymerase then synthesizes full-length negative stranded viral RNA, as well as N, P, and L proteins from the transfected plasmids. Research demonstrates that
manipulating the VSV genome, by changing the order of the genes, mutating, deleting, or inserting trans-genes, especially between the VSV G and L genes, does not prevent the recovery of infectious viral particles. Furthermore, VSV accommodates transgene insertion by increasing the size of the viral particle, as seen by electron microscopy. The first transgenic VSVs produced incorporated foreign genes such as chloramphenicol acetyl-transferase (CAT) or GFP and the transgenes were efficiently expressed(145).

Given VSV’s ability to activate a strong innate and adaptive immune response in vivo and infect many cell types, including mucosal surfaces, rVSV was initially developed as a vaccine vector(182). rVSV’s have been engineered to express an array of foreign proteins including HCV, influenza, and HIV antigens(183-186). Infection with these viruses generates an immune response against both VSV and the foreign protein, leading to production of antibodies and protection against both VSV and the vector of origin for the viral protein(184, 187, 188). This line of research continues to progress and new vectors that express proteins from Hepatitis B virus, Ebola, and SARS have been found to activate CD8 T cells, thus protecting mice from infection with these viruses(183, 186).

*Development of rVSV as an Oncolytic Vector*

As previously described, transgenes can be inserted into the VSV genome and functional virus can be recovered from cDNA (Figure 2.2)(138). This, as well as other strategies, is currently being studied with the goal of increasing the
attenuation and anti tumor efficacy of oncolytic rVSV. The first rSVVs generated to increase anti-tumor efficacy, contained the herpes thymidine kinase suicide cassette (TK) or the cytokine interleukin-4 (IL-4) inserted between the VSV G and L proteins(174). These viruses replicated to high titers and were highly oncolytic in vitro. TK and IL-4 were efficiently produced and VSV expressed TK retained the ability to phosphorylate gancyclovir, highlighting that trans-genes expressed from VSV can be functional. Furthermore, both TK and IL-4 expressing viruses increased protection against melanoma and adenocarcinoma models(174). These viruses were also able to induce an anti tumor T cell response required for prolonged tumor clearance. Similar findings have been reported for a number of other rVSV constructs, including VSV designed to increase bystander effect, VSV containing mutations in the M protein, replication incompetent VSV, and VSV that express genes involved in innate immunity(173, 179, 189-191). However, while many rVSV enhance the oncolytic ability and anti-tumor response, other constructs have been found to mitigate this effect. For example, VSV-CD40L was predicted to maximize the anti-tumor effect by providing co-stimulation to T cells by expressing the co-stimulatory molecule CD40L(192). However, this virus showed no discernable increase in anti-tumor efficacy when compared to VSV-GFP, whereas a replication incompetent AV-CD40L provided an increased survival benefit against B16 expressing ovalbumin(192). It is hypothesized that this finding is attributable to the skewing the immune response toward VSV antigens; the virus replicates efficiently in the tumor microenvironment providing
high levels of viral antigen and masking tumor antigens that are released during oncolysis.

**Oncolytic rVSV can Increase Bystander Effect**

In addition to the ability of oncolytic rVSV vectors to kill tumor cells, this strategy can also contribute to the bystander effect by expressing genes that interact with pro-drugs or by increasing the ability of drugs to infiltrate the tumor microenvironment. For example, VSV-TK as discussed, and VSV-cytosine deaminase (CD)/uracil phosphoribosyltransferase (UPRT) express high levels of CD/UPRT and increased the bystander effect when combined with 5-flourocystein (FC). 5-FC is a relatively non-toxic chemical that can be modified by CD to become the potent chemotherapeutic 5-florouricil (FU), which inhibits cell metabolism. Additionally, co-expression of CD and UPRT was found to increase the reactivity of 5-FC up to 168 times compared to CD alone and the VSV-CD/UPRT 5-FC combination therapy performed better in tumor models than 5-FU treatment alone.

**rVSV Expressing Genes that can Modulate the Immune Response**

As discussed, the innate immune response against VSV in normal cells is characterized by the production of IFN-β. Based on the success of this
mechanism, we constructed new rVSV, which expressed the murine or human IFN-β gene (VSV-m/hIFN-β)(173). Despite the fact that many transformed cells have defects in their ability to produce or respond to IFN induction, the newly generated rVSV was able to efficiently replicate, produce high levels of transgenic IFN-β and induce cell death(163, 173). Normal cells however, also respond to the expressed IFN-β, which can act in an autocrine or paracrine manner, and induce an antiviral state. As a result, VSV-IFN-β is more attenuated in vivo and may be useful in targeting cells that have defects in the innate immune response(163, 164). Furthermore, expression and subsequent production of IFN-β, from VSV-IFN-β, can stimulate anti-tumor host defense by bolstering the adaptive immune response(173). Other studies using a similar strategy have been conducted utilizing a variety of other transgenes including GMCSF, IL-12, and IL-23(196-199). This virus did not potently activate the interferon system when compared to VSV-EGFP, however, VSV-GMCSF did induce a more robust anti-viral T-cell response and increased the number of CD11b cells in the BAL fluid of infected mice(197). Additionally, VSV-GMCSF mediated T cell dependent clearance and generation of lasting memory against the murine mammary tumor line D2F2/E2, which expresses Her2/neu, in a Her2/neu dependent manner(197). In contrast, VSV23, which expresses IL-23, exhibits significant attenuation in vivo when administered IN(196). VSV23 replicated to lower titers in the brain, did not cause significant weight loss, and induced Nitric Oxide in the brain(196, 200). Lastly, VSV-IL-12 was shown to
increase the efficacy of VSV treatment against a murine head and neck carcinoma through an as yet unidentified mechanism (201).

**Genetically Modifying VSV Proteins (VSV-ΔM)**

Many viruses, including VSV, have developed strategies to block the immune response. The VSV peripheral Matrix protein (M), a multifunctional protein necessary for the proper assembly and propagation of new virus particles, is critical for the disruption of the immune response generated against VSV (137). In order to ablate the immune response, M enters the nucleus and inhibits mRNA export by binding to Rae1/Nup 98 mRNA export complex (142, 202). M also inhibits the transcription of genes transcribed by RNA polymerase II (203). Preventing transcription and translation by inhibiting export of mRNA is an effective method of disrupting the immune response after viral detection. Despite the presence of the appropriate signals in response to infection and the entry of transcription factors such as IRF3 into the nucleus, mRNA is unable to exit the nucleus, thereby inhibiting protein production. This leaves the host translation machinery available, which facilitates increased translation of viral proteins.

Another promising strategy for attenuating VSV in vivo is the mutation of the VSV M protein in order to recover host mRNA export, researchers independently identified the essential amino acid sequence, 51-MDTY-54, in M that facilitate the inhibition mRNA export (204, 205). Mutations of these critical amino acids also
released the block on host mRNA export without disrupting the trafficking and packaging functions of the M protein (205). Thus, it was postulated that a VSV-ΔM vector, in which these critical amino acids were mutated, would be attenuated in vivo because it will enable the escape of viral triggered innate immune mRNA such as type I IFN, and possibly induce a stronger anti-tumor response. To date, three different ΔM containing VSV vectors that allow efficient mRNA export have been created. For instance, our lab generated a triple mutant in which 52-DTY-54 was mutated to 52-AAA-54 using site directed mutagenesis which effectively inhibits the ability of M protein to block mRNA export (unpublished data). Additionally, Dahlberg et al. and Woo et al. have generated additional VSV-ΔM mutations including a methionine to arginine M51R mutation or deletion of the M at position 51 (Figure 2.2) (205, 206). All of these viruses are unable to block mRNA export, induce a more robust interferon response, and exhibit reduced toxicity 	extit{in vivo}. These three viral vectors are also effective against a variety of human and murine tumor models such as 4T1, PC-3, and CT-26 colon cancer, a
variety of glioma cell lines and ES-2 ovarian carcinoma models\(159, 190, 207\). These strategies have also been combined and different transgenes have been inserted into the VSV-\(\Delta\)M background. For example, one vector, \(rVSV(M\Delta51)-M3\), expresses Gamma Herpes Virus 68 M3 protein which is a high affinity chemokine binder. Infection with this virus increased numbers of infiltrating NK cells and neutrophils and protected mice from hepatocellular carcinoma\(206\). \(VSV\Delta M51\) has also shown efficacy against sub cutaneous rhabdoid tumor xenografts in CD-1 nude mice\(207\).

Deletion of VSV proteins (VSV-\(\Delta G\))

Although the previously mentioned \(rVSV\) vectors (i.e. \(VSV\)-IFN-\(\beta\), \(VSV\)-GMCSF, \(VSV(M\Delta51)-M3\)) are less toxic than wild type \(rVSV\) vectors, the use of live viruses as therapeutics raises concerns due to potential toxicity. Thus, alternative methods need to be thoroughly explored to ensure that the risk of toxicity is minimal. One potential method is to create replication deficient VSV vectors by deleting the surface glycoprotein G. G can then be added in trans, which creates a single cycle viral vector capable of only one round of infection, as the progeny virus cannot infect new cells as it lacks virally produced G protein\(208, 209\). This strategy shows promise in both tumor and vaccine studies. For example, our lab demonstrated that \(VSV-\Delta G-CE1/E2\), a virus that expresses HCV core, E1, and E2 proteins, was able to induce detectable titers of antibody against Core, E1 and E2 proteins as well as generate specific T cells.
against these proteins as well, if not better than wild type virus(184, 185). Additionally, replication deficient vectors represent a very safe option for use in tumor therapy(179, 210). Even though these viruses are replication incompetent it was shown that the mere presence of viral proteins in the tumor environment was sufficient to induce a host T cell response against the viral proteins and tumor antigens(179).

