Vesicular Stomatitis Virus is a Malleable Oncolytic Vector for the Treatment of Various Malignancies

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VESICULAR STOMATITIS VIRUS IS A MALLEABLE ONCOLOYTIC VECTOR FOR THE TREATMENT OF VARIOUS MALIGNANCIES

By

Dillon M. Betancourt

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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VESICULAR STOMATITIS VIRUS IS A MALLEABLE ONCOLYTIC VECTOR FOR THE TREATMENT OF VARIOUS MALIGNANCIES

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Oncolytic virotherapy is an exciting field that is currently generating a significant amount of interest. Several viruses have recently been approved for clinical use and there are many more in clinical trials awaiting approval. Vesicular Stomatitis Virus (VSV) has performed remarkably well in many preclinical studies and several strains have entered clinical trials across the world. VSV is a negative strand ssRNA virus in the Rhabdoviridae family and mainly infects livestock which produce sores on the mucus membranes. VSV infection in humans is extremely rare and most often asymptomatic due to its inability to overcome the innate and adaptive immune response of healthy individuals. However, upon infection of many types of cancerous cells VSV displays rapid growth kinetics and significant oncolytic potential due to defects in innate immune and translational control pathways commonly seen in cancer cells. VSV is of significant research interest partly due to its simple genome that is highly malleable. The entire 11kb genome has been cloned into a cDNA plasmid and the insertion and deletion of different genes allows for a highly customizable virus that researchers can take advantage. One such modification in our lab is the replacement of the VSV glycoprotein with a fusion protein created using domains of HIV-1 gp160 and VSV-G. The fusion protein dubbed gp160G was properly incorporated into VSV virions and successfully altered the tropism
of the virus. VSV-gp160G was selective for CD4+ cells and induced significant amounts of syncytia and apoptosis during infection of Hela CD4+ and adult T cell leukemia (ATL) cells. When used as a therapeutic in an ATL mouse model, VSV-gp160G improved survival time of tumor bearing mice and significantly reduced the onset of metastasis. The success of VSV-gp160G as a targeted therapy in ATL mouse models warrants further study as the virus could be an important therapeutic option to treat ATL in the clinic.
DEDICATION

I dedicate this thesis to my mother.

You were taken from me far too soon.

I hope I’ve made you proud.
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Chapter 1: Cancer: Ancient disease to modern times

Background

There are few words that inspire as much fear as cancer. It is a disease that has been with humanity likely our entire history, with some of the earliest texts of cancer dating back to 1500 BC from ancient Egypt. The word cancer came from the Greek word karkinos when the ancient Greek physician Hippocrates described carcinoma tumors in his texts (1). However, despite our ancient knowledge of this disease, for much of our history we were powerless against it and could only offer palliative remedies to the afflicted. Our initial lack of progress can be attributed to our lack of understanding of the nature of cancer. One of the earliest theories of disease was the humoral theory from Hippocrates. This theory postulates that the body contained 4 humors or body fluids: blood, phlegm, yellow bile, and black bile. Any imbalance in these fluids was thought to cause disease and that an excess of black bile was the cause of cancer.

This theory stood for thousands of years until the work of Richard Virchow eventually discredited humourism as a flawed theory and ushered in the development of cellular pathology (2). However, despite Virchow’s contribution it wasn’t until Watson and Crick discovered DNA’s structure and Hershey and Chase identified its role as the inheritable molecule would we able to make dramatic strides in understanding tumorigenesis. DNA integrity and fidelity is vital for the successful passing of genetic information from parent to offspring. It is DNA that directs the synthesis of proteins that carry out the biochemical reactions necessary for life and so the preservation of the sequence of DNA is vital to the survival of the organism. Unfortunately, during an
organism’s lifespan, DNA is continuously exposed to myriad insults from both exogenous and endogenous sources. Radiation, ultraviolet light, carcinogenic chemicals, and viruses are all exogenous sources of DNA damage while reactive oxygen species (ROS) produced in metabolic process, telomere shortening induced by cell division, and mistakes during DNA replication and all endogenous sources of DNA damage. The evolution of multiple DNA repair mechanisms emerged out of necessity to combat and mitigate DNA damage; these are predominantly the homologous recombination (HR) and non-homologous end joining (NHEJ) repair pathways. HR is mostly error-free, but requires an intact sister chromatid as a template while NHEJ is error prone since it lacks an intact template. In addition to repair mechanisms, there is a DNA damage response responsible for detecting strand breaks and initiating the repair mechanism (3). However, despite our repair and damage responses, these systems aren’t fully efficient at repairing DNA damage and occasionally mutations do occur.

Luckily, the vast majority of mutations are silent and do not significantly affect an organism, however certain genes can have drastic effects if they are altered. For example, 2 classes of genes: oncogenes and tumor suppressor genes are considered the primary drivers and inhibitors of tumor progression, respectively. Oncogenes (proto-oncogenes before the mutation occurs) are responsible for driving cell proliferation and determining the degree to which a cell differentiates while tumor suppressor genes control cell division, DNA repair, and apoptosis. Mutations that up regulate the activity of oncogenes or mutations that down regulate the activity of tumor suppressors genes can initiate tumorigenesis. Typically, a single mutation in either of the genes isn’t sufficient to cause
cancer due to redundancies in tumor suppression mechanisms as postulated in Knudson’s Two hit hypothesis (4).

Cancer was first proposed as a genetic disease in 1914 (5). This was heavily supported by the subsequent discovery that ionizing radiation long known to be carcinogenic, is also mutagenic (6) and the long latency period between exposure and the appearance of inducible cancers indicated that multiple mutations may be required for tumorigenesis (7). The Two hit hypothesis was first proposed following Knudson’s work with retinoblastoma in children. Knudson used a retrospective analysis and mathematical modeling to identify that bilateral and unilateral retinoblastoma cases followed one-hit and two-hit curves, respectively (4). Typically, after a cell has transformed into a cancerous cell, genetic mutations occur more rapidly resulting in tumor progression into a more malignant and aggressive phenotype.

**Cellular transformation and hallmarks of cancer**

The transformation process from a primary cell to a transformed cell requires the cell to acquire certain hallmarks or capabilities during its evolution (Figure 1.1). As the cell acquires more genetic hits, its phenotype changes into a more evolved tumor. Perhaps the most fundamental trait of cancer cells is their ability to sustain a proliferative state. Normal cells have tight regulation on cell cycle progression and number of divisions the cell will enter before entering senescence or undergoing apoptosis. However, cancer cells deregulate these processes and essentially direct their own growth and survival. Cancer cells produce and respond to their own secreted growth factors acting in both autocrine and paracrine manner and express constitutively active mutants of signaling proteins that render them independent of growth factors. Tumor cells
downregulate tumor suppressor genes like pRB and p53. The tumor suppressor genes pRB and p53 are the prototypical tumor suppressor genes and are vital for proper cell regulation. Cancer cells with defects in pRB lack an essential negative regulator in cell cycle progression while p53 is an important sensor of stress and abnormal conditions and will trigger cell cycle arrest or apoptosis if conditions do not improve (8).

In addition to sustained proliferative signaling, cancer cells have also achieved unlimited replicative capacity. During S-phase in the cell cycle, there is an end of strand replication problem and the end of the DNA strand is not replicated and lost during each round of cell division. Telomeres have evolved as a remedy to this problem and are nucelo-protein complexes composed of DNA repeats and associated telomere binding proteins (9). The length of a telomere dictates how many rounds of cell division a cell can
undergo as it progressively shortens for each division. Once a telomere reaches a critically short length, it loses its protective capabilities and the afflicted cells experience senescence, permanent cell cycle arrest, and activation of DNA damage response pathways (10). Cancer cells overcome this barrier by reactivating the telomerase enzyme. Normally, telomerase expression is restricted to embryogenesis and immature germ cells and certain stem cells compartments (9) but during tumorigenesis, cancer cells have reactivated telomerase expression and are able to replicative indefinitely without triggering senescence or cell crisis.

Cancer cells normally form large tumor masses because of their unlimited replicative capacity and perpetual cell cycle progression. The tumor cells required nutrients and oxygen to survive as normal tissues do and so cancer cells must evolve the ability to stimulate tumor associated angiogenesis. Angiogenesis is the proliferation of endothelial cells and their assembly into tubes to serve as a blood vessels. During embryogenesis, the development of new vasculature is extensive forming the circulatory system, however subsequently angiogenesis is largely quiescent and only a small part of physiological processes such as wound healing and female reproductive cycling trigger angiogenesis (11, 12). Tumor cells trigger angiogenesis through expression of proangiogenic signaling molecules like VEGF and FGF (13, 14). The vascular created by the nascent tumor is often atypical, lacking organization with eccentric capillary sprouting and excessive vessel branching. The vessels themselves have erratic blood flow, are hemorrhaging, and the endothelial cells are proliferating and undergoing apoptosis at abnormal rates (15). Recent evidence indicates that angiogenesis is a rather
early event during development of invasive cancers. The angiogenic switch typically occurs before macroscopic tumor formation or onset of malignancy (16).

Perhaps the most destructive characteristic of cancer cells is their ability to invade surrounding tissue and form metastatic colonies, as an estimated 90% of all cancer deaths are related to metastasis (17). Metastasis is an extreme process that involves multiple steps before a cancer cell can form an independent colony from the primary tumor. A tumor cell must (i) infiltrate adjacent tissue, (ii) perform intravasation and migrate into the circulatory system (iii) survive the extreme conditions of the circulatory system (iv) perform extravasation (v) and finally colonize and proliferate in competent organs (18).

The mechanisms underlying the process of successful metastatic progression were largely unknown until the early 2000s. Eventually, a developmental process in embryonic morphogenesis known as the epithelial-mesenchymal transition (EMT) was implicated as a mechanism by which transformed cells acquire the ability to invade, resist apoptosis, and to disseminate to distal organs (19). One of the best-described characteristics of the EMT is the loss of E-cadherin. E-cadherin is a key molecule in cell-cell adhesion and is necessary for the formation of cell sheets and maintaining the quiescent state of these cells during homeostasis (20). Additionally malignant tumors often express several transcription factors such as Snail, Slug, Twist, and Zeb ½ which coordinate the EMT and migratory processes and have been implicated in promoting invasion and metastasis when ectopically expressed (21). The expression of these transcription factors induce the loss of E-cadherin, conversion to a fibroblast morphology, expression of ECM degrading enzymes, increased motility, and apoptotic resistance, all traits that will enhance a cell’s ability to undergo invasion and metastasis (22).
The majority of cancer cells that disseminate from the primary tumor do not survive to successfully colonize a distal metastasis. It is estimated that <0.1% of disseminated cancer cells survive to develop metastatic foci and most of them remain as dormant solitary micrometastases either due to cell cycle arrest or a balance between apoptosis and proliferation (23). If metastatic foci can achieve sustained growth and develop into clinically detectable macrometastases, it drastically worsens the prognosis for the host. Metastatic cancer cells compete with primary cells for nutrients, space, and oxygen and often disrupt normal organ structure and function. If the cancer cells continue to grow they can eventually cause significant organ cell death and be extremely damaging to the host.

Metastatic cancer cells often colonize vital organs such as lung, brain, liver (24). The preferential organs of most metastasis have been known for over a century but the mechanisms by which metastatic cells colonize distal organs remained a mystery. Many believe that the primary factor that determined the pattern of metastasis was dependent on the circulatory and lymphatic drainage from the primary tumor and that the tumor cells colonize in whichever organ they encounter. Eventually, Paget proposed a “seed and soil” hypothesis in which metastatic tumor cells preferentially colonize certain microenvironments rather than passively settle due to the mechanical forces of the circulatory and lymphatic system (25). The current paradigm is that neither the seed and soil or the anatomical mechanical hypothesis need to be mutually exclusive and both the circulatory and lymphatic system and the organ microenvironment need to be considered in investigations of metastasis.
After establishing a viable colony in foreign tissue, a disseminated cancer cell is likely to be poorly suited for its new environment. It must activate colonization programs to thrive in its new environment. The colonization programs culminate into the expression of metastatic signature genes and are dependent on the microenvironment of the disseminated cancer cell. Metastatic signature genes can be grouped as: metastasis initiation genes, metastasis progression and metastasis virulence (26). Genes that allow the transformed cells to invade the surrounding tissue and attract supportive stroma would be considered metastatic initiation genes. They will promote cell motility, the EMT, ECM degradation, angiogenesis, or immune evasion. Genes that allow the infiltration of distant organs and survival of nascent colonizing tumor cells would be metastasis progression genes. They could have different functions depending on the microenvironment but will enable the cancer cell passage through the capillary walls and to survive in the newly invaded parenchyma. Genes that facilitate the outgrowth of metastatic colonies and are detectable only after colonizing distinct organs are metastatic virulence genes. For example, osteoclast mobilizing factors like parathyroid hormone related protein PTHRP and IL-11 enable disseminated tumor cells to establish osteolytic metastases in bone but provide little benefit to primary breast tumor (27).

The plasticity of gene expression is essential for metastatic cancer cells to acquire the necessary abilities to progress from a benign hyperplasia into an invasive malignant tumor. This is facilitated by an unstable genome caused by progressive loss of DNA repair factors and increased sensitivity to mutagenic agents (8). The multistep tumor progression can be thought of as an evolutionary process where each mutation can confer a growth advantage and allow the subclonal expansion of cells for each chance
acquisition. In addition to mutational changes, different phenotypes can be passed down through epigenetic modifications (i.e. DNA methylation and histone modifications) that affect the gene expression of cancer cell progeny.

**Oncogenic viruses and induced mechanisms of oncogenesis**

The accumulation of oncogenic hits that confer these hallmarks are typically a chance occurrence through random mutation. However, there are viruses that can induce an oncogenic hit and push the infected cell towards transformation. It is estimated that approximately 12% of human cancers are a result of viral infection, with the majority in the developing world (28). The phenomenon of viral induced cancers was first observed in 1911. Peyton Rous reported that a filterable agent in cell extracts could induce tumorigenesis in healthy chickens, this agent was later determined to be a virus and was the first oncogenic virus discovered (29). The first human tumor virus was discovered nearly half a century later in Burkitt’s lymphoma patients and marks the starting point of human tumor virology (30).

As the field evolved and more oncogenic viruses were discovered, common traits began to emerge. Viral induced oncogenesis requires oncoviruses but they are not sufficient for cancer development. Typically, cofactors are necessary for tumorigenesis; this includes immune suppression, chronic inflammation, or additional mutagens (31, 32). The onset of tumorigenesis in the context of viral infection typically occurs years to decades after the primary infection with the immune system being unable to clear the virus. The immune system can also play either a protective or deleterious role, where chronic inflammation can be a carcinogen as the immune system vainly attempts to clear the virus but in actuality causes chronic cellular stress that mediates transformation (33)
or the immune system can control the viral infection and a deleterious effect only occurs if the individual become immune compromised due to complications like AIDS or transplantation (34, 35).

Currently there are 7 known human tumor viruses. The first identified human oncovirus was Epstein Barr virus, and is the etiological agent for Burkitt’s Lymphoma, Nasopharyngeal carcinoma (NPC), and is associated with a wide variety of lymphoid and solid tissue malignancies (36) (30, 37). Since then, Hepatitis B virus and Hepatitis C virus were identified as contributing factors to hepatocellular carcinoma (HCC) (38, 39), human papillomaviruses were established as etiological agents of cervical carcinoma (40), Kaposi’s Sarcoma-associated herpesvirus was identified as the causative agent of Kaposi’s Sarcoma (41) and Human T-cell lymphotropic virus type 1 (HTLV-1) was identified as the first oncogenic retrovirus and is the etiological agent for Adult T cell leukemia (ATL) (42) and the most recent oncovirus discovered was Merkel cell polyomavirus as the causative agent of Merkel cell carcinoma (MCC) (43). Establishing each of these viruses to their associated cancer was a rather difficult process due to the barrier of determining causality. The classic standard for determining causality is the application of Koch’s postulates (44). Briefly stated, they are (i) the microorganism must be found in all cases of the disease and not in healthy individuals unless they are asymptomatic carriers, (ii) the microorganism must be isolated from the disease and propagated in culture, (iii) the cultured microorganism should cause disease if reintroduced, (iv) the microorganism must be isolated from the inoculated host and be identical to the original agent.
Obviously, these postulates are difficult to apply to human viruses and cancer for ethical and practical reasons. In effort to better relate a virus to a human cancer different guidelines were established (32). First, the geographical distribution of infection should coincide with cancer incidence. Second, cancer patients should have higher CTL or antibody titer against viral markers than in controls. Third, the viral markers should precede the onset of tumorigenesis. Fourth, a reduction in viral infection either through vaccination or preventative practices should cause a reduction in tumor incidence. Fifth, the virus should be able to induce transformation in human cell culture. And finally, the virus should induce tumorigenesis in animal models that can be prevented by neutralizing the virus.

**Evolutionary arms race between oncoviruses and their respective hosts**

The coevolution of oncoviruses and their human hosts has been a conflict that’s as ancient as the earliest humans. During our evolution, humanity has developed highly complex immune defenses to prevent and remove viral infections and viruses have evolved myriad mechanisms to subvert and evade immune responses to establish persistent infections. Human oncoviruses are interesting because they typically improve the fitness of the individual infected cell at the expense of the fitness of the host. Human oncoviruses often employ powerful anti-apoptotic and proliferative factors to prevent premature cell death and increase their genome copy (45). Many viruses encode mutated versions of cellular proteins that act in a dominant manner to upregulate growth or survival. This signaling mimicry ensures that the cell survives to propagate the virus while simultaneously pushing the cell towards transformation (46). In addition to signaling mimicry, viruses often subvert the DNA damage response to promote
unrestricted cell cycle growth. The cell cycle inhibitor pRB and the tumor suppressor p53 are common targets of viral oncoproteins as they are critical regulators of proper cellular growth and survival (47).

A classic example of signaling mimicry is the latent membrane protein 1 (LMP1) from EBV. LMP1 is viral transmembrane protein that mimics activated CD40 to drive B cell proliferation. LMP1 recruits and serves as a docking site for multiple adaptor proteins including TRAFs, TRADDs, RIP, BS69, and JAK-3. These adaptors signal to induce the activation of NF-κB, JNK/p38-SAPK, PI3K/Akt, ERK-MAPK and JAK/STAT pathways providing significant gene expression supporting proliferation, survival, and invasion (48). LMP-1 is ligand independent and results in the chronic stimulation of these pathways driving carcinogenesis through chronic replicative stress and down regulation of tumor suppressor genes (TSGs) and anti-apoptotic genes through NF-κB stimulation.

Suppression of the DNA damage response (DDR) is a key adaptation of many tumor viruses. The replication of tumor viruses relies on their ability to drive cell proliferation in cells that are otherwise quiescent (47). A consequence of aberrant proliferation is replicative stress that will normally halt the cell cycle without interference from viral proteins. A classic example is the suppression of pRB by E7 from HPV (49). Normally, pRB functions to repress G1/S entry and cell cycle progression by binding to E2F factors and inhibiting them from promoting transcription of genes necessary for S-phase. E7 preferentially binds to pRB disrupting the pRB-E2F complex allowing E2F to constitutively drive cell-cycle progression. Through the chronic cell cycle progression and aberrant check point regulation, replication-based mutations are not corrected and
will be passed along to the cell progeny, compromising the stability and integrity of the genome, which is a well-known cancer hallmark (8, 50).

The p53 protein is another key target for tumor viruses. Hailed as the “guardian of the genome” p53 is one of the most celebrated, studied, and important proteins in the field (51). It is widely recognized as a key tumor suppressor protein as evidence by its functional inactivation in more than half of all sporadic human cancers (52). Tumors that are p53 deficient are commonly more resistant to apoptosis, undifferentiated, genetically unstable and have increased invasiveness and metastatic potential (53). P53 acts a stress sensor that can inhibit cell cycle progression, initiate senescence, or induce apoptosis in response to DNA damage, hyperproliferation, hypoxia, oxidative stress, nucleotide depletion or nutrient starvation (54). As stated earlier tumor viruses often inhibit pRB to push cell cycle progression to enhance their own replication. However, forced S-phase entry activates p53 to induce apoptosis and so tumor viruses have to concomitantly silence p53 function to finish their lifecycle without the cell engaging in premature apoptosis (55). Viruses overcome p53 through several mechanisms but the most common is the binding of viral proteins to p53. For example, adenovirus E1B gene encodes E1B55K and E1B19K. E1B55K binds to p53 and inhibits its transcriptional function and E1B19K reduces p53-mediated transcription repression (56, 57).

**Viral immunity**

In addition to promoting cell cycle progression and resisting apoptosis tumor viruses must establish a chronic infection and evade both the innate and adaptive immune system. Cells have evolved numerous mechanisms to detect and respond to viral infection and viruses have adapted to evade and override many of these mechanisms. Cell intrinsic
defense mechanisms involve a vast array of restriction factors that can inhibit all steps of viral replication from viral entry to viral budding. The restriction factors often are non-specific between virus and host and thus need to be tightly regulated. For example, Protein Kinase R activation causes a global shutdown in protein translation and RNaseL cleaves cytoplasmic RNA (58). Unregulated expression of PKR and RNaseL would be severely detrimental to the well-being of a host cell and to account for collateral damage, the antiviral effectors are typically inactive until a viral trigger stimulates their activity or until their expression is upregulated in response to interferon (IFN).

The IFN pathway is extremely important in both adaptive and innate arms of the immune system (Figure 1.2). The expression of type I and III IFNs can be produced by all cells upon viral infection and IFN acts in a paracrine manner creating a local response to induce cells to enter an antiviral state through JAK/STAT signaling that induces a myriad number of IFN-stimulated genes (ISGs) that function as restriction factors to the virus lifecycle. In addition to restriction factors the IFN system also induces the expression of multiple cytokines and chemokines that will stimulate the initiation of the adaptive immune system. In effect IFN is the primary switch for activating antiviral immunity in vertebrates (59).

The activation of the IFN pathway is dependent about several different mechanisms that have evolved to sense foreign material whether from viruses, bacteria, parasites, or fungi. The molecules responsible for detecting foreign invasion are pattern recognition receptors (PRR) and they detect pathogen associated molecular patterns (PAMPs). The first mechanism for detecting PAMPs discovered was the Toll-like-receptors (TLRs) (60). The TLRs are a family of membrane bound receptors that bind to
various pathogen structural proteins or nucleic acids that are found in the extracellular milieu or within endosomal compartments (Figure 1.3). There are 10 different TLR proteins in humans with each one recognizing a specific PAMP. Several of them are able to recognize viral components to stimulate an antiviral response, specifically TLR2, TLR3, TLR4, TLR6, TLR7, TLR8, and TLR9 recognize lipoprotein (TLR2), dsRNA (TLR3), LPS (TLR4), diacyl lipoprotein (TLR6), ssRNA (TLR7/8), and CpG-DNA (TLR9), respectively (60). Upon binding to their specific ligand, TLRs dimerize and

Figure 1.2 Diagram of type I and type III IFN signaling through IFN JAK/STAT pathway leading to the induction of antiviral genes collectively known as interferon stimulated genes (ISGs)
undergo conformational changes to recruit downstream adaptor molecules including MyD88, IRAK, TAK1, TAB1, TAB2, TRAF6, TRAM and TRIF (60, 61). TLR signaling can be divided as either MyD88 dependent or TRIF dependent. Most TLRs depend on
MyD88 except TLR3 which depends on TRIF. Signaling from TLR can activate proinflammatory pathways like NF-κB or the induction of type I IFNs. The TLRs are essential for recognizing extracellular pathogens or pathogens within endosomal compartments. However, TLRs do not detect ligands within the cytoplasm and so other pathways have evolved to detect cytoplasmic pathogens.

The receptors involved in detecting viral RNA in the cytoplasm are the RIG-I-like receptors (RLRs). There are 3 central members of the RLR family; RIG-I, MDA-5, and LGP2, and each are found in the cytosol of most cell types (Figure 1.4). They are basally expressed at relatively low levels but expression can be strongly upregulated in response to IFN in a positive feedback loop of viral detection. The RLRs each have specific RNA structures that they preferentially bind. RIG-I for example, binds ssRNA or dsRNA with a triphosphate moiety at the 5’ end (62). The necessity for a 5’PPP moiety provides a method for discriminating between self and viral RNA. During RNA synthesis, the host RNA begins with a 5’ PPP but through post transcriptional modifications that moiety is masked by a 7-methyl-guanosine cap or removed before export (59). Therefore, in an uninfected cell there is virtually no 5’PPP RNA in the cytosol. In virally infected cells, the viral RNA does not undergo the same post transcriptional modifications, as the virus uses primer independent mechanisms for genome replication. MDA-5 is not as well understood as RIG-I but evidence suggests that it preferentially binds to longer segments of dsRNA and functions as a sort of “molecular ruler” as relatively short dsRNA do not effectively activate MDA-5 and longer dsRNAs are highly effective at activating MDA-5 (63).
Once RIG-I and MDA-5 become activated through RNA binding, they in turn bind to the mitochondrial adaptor MAVS via a CARD-CARD interaction on the mitochondria (60). The mitochondrion acts as a scaffold for the assembly of large aggregates of MAVS protein allowing for a relatively small amount of cytoplasmic RNA to initiate a large-scale signal amplification facilitating a robust response (64). This assembly recruits other factors like TBK1-IKKε to activate IRF-3/7 for IFN induction and IKKα/β for the induction of the NF-κB proinflammatory pathway. Additionally, many proteins also serve to modulate and regulate the RLR signaling pathway (65). The extensive network of RLR signaling regulation is a testament to both its importance in provided protection against infectious pathogens and the severe implications to the cell if the signaling network isn’t properly controlled.