Retargeting VSV to Restrict Viral Tropism

While the previously described strategies attenuate rVSV, they do not address the fact that rVSV has an extremely wide tropism, including the CNS and neurons(153, 154). Although the \( \Delta G \) deletion prevents replication and attenuates the virus, a fully retargeted vector would offer an additional level of specificity. Previously, it has been demonstrated that the VSV G protein can be pseudotyped with other variants of VSV G protein, such as New Jersey versus Indiana serotype, or surface glycoproteins(106, 211, 212). In addition, with the solving of the crystal structure of the G protein, it may be possible to insert specific binding sequences or single chain antibodies into critical loops of the G protein, thus disrupting its normal binding ability and targeting the virus to a specific cellular receptor(104, 213). A similar strategy has proven effective using measles virus, however, because the G protein is responsible for both binding to an as yet undetermined receptor and fusion within a target cell, it is necessary that these manipulations ablate targeting without disrupting fusion.
A second method of restricting VSV tropism that is under development, is the incorporation of microRNA (miRNA) binding sites into the 3’ UTR of the VSV genome or individual VSV proteins. miRNA expression is often tissue specific and miRNA expression profiles are often altered in malignant tissues\(^{(214, 215)}\). For example, it was found that mir-124 and mir-125 are highly expressed in normal neurons but are down regulated in neural malignancies like glioma\(^{(216)}\). Therefore, if the miRNA corresponding to the binding site inserted into the VSV genome is expressed, the translation of VSV proteins will be inhibited, and viral replication will be attenuated\(^{(105, 216)}\). VSV bearing mir-125 (VSV-125r) binding sites are highly attenuated \textit{in vivo} and the tropism is restricted to mir-125 non-expressing neurons\(^{(105)}\). Mice infected with VSV-125r displayed restricted neural tropism, tolerated administration, and showed an anti-tumor efficacy against CT-26 colorectal carcinoma\(^{(105)}\). Similarly, the incorporation of a mir-let-7a binding site attenuated VSV replication in mir-let-7a expressing cells\(^{(217)}\). Another strategy is to combine various approaches such as miRNA restricted tropism, mutation of VSV genes, and transgene insertion, in order to develop new therapeutic strategies using rVSV as an oncolytic vector.

\textit{Combination Therapy Using VSV}

It is possible that a combination of different anti-cancer treatments may exert enhanced therapeutic efficacy compared to a single treatment strategy\(^{(218, 219)}\). There are many different drug cocktails available for cancer treatment and
these medications can be prescribed in conjunction with immunotherapy, small molecules, surgery, and radiation. For example, ovarian cancer is often treated initially by surgical debulking of the tumor followed by administration of platinum based drugs cocktails that include paclitaxel plus cisplatin or carboplatin. Breast cancers are treated with surgery, radiation, and monoclonal antibodies or tyrosine kinase inhibitors(220-222). Given this, it is possible that current treatment strategies could be combined with rVSV. For instance, research has demonstrated that VSV, in combination with a small molecule BCL-2 inhibitor, Obatoclax, increased apoptosis in A20 B lymphoma cells, and this combination was found to significantly delay tumor progression in mice treated with SC A20 xenografts(223). Furthermore, a combination of treatments consisting of Histone deacetylase inhibitors (HDIs), which prevent transcription of antiviral genes in response to infection, and VSV sensitized refractory PC-3 human prostate cancer, HT29 human colon adenocarcinoma, SW620 human colon cancer, M14 human melanoma and 4T1 murine breast cancer cells in vitro, and enhanced oncolytic VSV infection in primary human tumor samples and in animal models(224). Additionally, rVSV expressing the fusion associated small-transmembrane (p14FAST) protein or harboring the ΔM51, treatment was shown to be more efficacious when combined with a double deleted vaccinia virus (VVDD) even though the incorporation of p14FAST lead to an increase in neuropathogenesis(225). The observed increase in rVSV titer was dependent on the VV B18R gene product(226). Also, VSVΔM51 has also been used successfully against gliomas that are non-permissive to VSV infection due to
their ability to produce and respond to IFN-β (207). This was overcome by impairing mTORC1 dependent IFN-β by pretreatment with Rapamycin that facilitated VSV mediated oncolysis in these non-permissive cell lines (227).

There are many other potential combination treatments that could be used in conjunction with rVSV, such as: 1) rVSV could be used post-operatively to seek out and destroy metastasis not removed during surgical resection of a tumor (228). 2) rVSV vectors may be able to target chemotherapeutic resistant cancer cells if these cells have defects in either innate immune responses or translation regulation (229). Currently, additional studies should attempt to add a number of transgenes to enhance the antiviral and anti-tumor response by further activating and modulating the immune system. Furthermore, it may be possible to increase the efficacy of rVSV by using viral vectors that express different transgenes in combination or sequentially. Finally, development and study of treatment regimens such as giving multiple doses at varying time points of the same or pseudo-typed rVSV vectors is essential. Currently, data indicate that a single dose of replication competent rVSV, given 3 or 4 days after IV tumor inoculation or IT into small SC tumors is most effective, however further research into dosing schedules and concentrations are needed. An additional strategy being developed to circumvent the immune response to VSV, which may allow for additional doses of VSV to be administered, is the use of a carrier cell line that could deliver VSV to the tumor site without activating a secondary immune response. In fact, it was demonstrated that, in pre-immunized mice harboring CT-26 tumors, the use of a syngeneic leukemia cell line significantly enhanced
the oncolytic potential of a second administration of VSV leading to marked tumor regression and delayed the secondary immune response against VSV(230). A similar method has been used to increase the delivery of VSV into the immunosuppressive tumor microenvironment by loading VSV onto antigen specific OT-I T cells thereby targeting the delivery of VSV to ova expressing tumor cells. This method also highlighted the necessity of host T cells and NK cells in mediating maximal anti tumor response(231-233).

Conclusions

Evidence indicates that oncolytic virotherapy has the potential to enhance the treatment of malignant disease. There are a number of viruses that exhibit oncolytic activity including reovirus, measles, Newcastle disease virus, mumps, influenza, and adenovirus. Some of these viruses are currently being tested in clinical trials, and positive initial findings have helped to generate interest in oncolytic VSV(84, 234-237). Also, the production of clinical grade rVSV and the positive results from toxicology studies in rats and non human primates have set the stage for a Phase I clinical trial of VSV-IFN-β for cancer treatment(234, 236, 238, 239). Although progress has been made in determining the mechanisms of direct viral oncolysis, much work remains in order to fully understand this phenomenon. Oncolytic rVSV exhibits the preferential ability to replicate in and kill most malignant cells. It is clear that both defects in translation regulation, highlighted by work on the NFARs, PKR, and eIF2B complex, as well as defects
in innate IFN signaling pathways that have been characterized in a number of tumors facilitate oncolysis (32, 116, 134, 160, 240, 241). Furthermore, rVSV has many other characteristics that make it an optimal oncolytic vector including genetic malleability, non-integrating or transforming genome, and low pathogenicity in an immunocompetent setting. Despite these benefits, there are risk factors associated with rVSV due to its wide tropism and its ability to infect the CNS. However, current research is focusing on new strategies, including receptor targeting, trans-gene insertion, genome manipulation, and miRNA targeting, to address these risks and create safer and more effective oncolytic rVSV.

Specific Aims

We hypothesize that the insertion of a tumor suppressor gene that also has immunomodulatory function, like p53, into VSV that specifically harbors the ΔM mutation will generate a more effective oncolytic vector. In order to test these new vectors we propose the following aims:

**Specific Aim 1: Characterization of the efficiency and kinetics of rVSVs in cell culture.**

a. Determine whether new rVSV is able to preferentially replicate in transformed cells and express the trans-gene; IPS-1, TRIF, mp53.
b. Examine the functional effects of IPS-1 and TRIF gene expression from VSV.

c. Determine the activation status and gene expression profile of p53 in the context of delivery by VSV.

Specific Aim 2: To use the information gained in Aim 1 to test the efficacy and verify the mechanism of VSV-mp53, VSV-ΔM-mp53, VSV-IPS-1, and VSV-TRIF in a mouse model.

a. Perform a series of survival studies to determine if rVSV can protect mice from metastatic colonization of the lungs using the BALB/c syngeneic mouse mammary adenocarcinoma TS/A that expresses the luciferase gene (TS/A-luc).

b. Develop a solid tumor model using TS/A-luc in order to determine the role of the innate immune system in tumor regression and verify gene induction in-vivo.

c. Examine the ability of rVSV to induce cross presentation of tumor specific antigen in vivo.
Chapter 3: VSV-$\Delta$M Expressing mp53 is a Safe and Effective Oncolytic Vector

Background

Cancer continues to be a leading cause of morbidity and mortality worldwide, necessitating the continuing development of new therapeutics in order to combat this disease. Current cancer therapeutic strategies include surgery, radiation, chemotherapy, and immunotherapy. These treatments are designed to exploit specific characteristics of cancer cells(242-244). However, current treatment modalities that take advantage of these defects can lead to debilitating side effects as well as the selection of more aggressive malignant disease(245). Viral oncolysis is possible due to acquired genetic defects in innate immune signaling and translation regulation pathways acquired during transformation(116). Oncolytic virotherapy has the potential to prevent metastasis and initiate a host derived anti tumor response making it an attractive candidate for development(246). Furthermore, mutating and/or inserting transgenes into the viral genome can generate highly attenuated oncolytic viruses.