RIG-I and MDA-5 have evolved to detect cytoplasmic RNA species and for a long time the receptors responsible for the detection of cytoplasmic DNA were unknown. The TLR9 protein was known to recognize CpG (cytidine-phosphate guanosine) DNA, a DAMP associated with bacteria and some viruses. However TLR9’s expression is limited to primarily leukocytes and the topology of TLR9 restricts it to extracellular sensing (59) ruling out this molecule as a player in most cell types ability to recognize cytoplasmic DNA to produce IFN. An ER localized protein STING (stimulator of IFN genes) was discovered to be essential for an IFN response to cytosolic DNA (66). It was observed that STING is capable of binding DNA strands directly but the physiological relevance isn’t completely elucidated. The initial DNA sensors that serve to engage STING dependent transcription of IFN were difficult to identify. But recently, a study suggested that it is not actually strands of DNA that bind STING to initiate the cascade but rather a
second messenger termed cyclic-GMP-AMP created from cGAS (cyclic GMP-AMP synthase) (67).
The complexity of antiviral immunity is indicative of the importance of protecting oneself from viral infection. Viruses are ubiquitous and ancient, predating millions of years before the earliest vertebrates (68). However, the complexity of our relationship cannot be simplified as viruses are dangerous pathogens and must be avoided at all costs. While many viruses are pathogenic and destructive, most are relatively benign and viral manipulation of the genome was instrumental in catalyzing evolution from single celled organisms to highly complex vertebrates and other higher organisms (69).

**Human T-cell lymphotropic virus type 1 and Adult T cell leukemia**

In the 1970s, clustering of a new form of leukemia was seen in southwest Japan (70). This new form of leukemia was classified as Adult T-cell leukemia (ATL) and presented as an aggressive form of CD4 T cell malignancy. ATL gives rise to multilobulated nuclei giving them a unique flower like appearance. ATL cells are extremely invasive and infiltration into the lymph nodes, liver, spleen, and skin lesions are the most common, though ATL involvement into the digestive tract, lungs, CNS, bone, and other organs is occasionally seen. Alongside organ complication ATL patients suffer from hypercalcemia, opportunistic infection, diarrhea, ascites, jaundice, dyspnea, pleural effusion, cough, sputum, and organometallic (71). The diversity of clinical manifestations led to the classification of ATL into 4 subtypes: acute, lymphoma, chronic, and smoldering based on prognostic factors and clinical features (72).

The geographic clustering of ATL cases led clinicians to believe that ATL may have a viral etiology and several years after the initial diagnosis of ATL, a novel RNA retrovirus was isolated from an ATL cell line established from an ATL patient. This novel virus, HTLV-1 was found to be endemic in the same locations as ATL and
antibodies against the virus were found in all ATL patients (73). Shortly after HTLV-1’s discovery it was also associated with several inflammatory disorders, most notably, HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM-TSP).

HTLV-1 belongs to a larger family of viruses known as primate T-cell leukemia viruses (PTLVs). HTLV-1 is closely related to the Simian T-cell leukemia virus type 1 (STLV-1) and it speculated to have been transmitted to humans from primates some 50,000 years ago in multiple separate independent events (74). Currently an estimated 10-20 million people worldwide are infected with HTLV-1. It is endemic in southwestern Japan, Africa, the Caribbean, and South America (75). Among the infected ATL only develops in approximately 6.6% of males and 2.1% for females and only after a long latency period lasting for several decades. The reason behind the relatively low incidence of leukemogeneis among the infected is not fully explained but it likely due to host factors such as major histocompatibility antigens and gene polymorphisms as well as maintenance of immune competence. The mechanism of leukemogenesis has been extensively studied and the viral gene Tax and HBZ have been heavily implicated as causative agents for ATL development.
**HTLV1- oncogenic protein Tax and cellular factors**

HTLV-1’s genome is arranged in a similar fashion as other animal retroviruses. The 9kb genome consists of the structural *gag*, *pol*, and *env* genes bracketed by long terminal repeat (LTR) sequences on either side of the genome. The *pol* region encodes reverse transcription, protease, and integrase related genes. The *gag* region encodes virion core proteins. And the *env* region encodes envelope proteins essential for viral infectivity (76). The 5′-LTR is the promoter region for the HTLV-1 genome. Additionally, HTLV-1 has a pX region downstream of the *env* and upstream of the 3′-LTR. This pX encodes several regulatory viral factors, Rex, p12, p13, p30, p21 and the oncogenic proteins Tax and HBZ. HBZ is encoded in the minus strand of pX while the other genes are from the plus strand of the HTLV-1 genome.

The HTLV-1 Tax oncoprotein is a transactivator that stimulates HTLV-1 transcription through triplicate enhancer elements (TxRE) found in the U₃ region of the 5′-LTR. Tax itself has poor affinity for DNA but interacts with a wide assortment of proteins (Figure 1.5). Tax is able to promote transcription from the 5′ LTR by binding with a variety of the cAMP-responsive element (CRE)-binding/activating transcription factors (CREB/ATF) promoting the formation of a Tax-CREB/ATF-TxRE ternary complex through the b-ZIP domain of CREB/ATF factors (77). Tax then recruits coactivators (CBP/p300 and P/CAF) to this complex which initiates transcription of HTLV-1 genes. In addition to binding and utilizing CREB/ATF for its own transcription, Tax has an impressive array of interactions in multiple pathways to induce a more hospitable environment for the virus. Tax is able to interact with the cofactors of AP1 (Fos and Jun), various members of the NF-κB pathway, various transcription factors like
NF-YB, C/EBPβ, Sp1/Egr1, Sp1/Ets, nuclear respiratory factor 1 (NRF1), TFIIA, 2 subunits of TFID (TBP and TAFII28), Histone modifying enzymes including the HATs; p300, CBP, and P/CAF, the HDAC HDAC1, Histone methyl and dimethyl transferases like SUV39H1, JMJD2A, CARM1, proteins of the switch/sucrose non fermentable complex (SWI/SNF): BRG1, BAFs 53,57, and 155, the positive transcription elongation factor b (P-TEFb) and splicing factor sc35, a Tax1-binding protein (Tax1BP1), post transcriptional and translation regulators TTP, Int6, and TRBP, Mitogen-activated kinases; MEKK1 and TAK1, the G protein pathway repressor 2 (GSP2), the GTP-binding proteins from the Rho family (RhoA, Rac, Gap1m, Cdc42), the cytoskeleton structural and modifying proteins; internexin, cytokeratin, actin, gelsolin, annexin, and gamma-tubulin, the beta subunit of the CXCR4 GPCR chemokine receptor, the kinase PI3K, the Smad proteins Smad2,3 and 4, the Cas proteins p130 Cas and CasL, the cyclins-D1,-D2,-D3, CDK4, and CDK6, p15INK4b, p16INK4a, pRb, the DNA repair pathway proteins Chk1,Chk2, Rad51, and DNA Topoisomerase 1, the centrosome associated and spindle assembly checkpoint proteins RanBP1, Tax1 BP2, Mad 1, and cdc20, numerous PDZ containing proteins; pro-IL16, Hdlg, PSD-95, β-syntrophin, lin-7, Tip1, MAGI3, hTid1, hScrib, and numerous proteins of the nuclear import, cytoplasmic export, and secretory pathways. Tax is able to interact with such a variety of proteins due to the multiple domains contained within its structure (77). Structurally, HTLV-1 Tax has multiple protein binding domains that enable it to interact with numerous cellular targets. Tax has an N terminal zinc finger motif, both NLS and NES sequences, a domain for interaction with CBP/p300, Chk2, and Gβ2, a dimerization domain, 2 leucine zipper like domains, a
carboxy-terminal activation domain, motifs for Golgi localization and secretion, and multiple sequences shown necessary for binding its various partners (78).

The Tax associated CREB/ATF transcription factors play a role in cell growth, survival, and apoptosis through CRE-directed gene transcription. The CRE element has been found to upregulate a wide array of genes including transcription factors, signaling molecules, neuron-associated molecules, metabolic factors, and factors involved with cell cycle and proliferation (79). Additionally, CREB induces the transcription of immune related genes such as IL-2, IL-6, IL-10, TNF-α, cyclooxygenase-2, and macrophage migration-inhibitory factor. The interaction of Tax with CREB causes a constitutive activation of CREB signaling, severely disrupting multiple regulatory pathways. Tax mutants deficient in CREB activation fail to induce transformation or aneuploidy in infected cells indicting that Tax induction of CREB signaling is a contributor to oncogenesis (80).

HTLV-1 Tax also induces the expression of activator protein 1 (AP1), a homo or heterodimer complex of Fos (c-Fos, FosB, Fra1 and Fra 2) and Jun (c-Jun, JunB, and Jun D). Tax binds with SRF increasing its affinity for the SRE motif found in the promoter region of Fos and Jun. Tax recruits CBP/p300 and P/CAF to the SRE-Tax-SRF complex and induces transcription (81). AP1 activation induces cell cycle progression as AP1 proteins bind to the cyclin D1 promoter inducing transcription (82). It stimulates angiogenesis through the activation of proliferin (FL1) gene expression (83).

A principle pathway affected by HTLV-1 Tax expression is NF-κB. The NF-κB pathway was first identified as a nuclear factor that bound to the enhancer element of the immunoglobulin kappa light chain of activated B cells (84). Since then it has been
realized as a highly conserved pathway across nearly all cell types in multiple species and a principle pathway in inducing an immune response. NF-κb activates the transcription of over 150 target genes which most of them participating in host immunity. Among the NF-κB target genes are 27 different cytokines and chemokines, immune recognition receptors i.e. MHC molecules, proteins for antigen presentation and receptors for neutrophil adhesion and transmigration across blood vessel walls (85).

The NF-κB pathway is divided into 3 families of proteins. There is the NF-κB/Rel family of transcription factors consisting of RelA (p65), RelB, c-Rel, and NF-κB1 and NF-κB2 which share an N-terminal Rel homology domain (RHD) responsible for DNA binding and dimerization with other members. The IκB family consisting of IκBα, IκBβ, IκBε, IκBγ, BCL-3, and the first three of which (IκBα, IκBβ, IκBε) suppress NF-κB by sequestering the NF-κB proteins in the cytoplasm and must be degraded before releasing the NF-κB units to the nucleus. The IκB proteins share ankryin repeat domains which bind to the RHD domains of the NF-κB members functionally making them transcriptionally inactive (86). And there is the IKK family consisting of kinases IKKα, IKKβ, and the non-enzymatic scaffolding protein NEMO (IKKγ) which phosphorylate the IκBs signaling the induction of NF-κB activation.

NF-κB activation induces the transcription of various genes through the dimers binding to κB consensus sequences in promoter and enhancer regions. The κB consensus sequence is 5’ GGGRNWYYCC 3’ (N- any base, R- purine, W- adenine or thymine; and Y- pyrimidine) (87). The degenerate nature of the κB sites as well as the variety of NF-κB dimers afford a degree of complexity to this transcriptional regulation system as
different dimers have different binding preferences to the κB sites. In addition, the NF-κB dimers contain several sites for phosphorylation and other post translational modifications (PTMs) allowing for unique activation profiles and crosstalk with other signaling pathways. The activity of NF-κB is complex as its influenced by not only phosphorylation and PTMs but also by an extensive network of protein-protein interactions creating interdependencies and feedback loops (88).

HTLV-1 Tax associates with RelA, c-Rel, p50, and p52 once they enter the nucleus and stabilizations their dimerization increasing the signaling output from the transcription factors (Figure 1.6). Additionally, Tax recruits CBP/p300 and PCAF coactivators to further promote transcriptional activation in the NF-κB pathway. Cytoplasmic Tax binds NEMO which is in complex with IKKα and IKKβ inducing their phosphorylation and subsequent kinase activity on IκBα releasing NF-κB to migrate to the nucleus (89). Tax also stimulates the non-canonical pathway through binding IKKα, IKKγ and p100 and inducing the cleavage of p100 into its active p52 form.

The cofactors CBP and p300 are vitally important in HTLV-1 infection. Tax requires their recruitment to the Tax/CREB complex for viral transcription from the 5’LTR. CBP and p300 increase transcription from the 5’ LTR by mediating chromatin remodeling through the acetylation of both histone and non-histone substrates (90). These cofactors interact with numerous other Tax binding proteins to globally change gene expression in HTLV-1 infected cells. The SWI/SNF complexes closely interact with Tax-CBP/p300 for Tax transactivation and histone hyperacetylation (91). And additionally, the chaperone protein nucleosome assembly protein 1 (NAP1) aids in nucleosome eviction from the HTLV-1 promoter. Tax mediated chromatin unfolding also results in a
global reduction in histone levels and is dependent on the HAT-activity of CBP/p300.

The loss of histones reduces chromatin folding and allows promoter access for transcription factors to bind (92).

Tax also interferes with the DNA damage response resulting in the accumulation of genetic changes in the infected cells. Tax can target several DDR proteins and inhibit
base excision repair, nucleotide excision repair, mismatch repair, non-homologous end joining, and damage response signaling via ATR/CHK1 (93). Tax inhibits BER by preventing DNA polymerase β function, reducing enzyme levels significantly. Tax also targets p53 and proliferating cell nuclear antigen (PCNA) to disrupt NER. Elevated Tax levels functionally inactivate p53 and renders it incapable of stimulating NER while Tax activates the PCNA promoter and interferes with the repressor PIR causing an increase in PCNA transcription and impairing NER activity. Tax expression targets the DNA-dependent kinase complex by localizing with active phosphorylated DNA-PKcs impairing the ability of cells to sense DNA damage (94). Tax also suppresses the expression of Ku80, the kinase domain of DNA-PK, reducing its availability and allowing the accumulation of dsDNA breaks within the nucleus. Tax inhibits the DNA damage sensor ataxia telangiectasia mutated (ATM) and its substrate CHK2, by causing the dephosphorylation of ATM, inactivating the kinase and binding directly to CHK2 inactivating its function (95).

The HTLV-1 Tax protein is quite the paradox in clinical ATL. It has been extensively studied and the numerous oncogenic properties have been described. However, despite its established role in cellular transformation and its requirement for leukemogenesis the Tax protein is absent in the majority of ATL cases (96). Tax is an immunodominant protein and a favored target of the CTL response in ATL patients, leading to the current hypothesis that Tax is eventually selected out of the ATL cell population as it may no longer be needed to maintain the transformed phenotype. The silencing of Tax occurs through 3 known mechanisms: 1) genetic mutation of the tax gene either through nonsense mutation, deletion, or insertion 2) deletion of the 5’ LTR
and the tax promoter and 3) DNA methylation of the 5’ LTR, silencing viral expression. By preventing tax expression, this may aid the ATL cells to escape from the host CTL and can progress to clinical disease (97).

**HTLV-1 HBZ expression and cellular factors**

Interestingly, one HTLV viral product is consistently expressed in ATL cases, the HBZ gene. HTLV-1 HBZ is the only gene transcribed from the anti-sense strand of the HTLV-1 genome. Its promoter lies within the 3’ LTR and hasn’t been observed to be silenced in clinical ATL, leading researchers to believe that while tax is no longer required for maintenance of the malignant phenotype, HBZ may be indispensable. In fact, HBZ was recognized as the first gene that is uniformly expressed in the leukemic cells of all ATL patients (98).

HBZ is expressed in 2 two forms; a spliced (sHBZ) and an unspliced (usHBZ) variant with the spliced variant being the dominant form with both mRNA and protein expression levels being much higher than that of the unspliced form. The spliced form has a small exon in the U5 and R regions of the 3’ LTR while the unspliced form initiates within the Tax gene. The proteins differ only in the first few amino acids at the N terminal region, with sHBZ beginning with MAAS and the usHBZ beginning with MVNFVSA before becoming homologous. The promoter regions for both variants are TATA- less and thus rely on the transcription factor Sp1 for transcription (99).

Structurally, HBZ has three domains: activation, central, and bZIP. The activation domain has transactivation potential and contains two LXXLL-like motifs that bind the KIX domain of CBP/p300 (100). By binding CBP/p300, HBZ inhibits viral transcription by preventing CBP/p300 from being recruited to the 5’ LTR HTLV promoter. The
LXXLL motifs activate TGF-β/Smad signaling, necessary for HBZ mediated Foxp3 expression, which contributes to the immunosuppressive phenotype of HTLV-1 infected T cells (101). The activation domain also inhibits c-Jun signaling through 3 known mechanisms; 1) inhibiting DNA binding, 2) the unspliced HBZ AD delivers c-Jun to the proteasome and induces its degradation through a ubiquitin independent mechanism, and 3) sequestering c-Jun through the formation of nuclear bodies (98, 102). The central domain navigates HBZ to the nucleus and has multiple NLS motifs identified (103). The bZIP domain mediates inhibition of c-Jun, Jun B, CREB, and CREB2, and activates JunD (98) through the formation of heterodimers with each transcription factor. Interestingly, HBZ inhibits c-Jun, JunB through binding but JunD it activated through binding the bZIP domain with the activation domain of HBZ necessary for activation (104). The bZIP and activation domain is also responsible for binding to p65 and mediates the suppression of the classical NF-κB pathway (105).

**HTLV-I immune evasion and mechanisms of persistence**

The persistence of HTLV-1 in an infected individual despite being immunocompetent is not yet fully understood. The current paradigm describes HTLV-1 as a rather latent virus that replicates its genome through the replication of infected cells with minimal expression of viral proteins allowing it to elude detection by the immune system. This method of genome replication allows the virus to increase its copy number without having to rely on the production of viral particles to infect neighboring cells (106). Additionally, HTLV-1 can spread without the need for infectious virions through cell-cell transmission via the formation of a virological synapse in the infected cell, an effect mediated by Tax. This occurs when an infected cell contacts a naïve cell and a
microtubule organizing center (MTOC) forms at the cell-cell junction. HTLV-1 gag complex proteins and viral genomic RNA accumulate at the contact point and are secreted into the uninfected cell (107).

The role of Tax and HBZ in viral persistence is best characterized as a subtle equilibrium between Tax mediated viral expression and cellular effects and HBZ mediated silencing of viral expression to allow immunoevasion of HTLV-1. HTLV-1 Tax is heavily implicated in viral persistence through its cellular effects describe earlier (immune suppression, 5’ LTR transactivation, aneuploidy, DDR response attenuation) but it also heavily targeted by the CTL response (108) counteracting its effects on cell proliferation. HBZ represses Tax expression preventing an over exaggeration of Tax levels, thus preventing their destruction by the immune system. By limiting the duration of Tax expression into short periods, the cells slowly accumulate genetic changes mediated through Tax and they eventually exhibit enhanced proliferation without the need for Tax expression.

In addition to the roles Tax and HBZ described above, HTLV-1 accessory proteins have also been implicated in infectivity, spread, and persistence. The accessory p12 interferes with MHC class I presentation by shuttling MHC heavy chains for degradation by the proteasome and a cleaved p12 is recruited to the TCR complex following ligation and downregulates signal transduction from the TCR thereby blunting the cell’s ability to function in an immune response (109). And p30 retains tax/rex mRNA in the nucleus preventing viral expression and inducing viral latency, making the infected cell immunologically silent (110) Additionally, p30 forms a complex with
p300/CBP and TIP60 augmenting the expression of genes involved in T-cell activation, apoptosis, and cell cycle regulation (111).
Chapter 2: Oncolytic virotherapy: recruiting viruses to the fight against cancer

The role of viruses in the context of cancer is particularly interesting. Oncogenic viruses have already been discussed but there are other viruses that can exert powerful oncolytic effects and are currently under investigation as cancer therapeutics. This phenomenon was first reported in a series of case studies where tumor regression coincided with natural virus infection. The most widely cited example describes a woman suffering from myelogenous leukemia experiencing a reduction in tumor burden following a presumed influenza infection (112). In the mid twentieth century, there were several clinical trials performed using an assortment of viruses against a panel of malignancies. Unfortunately, the majority of the trials produced less than desirable results and the field was relatively abandoned for decades (113).

Advances in multiple fields of study have led to a resurgence of interest in oncolytic virotherapy in the past few decades. The advancement of recombinant DNA technology has greatly benefitted the field of virotherapy as it allows researchers to manipulate viral genomes and create highly specialized vectors while significant advances in cancer biology have led to greater understanding of cancer mechanisms and enables researchers to intelligently design new vectors based on recent insights.

A successful oncolytic virus will typically possess certain desirable features. Ideally, the virus should selectively infect, replicate and kill cancer cells, preferentially including the non-mitotic cancer cells as well. The virus must be relatively harmless to the host either as a natural attribute or attenuated through genetic engineering. Other desirable factors include a non-integrating lifecycle to prevent unpredicted genomic
integration, low seroprevalence in the target population to reduce the likelihood of preexisting immunity eliminating the virus before it can exert oncolytic effects, and able to replicate to high titer under Good Manufacturing Practices (GMP) guidelines (114).

**Clinical oncolytic viruses**

Currently, the field of oncolytic virotherapy has matured past its infancy and is reaching adolescence. The approval of H101 as the first oncolytic vector for treatment of cancer patients in China is an important milestone in the field and major advancements have been made since (115). The list of oncolytic virus vector is continually growing as researchers can easily integrate new genes into a number of viral species. Some of the more common viral species are Adenovirus, Herpes Simplex Virus, Poxviruses, Vesicular Stomatitis Virus, Measles virus, Newcastle disease virus, Influenza A, Parvovirus, Reovirus, and Coxsackie virus (116). The list is by no means exhaustive but several of the viruses will be discussed below.

Adenovirus is one of the more popular oncolytic viruses used in oncolytic virotherapy. Its lifecycle naturally assumes the above-mentioned prerequisites for a strong oncolytic vector. The virus itself has a linear dsDNA genome of approximately 38kB. Virions form nonenveloped icosahedral particles less than 100nm in size with an outer protein shell that encases a nucleoprotein core (117). There are a total of 51 distinct serotypes of human adenovirus that are classified into groups A-F. (118). Adenovirus serotype 2 and 5 (Ad2/5) are the most well studied vectors and are used for oncolytic virotherapy. During infection most Adenovirus groups achieve entry through coxsackie virus B and adenovirus receptor (CAR) and integrin binding (117). Group B Adenovirus
utilizes the CD46 molecule, which allows this subset of adenovirus to effectively infect hematopoietic stem cells, dendritic cells, and malignant tumor cells (117). After the initial attachment, an exposed RGD motif on the Adenovirus penton base interacts with integrin to trigger clathrin dependent, receptor mediated endocytosis (119). Then acidification of the endosome triggers escape of virions into the cytoplasm by mechanisms that still need to be elucidated. Virions then traffic to the nucleus alongside microtubules and dock with the nuclear pore complex (NPC) (120). There the capsid dissembles and the viral genome can import into the nucleus and begin viral gene transcription.

The adenovirus replicative cycle is 2 phased: Early (E1-E4) and Late (L1-L5). The early viral transcription units E1-E4 encodes gene products that function mostly to induce cellular changes in the host cell to make it more conducive to viral replication. The products from E1A include 12S and 13S which induce chromatin remodeling and dissociation of E2F from pRB, allowing E2F mediated transcription of the E1B, E2, E3, and E4 gene products. The E1B product 55K inhibits p53 mediated apoptosis that would normally respond to the aberrant E2F activity and this protein was originally deleted to create the ONYX-015 oncolytic strain of Adenovirus. The rationale behind the deletion of 55k is that such action would render the virus highly dependent on p53 inactivation for successful viral replication (121). The E2 region encodes the DNA polymerase, the preterminal protein, and the ssDNA binding protein all required for replication of the viral genome. The E3 region is a nonessential region that functions to subvert and evade the immune response. The products downregulate MHC I and protect the cell from the lytic effects of TNF-α. This nonessential region is often deleted in oncolytic strains to
allow the insertion of additional therapeutic transgenes. The E4 region encodes 6 products that function to further augment the cell in favor of the virus. These products affect the cell cycle, host protein translation, host transcription, and DNA replication. Finally, after sufficient expression of the Early transcription genes, the major late promoter initiates the transcription from the late transcription program. The products from L1-L5 mostly encode the structural products necessary to form nascent virions. (122).

Onyx-015 (dl1520, CI-1042 Pfizer, Inc.) was the first virus used in humans as a genetically engineered oncolytic virus with replication-selectivity. With the deletion of E1B-55K, Onyx-015 was thought to be highly dependent on the functional inactivation of p53, which is lost in a high percentage of tumors due its importance in safeguarding the cell from DNA aberrations. However, conflicting data has indicated that tumor selectivity may not be dependent on p53 inactivation but rather the severe attenuation of Onyx-015 from the loss of E1B-55KD. E1B-55KD has other important viral functions separate from inhibition of p53 such as viral mRNA transport and host cell protein synthesis shut-off (123, 124) and the loss of this protein drastically affects multiple levels of the viral lifecycle. Nevertheless, Onyx-015 entered into numerous phase I-III clinical trials in the 1990s with hundreds of cancer patients treated suffering from a variety of malignancies. The phase I trials indicated an excellent safety profile for Onyx-015 with no maximally tolerated dose reached and relatively mild adverse effects such as fever, chills, nausea, asthenia, or local injection site pain (125). The phase II trials were initially designed to test Onyx-015 as a single agent against recurrent head and neck cancer (126, 127) and unfortunately the majority of cancer did not respond to treatment of Onyx-015 alone.
Tumors are rapidly evolving diseases and oftentimes they become resistant to cancer therapies through mutation and selective evolutionary pressure generated by treatment. To achieve maximal therapeutic benefit clinicians will resort to combining multiple treatment options that affect tumor growth through different mechanisms to reduce the likelihood of a tumor achieving resistance to single treatment regimen. This makes oncolytic viruses excellent candidates to use in addition to standard chemotherapy. Following the disappointing results of Onyx-015 as a single agent, clinical trials combining the adenovirus with chemotherapy were explored. The results from these trials were far more encouraging, as the combination of Onyx-015 and cisplatin and 5-fluorouracil generally produced a significant reduction in tumor volume and disease progression (128).

The efficacy of adenovirus will need to improve if it is to be considered as a single agent therapy. The failure of Onyx-015 as a single agent therapy raises the need for more potent second generation constructs. An example of a promising vector is an adenoviral E1A mutant that exhibits tumor selectivity but with significantly greater anti-tumor efficacy. It has performed well in a variety of cancer models and is being tested in multiple preclinical models (129)

Herpes Simplex Virus type 1 (HSV-1) is another well studied and developed oncolytic virus. The first replication competent oncolytic HSV was described in 1991 for the treatment of gliomas (130). It several features that give it several advantages over Adenovirus as a vector. HSV-1 can complete its replication cycle within 20 hours while adenovirus typically takes 48-72 hours. This faster replication time translates into more oncolytic effect before antiviral immunity severely reduces the efficacy of the virus
Additionally, HSV-1 virions are capable of direct cell to cell spread through cell junctions and induction of syncytia formation in addition to spreading through the extracellular milieu. Syncytia involves the fusion of cellular membranes creating multi-nucleated cellular structures that facilitate spread of the virus without the need to enter the extracellular environment enhancing the spread of the virus throughout a tumor mass. HSV-1 also has a much wider tropism than human adenovirus vectors, allowing it to replicate in most laboratory animals greatly simplifying preclinical evaluation compared to adenovirus.