Numerous viruses are now known to have the ability to selectively kill cancer cells. Although the mechanisms of oncolysis remain unclear, it is possible that intracellular innate immune responses and/or translation regulation pathways are defective in these cells(32, 33, 70). Viral oncolysis also efficiently promotes cell death thus providing a supply of tumor antigens, which may be
recognized by the host immune system\(^{(246)}\). These characteristics have enabled the use of oncolytic virotherapy as a treatment option. Oncolytic viruses show promise as a new therapy because these agents have potential to induce a host anti-tumor response aside from exerting direct oncolytic ability\(^{(173)}\). Viruses being considered as oncolytic agents include MV, AV, HSV, CV, and VSV.

VSV is a 11kb negative-stranded RNA virus of the rhabdoviridae family. It is non-transforming, has a low seroprevalence, causes minimal morbidity and mortality in humans, and is genetically malleable allowing for trans-genes of interest to be incorporated into the genome generating rVSV. For example, previously our lab demonstrated that rVSVs designed to express cellular genes that modulate immunity, such as IFN-\(\beta\) and exhibited effective oncolytic activity with increased immune activation\(^{(173, 189)}\).

Previous work by our lab and others have shown that the insertion of transgenes such as Type I IFN-\(\beta\), Thymidine Kinase (TK) or Green Fluorescent Protein (GFP) into VSV between the glycoprotein (G) and the large polymerase protein (L) does not attenuate viral replication in permissive cell lines and leads to high expression of the incorporated trans-gene \(^{(173, 174, 189, 197, 205)}\). Further, it has also been shown that mutation of the matrix (M) protein at critical amino acid residues produces a virus that is incapable of blocking mRNA export from the nucleus and facilitates a more robust innate immune response characterized by an increase in IFN-\(\beta\) and ISG production\(^{(202, 206, 247)}\).
The stimulation of innate immune responses is essential to facilitate up regulation of antigen presentation machinery, cytokines, chemokines, and adhesion molecules (248, 249). P53 is a well-documented regulator of many ubiquitous cellular processes and is mutated or deleted in more than 50% of all cancers. It can modulate signal transduction pathways including cellular senescence and cell cycle pathways (248, 250). Additionally, it can affect the expression of several transcriptional gene expression profiles including DNA repair and apoptosis (251). Recently it was described that p53 inhibits tumor formation through mechanisms that stimulate innate immunity such as cross talk with the IFN system (249). We therefore incorporated the murine p53 (mp53 gene) into VSV hypothesizing that this could yield a safer and more effective oncolytic vector.

VSV infection is a weak activator of the type I IFN response in part due to the matrix (M) proteins ability to inhibit mRNA export (142). In order to enhance VSV’s ability to induce the innate immune response we developed an rVSV in which amino acids 52-54 of the M protein were mutated from DTY to AAA yielding VSV-ΔM. This mutation inhibits M protein from binding to the Rae1/Nup98 mRNA export complexes and allows cells infected with VSV-ΔM to efficiently export mRNA from the nucleus, facilitating the initiation of a stronger immune response in cells with intact innate immune signaling pathways. Additionally, we choose to incorporate the murine tumor suppressor protein 53 (mp53) into the VSV-ΔM and original VSV-XN2 background creating VSV-ΔM-mp53 and VSV-mp53.
Results

Generation of Oncolytic VSV-ΔM-mp53 and VSV-mp53

To evaluate novel VSVs that exhibit increased safety and efficacy \textit{in vivo}, the murine p53 gene (mp53) was cloned between the G and L proteins of VSV (VSV-XN2) or VSV with mutated M protein (VSV-ΔM), which is impaired in blocking host mRNA export. The resulting plasmids were then used to recover functional viral particles as previously described (Figure 3.1A)(138). To verify that the resulting VSV-ΔM-mp53 and VSV-mp53 retain oncolytic specificity, C57BL/6 mouse embryonic fibroblasts (MEFs), a murine mammary adenocarcinoma TS/A, and the murine melanoma B16(F10) cell lines were infected at MOIs of 0.01, 0.1, 1, and 5 for 24 hours. Cytopathic effects (CPEs) were observed using brightfield microscopy (Figure 3.1B). Our results indicated that infected cells round up and detach which are both characteristics of CPEs in VSV permissive cell lines TS/A and B16(F10) whereas MEFs show minimal CPE(116). The cells were lysed and subjected to immunoblotting to confirm the expression of the mp53 transgene and VSV proteins in infected malignant cells (Figure 3.1C). This analysis indicated that VSV-mp53 and VSV-ΔM-mp53 efficiently expressed the mp53 transgene in cell lines that are permissive for VSV replication and provided evidence that normal MEFs do not support robust viral replication.
Figure 3.1: VSV-ΔM-mp53 and VSV-mp53 retain oncolytic ability and express mp53. A, Schematic representation of VSV p53 constructs. B, Immuno blot analysis for VSV and mp53 protein expression in infected C57BL/6 MEF, TS/A or B16(F10) cells infected at an MOI of .01, 0.1, 1, and 5 24 hours post infection. C, Brightfield microscopy of VSV-ΔM-mp53 and VSV-mp53 infected cells at an MOI of .01 and 5 24 hours post infection.
To evaluate whether the new VSV-mp53 and VSV-ΔM-mp53 retained the ability to replicate efficiently in and kill transformed cells, C57BL/6 MEF, TS/A murine adenocarcinoma, and B16(F10) melanoma cells were infected with rVSVs. Our data indicated that VSV-mp53 and VSV-ΔM-mp53 as well as control VSV-GFP and VSV-ΔM-GFP, effectively killed transformed TS/A and B16(F10) cells and replicated to similar titers within 24 hours. Importantly, VSV-mp53 and VSV-Δ-mp53 retain oncolytic specificity as C57BL/6 MEF remains largely unaffected by rVSV infection at low or high MOI 0.1 and 5 (Figure 3.2A). In contrast, VSV-mp53 and VSV-ΔM-mp53 exerted extensive oncolytic activity against TS/A and B16(F10) to a slightly lesser extent cells over 24 hours (Figure 3.2B and C). It has also previously been shown that wild type cells respond to IFN production in response to infection and can inhibit viral replication by activating PKR, which phosphorylates eIF2α to inhibit translation. Our analysis indicates that C57BL/6 MEFs are less permissive to VSV infection than transformed TS/A and B16(F10), likely the result of intact translation regulation mechanisms (Figure 3.2D, E, and F). C57BL/6 MEFs allow 2-3 logs less virus to be released up to 24 hours post infection compared to TS/A or B16(F10) which allow large amounts of viral progeny to be produced. These data clearly indicate that insertion of mp53 into either XN2 or ΔM background does not inhibit the killing or replication abilities of these viruses in-vitro.
Figure 3.2: VSV-ΔM-mp53 and VSV-mp53 replication in vitro. C57BL/6 MEF, TS/A, or B16(F10) cells were infected with rVSVs at an MOI of 0.1, 1, or 5. Cells and supernatants were collected 6, 12, 18, and 24 hours post infection. A, B, and C, indicate cell death at each time point as determined by Annexin V/Propidium iodide staining. D, E, and F, supernatants were analyzed by the standard plaque assay to determine the replication of rVSVs.
Expressed mp53 is phosphorylated and activates transcription of target genes.