HSV-1 is an enveloped double stranded DNA virus. The genome is relatively large at 152kb and encodes approximately 90 different gene products. The genome is arranged as L (long) and S (short) components that are covalently linked together. The components are bracketed by inverted repeats that facilitate homologous recombination inverting the components to create different isomers designated as prototype (P), Inversion of L (IL), Inversion of S (IS), and Inversion of L and S (ISL) (131). The mature HSV-1 virions consist of four elements: (a) a densely packed core with the dsDNA genome wrapped as a toroid (b) a capsid surrounding and protecting the DNA core composed of 162 capsomeres arranged in a icosadeltahedral. The capsid shell contains channels controlled by tegument proteins to control the transport of DNA through the channel. (c) an unstructured tegument surrounds the capsid and is composed of an assortment of proteins that aid in many functions for the virion. (d) An outer envelope exhibiting spikes on the surface. The envelope consists of a lipid bilayer with over a dozen different viral glycoproteins that mediate binding and fusion with the target host cell (131).
When HSV-1 first encounters, a target cell the initial attachment is mediated through the interaction of viral envelope glycoproteins gC and gB with glycosaminoglycan moieties of cell surface heparin sulfate (132). Following the initial attachment, viral glycoprotein gD commits the virion to fusion by binding one of several receptor targets such as nectin-1a, 1b,2a,2d, or HveA (133). Once the virion has fused with the cell, the capsid is then transported to the nuclear pore through the microtubular network (134).

Once inside the nucleus, the HSV-1 genome circularizes and transcription of the immediate early genes ICP 0,4,22,27, and 47 commences rapidly (135). Host transcription factors, and VP16 stimulate the transcription of the IE genes. The majority of the IE gene products are involved in transcription regulation to inhibit host gene expression and switch the gene expression from cellular to viral gene expression. Subsequently expression of the early viral genes function to stimulate nucleotide metabolism and viral DNA replication and include the viral DNA polymerase, ssDNA binding protein, origin binding protein, and DNA helicase-primase. The viral E gene products localize to the viral genome inside the nucleus and begin origin dependent transcription of the viral DNA. Viral DNA synthesis uses a rolling-circle mechanism and produces head-to-tail concatemeric molecules. Following viral DNA replication, the expression of late gene products begins. The late genes encode primarily the structural components of the virion.

HSV-1 constructs have performed remarkable well in preclinical and safety studies and several variants of the virus are currently in clinical studies. One such HSV vector currently known as T-VEC has recently been FDA approved for the treatment
melanoma lesions in the skin and lymph nodes after a successful clinical trial (ClinicalTrial.gov Identifier NCT00769704) (136). T-VEC is a modified HSV virus that is designed to selectively replicate and lyse tumor cells while simultaneously promoting both local and systemic antitumor immunity. The selectivity of T-VEC is achieved through the deletion of several viral genes. The deletion of the neurovirulence factor gene (ICP34.5) attenuates viral pathogenicity and enhances its tumor-selectivity and the deletion of the ICP47 gene reduces the ability of the virus to evade the immune system, priming infected cells for immune attack. T-VEC is also modified to encode human granulocyte macrophage colony-stimulating factor (GM-CSF) which allows local cytokine production to recruit and activate antigen presenting cells (APCs) boosting a tumor specific immune response.

T-VEC has demonstrated an acceptable safety profile (low grade fever, chills, myalgia, and injection site reaction) after intralesional administration during phase I clinical trials (137). Then in a phase II trial, an overall response rate (ORR) of 26% was reported in patients with stage IIIC to IV melanoma both at the injected site and distal lesions (138). In the phase III OPTiM trial, T-VEC was shown to produce a durable response rate (DRR) significantly higher than GM-CSF treatment alone (16.3% vs 2.1%) and also displayed a better overall response rate (ORR) and a higher median overall survival duration than GM-CSF alone (139).

**Research strategy to improve oncolytic vectors**

The FDA approval of T-VEC marks an important milestone in the field of oncolytic virotherapy and provides a tremendous opportunity for the development of more potent oncolytic viruses and treatment modalities. Perhaps the most promising
strategy involves the combination of an oncolytic virus with another therapeutic option. An assault on tumor cells from multiple treatment modalities that affect different pathways greatly decreases the likelihood of the target tumor to become resistant to the treatment strategy. Currently there are three basic strategies for the creation of combination therapy using an oncolytic virus. The quickest route to clinical relevance would be to simply add the virus to a current standard of care. Various chemotherapies have shown a high potential for synergy in a multitude of tumor models. However, several models have also shown that certain chemotherapeutics and radiation modalities can be antagonistic to certain viral replication lifecycles. Therefore, it is important to understand the mechanisms and pathways affected by both the virus and the therapy in question. Another strategy would be to identify the barriers to a viral activity and select therapies that reduce that barrier increasing the efficacy of the virus. And finally, another approach would be to combine the oncolytic virus with some form of immunotherapy to significantly activate the immune system to target the tumor.

Radiation therapy is a widely-used approach to induce oncolysis through the induction of DNA damage. The form of radiation used is ionizing radiation (IR) in that it forms ions which deposit energy in the targeted cells. IR reacts strongly with DNA within cells and can cause DNA damage through single and double strand breaks which severely limit the affected cell’s ability to proliferate and often results in cell death (140). Cancer cells typically have defects in their DNA repair pathways making them more susceptible to IR induce radiolysis (141), and when combined with highly advanced computer imaging and targeting techniques clinicians are able to maximize the exposure of cancer cell to the IR while minimizing damage to the surrounding normal tissue (142).
The effects of combining radiation therapy with an oncolytic virus has been extensively studied using multiple viruses across a multitude of tumor models. As the relationship between these two treatment modalities becomes better understood, researchers have developed new strategies and refined previous techniques to enhance the synergistic output of combination therapy. Synergy, where the combined effect is greater than the sum of its parts, is observed through a variety of mechanisms depending on the construct used. The relationship between HSV-1 and radiation therapy is one of the more well studied and characterized. Radiation exposure is able to significantly improve HSV titers in a variety of cancer cells and in vivo (143). The mechanism behind the increase in titer is believed to be due to the radiation induced induction of cellular GADD34. GADD34 functions as a DNA damage and growth arrest inducible gene that protects cells from genetic damage. Interestingly, a region of GADD34 show significant homology to HSV \( \gamma 34.5 \) protein (144). HSV-1 \( \gamma 34.5 \) works to dephosphorylate the cellular translation initiation factor eIF-2\( \alpha \) resulting in continued protein synthesis and cell survival even during a stress response. HSV-1 \( \gamma 34.5 \) is often deleted in HSV-1 constructs to reduce neurovirulence but it also severely attenuates viral replication and cytotoxicity in tumor cells compared to \( \gamma 34.5 \) positive strains (145). GADD34 prevents the phosphorylation of eIF-2\( \alpha \) and can functionally rescue \( \gamma 34.5 \) function without increasing the risk of neurotoxicity.

Oncolytic viruses can also be genetically modified to enhance the synergistic effect of radiation therapy. Many viruses have promoters that are activated by exposure to radiation and encode gene products that will sensitize the cells to radiation induced cell death. An adenoviral construct CRAd-S-pk7 was modified with the survivin promoter
and when combined with external beam radiotherapy (XRT), researchers reported a significantly delay in glioma tumor growth compared to radiation or WT adenovirus alone. Additionally, viral titers in tumor 6 days post infection were increased by nearly 100-fold when combined with XRT, proving useful in overcoming the challenge of adequate viral replication and spread (146).

Another approach to enhance radiosensitivity is the addition of the tumor suppressor gene p53. Lack of functional p53 is a common characteristic in malignancy and re-expression of p53 can render tumor cells more susceptible to cytotoxic treatments including radiation therapy (147). This strategy was utilized using an adenoviral vector AdΔ24 expressing p53. Numerous studies indicate that adenovirus mediated tumor lysis is more effective in cells that express p53 and combination of AdΔ24-p53 with XRT displayed synergistic cytotoxicity in human glioma cell lines compared to either treatment alone (148).

Targeted radionuclide therapy is another proven method that works very well with oncolytic virotherapy. Certain malignancies overexpress specific cell-surface receptors that can enable an accumulation of their substrate within the tumor mass. A well-known example of this is thyroid cancer expressing the sodium iodide symporter (NIS). By administering radiolabeled iodine, the tumor will effectively sequester the radiolabeled-iodine exposing the tumor to cytotoxic levels of radiation while effectively sparing cells that do not express NIS. Oncolytic viruses can be modified to express NIS forcing the expression of NIS in infected cells and enabling therapeutic administration of radiolabeled-iodine.
An adenoviral construct Ad5-yCD/mutTK<sub>S</sub><sup>rep</sup>-hHIS is currently in clinical trials for the treatment of clinically localized prostate cancer. This construct expresses a highly efficient fusion protein that encodes the catalytic domains of 2 therapeutic suicide genes cytosine deaminase and thymidine kinase as well as the hNIS reporter gene. In this study the hNIS gene was used to image viral gene expression through the uptake of Na<sup><sup>99m</sup>TcO<sub>4</sub></sup> in infected cells using single photon emission-computed tomography (SPECT). This technology allowed researchers to monitor the activity of gene therapy following administration to patients, providing valuable insight to the local and distant spread of the viral vector as well as its persistence in the target tissue (149).

The suicide genes cytosine deaminase (CD) and thymidine kinase (TK) are excellent examples of using chemotherapy alongside oncolytic viruses to treat malignancy. Chemotherapy is the use of cytotoxic agents that preferentially kill tumor cells while being less harmful to normal cells. The difference between tumor and host toxicity is the therapeutic index and is the difference between the drug concentrations required to kill a tumor cell compared to a normal cell (150). A large therapeutic index is a requirement for any successful treatment and many chemotherapies are DNA damaging agents that are highly toxic to actively mitotic cells, a common characteristic of transformed cells.

Cytotoxic agents are unable to differentiate between cancerous and rapidly dividing healthy cells such as hematopoietic stem cells and gastrointestinal mucosal epithelial cells. Therefore systemic administration of chemotherapy is associated with a range of side effects and can cause long-term organ damage and increased risk of leukemia (151, 152). To localize the effects of chemotherapy, gene directed enzyme
prodrug therapy (GDEPT) was developed to introduce genetic material into tumor cells that will render them susceptible to cytotoxic effects from otherwise inert prodrugs. The prodrug is administered to the tumor and in its native form it has minimal cytotoxic effects. However, it can be converted to a highly toxic agent via an exogenous enzyme in the tumor from GDEPT. Both CD and TK are examples of GDEPT and activate the prodrugs 5-fluorocytosine (5-FC) and ganciclovir (GCV), respectively.

GCV is a synthetic nucleotide analogue of 2’-deoxy-guanosine that was first synthesized as an antiviral agent against HSV-1 (153). GCV is phosphorylated by TK to a monophosphate (GCV-MP), and it is then phosphorylated by cellular kinases to a triphosphate (GCV-TP). GCV-TP is similar to deoxyguanosine triphosphate (dGTP) and is incorporated into DNA during replication, causing inhibition of DNA polymerase, rapid chain termination, and SSBs, leading to cell death (154). HSV-TK/GCV has proven to be a very successful strategy and has reached phase III clinical trials (155) in treating glioblastoma multiforme using a replication defective retrovirus combined with radiotherapy. A subsequent phase II trial utilized a replication defective adenovirus expressing HSV-TK to treat glioma and produced a significant survival benefit over standard therapy (150).

The administration of the nucleotide analogue 5-FC following the exogenous expression of cytosine deaminase is an ingenious method to localize the effects of 5-FU. 5-FC is a relatively harmless compound that can be converted into highly toxic 5-FU by CD. CD is an enzyme only found in yeast and bacteria (156). Fluorouracil (5-FU) is a common chemotherapeutic agent for a variety of cancers. It works in a similar function as GCV in that intracellular enzymes convert 5-FU to the active cytotoxic metabolites 5-
fluorodeoxyuridine-5’ triphosphate (5-FdUMP), 5- fluorodeoxyuridine-5’ triphosphate (5-FdUTP) and 5-fluorouridine-5’triphosphate (5-FUTP). 5-FUTP is incorporated into DNA causing damage, and prevents nuclear processing of rRNA, tRNA, and mRNA. 5-FdUMP inhibits thymidylate synthase (150). FdUTP is also incorporated into DNA, leading to strand nicked DNA and inhibition of replication leading to cell death (150).

The CD/5-FC system also produces a substantial bystander effect compared with the HSV-TK/GCV system. 5-FU is able to freely diffused in and out of cells without the need for gap junctions or any sort of receptor (150). In vivo studies have indicated that even as little as 2% of CD+ tumor cells in a tumor mass is able to produce significant tumor reductions (150). The CD/5-FC system has also been tested in several clinical trials and was determined to be a safe therapeutic option (150).

In addition to nucleotide analogues, there are many different pathways targeted by different chemotherapy drugs. For oncolytic virotherapy to be successfully incorporated into the clinic, it must at least be compatible with the most common chemotherapies and ideally it would be synergistic with existing treatment modalities. Some of the more common drugs can be categorized as alkylating agents (cisplatin, cyclophosphamide, and mitomycin C), DNA intercalators (doxorubicin), nucleotide analogues (5-FU and GCV), cytoskeleton modifiers (paclitaxel and docetaxel) and cytostatic agents (rapamycin) (143). As each of these drugs affect different pathways, it is vital that special consideration be given when determining the optimal chemotherapy and virus combination to use in each cancer.
Alkylating agents were among the first compounds to treat cancer effectively and they are still in common use. These cytotoxic agents cause DNA damage by attaching alkyl groups to either the N- or O- atoms in DNA bases. The alkyl groups added to DNA can be a simple methyl group (-CH3) or be complex hydrocarbon chains depending on the class and specific agent. Alkylating agents create a pattern of DNA lesions depending on the number of reactive sites and its specific chemical reactivity. Agents are either monofunctional or bifunctional and react through either SN1 or SN2 type nucleophilic substitutions (157). The monofunctional agents contain one active chemical moiety to attach the alkyl group to either the N or O atom while bifunctional agents contain two active sites and mediate interstrand crosslinks. The SN1 agents attach to either the N- or O- atom while the SN2 agents primarily react with the nitrogen atoms. Primarily, chemotherapeutic alkylating agents are SN1 type and can be either monofunctional or bifunctional.

Cyclophosphamide (CPA) is a common alkylating agent used to treat lymphoma, leukemia, breast, ovarian and bladder cancers. CPA is converted into the active metabolites, 4-hydroxycyclophosphamide and aldophosphamide by liver oxidases and the aldophosamide is then converted into phosphoramid mustard that causes DNA cross-linking leading to apoptosis. CPA can be used to enhance the effect of oncolytic virotherapy and it is used in 2 main strategies. First, CPA can act as an immunosuppressant to enhance viral infectivity, replication, and spread of the oncolytic virus. And a second strategy is to use an OV encoding cytochrome P450 (CYP2B1) to convert CPA to its active metabolites, which will concentrate the toxic metabolites in the OV infected cells (143).
The immunosuppressive effects of CPA are multifaceted, affecting multiple aspects of the immune system including the humoral and cellular mediators of the innate and adaptive immune system. CPA treatment at high doses results in global immunosuppression characterized by significant decreases in total white blood cells, i.e. lymphocytes, neutrophils, and monocytes. Additionally, the serum from treated animals show neutralizing antibody titers below the limit of detection and a reduced inhibition of viral infectivity (158). The use of CPA as an immunosuppressant has improve the efficacy of several vectors in a variety of models including HSV(159-161). Adenovirus (162), measles virus (163), Reovirus (164, 165), and VV (166).

Cisplatin is another bifunctional alkylating agent that has been extensively tested with a variety of oncolytic viruses including adenovirus(167), HSV (168) parvovirus (169), VV(170), and VSV(171). Adenovirus with a deletion of the E1B 55/19kD protein performed especially well with cisplatin in both in vivo and in vitro models (172). Parvovirus can sensitize p53-negative cells to cisplatin (169). Cisplatin increases GADD34 expression and can sensitize cells to HSV oncolytic vectors (168).

Another example of chemotherapeutic drugs that target DNA are the DNA intercalating agents. These agents are able to insert between base pairs in DNA through a planar aromatic moiety, causing DNA structural changes and functional arrest (173). DNA intercalators are used in a variety of clinical settings including anticancer, antiparasite, and antimicrobial. The use of these compounds can directly inhibit DNA dependent enzymes depending on the specific compound. Anthracycline and amsacrine inhibit topoisomerases II inhibitors, whereas ellipticine can inhibit topoisomerases, DNA polymerase and RNA methylase (174) and others like ethidium bromide, proflavine, and
actinomycin D inhibit RNA polymerase (174). The intercalation puts stress on the structure of DNA and several intercalators can induce strand breakage of the DNA. Combination of MAP (Doxorubicin, mitomycin C, and cisplatin) and adenovirus was used in a clinical trial to treat advanced sarcomas. The therapy was well tolerated and a partial response occurred in one patient (175).

**Immunomodulators and oncolytic virotherapy**

The role of the immune system in oncolytic virotherapy is multifaceted and must be carefully considered when designing treatment options. It represents a double-edged sword in that the immune system can prematurely clear the virus before significant oncolysis has occurred but conversely the direct oncolysis can also stimulate the immune system and create an antitumor immune effect. Naturally, immunomodulatory agents would be a primary consideration when developing therapies to utilize these powerful oncolytic agents. Histone deacetylases (HDACs) inhibitors and immune checkpoint modulators are important immunomodulatory agents being investigated in a variety of tumor models.

Chromatin is the condensed and packaged form of DNA, allowing the entire organism’s genome to fit neatly within a nucleus. A side effect of this efficient packaging is the inaccessibility of the genome to transcription factors and so chromatin must be remodeled to allow expression of genes. Chromatin is packaged around nucleosomes, which is 147bp of DNA wrapped around a histone core containing the twin subunits of H2A, H2B, H3, and H4 (176). Chromatin structure is highly dependent on the epigenetic modification that occur in both the DNA and the histone tails protruding from the
nucleosomes. Histone modification is a complex process and includes acetylation, methylation, phosphorylation, and SUMOylation (177).

The role of histone modification in cancer is evident in the mutations and aberrant expression of various HDACs, making them an important player and therapeutic target for treatment. Several HDAC inhibitors (HDIs) have thus found themselves in a clinical role for the treatment of several malignancies. They have been shown capable of inducing growth arrest, differentiation, senescence and death in cancer cells with attenuated effects in normal cells. For example, vorinostat, romidepsin, and belinostat have already been approved for clinical use and several others are in clinical trials (178).

HDIs are excellent standalone therapies for their anti-neoplastic effects but they are also capable of weakening intrinsic cellular anti-viral immunity (179, 180). Several groups have investigated combining OV-based therapy with different HDIs to enhance the effect of the virus while simultaneously utilizing the anti-neoplastic potential. VSV, HSV, Adenovirus, VV, and Western Reserve have all been extensively studied with different HDIs and various mechanisms of synergy were observed (178).

VSV is a powerful oncolytic virus and one such variant VSVΔ51 displayed remarkable synergy with the HDIs vorinostat and MS-275 (181). VSVΔ51 possesses a defect in the M gene that renders it incapable of counteracting anti-viral IFN responses. This defect makes VSVΔ51 an excellent and extremely safe option for treatment. However, some cancers still retain residual anti-viral IFN activity and will be refractory to VSVΔ51. When VSVΔ51 was tested in combination with the HDIs, IFN inducible genes like IRF3, IRF7, and MxA were suppressed and VSVΔ51 was able to direct a
significant oncolytic effect through lytic replication and activation of intrinsic apoptotic pathways (182). Interestingly, MS-275 co-treatment was able to induce an immunosuppression in regulatory and naïve T cells without reducing the secondary response towards tumor associated antigens (TAA) (183).

Immune checkpoint modulators take advantage of the elaborate regulatory mechanisms of the immune system to promote antitumor immunity. In order for cancer to develop into a pathological disease it must find a way to subvert and evade the immune system and a common approach is the expression of immunosuppressive cytokines and ligands (8). Tumor cells often express numerous factors that drive the evolution of an immunosuppressive network which protects the primary tumor from the immune system and ultimately allows for the colonization of metastatic sites (184). Key factors in this network include several different types of tumor-derived soluble factors (TDSFs). The list is by no means exhaustive but they include the immunosuppressive cytokines TGF-β, IL-10, small molecule prostaglandin E, angiogenic molecule VEGF, soluble Fas ligand (FasL), soluble MHC class I-related chain A gene (sMICA), soluble phosphatidylserine (sPS) (185-189). In addition to soluble factors tumor cells will also express PD-L1 which is a potent immunosuppressive cell surface receptor. PD-L1 expressed on tumor cells are able to bind to PD-1 and CD80 on T cells, mediating inhibition (190, 191). PD-L1/PD-1 interaction inhibits Akt phosphorylation by preventing CD28-mediated activated of phosphatidylinositol 3-kinase (PI3K). The inhibition of Akt limits glucose metabolism, prevents GSK-3 phosphorylation, and limits IL-2 production all contributing to T cell inhibition (192).
The soluble factors interact with immune cells through several mechanisms to provide a suitable environment for the tumor to proliferate. VEGF acts as a chemoattractant for the production of immature myeloid cells in the tumor environment where they can expand into tumor associated dendritic cells (TiDCs) and macrophages (TAM) through the exposure of TDSFs and facilitate an immunosuppressive environment (193). TGF-β, IL-10, and PGE₂ can inhibit the maturation of DCs and T cell function (194, 195). Soluble FasL and sMICA can inhibit Fas and NKG2D mediated cell killing of immune cells thereby protecting the immunosuppressive network cultivated by the tumor and the TDSFs (186, 196).

Immune checkpoint blockade aims to circumvent some of the mechanisms of immune evasion employed by tumor cells. The two most clinically relevant blockade strategies are CTLA-4 and PD-1 blockade. The use of antibodies directed against CTLA-4 (ipilimumab) and PD-1 (nivolumab and penbrolizumab) are FDA approved therapies and are the standard of care for advanced metastatic melanoma (178). The blockade of CTLA-4 and PD-1 can result in serious side effects as the signaling cascade of CTLA-4 is exceptionally vital in modulating the immune system and studies with CTLA-4 knockout mice will result in a rapidly lethal systemic immune hyperactivation (197). PD-1 knockout results in a more mild organ specific inflammation that is late onset (198, 199). This is consistent with the clinical observation that the autoimmune side effects of PD-1 blockade drugs are generally more mild and less frequent than CTLA-4 blockade. Nonetheless, immune checkpoint blockade has produced extremely effective and durable response rates in some melanoma patients that justifies their use (200).
The elaborate immunosuppressive networks generated by the tumor microenvironment (TME) prevents an immune response against the tumor and some networks are even able to resist the effects of immune checkpoint blockade in more aggressive cancers. In these cases, strategies need to be developed to transform a non-immunogenic TME into an immunogenic one (201). Oncolytic viruses are ideal candidates to complement immune checkpoint blockade as by their very nature they create an immunogenic form of cell death (ICD). The viral antigens coupled with the release of TAAs and the release of cytokines that are a normal response to viral infection create a highly immunogenic environment and when combined with checkpoint blockade antibodies it should break the established immunotolerance and generate antitumor immunity(202, 203).

The efficacy of combining oncolytic vectors with immune checkpoint blockade is under extensive investigation in both pre-clinical and clinical settings. VSV, NDV, MV, Ad and VV have all demonstrated remarkable synergy when combined with immune checkpoint blockade. VSV targeting Her1/neu expressing tumors, followed by systemic CTLA-4 blockade elicited a strong anti-tumor CD4+ and CD8+ T cell response and generated a complete and long lasting remission with the development of anti-tumor memory (204). NDV with systemic CTLA-4 blockade drastically improve the NDV mediated immune response in a bilateral B16-F10 murine melanoma model compared to NDV alone (205). The effect was largely dependent on NK cells, CD8+ cells, and type I and II IFN.

Additionally, the timing of the treatment regimen needs to be considered and will determine the success of the combination therapy. In the VSV model discussed earlier,
the benefit of CTLA-4 inhibition was reduced when treatment was delayed to 3 days after VSV administration and completely lost when given 7 days after VSV therapy (204). The important of timing was also demonstrated using VV in syngeneic murine models of renal and colon adenocarcinoma. If CTLA-4 blockade was given concurrently with systemic VV therapy, the immune system would target the virus too quickly and restrict viral replication that limited its oncolytic efficacy. However, if CTLA-4 therapy was given 4 days after VV therapy the effect was significantly improved over monotherapy and correlated with a rise in CTLs recognizing tumor cell antigens and was mediating by both CD8+ T cells and NK cells (206).

An alternative approach to co-administration of immune checkpoint blockade is to directly insert the genes encoding antibodies against CTLA-4, PDL-1, or PD-1 into the OV. By inserting the gene into the OV itself, it restricts the expression of the antibody to the TME, thus reducing systemic immune-related side effects. An oncolytic Adenovirus was constructed expressing an anti-CTLA-4 antibody and when injected into human lung carcinoma xenografts, it induced a drastic rise in antibodies levels at the tumor site over plasma (207). A MV encoding anti-CTLA-4 antibody was also able to mediate a greater anti-tumor response in an immunocompetent murine model of melanoma. The improved response corresponded with increased levels of activated CD8+ T cells and reduced tumor infiltration of Treg cells. However, despite the MV-anti-CTLA-4 Ab improvement over unarmed virus it was still less effective then MV combined with systemic CTLA-4 blockade. This indicates a possible drawback in arming the oncolytic virus as there is little flexibility to optimize the timing of the OV and the immune checkpoint blockade (208).
Chapter 3: VSV virology and its utilization as an oncolytic vector

After the landmark discovery that natural viral infection can result in tumor regression, researchers everywhere began experimenting with different viruses to determine their efficacy in cancer therapy (112). Eventually, several viruses began to emerge as superior agents in maintaining the balance between oncolysis and avirulence to the host. The favored viral families include Herpesviridae, Adenoviridae, Poxviridae, Parvoviridae, Paramyxoviridae, Picornaviridae, Rhabdoviridae, Retroviridae, and Reoviridae (209). The vast majority of these viruses have been genetically engineered in countless ways allowing researchers to design therapies to take advantage of their augmented viruses. VSV from the Rhabdoviridae family is a prime example of a heavily genetically modified virus that has reached clinical trials and has been tested extensively in preclinical models.