It is well known that mp53 undergoes a variety of post-translational modifications such as phosphorylation, ubiquitination, sumoylation, acetylation, and neddylation in order to regulate its activity (252, 253). For example, murine p53 is primarily activated by phosphorylation on serine 18 leading to dissociation from its primary negative regulator MDM-2. In addition serine 389 is phosphorylated which induces p53 oligomerization necessary for DNA binding and transcription activation (254, 255). To evaluate whether mp53 expressed by VSV-mp53 and VSV-ΔM-mp53 was active, mock and infected cell lysates were immunoblotted using a murine p53 antibody. This analysis confirmed efficient expression of the mp53 transgene that correlates well with the ability of rVSV to preferentially replicate in transformed cells (Figure 3.3A). Additionally, membranes were probed with phospho-serine 18 or 389 specific antibodies to determine if virally expressed mp53 is phosphorylated on serine residues necessary for stabilization and transcription activation (Figure 3.3A). Serine 18 is phosphorylated by DNA-PK in response to cellular stress causing dissociation from MDM-2, its E3 ubiquitin ligase negative regulator, thus promoting the stabilization of p53. Additionally, it was shown that the DNA binding and transcription activation ability of p53 is dependent on phosphorylation of serine 389 by using p53 knockout cells that were reconstituted with p53 mutants at critical serines. Confirmation that mp53 is phosphorylated on serines 18 and 389 provided initial evidence that mp53 produced during viral replication is
capable of being activated and may be able to initiate transcription of target genes. To next ascertain if phosphorylated VSV expressed mp53 is transcriptionally active we performed a p53 luciferase reporter assay (Figure 3.3B). Essentially, VSV-mp53 and VSV-ΔM-mp53 were used to infect cells transiently expressing a luciferase gene under control of a p53 promoter element. Although TS/A cells show higher levels of luciferase expression, likely the result of their ability to support higher levels of viral replication, C57BL/6 MEF also show induction of the p53 reporter indicating that even though virally expressed mp53 is undetectable in normal cells by immuno blot, it can still activate transcription of target genes. Another observation from this experiment is that the luciferase expression was higher in cells infected with VSV-ΔM-mp53, likely due to the ΔM mutation, which facilitated enhanced export of mRNA message from the nucleus(202, 204). Use of the VSV-ΔM background is particularly relevant for mp53 incorporation since it acts primarily as a transcription factor to induce p53 target genes and ΔM should permit mp53 induced gene products to be exported and translated more readily than those induced by VSV-mp53. Although VSV efficiently blocks mRNA export and can inhibit the transcription of RNA polymerase II transcripts, low levels of luciferase expression were observed from VSV-mp53 indicating that either the block on mRNA export is not absolute or that high levels of mp53 expression can overcome the M proteins block on nuclear export (Figure 3.3B)(202, 203). Furthermore, it has also been shown that viral infection, specifically VSV, can induce activation of mp53 through a cross-talk mechanism between the type IFN response and mp53, accounting for
Figure 3.3: Expressed mp53 is functional. A, C57BL/6 MEF, TS/A, or B16(F10) cells were infected with rVSV at an MOI of 0.1 and 5. Cells were lysed 24 hours post infection and used for immuno blot analysis. B, C57BL/6 MEF or TS/A cells were transfected with a p53 luciferase reporter and then infected at an MOI of 0.1 or 5. Luciferase levels were assessed 24 hours post infection. C, C57BL/6 MEF or TS/A cells were infected at an MOI of 10 or 1 respectively and mp53 target gene mRNA expression was determined. Red box MDM-2, Blue box CDKN1a (p21).
the low level of luciferase activation seen from VSV-ΔM(249). To examine the ability of virally encoded mp53 to activate transcription of target genes we assessed a panel of 112 mp53 targets using a p53 microarray (SA Biosciences) (Figure 3.3C). MEF and TS/A cells were infected at MOIs of 10 or 1 for one hour respectively and incubated for 8 hours. VSV infection in general activated a number of p53 associated genes however, VSV-ΔM-mp53 induced robust production of MDM-2 (red box) and p21 (blue box) mRNA 8 hours post infection. Both genes are well known transcriptional targets of p53. This data highlights the functional advantage of using the VSV-ΔM background since MDM-2 and p21 mRNAs are only observed in cells infected with VSV-ΔM-mp53. These data also indicate that virally expressed murine p53 especially from VSV-ΔM, is phosphorylated and transcriptionally active.

VSV-ΔM-mp53 is highly attenuated in Balb/c mice

To begin to evaluate the in vivo affects of VSV-ΔM-mp53 we carried out preliminary toxicity assays. Balb/c mice (n=7) were infected intravenously (IV) with escalating doses of 1x10^8-1x10^9 pfu of VSV-ΔM-mp53, VSV-mp53, VSV-ΔM-GFP, VSV-GFP, and VSV-IFN-β (Table 3.1). Mice infected with VSV-GFP or VSV-IFN-β exhibited toxicity only at high doses. VSV-mp53, VSV-ΔM, and VSV-ΔM-GFP exhibit more attenuation as mice treated with these viruses succumbed to higher doses of 5x10^8 or 1x10^9 pfu. Surprisingly, 84% (6/7) of the mice
treated with VSV-ΔM-mp53 compared to 14% (1/7) of mice treated with VSV-ΔM survived infection with 1x10^9 pfu twenty times more virus than the treatment dose used in the tumor model. This experiment highlighted that the combination of VSV-ΔM with mp53 was having an additive effect since mice infected with VSV-ΔM or VSV-mp53 have increased mortality compared to VSV-ΔM-mp53. This data also suggests that VSV-ΔM-mp53 is highly attenuated and can be used for in vivo tumor models.

To assess the anti-tumor efficacy of VSV-ΔM-mp53 and VSV-mp53 we chose a Balb/c syngeneic tumor model using TS/A mammary adenocarcinoma stably transfected with a luciferase reporter (TS/A-luc) developed by our lab(174, 256). Wild type Balb/c (n=7) mice were injected IV with 1x10^5 TS/A-Luc cells. Three days later mice were treated with 5x10^7 or 5x10^8 pfu of VSV-ΔM-mp53, mock, or 5x10^7 pfu of control viruses VSV-GFP, VSV-mp53, and VSV-ΔM. One advantage of TS/A-luc is that this reagent allows for in vivo observation using the in vivo imaging system (IVIS). Measuring luciferase activity can thus be used to assess relative tumor burden. Representative images along with luciferase signal quantification, from day 14 and 28 after tumor administration, are shown for mock, VSV-ΔM-mp53 and VSV-

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Table 3.1: VSV-ΔM-mp53 is highly attenuated in vivo. Balb/c mice (n=7) were infected IV with escalating doses of rVSV and mortality was monitored.
mp53 treated mice (Figure 3.4A). Noticeably, luciferase activity is absent in mice treated with VSV-ΔM-mp53 up to 28 days post tumor inoculation. This strategy, of using a single dose of VSV-ΔM-mp53, significantly protected Balb/c mice from TS/A-luc tumor formation when treated with 5x10^7 pfu compared to control rVSVs (n=7, p < .05). Mice treated with VSV-ΔM, VSV-mp53, VSV-GFP and 5x10^8 pfu of VSV-ΔM-mp53 had median survival times ranging from 40 to 75 days however less than 50% of VSV-ΔM-mp53 succumbed over the 120 day experiment and a mean could not be determined (Figure 3.4B). Also, due to VSV-ΔM-mp53’s low toxicity we hypothesized that using an increased dose would lead to enhanced anti tumor efficacy, however treatment with 5x10^8 pfu protected only 14% of animals compared to treatment with 5x10^7 pfu in which 71% of mice survive (Figure 4B). It is counterintuitive that more virus should yield less protection however it is possible that the increased viremia skews the immune response to viral antigens and does not allow a functional host anti tumor response to occur. The surviving mice treated with VSV-ΔM-mp53 (n=4) and naïve mice were re-challenged with 2x10^5 TS/A-luc cells to determine if lasting immunity against the tumor was generated. This data suggests that immunologic memory is generated against TS/A-luc and total tumor clearance is likely dependent on a host immune response since 75% of re-challenged mice survived an additional 250 days until sacrifice. Additionally, one of the re-challenged mice did succumb to the tumor, likely indicting immune escape of the tumor, but only after doubling the median survival time of the naïve treated mice (60 vs. 30.5 days, p=.004) (Figure 3.4C).
Our analysis indicated that immunologic memory was generated by mice treated with VSV-ΔM-mp53 (Figure 3.4C). We therefore sought to determine if VSV-ΔM-mp53 was able to modulate the immune compartment and host anti-tumor response in treated mice (Figure 3.5A and B). We therefore inoculated...
mice (n=6) with VSV-ΔM-mp53, VSV-ΔM, VSV-mp53 and VSV-GFP and measured splenic CD8+ cells (Figure 3.5A). We then performed an Enzyme Linked Immunospot (ELISPOT) assay using mitomycin C treated TS/A-luc cells as the target to determine if VSV-ΔM-mp53 increased the number of tumor specific IFN-γ secreting T-cells compared to control viruses (Figure 3.5B). Indeed, tumor bearing mice receiving VSV-ΔM-mp53 had elevated numbers of tumor specific CD8+ T cells compared to control VSV-ΔM and VSV-GFP. We then repeated the toxicity and tumor model experiments in athymic Balb/c Nude mice to further establish the necessity of an intact adaptive immune compartment for complete tumor clearance. It has previously been reported that mice lacking T cells succumb to VSV infection at low doses(119). To determine the role of T cell activity in p53 action, athymic Balb/c nude (n=5) mice were infected IV with 5x10^7 pfu or IN with 5x10^8 pfu VSV-ΔM-mp53, VSV-mp53, control rVSVs and their survival was monitored (Figure 3.5C). Surprisingly, these mice tolerated IV treatment with 5x10^7 pfu of VSV-ΔM or VSV-ΔM-mp53. This analysis indicates that protection from modest doses of VSV-ΔM-mp53 is likely due to modulation of the innate immune response and not on T cells. It is known that p53 can be induced by IFN signaling, enhance IFN signaling, and plays a role in the antiviral response to VSV since p53 knockout mice display increased susceptibility to VSV infection(249, 257). However, all mice succumbed to IN inoculation (Figure 3.5C). This demonstrates that the route of administration may also be an important factor in rVSV’s toxicity. We next assessed rVSV anti tumor efficacy using TS/A-luc in athymic Balb/c Nude (n=5) mice using the treatment schedule
previously described (Figure 3.5D). Interestingly, all nude mice treated with rVSV succumbed to tumor formation highlighting the different requirements for an intact T cell compartment in eliciting protection from TS/A-Luc tumor formation versus involvement in the control of VSV-ΔM-mp53 infection.