VSV biology

Wild type VSV exists as two major serotypes, Indiana and New Jersey. WT VSV is endemic to central and South America and certain parts of the USA and primarily affects horses, cattle, pigs, and insect vectors (210). VSV infections are rarely lethal and manifest as fever and blister like lesions of the mouth, feet, and teats in livestock (211). In humans, VSV infection is generally asymptomatic and limited to agricultural and laboratory workers. Under certain conditions VSV can result in neurotoxicity if it accesses the CNS. Neurotoxicity has been observed in mice or rats when administered intracranially, intranasally, intravascularly, and intraperitoneally (212). Additionally, VSV-mediated encephalitis has been observed in non-human primates (NHP) although neurotoxicity was only observed when VSV was injected intrathalamically (213).
Neurotoxicity from VSV infection is a direct result from the cytopathological nature of VSV infection and not from immune cell mediated inflammation. Animal models demonstrate that athymic and immune-deficient SCID mice succumb quickly to VSV mediated encephalitis (214, 215). VSV is able to efficiently infect and replicate within neuron cells resulting in lytic cell death causing neuropathogenesis and can only be effectively removed from the CNS if both innate and adaptive immune mechanisms are intact (216).

VSV has a relatively simple genome that encodes the nucleocapsid (N) protein, phosphoprotein (P), matrix (M), glycoprotein (G), and large polymerase (L) protein. The genome itself is 11kb and is encased within 185x75mm bullet shaped virions encompassing all 5 viral proteins (Figure 3.1) (217). Each protein plays an essential role in the viral lifecycle. The nucleocapsid protein tightly encapsidates the VSV genome forming a nuclease resistant nucleocapsid. This nucleocapsid serves as the template for the virally encoded RNA polymerase formed by the phosphoprotein and large polymerase protein facilitating both viral transcription and genome replication. The G protein is essential for binding the virion to the host cell and mediating membrane fusion to insert the viral components into the cytoplasm to initiate viral transcription (218). The matrix protein is a complex multifunctional protein that plays a role in immune evasion by limiting host cell mRNA export. It localizes to the nuclear membrane and interacts with Rae1 and Nup98 of the nuclear pore to inhibit nucleocytoplasmic trafficking of host
mRNA and snRNAs to limit a cellular antiviral response (219). The M protein also interferes with spindle malformation during metaphase and triggers apoptosis through its interaction with Rae1-Nup98 (220).

The VSV infection lifecycle is fairly typical among RNA viral species. Following attachment of the G protein to the cell surface, the virion is absorbed through clathrin-mediated endocytosis and enters endocytic pathway (218). Once the pH in the endosome drops, conformational changes in the G protein mediates the fusion of the viral and endosomal membrane, resulting in the eventual release of the viral ribonucleoprotein components into the cytoplasm. The VSV polymerase complex (the N, P, and L proteins) transcribes viral genes directly from the RNA viral genome. It initiates binding at the extreme 3’ end of the genome at the leader sequence of the genome, where it will begin transcribing in a sequential manner, each of the 5 VSV genes along with the leader and
trailer region. It will stutter at each intergenic region during the polyadenylation of the transcript and have a probability to dissociate from the genome. This results in a decreasing gradation of the viral products so that the N protein will be the most abundant, followed by decreasing amounts P, M, G and L proteins (221). The availability of N protein bound to the VSV genome is believed to help the polymerase complex remain associated with the genome to transcribe the later VSV genes. Although the exact mechanism by which the polymerase acts as a replicase or a transcriptase remains poorly understood (222).

Once the levels of viral proteins have reached adequate levels, the polymerase complex begins transcribing the antigenome of VSV. The antigenome will then act as a template to allow the VSV polymerase complex to replicate the VSV genome. The nascent genome is encapsidated in soluble N protein and the P and L proteins associate with the RNP core. This complex is assembled into mature VSV virions through mechanisms that are not fully elucidated but it is known that the M protein and G are essential in the assembly and budding process (223-225). In the mature virions, the M protein is located beneath the viral membrane and serves to bridge the nucleocapsid with the lipid bilayer. The G protein is an integral transmembrane protein that is anchored in the lipid membrane. These mature infectious virions bud from cell surface removing host cell derived lipid envelope during the process and can begin the infection cycle anew.

**Host response to VSV**

Despite its wide tropism VSV infection is rarely pathogenic in humans and is known to be extremely vulnerable to the defenses of the innate and adaptive immunity.
The host response to VSV infection is largely dependent on two pathways, specifically, the RLR and TLR pathways. RLR signaling depends on the RIG-I helicase recognizing viral RNA. RIG-I recognizes 5’ triphosphate RNA that is typically only present in the cytosol during viral infection. VSV replication produces a leader sequence that is upstream of the N gene and this sequence along with the genome and antigenome produced is believed to represent the major IFN inducing patterns. RIG-I consists of 2 N-terminal CARD domains that mediate downstream signaling, a central subfamily 2 type DECH box ATPase domain, and either a specific short C-terminal domain or a regulatory domain (RD) (226). RIG-I remains inactive until 5’ triphosphate RNA binds to the RD inducing ATPase-dependent conformational changes. After binding, RIG-I self-associates as the CARD domain is liberated allowing CARD-CARD interactions. RIG-I is ubiquitinylated by the E3 ubiquitin ligases TRIM25 and riplet/RNF135 allowing association with the CARD domain of the adaptor protein MAVS (CARDIF, IPS-1, VISA) on the outer mitochondrial membrane (227). The ubiquitinated complex leads to the formation of the multiunit complex consisting of RIP1, TRADD, FADD, TBKBP1, NAP1, and TANK on the mitochondrial membrane. This complex activates the kinases TBK1 and IKKi, which phosphorylate and activate IRF3. The pIRF3 form dimers that translocate to the nucleus and activate the IFN-β promoter alongside the NF-κB and AP1 transcription factors (59). Recently STING was determined to play a role in downstream RIG-I signaling but its exact role remains elusive but is believed to form a complex with MAVS, RIG-I and TBK1 to participate in signal transduction (228).

In addition to RIG-I, TLR signaling is known to be involved in the detection of VSV. The TLR receptors known to detect VSV components are TLR7, TLR8, TLR4,
TLR3. TLR7 binds ssRNA and is predominantly expressed in plasmacytoid dendritic cells (pDCs) and B cells (229). TLR7 detects within endosomal compartments of the pDCs and requires the endocytosis of extracellular viral particles followed by autophagy to transport the viral replication intermediates into the lysosome (230). TLR8 is phylogenetically similar to TLR7 and also recognizes ssRNA within endosomes. TLR8 is primarily expressed in monocytes/macrophages and myeloid dendritic cells (DCs) (231). TLR4 is expressed in monocytes, fibroblasts, epithelial cells and promononuclear leukocytes (232). TLR4 is the prototypical receptor for LPS but other ligands have been discovered for TLR4 and include respiratory syncytial virus protein F, HSP60, proinflammatory extra domain A of fibronectin (233). The VSV-G protein was reported to induce a CD14/TLR4 dependent response that activates IRF7 in both mDC and macrophages (234). And studies have shown that mutations in CD14 or TLR4 rendered macrophages unable to produce IFN during VSV infection. TLR3 recognizes dsRNA enabling it to bind to the genome of some RNA viruses as well as viral replication intermediates of ssRNA viruses. Its expressed in the intracellular membrane of macrophages, B cells, and dendritic cells, as well as intracellularly and surfaces of NK cells, epithelial cells, and fibroblasts (229).

Downstream signaling from the TLR receptors share several key adaptor molecules to propagate the signal through 3 major signaling pathways: mitogen activated protein kinases (MAPKs), IRFs, and NF-κB. The IRFs activate the IFN response, while MAPKs activates activator protein 1 (AP-1), which acts with NF-κB to induce the expression of a plethora of inflammatory and adaptive immune activating genes, i.e. IL-1β, IL-6, IL-18, and TNF (61). After binding to their ligand TLR7/8 recruit MyD88 to
their cytoplasmic TIR domain through the TIR domain on MyD88’s C-terminal region. The death domain on MyD88 forms a complex with IRAKs 1 and 4, which then binds to TRAF6 and this IRAK-1/TRAF6 complex dissociates from the TLR. Activated TRAF6 performs K-63 linked polyubiquitination to both TGF-β activated kinase (TAK1) and IKKγ. IKKγ then binds with IKKα and IKKβ, leading to IKKβ phosphorylation by the activated TAK1 associated with TAK1 binding protein 1,2, and 3 (TAB 1,2 and 3). The IKK complex then phosphorylates IKB, which releases NF-κB, allowing it to localize to the nucleus to induce gene expression (229). The TAK1-TAB1/2/3 complex also triggers MAPK signaling leading to the formation of AP-1 where it can act alongside NF-κB to trigger the expression of proinflammatory genes. IRF5 and IRF7 also interact with the IRAKs and TRAF6 leading to the nuclear translocation of the IRFs. IRF5 is primarily involved in the induction of proinflammatory cytokines like IL-6 and IL-12p40 (235) and is essential for in vivo resistance to VSV and HSV, where IRF5 deficient mice failed to produce high serum levels of IL-6 and type I IFN and had an increased mortality rate (236). IRF7 is a key mediator in TLR7/8 dependent type I IFN production (237) and mice deficient in IRF7 are more susceptible to viral infection.

The activation of the RLR or TLR pathways invoke the production of copious amounts of type I and type III IFN. The type I IFN consists of the IFN-α subgroup consisting of multiple members (13 in humans) and the IFN-β subgroup (1 in humans). The IFN cytokines are secreted from the cell where they can act in both an autocrine or paracrine manner. Extracellular type I IFN binds to IFNAR1/2 found on the cell surface. The binding of IFN to its receptor induces oligomerization of the IFNARs. In the inactive state, Tyk2 and JAK1 are bound to the cytoplasmic tail of IFNAR1 and 2 respectively.
Once the receptors have dimerized Tyk2 undergoes a conformational change allowing it to phosphorylate STAT2 and JAK1 phosphorylates STAT1. Phosphorylated STAT1/2 then dimerize with each other and this exposes a NLS domain allowing it to be transported to the nucleus. The STAT1/2 dimer associates with IRF9 and co-factor CBP to form the complex ISGF3. This complex binds to DNA at the IFN-stimulated response element (ISRE) which it aids in the transcription of various interferon stimulated genes (ISGs) (238). Type III IFN consists of IFN-λ1, 2, 3 and appear to follow a similar pattern of activation as type I IFN (239). Type III IFN binds to IFNGR1 and 2 on the cell surface inducing their dimerization. A different set of JAK/STAT adaptors associates with the cytoplasmic region of the IFNGRs. JAK1 and JAK2 are phosphorylated by the dimerization and then phosphorylate a region on IFNGR1 that creates a pair of binding sites for STAT1. STAT1 forms a homo-dimer via their SH2 domains and are phosphorylated via their SH2 domains and are phosphorylated themselves. This STAT1 homodimer translocates to the nucleus and binds to the gamma-activation sequence (GAS) and stimulates the transcription of a different set of ISGs (240).

The exposure of cells to IFN results in the overexpression of a plethora of ISGs, several hundred genes in magnitude. Several ISGs such as MxA, PKR, PCBP1/2, PML, and others have been proven to directly inhibit VSV replication (212). MxA belongs to a family of large GTPases similar to dynamin. MxA is capable of recognizing the nucleocapsid of several viruses and interferes with viral mRNA synthesis (241). Protein Kinase (PKR) is expressed in an inactive form until it binds to its cofactor dsRNA or PKR activating protein (PACT). Once bound to its cofactor, PKR phosphorylates the alpha subunit of the eukaryotic translational initiation factor 2 (eIF2α). This event
prevents the recycling of eIF2α during translation, effectively halting protein translation, preventing viral replication from occurring (242). PCBP 1/2 are the poly(C) binding proteins known to interact with VSV P and overexpression of either PCBP 1 or 2 caused an inhibition of VSV replication (243). PCBP 1 inhibits VSV primary mRNA transcription without affecting viral genome replication while PCBP 2 down regulated viral mRNA and genome replication. ProMyelocytic Leukemia (PML) protein belongs to the TRIM family of proteins and multiple splice variants exist to express different PML isoforms. Their functions are still under investigation but they have a role in chromatin structure, stress response, and antiviral response as overexpression of PMLIII can inhibit VSV replication (240, 244).

**VSV oncoselectivity and oncolysis**

Once it was discovered that a large and diverse panel of tumor cell lines are extremely susceptible to VSV, the mechanisms of oncolysis and oncoselectivity were an important goal (242). The wide array of tumor cell lines being susceptible to viral infection indicates that the mechanisms that promote transformation may concomitantly leave the tumor cells highly susceptible to viral infection. During the transformation process cancer cells often acquire numerous defects in their antiviral and translational control pathways (245). This gives the transformed cell a survival and growth advantage over their non-transformed counterparts as the antiviral pathway induces growth arrest and even apoptosis to restrict viral spread, while the translational control pathway regulates the production of proteins and cell growth in response to external stimuli. However, many tumor cells will often be nonresponsive to the PAMPs associated with
viral infection and continue with their agenda of growth and replication, creating a
perfect host cell for unrestricted viral replication.