**Figure 3.5:** VSV-ΔM-mp53 modulates T cells and is attenuated in Athymic Balb/c Nude mice. A, Balb/C mice n=3 were infected with 5x10^7 pfu rVSV. 96 hours post infection spleens were removed and total splenocytes were assessed for the percentage of CD8+ cells by flow cytometry (* p<.05, ** p<.005 Bonferroni post test). B, Balb/c mice (n=6) were initially given 1x10^5 TS/A-luc cells IV on day 0 and were then either mock or rVSV treated with 5x10^7 pfu on day 3. On day 13 mice were sacrificed and total splenocytes were used for an IFN-γ ELISPOT using mitomycin C treated TS/A-luc cells as the target (* p<.05 Dunnett’s Multiple Comparison Test). C, Athymic Balb/c Nude mice (n=5) were infected with 5x10^7 pfu IV and 5x10^8 pfu IN and mice were observed for mortality. B, Athymic Balb/c Nude mice (n=5) were injected with 1x10^8 TS/A-luc cells on Day 0 and mock or treated with 5x10^7 pfu rVSV on Day 3 and then observed for survival.
In addition to modulating CD8+ T cells it was also possible that treatment with VSV-ΔM-mp53 could be altering additional immune effector cells and cytokine profiles in treated mice. To elucidate a further mechanism of protection and reduced toxicity we sought to characterize the splenic and cytokine profile of mice after rVSV administration (Figure 3.6). In addition to CD8+ T cells (Figure 3.5A) the population of CD49b+ cells, an NK marker, also increased in the spleens of mice infected with VSV-ΔM-mp53 96 hours post infection (Figure 3.6A). Serum cytokine levels were then tested using a Multiplex and IFN-β ELISA. Treatment with VSV-ΔM-mp53 and control rVSVs lead to expected increases in antiviral induced cytokines like TNF-α and RANTES(42) (Data not shown). However, treatment with VSV-ΔM-mp53 appeared to blunt the production of inflammatory cytokines IL-6 and IP-10 as reduced levels were observed in VSV-ΔM-mp53 infected mouse serum (Figure 3.6B and C)(42, 133). Furthermore, VSV-ΔM-mp53 significantly increased the amount of IFN-β present in the serum 6 hours post infection (Figure 3.6D). The modulation of cytokine profiles in these mice may begin to explain the reduced toxicity displayed by VSV-ΔM-mp53 by limiting the inflammatory response and increasing IFN-β production in these mice which limits virus spread and may also help to facilitate increased tumor clearance(173).
Discussion

Increasing evidence indicates that there are strategies, which upon combination, may be used to increase the attenuation and efficacy of rVSV.
These include mutating VSV proteins (VSV-ΔM) and inserting transgenes that modulate the innate and adaptive immune response (173, 174, 179, 197, 216). Here, our data clearly demonstrates that the insertion of mp53 into the VSV-ΔM background produces a highly attenuated and effective rVSV. It is well known that rVSV preferentially replicates in and kills transformed cells in-vitro and in-vivo, and VSV-ΔM-mp53 retains this ability(160). Furthermore, the mp53 transgene is efficiently expressed and phosphorylated on serines 18 and 389. Phosphorylation on serine 18 enables dissociation of mp53 from MDM-2, an E3 ubiquitin ligase that negatively regulates mp53 by targeting it for proteasomal degradation. Phosphorylation on serine 389 allows oligomerization and DNA binding to occur, a necessary step for transcriptional activation as was demonstrated using phospho-mimetic mutant p53(258-261). Phosphorylated mp53 expressed from rVSV activated a luciferase reporter, more potently when expressed from the VSV-ΔM background, and induced MDM-2 and p21 mRNA expression, known mp53 targets. This indicated that p53 expressed in the context of the VSV genome is functional and active. mp53 has the ability to exert many effects when transcriptionally active in cells. For example, p53 reactivation in heptocellular carcinoma lead to induction of innate immune signaling, trafficking, and activating molecules and lead to tumor regression(248). It has also been reported that p53 can cross talk with the IFN system and plays an essential role in anti viral immunity. IFN inducible ISGF3 can activate transcription of p53 and TLR3 production can be initiated by p53(262). Additionally, IFN treatment can activate apoptotic pathways in both p53
dependent and independent manners(263). Interestingly, mice that have an extra copy of the p53 gene, “Super p53” mice are remarkably resistant to VSV infection whereas p53 knockout mice are highly susceptible to infection highlighting the potential role for p53 in anti-viral immunity(257). Furthermore, p53 is known to regulate, cell cycle, apoptosis, miRNAs, and senescence, all of which may be a benefit when expressed from VSV-ΔM-mp53 in the tumor microenvironment(251, 264, 265).

VSV-ΔM-mp53 was highly attenuated in vivo and showed remarkable ability to protect Balb/c mice from TS/A-luc tumor formation. The reduced toxicity associated with this virus allowed administration of twenty times more virus without mortality. However, increasing the treatment dose ten fold to 5x10^8 pfu did not increase VSV-ΔM-mp53 anti-tumor efficacy. It is possible that by increasing the viral load the secondary immune response becomes more heavily focused on viral antigens and does not respond to released tumor antigens. Furthermore, it is possible that infection at higher doses may lead to an increase in regulatory T cells or myeloid derived suppressor cells (MDSC) which would dampen an anti-tumor response. Surprisingly, VSV-ΔM-mp53 and VSV-ΔM appeared to be somewhat attenuated in athymic Balb/c nude mice indicating that viral clearance is not completely dependent on T cells and that VSV-ΔM-mp53 was likely modulating the innate immune response in order to control viral infection.

It has previously been shown that oncolytic virotherapy using VSV as well as other viruses, can lead to cross presentation of tumor antigens and induction
of a host anti-tumor response.(246) We therefore determined whether VSV-ΔM-mp53 treatment might modulate components of the innate and adaptive immune systems in order to elicit a cross presentation response and facilitate tumor clearance. Indeed, mice treated with VSV-ΔM-mp53 showed increased numbers of tumor specific CD8+ T cells and CD49b+ NK cells in the spleen 96 hours post infection. Increasing the number of CD49b+ NK cells is advantageous as these cells have cytolytic abilities and can directly kill virus infected tumor cells, leading to the production of tumor cell debris, which can be processed and displayed by antigen presenting cells leading to the generation of tumor specific CD8+ T cells that can enhance tumor protection(23, 79, 266-269). It is thus not surprising that athymic Balb/c nude mice were not protected from TS/A-luc tumor formation since these mice lack T cells.

VSV-ΔM-mp53 was found to modulate the cell populations in the spleen as exhibited by CD49b+ cells. VSV-ΔM-mp53 also altered the serum levels of cytokines and chemokines. Normally viral infection leads to production of inflammatory and antiviral cytokines and chemokines like IL-6, IP-10, TNF-α, and IFN-β. However, treatment with high doses of rVSV can lead to increased expression of these cytokines and chemokines, which can cause lethality by inducing a fatal cytokine storm(49, 270). Our data indicated that VSV-ΔM-mp53 administration lead to a reduction in the serum levels of IL-6 and IP-10 24 hours after treatment while serum IFN-β levels were elevated 6 hours post infection. mp53 has been reported to be a potential negative regulator of IL-6 and suppressor of macrophage activation(271). IL-6 can act as a pro-inflammatory
cytokine that signals through IL-6rα/gp130 receptor complex and activates janus kinases (JAKs) which in turn activates a number of signal transducer and activator of transcription (STAT) molecules (272). IL-6 is also the primary mediator of the acute phase fever response and is one of the components associated with fatal cytokine storm caused by infection with increased doses of rVSV. IP-10 (CXCL10) levels were also reduced in the serum of VSV-ΔM-mp53 treated mice. IP-10 is a proinflammatory chemokine that functions as a chemo attractant for macrophages, DC and NK cells by binding to CXCR3 (78, 273). The reduction in serum IP-10 may reduce immune cell infiltration into sites where increased inflammation may be deleterious to the host. Furthermore, the decreased inflammation associated with VSV-ΔM-mp53 could allow for a more finely tuned anti tumor response to occur by not skewing the immune response against the viral infection. Thus, it is possible that a reduction in the expression of IL-6 and IP-10 along with the suppression of macrophage activation in the presence of increased levels of IFN-β may facilitate a reduction in VSV-ΔM-mp53 toxicity and enhance anti tumor efficacy.

Collectively, our data indicates that VSV-ΔM-mp53 is an oncolytic vector with enhanced safety and efficacy. We demonstrate that this virus retains its specificity to preferentially replicate in and kill transformed cells and efficiently expresses the inserted transgene, which upon expression becomes phosphorylated and transcriptionally active. Animals tolerate increased doses of VSV-ΔM-mp53 and treatment with this virus facilitated better tumor protection in Balb/c mice. Thus, VSV-ΔM-mp53 enhances the oncolytic properties and
potential of VSV and can be considered a valid therapeutic strategy for translational development.

Materials and Methods

Cells and Transfection

C57BL/6 and 129/B6 MEF cells were maintained in DMEM supplemented with 10% FBS, 5% Penicillin/Streptomycin, and 1% Non-Essential Amino Acids. B16(F10) cells were maintained in Low Bicarbonate DMEM and supplemented with 10% FBS, 5% Penicillin/Streptomycin, and 1.5g/L Sodium Bicarbonate. BHK cells were maintained in DMEM supplemented with 10% FBS, 5% Streptomycin/Streptomycin TS/A and TS/A-luc were maintained in RPMI-1640 supplemented with 10% FBS, 5% Penicillin/Streptomycin, and 10mg/mL Puromycin for TS/A-luc. C57BL/6 MEF were transfected using Lipofectamine LTX and PLUS reagent (Invitrogen), whereas all other cell lines were transfected using Lipofectamine 2000 (Invitrogen) per the manufacturers protocol.

Generation of VSV-mp53 and VSV-DM-mp53

In order to obtain the mp53 cDNA PCR was performed using Pfx super mix (Invitrogen) and oligos: FWD 5’-TTATGTCGACATGACTGCCATGGAGGAGTC-3’ AND REV 5’-GCTAGCAGCCCTGAAGTCATA-3’. The PCR product was then ligated into pCR-Blunt II Topo (Invitrogen) and sequence verified. mp53 was then sequentially digested using Sall and Nhel restriction enzymes (NEB) and
subsequently gel purified. The purified fragment was then cloned into the $XhoI$ and $NheI$ sites of VSV-XN2(138) and VSV-$\Delta M$. VSV-$\Delta M$ was generated by mutating amino acids 52-54 of the M protein from DTY to AAA using a site directed mutagenesis kit (Stratagene) using oligos for amino acid 52 D to A FWD 5'-GGAGTTGACGAGATGGCCACCTATGATCCGAATC and REV 5'-GATTCCGGATCATAGGTTGCCCCATCTCGTCAACTCC for amino acid 53 T to A FWD 5'-GGAGTTGACGAGATGGACGCACCTATGATCCGAATC and REV 3'-GATTCCGGATCATAGGTTGCCCCATCTCGTCAACTCC and for amino acid 54 Y to A FWD 5' - TTTGGAGTTGACGAGATGGGACCGCTGATCCGAATCAATTAAG and REV 5'-CTTAATTGATTCCGGATCAGCGGTGTCCATCTCGTCAACTCCAAA. Virus was then grown using a cDNA viral recovery protocol. Briefly, 10 cm plates were seeded with BHK cells and grown to 70-80% confluence. Cells were washed 1X with PBS and then infected with Vaccinia virus that expresses T7 Polymerase (VtF7) at an MOI of 5 for 45 min in 3mL of serum free DMEM. Cells were then transfected as previously described with pBluescript plasmids that express VSV-N (2.8ug), P (5ug), and L (2ug) proteins and the rVSV genome also in a pBluescript vector (10ug) all under control of a T7 promoter. After 48 hours supernatants are collected and filtered through 0.2 µm filters 2x and plated onto fresh 70% confluent BHK cells. Supernatants from these infected cells are then aliquoted and stored at -80C for future use.
**Purification of rVSV**

4- 15cm cell culture dishes were seeded with BHK cells grown to 70% confluence. 100ul of cDNA recovery supernatant was added to each plate. After 24-30 hour incubation supernatants are collected and centrifuged into a 10% sucrose cushion at 27k rpm for 1 hour 30 min. Pellets are then suspended in 300 µl sterile PBS and frozen at -80°C in 15 µl aliquots.

**Virus Infections**

MEF, TS/A, and B16(F10) cells were seeded in 6 or 12-well plates and grown to 70% confluence. After washing with 1X PBS, cells were infected with rVSVs using the indicated MOI which is calculated (# of cells * MOI)/titer = mL virus needed. Cells were incubated with virus for 1 hour at 37°C in serum free DMEM with rocking every 15 minutes. After removal of the media-containing virus, cells were washed with PBS 2X and complete media was added to cells. Supernatants and total protein was collected at various time points.

**Virus Titers from Supernatant**

BHK cells were plated to 90% confluence and infected with 6 dilutions of the collected supernatant or purified virus in a volume of 300ul rocking every 15 min. After one hour incubation with virus, 1.0% low melting point agarose in complete media was added to the cells and plates are incubated for 24-48 hours. After plaques form the agarose was melted for 13 sec in a 600W microwave oven and removed by suction. Plaques were fixed and visualized with crystal violet
solution (0.1% crystal violet and 30% methanol in sterile water). Viral titers are in pfu/ml.

**Cell Viability**

C57BL/6 MEF, TS/A, and B16(F10) cells were grown to 70% confluence in 12-well plates. Cells were then infected as described at the indicated MOIs. After incubation for 6, 12, 24, and 48 hours cells were collected, washed with PBS 2X, and suspended in AnnexinV buffer (BD #51-66121E). Cells were then stained using Annexin V-eFluor 450 1 ug/mL (eBioscence) and Propidium Iodide (1ug). Stained cells are then used for flow cytometry.

**Western Blotting**

C57BL/6 MEF, TS/A, and B16(F10) cells were grown to 70% confluence in 6-well plates. Cells were then infected as described at the indicated MOIs. After incubation for 12 and 24 hours cells were collected and lysed in radio-immunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors for 5 min at 4C and the centrifuged at 13k RPM for 2 min. Lysates were kept on ice and stored at -80C. Protein concentrations were then determined using Coomassie Plus Protein Assay Reagent (Thermo #1856210) and the OD was read at 595nm. Equal amounts of protein were subjected to SDS-PAGE using 10 or 12% polyacrylimide gels. Following PAGE proteins were transferred onto a PVDF membrane and blocked using 5% Milk in PBS-Tween (0.1% Tween-20). Membranes were then immunoblotted using antibodies
against p53 (Santa Cruz SC-99), p53 phosphorylated at serines 18 and 389 (Cell Signaling #9284 and #9281), VSV-G (Sigma #V5507) rocking overnight at 4C. Membranes were then washed using PBS-T 3X for 10 minutes and probed with a secondary antibody goat anti-mouse, goat anti-rabbit, donkey anti-goat (Santa Cruz) at 1:5000 for 1 hour at room temperature. The image was resolved using chemiluminescence (Super Signal) and captured by autoradiography (Kodak Film).

**p53 Luciferase Assay**
Firefly, p53-luc (Clontech) (250ng) and renilla pRLTK (50ng), were transfected as described into 70% confluent C57BL/6 MEFs, TS/A, and B16(F10), grown in 12 well plates 4 hours prior to infection. Cells were then infected as described at desired MOI. After 24 hours cells were lysed using 1X CCLR and luciferase assays were performed using a Luciferase Assay System and Renilla Assay System (Promega).

**p53 DNA Microarray**
C57BL/6 MEF and TS/A cells were grown to 70% confluence in 6-well plates and infected as described at the indicated MOI. After 8hour incubation total RNA was collected using ArrayGrade Total RNA Isolation Kit (SA Biosciences). cDNA and biotin labeled cRNA was then generated using TrueLabeling-AMP 2.0 (SA Biosciences). Labeled cRNA was then hybridized to an Oligo GEArray DNA
Microarray (OMM-027) and resolved using chemiluminescent detection and autoradiograph capture.

**Mouse Studies**

Female Balb/c (6-8 week old) were acquired from Jackson Labs. Female Balb/c athymic nude (CAaN.Cg-Foxn1\textsuperscript{nu}/Crl) mice were acquired from Charles River. All mice were housed in pathogen free conditions.

**Toxicity**

To determine toxicity of VSV (n=7) Balb/c or (n=5) Nude mice were injected with the indicated amount of VSV IV in 100ul in the tail vein. Mice were then monitored for survival. Additionally mice were sacrificed if they displayed gross morbidity or if they developed hind limb paralysis, which can occur due to virally induced encephalomyelitis.

**Tumor studies**

Balb/c (n=7) or Nude (n=5) mice were injected with 1x10\textsuperscript{5} TS/A-luc cells IV. Three days later mice are injected with 5x10\textsuperscript{7} or 5x10\textsuperscript{8} pfu of rVSV. Survival of mice was monitored daily. Surviving mice were re-challenged with 2x10\textsuperscript{5} TS/A-luc cells and monitored daily for survival.
Production of tumor specific IFN-γ secreting cells was assessed using the IFN-γ Enzyme-linked immunospot (ELISPOT) assay. 96 well nitrocellulose plates were pre-coated with a monoclonal IFN-γ antibody (R&D). Female Balb/c (n=5)) mice were injected intravenously (IV) in the tail vein with 1x10^5 TS/A-luc cells. Three days later mice were infected with 5x10^7 pfu of the indicated rVSV IV. 10 days post-infection mice were sacrificed and spleens were removed and layered over a Ficol-paque gradient. Mononuclear cells were then harvested and incubated for 2 hours at 37°C with TS/A-luc cells previously treated with mitomycin C 25 µg/mL at varying ratios 1:10, 1:25, and 1:100. After washing a biotin conjugated monoclonal IFN-γ detection antibody was added for 90 minutes at room temperature followed by the addition of steptavidin-alkaline phosphates and 5-bromo-4chloro-3-indolylphosphate to develop the spots. Spot development was stopped 10 minutes later by washing and plates with dH20. Plates were analyzed the following day using an ELISPOT reader.

**Multiplex ELISA and Flow Cytometry**

In order to establish the cytokine and immune compartment profiles generated by rVSV infection in-vivo Female Balb/c (n=6) mice were infected with 5x10^7 pfu rVSV IV. 24 (n=3) and 96 (n=3) hours post infection serum, spleen, and thymus was collected. Serum was stored at -80°C and then used for Multiplex Elisa (Millipore). Briefly, serum was incubated overnight at 4C with agitation with a mix
of 32 different-sized antibody coated beads. The next day samples were incubated with detection antibodies for 1 hour at room temperature, and run on a Luminex 100 machine, which quantifies the size, and mean fluorescence of each bead and calculates cytokine concentration in pg/mL. Spleens and thymuses were passed through a 0.2 μm mesh and cells were recovered in 3mL of RPMI-1640. Cells were then centrifuged 1400 rpm and red blood cells lysed by treating with 1 mL of ACK buffer for 5 min, centrifuged again and washed with PBS 1X. Cells were counted and two groups of 1x10^6 cells were stained for 4 color flow cytometry using CD4-PE (BD 557308), CD8-PerCP (BD 553036), B220-Fitc (BD 553088), CD49b-APC (eBioscience 17-5971-81) or CD11c-Fitc (BD 553801), CD11b-APC (BD553312) , F4/80-PE (eBioscience 12-4801-80), MHCII-eFlour450 (eBioscience 48-5321-80) on ice in darkness for 20 minutes. Cells were then washed 1X with PBS and fixed with 1-4% paraformaldehyde and stored in the dark at 4C until samples were run on flow cytometry (BD-LSRI).

**IFN-β ELISA**

Balb/c mice (n=3) were mock or infected with 5x10^7 pfu rVSV. 6 hours post infection blood was collected by sub-mandibular bleed. Blood was then incubated for 1 hour at 4C and centrifuged at 13,000 rpm in order collect serum. Samples were diluted 1:50 and assessed by IFN-β ELISA (PBL Interferonsource).
In Vivo Imaging of Mice

Mice previously treated with tumor cells and rVSV were anesthetized and injected with D-luciferin (Caliper). Animals were placed on a warmed stage (37°C) and imaged using an IVIS 200 (Xenogen). Bioluminescence was monitored by detecting photon emission using a ccd camera. Images of treated mice were displayed as a pseudo color image representing photon intensities superimposed over the white light image of the mouse. Photon radiance was quantified using Living Image software (Caliper).
Vesicular Stomatitis Virus (VSV) is emerging as an effective oncolytic virus in preclinical models of cancer (173, 179, 195, 274). VSV is a prototypical single stranded negative sense RNA rhabdovirus, comprised of 5 genes, N, P, M, G and L (275). Although wild type VSV can cause oncolysis of malignant cells while sparing normal tissues, there are still concerns about the safety of VSV. Despite this, VSV has many characteristics that make it an optimal oncolytic vector including low seroprevalence, minimal morbidity in humans, and a simple non-integrating genome. In order to improve the anti tumor efficacy and reduce VSV associated toxicity, transgenes, like IFN-β, which can modulate the immune response are inserted into the VSV virus and infectious viral particles are recovered from the cDNA viral packaging system (138, 173).

IFN-β is a critical mediator of the antiviral response. IFN-β is produced in response to pathogenic stimulus, such as viral infection, through the RLH or TLR pathways, and initiates a JAK/STAT signaling cascade (59). STAT phosphorylation leads to dimerization and association with IRF9, to form the transcription initiation complex ISGF3 that leads to the induction of over 100 ISG’s (71, 276). VSV like many viruses attempts to subvert the immune response in order to facilitate better replication. VSV M protein inhibits mRNA export and suppresses IFN-β production (202). Thus, VSV infection is not a strong inducer of the IFN response. However, VSV is highly susceptible to PKR phosphorylation of eif2α that leads to translation inhibition (116, 134). However, in animals PKR...
activation alone is not sufficient to completely control VSV infection, rather it provides a sufficient delay in viral replication that allows the immune response to “catch up” and mediate clearance of the virus(32).

Currently, there are many strategies being used to improve the efficacy and safety of VSV as an oncolytic vector(105, 179, 195, 206). In particular the insertion of immunomodulatory transgenes can both attenuate toxicity while simultaneously increasing the anti-tumor response(197, 277). Previously, our lab inserted the human or mouse IFN-β gene into the VSV genome generating VSV-h/m-IFN-β(173). In vitro VSV-IFN-β maintained its oncolytic capacity to preferentially replicate in and kill malignant cells. It also expressed biologically active IFN-β at high levels. In vivo, VSV-IFN-β was attenuated with no viral associated toxicity at treatment dose of 5x10⁷ pfu, and enhanced the host anti-tumor response making it more effective in murine models of cancer. VSV-hIFN-β has also completed toxicity studies in non-human primates with positive results and is poised to begin phase I clinical trials(234).

As VSV-hIFN-β rapidly approaches the beginning of clinical trials additional vectors that express transgenes to modulate the IFN response are in development. Previously, it has been shown using luciferase reporters and ELISA that over expression of signaling molecules, IPS-1 and TRIF, in cell culture leads to strong induction of INF-β(127, 278). IPS-1 and TRIF are both upstream signaling adaptors that facilitate type 1 IFN induction. IPS-1 is a mitochondrially associated protein, involved in the RLH pathway that leads to IFN production. IPS-1 is activated by either RIG-I or MDA-5 through its CARD
Following activation IPS-1 recruits a number of additional molecules including TRAF2/3, TANK, TRADD, FADD, and RIP-1, which lead to induction of both type 1 IFN and NF-κB (47, 280). TRIF, which is stimulated by TLR-3 directly or TLR-4 with the help of TIRAP, recruits TRAF3, TRAF6, RIP-1, and TAK and also activates IFN and NF-κB production (56, 281). We therefore hypothesized that incorporation of IPS-1 or TRIF into VSV would generate a vector capable of inducing high levels of IFN-β leading to a safer and more effective oncolytic vector.

Initially, the wild type VSV genome was altered to produce unique Xhol and Nhel cloning sites between the G and L proteins. The murine IPS-1 or TRIF genes were then inserted into this site (Figure 4.1A). In order to recover infectious viral particles BHK-21 cells were first pre-infected with vaccinia virus that expresses T7 polymerase (VTF7-3). These cells were then transfected, using Lipofectamine 2000, with pBlueScript vectors containing the VSV-N, -P, -L, and full length VSV genome all under control of the T7 promoter and incubated for 48 hours. After incubation, the supernatants were passed through a 0.2 um filter to remove the VTF7-3 while the newly generated rVSVs flow through and are plated on fresh BHK-21 cells. Recovered virus was then purified by ultracentrifugation into a 10% sucrose cushion, titered by the standard plaque assay and assessed for activity. Newly generated VSV-IPS-1 and VSV-TRIF retained the ability to preferentially kill transformed cells. Primary C57BL/6 MEF, TS/A, and B16(F10) cells were infected at an MOI of 5 and brightfield microscopy pictures were taken 24 hours post infection (Figure 4.1B). This analysis
indicated that VSV-IPS-1 and VSV-TRIF readily killed transformed cells as infection caused extensive CPE in TS/A and B16(F10) whereas almost no CPE is observed in C57BL/6 MEF. Next we confirmed efficient transgene expression in BHK-21 cells. Cells were infected at an MOI of 1 and 24 hours post infection IPS-1 or TRIF expression was confirmed by immunoblotting (Figure 4.1C). These data indicated that our new rVSV’s were oncolytic and efficiently expressed the IPS-1 or TRIF transgene.

![Diagram](image)

Figure 4.1: VSV-IPS-1 and VSV-TRIF retain oncolytic ability and express the inserted transgene. A, Schematic representation of VSV-IPS-1 and VSV-TRIF constructs. B, Brightfield microscopy of VSV-IPS-1 and VSV-TRIF infected cells at an MOI of 5 24 hours post infection. Immuno blot analysis for C, IPS-1 and D, TRIF protein expression from VSV in infected BHK-21 cells.

Given the apparent oncolytic ability of VSV-IPS-1 and VSV-TRIF, we quantified the killing and replication capabilities of these rVSVs in transformed and normal cells (Figure 4.2A and B). C57BL/6 MEF, TS/A, and B16(F10) cells were infected at an MOI of 0.1 or 5 for one hour. 24 hours post infection cells
were stained with Annexin V and Propidium Iodide. Annexin V binds to phosphotidyl serine, which is exposed as cells begin to undergo apoptosis and PI can enter permeabilized cells and bind to DNA, an additional indicator of apoptosis. The insertion of an IFN stimulatory transgene did not attenuate the viruses' ability to kill malignant cells in vitro compared to control VSV-GFP as 80-90% of cells infected with VSV-IPS-1 or VSV-TRIF died within 24 hours. C57BL/6 MEF cells remain largely unaffected by VSV infection because of an intact IFN system and antiviral response (Figure 4.2A). The supernatants from these infections were also used in a standard plaque assay to determine if permissive TS/A and B16(F10) cell lines allowed for increased viral replication (Figure 4.2B). All rVSVs replicated to high titers $10^5$-$10^8$ pfu/mL in TS/A and B16(F10) cells, but not in primary C57BL/6 MEF, which had titers that ranged from undetectable to $10^3$ pfu/mL. This investigation confirmed that VSV-IPS-1 and VSV-TRIF are oncolytic viruses that preferentially replicate in and kill malignant cells.

It has been previously reported that VSV is a weak inducer of IFN-β (159). Therefore, incorporation of IPS-1 or TRIF into VSV and their subsequent forced expression by VSV in tumor cells should result in increased IFN-β production. To prove this, we first performed a luciferase assay using an IFN-β luciferase reporter to verify if these new rVSVs could in fact activate the IFN-β promoter. C57BL/6 MEF, TS/A, and B16(F10) cells were first transfected with IFN-β luciferase (pIFN-β-luc) and renilla luciferase (pRLTK) transfection control at a ratio of 5:1. Transfected cells were then mock infected or infected with VSV-
GFP, VSV-IPS-1, or VSV-TRIF at an MOI of 0.1 or 5 for one hour and luciferase activity was determined 24 hours post infection (Figure 4.2C). As expected VSV-GFP infection minimally induced luciferase production while VSV-IPS-1 and VSV-TRIF lead to stronger luciferase induction compared to VSV-GFP. However, even though these viruses can activate the promoter it was necessary to prove that actual IFN-β protein was being secreted. C57BL/6 MEF, TS/A and
B16(F10) cells were infected with VSV-IPS-1 and VSV-TRIF at an MOI of 0.1 or 5 and supernatants were collected for ELISA 24 hours post infection (Figure 4.2D). As anticipated, both viruses induced secretion of IFN-β primarily in permissive cell lines indicating that increased expression of the IPS-1 or TRIF transgene facilitated stronger induction of IFN-β protein.

Having confirmed in vitro that VSV-IPS-1 and VSV-TRIF were able to induce IFN-β production we next assessed their toxicity prior to beginning in vivo
models. Female 6-8 weeks old Balb/c mice (n=5) were infected IV with increasing doses of 5x10^6-5x10^8 pfu of VSV-IPS-1 or TRIF (Figure 4.3A and B). Although, our previous work with VSV-IFN-β showed minimal toxicity up to doses of 1x10^8 pfu, surprisingly VSV-IPS-1 and VSV-TRIF were more toxic, with 80% of mice infected with 5x10^7 pfu VSV-IPS-1 succumbing rapidly to infection (Figure 4.3B). VSV-TRIF was safe at the optimal treatment dose of 5x10^7 pfu, however the ability to increase IFN-β production did not attenuate this virus as 100% of mice die with an increased dose of 5x10^8 pfu (Figure 4.3A). It is possible that because these genes are upstream signaling adaptors and have the ability to activate MAPK and NF-κB pathways that infection with these viruses leads to an increased inflammatory response and fatal cytokine storm which could account for the rapid mortality caused by these viruses. Regardless non-toxic doses were determined and used in a syngeneic Balb/c tumor model TS/A-luc. Initially, Balb/c mice (n=7) were injected IV with 1x10^5 TS/A-luc cells. Three days later mice were mock infected or infected with 5x10^6 pfu of VSV-IPS-1 or 5x10^7 pfu of VSV-TRIF or VSV-GFP (Figure 4.3C). Interestingly, these viruses were highly ineffective against TS/A-luc. This result was unexpected as previously our data indicated that VSV-IFN-β protected between 30-50% of treated mice(173). Although the mechanism is unclear it is possible that increased inflammation prevented a robust host anti-tumor response from occurring and the insertion of upstream signaling adaptors did not elicit increased anti-tumor efficacy.

The use of oncolytic viruses for the treatment of cancer is rapidly approaching clinical relevance. This study was designed to improve upon
current VSV vectors by inserting immunomodulatory transgenes IPS-1 and TRIF based upon the success of the VSV-IFN-β vector. However, newly developed VSV-IPS-1 and VSV-TRIF did not provide any survival benefit compared to the control VSV-GFP. Despite this setback many new oncolytic VSV vectors are being developed using the transgene insertion strategy that show excellent pre-clinical potential. Thus, continued research into the use of VSV as an oncolytic vector is ongoing and it is probable that additional successful vectors will be generated.
Chapter 5: Conclusions and Future Directions

Cancer is a complex social and medical issue and finding the “cure” has proven extremely difficult. This is in part because cancer is not a singular disease. In 1971 President Richard Nixon declared a war on cancer and signed the National Cancer Act in an attempt to increase the resources available to further cancer research and find a “cure”. Though research efforts to provide broad frontline therapeutics, like surgery, radiation and chemotherapy, have had some successes, these therapies are not effective for every type of malignancy. Thus, despite the increase in survival rates observed across many types of cancers, there is a great need for novel treatments that target all tumor types effectively.

As our knowledge of molecular biology and genetics has increased, our ability to design new, safer, less toxic, and highly effective treatment modalities against cancer has also been enhanced. Understanding the underlying molecular mechanisms of cancer cells has facilitated the development of specific targeted therapies, such as small molecule inhibitors of signaling pathways, monoclonal antibodies, cytokines and immunotherapy. However, while these treatment modalities have addressed issues of specificity and toxicity they are still limited in the number of malignancies that they can treat since they can only be used for altered signaling pathways or modified surface molecules. Additionally, because cancers have a high mutation rate and genomic instability and these treatments exert additional selective pressure on the tumor cells,
metastases, or relapses may become unresponsive or refractory to these treatments. As a result continued research and development of cancer therapeutics is needed.

Recently, the idea to use an infectious agent, namely viruses, to treat debilitating disease has gained a great deal of financial and research support. However, the use of viruses in this capacity is not a new idea. Previous findings have demonstrated that patients with both solid tumors and hematological malignancies experience remission after acquiring a secondary viral infection. As a result of this clinical observation it was discovered that many naturally occurring viruses have oncolytic ability. These viruses include Herpes Simplex Virus-1, Hepatitis B/C Virus, Measles Virus, Adenovirus, Vaccinia Virus, Seneca Valley virus, Vesicular Stomatitis Virus, and many others. A number of these viruses were used in clinical trials from 1940-1970. However, while there were some successful treatments, these trials were overall ineffective, as remission status was limited, there was significant toxicity associated with using unattenuated virus, and viral clearance by the immune system occurred. Since these trials, and through extensive research and gene manipulation, additional oncolytic vectors have been identified and generated. For example, some viruses, critical genes are mutated or deleted in an attempt to reduce viral replication or to target tumor cells. Viruses can be retargeted to tumor cells by altering their surface receptors with fusion proteins or single chain antibodies. Furthermore, viral genomes can be manipulated and have immune modulating or bystander effects by the transgenes inserted.
One virus that is on the frontline of oncolytic virotherapy research and has shown remarkable pre-clinical success is Vesicular Stomatitis Virus (VSV). Interestingly, nearly 80% of cancer cell lines tested are permissive for VSV replication and oncolysis. Although the mechanisms that facilitate VSV oncolysis remain unclear it is likely that they fall into two categories. First, many tumors develop defects in innate immune signaling pathways such that they are unable to mount an effective anti-viral response. Second, tumor cells are unable to regulate translation and cell division making these cells an ideal setting for viral replication that relies on host translation machinery.

There are many potential advantages to using VSV as an oncolytic vector. VSV can: 1) infect nearly all cell types through an unknown but ubiquitous cellular receptor(s). 2) Infect many different types of cancer cell lines. 3) VSV is also neurotropic, meaning that it can be used to treat central nervous system (CNS) malignancies. Another advantage to using VSV is that it has a simple 5 gene non-integrating genome that can be manipulated and functional virus can be recovered from cDNA. Thus, there are many strategies being explored to improve upon VSV as an oncolytic vector. VSV can have trans-genes inserted into the viral genome and then express them at high levels in tumor cells. There are many options for transgene insertion including genes that modulate the immune response, increase bystander effects, or reduce the immune response to VSV. The transgene insertion strategy has been widely used and in some cases has shown remarkable pre-clinical success. However, not all transgenes lead to improved vectors. Interestingly, VSV-IFN-β was attenuated and effective while
newly generated VSV-mp53, VSV-IPS-1, and VSV-TRIF were not. It may also be possible to express multiple transgenes in a single VSV construct, which could enhance the anti-tumor and antiviral response, associated with VSV treatment. This highlights the necessity to rigorously test a wide array of transgenes in VSV as the in vivo benefit can vary widely even though all of these vectors are quite effective in vitro.

Another strategy is to reduce VSV replicative ability by deleting and then supplying the G protein in trans or by mutating the M protein such that it can no longer inhibit mRNA export and thus facilitate a more robust anti-viral response against VSV. This method results in greatly attenuated vectors that cause minimal toxicity and appears effective in some tumor models. VSV can also be retargeted by making fusions with the G and other viral entry proteins, like gp160, or by inserting miRNA binding sites into the virus in order to restrict viral tropism. Fully retargeted vectors should be completely safe as the virus could only enter or replicate in cells that have a specific surface receptor or miRNA profile.

The next generation of frontline VSV constructs will likely employ combinations of these strategies to generate the most effective and safe oncolytic vectors. One such vector is the newly generated VSV-ΔM-mp53 that combines the viral mutation and transgene insertion strategy. This virus was remarkably attenuated allowing administration of up to 20 times more virus without toxicity. Additionally, VSV-ΔM-mp53 was effective in a metastatic mammary tumor model in immunocompetent mice by inducing an enhanced host anti-tumor response that included the generation of immunologic memory against
the tumor. In addition the ΔM mutation can be combined with other transgenes and lead to the generation of other safe and effective rVSVs. The mutation and transgene approach can also be performed with viral retargeting methods leading to specific, attenuated, and immunomodulatory viruses.

Surprisingly, in most cases the pre-clinical success of VSV has been generated in models using at most two VSV treatments. There are numerous combinations that could be assessed for preclinical efficacy using VSV. For example, it may be possible to use multiple doses of VSV given over a time period, to dispense a single dose that combines 2 or more viral constructs, or to employ sequential treatment strategies using the same or different viruses. Additionally, VSV could be administered through IV or IT administration. Lastly it may be beneficial to combine VSV with current standards of care. Interestingly, there have been very few animal models that use VSV in combination with other cancer treatments. The current standard of care for most malignancies combines a variety of treatment modalities including surgical debulking, radiation, and multidrug cocktails. Thus, it is likely that clinical VSV treatment will be combined with current standard of care. Therefore, new more clinically relevant animal models are needed in which VSV treatment is combined with standard treatments to determine if VSV provides a therapeutic advantage.
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