The wide tropism of VSV complicated efforts to identify the receptor that VSV
uses to gain cell entry. Genetic, biochemical, and immunochemical studies could identify
that VSV G is essential for binding, internalization, and fusion with the cell membrane.
But despite numerous studies the exact receptor of VSV G remained elusive and was the
subject of some controversy in VSV research. Early studies noted that proteolytic
digestion of cell surface proteins did not affect VSV binding. This led to the hypothesis
that VSV’s receptor was a plasma membrane lipid component, with phosphatidylyserine
being the leading candidate. However, recently studies have demonstrated evidence
against this hypothesis and so the search for VSV’s receptor continued. A breakthrough
in the mystery occurred in 2013. Finkelshtein et al reported that the LDL receptor is
responsible as the major entry port of VSV and VSV-G pseudotyped vectors (246). The
same study also identified several other LDLR family members that serve as entry routes
for human and mouse cells albeit with less efficiency.

The pantropic infectivity of VSV and its dependence on the ubiquitous LDL
receptor indicates that its oncoselectivity is dependent not on differential receptor
expression or cell-cycle state but rather the status of the tumor cell’s IFN pathway as a
key determinant of oncoselectivity (212). There is abundant data and studies available
that confirm the IFN pathway is essential in restricting VSV replication. Mice lacking
components of IFN signaling such as IFNAR1 or STAT1 are rendered remarkably
susceptible to normally innocuous doses of VSV and MEFs lacking IFN inducible genes
such as PKR are extremely permissive to VSV (242, 247). The loss of these genes grant
VSV a growth advantage that is sufficient to enable enough viral protein expression to effectively hijack a cell's translation machinery for its own uses.

The cell translation machinery in a transform cell is especially conducive to viral growth including VSV (248). The IFN inducible gene PKR is an important regulator of the translation machinery in a cell. Numerous studies have reported that PKR is nonfunctional in tumors such as chronic lymphocytic leukemia and that dominate negative mutants of PKR can transform cells (249-251). The substrate of PKR, eIF2α, is often dysregulated in cancers, being both overexpressed and non phosphorylatable (252, 253) thus preventing inhibition from PKR. The mRNA cap-binding factor, eIF4E, is upregulated in many different primary tumors and cell lines (254). Constitutively active mutants in the PI3K pathway or loss of the PI3K inhibitor PTEN can stimulate tumorigenesis. PI3K signaling can release eIF4E from the 4E-BPs, allowing translation initiation and constitutive activation of the pathway prevents translation shutdown in response to viral infection (255, 256).

Unfortunately, a transformed cell isn’t always rendered susceptible to VSV and on occasion cancer cells can resist VSV infection much like normal cells (257). Several cell lines from different organs can still respond to IFN-α treatment and capable of secreting IFN-β after infection (258). The defective IFN system isn’t a requirement for cellular transformation but it is a common pathway disrupted by transformation as it activation creates unfavorable conditions for tumor growth, opposing activities like proliferation, angiogenesis and being pro-apoptotic (259).
Genetically engineering VSV

VSV has been successfully cloned into a cDNA plasmid allowing the recovery of infectious virions from cDNA transfection through a typical reverse genetics approach (260) (261). In brief, cells expressing T7 polymerase are transfected with the desired VSV genome alongside a mixture of VSV-N, VSV-P, and VSV-L plasmids to aid in recovery. The virus will be assembled and propagated from the cells allowing the recovery and further amplification of nascent virions from the transfected cells. The vast majority of VSV based oncolytic vectors are derived from the rWT VSV from Rose lab and designated VSV-XN2 (260). It contains the L gene and N terminal region of N derived from the Mudd-Summers strain; with the rest being from the San Juan strain (both fall under the Indiana serotype). VSV-XN2 was engineered with restriction sites within the genome to facilitate the incorporation of foreign genes as well as the removal and/or replacement of the VSV-G glycoprotein (figure 3.2).

VSV safety and potency

The dependence of VSV’s oncoselectivity upon the IFN status of a cell is convenient in allowing its use in a wide variety of different cancers. However, this
method of oncoselectivity raises concern in the context of an immunocompromised patient. The virus is potentially neurovirulent and in a weakened host it may prove to be a pathogenic virus rather than a therapeutic. The development of additional safety measures through genetic manipulation of the virus is the subject of considerable research and through the understanding of both cancer and virus biology, studies have produced a wide variety of strategies that successfully attenuated the potential neurovirulence of the virus while maintaining potent oncolytic potential. These strategies include (i) mutating the VSV M protein; (ii) VSV-directed gene expression; (iii) attenuation of VSV through rearranging the order of its genes; (iv) mutating the VSV G protein; (v) introducing micro RNA sequences; (vi) pseudotyping VSV; (vii) experimental adaptation of VSV to cancer cells; and (viii) using semi-replicative VSV (212).

**VSV-ΔM**

VSV strains with a mutated M protein are attenuated in the sense that they are unable to evade the antiviral response. The VSV M protein is a multifunctional protein necessary for virus assembly and also functions to prevent the antiviral response by binding to Rae1/Nup 98 of the mRNA export complex (262) effectively shutting down host gene expression by preventing the export of host mRNAs from the nucleus. VSV M also interferes with RNA polymerase II function in RNA transcription initiation (263). Preventing the export of host mRNAs and transcription of mRNAs is an effective method of disrupting the immune response, as even though the signaling pathways are intact, without host mRNA export, the effectors of the response cannot be translated. This strategy also reduces the burden on translation machinery from the host allowing VSV directed translation to dominate gene expression. There are several variants of mutated M
protein that remove its immunoevasive function, one being the mutation or deletion of the
methionine reside at position 51 (264, 265) and the other has the residues 52-54 mutated
from DTY to AAA (266). Additionally, by allowing host gene response VSV is highly
oncoselective and this strategy allows for a more robust tumor antigen presentation since
host gene expression is enabled even through active VSV replication.

**VSV directed gene expression**

The VSV plasmid created is genetically malleable and foreign genes can be
inserted by utilizing the restriction sites located between the G and L genes. Several
studies have taken advantage of this property and myriad strains of VSV expressing
foreign genes exist for oncolytic, vaccine, and various research purposes. VSV strains
expressing foreign genes actually produce larger virions to accommodate the larger
genome, as seen by electron microscopy.

The first transgenic VSVs produced incorporated chloramphenicol acetyl-
transferase (CAT) or GFP (267) and quickly afterwards rVSVs were developed for
vaccine vectors (268). Recombinant VSV’s expressing various viral components from
different viruses have been created for use as vaccines against HCV, influenza, HIV,
Ebola, and various others (269-274). Following infection with rVSV, the immune
response generates antibodies and protection against both the VSV and the foreign gene
expressed as the target antigen.

The Barber lab has published several papers using VSV vectors expressing genes
aimed at increasing the antitumor efficacy of rVSV. Some of the first rVSVs aimed at
oncolytic virotherapy contained the herpes thymidine kinase suicide cassette (TK) or the
IL-4 gene inserted in the G-L site (275). The TK gene works alongside the nontoxic prodrug Ganciclovir, phosphorylating it and allowing it to become incorporated into cellular DNA during replication leading to DNA chain termination and apoptosis. The IL-4 cytokine has pleiotropic function. It can influence the development and activation of immune effector cells like eosinophils and APCs (276). It regulates T-helper cell development into Th2 cells and assists with B cell maturation into producing antibodies (277). Sufficiently high levels of IL-4 can also stimulate tumor rejection in the initial stages of tumor development (278). Immunomodulatory proteins like IFNβ (279) and tumor suppressor proteins like p53 (266) have also been inserted into the VSV genome.

The VSV-hIFNβ strain showed remarkable promise as an oncolytic therapeutic. The insertion of IFNβ into the VSV severely limits the replication of the virus in nontransformed cells with an intact IFN pathway. When normal cells are exposed to IFNβ they enter an antiviral state that serves to limit viral spread through the expression of ISGs that combat the virus at nearly every stage of the viral lifecycle. VSV-hIFNβ is an effective therapeutic because a common defect in tumor cells is in the IFN pathway rendering transformed cells still susceptible to VSV infection as they are nonresponsive to hIFNβ while the nontransformed cells are extremely resistant to the spread of VSV-hIFNβ. The expression of IFN also serves as an immunomodulatory agent to enhance antitumor immunity by stimulating the maturing of NKs, CTLs, and DCs (279).

The VSV-p53 strain also produced promising results in preclinical testing. The p53 is an essential tumor suppressor that is defunct in more than 50% of all cancers. The reconstitution of p53 by viral gene therapy can theoretically restore the pathway, enabling
p53 to exert its potent anti-tumor functions. P53 can modulate cellular senescence and cell cycle pathways. It affects DNA repair expression levels and apoptotic protein expression (280). Studies also indicate that reactivation of p53 stimulates innate immune antitumor activity (281). The pleiotropic functions of p53 made it a promising candidate gene to improve the efficacy of VSV as an oncolytic vector and considering the biology of its function, another VSVm-p53 construct was created. P53 functions primarily through intrinsic pathways as it is not secreted like IFNβ, to fully utilize its potential the VSVm mutant was used to allow p53 to exert its function on the tumor gene expression levels. By combining the properties of VSVm with the p53 protein, high levels of p53 were expressed in tumor cells and were able to drastically affect the gene expression of the target tumor cells when compared to wild type M VSV constructs expressing p53 (266). The VSVm-p53 constructs improved the survival of TS/a bearing mice, increased CTL and NK cell numbers in tumor bearing mice, and affected serum cytokine levels as well. TS/a bearing mice had higher levels of IFNβ, and reduced levels of the inflammatory protein IL-6 which can contribute to angiogenesis and tumor cell growth (282)

**VSV gene rearrangement**

VSV can be altered in many ways and still produce infectious virions. A clever approach is gene rearrangement. Any alteration to the order of the genes in the genomes causes attenuation of the VSV due to its replication cycle. The genome of VSV is in a fixed orientation of 3’-N-P-M-G-L-5’ and the transcription of both the virus genes and the full length genome begins at a single 3’ polymerase entry site (283). After
transcription of the initial N gene, the polymerase either continues onto the next gene or disengages from the template and must start transcription again from the 3’ site. This sequential transcription results in a gradient of expression with those genes located near the 3’ site being expressed more abundantly than those on the distal end of the genome (284). By rearranging the order of the genes or inserting a foreign gene up stream of the N gene, the resultant VSV will be attenuated as is it will be more difficult for the virus to express sufficient levels of the N protein to complete its lifecycle. VSV vaccines using this approach have been developed that display limited pathogenic potential and generated immunity against wildtype VSV (285). A VSV-p1-GFP strain expressing GFP upstream of the N protein was used in an intracranial xenograft model with U87 glioblastoma. This attenuated strain was compared against VSV-G/GFP and was shown to have no adverse neurological effects in infants mice as early as 16 days postnatal using intranasal inoculation while VSV-G/GFP was associated with 80% lethality (286).

**VSV G mutation**

The VSV-G protein is another target that be altered to attenuate VSV making it less neurovirulent and more oncoselective. The use of VSV as an oncolytic virus or as a vaccine vector has been very promising but infection of normal brain cells remain a concern as direct cranial VSV injections can cause neurological dysfunction or even lethal encephalitis (213, 287). Two mutant VSV-Gs were created to attenuate the neuropathic potential of VSV, VSV-CT1 and VSV-CT9. VSV-CT1 and CT9 have truncated cytoplasmic tails of VSV-G reducing its length from 29 amino acids (aa) to 1aa and 9aa, respectively (288). The truncated G variants proved to be attenuated both in *vitro* and *in vivo* yet still capable of inducing immunity against wild type VSV and
capable of inducing oncolysis against human glioblastoma. Additionally, a VSV CT9-M51 variant was created combining the truncated G mutant with the M51 M protein mutant. This double mutant was still capable of targeting cancer cells in vivo and displayed reduced neurovirulence upon intracranial injection compared to the single mutant (288).

**Introducing micro RNA sequences into VSV**

The aforementioned strategies of genetically modifying VSV resulted in attenuated constructs that were still able to induce oncolysis due to the cancerous cell’s intrinsic defects in innate immunity and translation control however attenuating the virus reduces it oncolytic potential. A more elaborate strategy is utilizing a microRNA (miRNA) targeting paradigm. This entails encoding miRNA sequences in the VSV genome where they can interact with miRNAs present in certain host tissues that are absent in cancerous cells, greatly attenuating the virus in normal tissue but it remains far more potent in cancerous cells.

MicroRNAs are a diverse set of small sequences of RNA only ~22nts long. They help to regulate a vast array of cellular activities and become dysregulated during cellular transformation. The miRNA sequences can target certain elements in cellular or viral RNAs through sequence-complementary binding and target them for degradation (289) or inhibit their translation by impeding the translation machinery. Expression of specific miRNA sequences depends on several factors such as cell lineage, activation status or differentiation. The miRNA signature of a specific cell will give insight on the cell type
as certain miRNAs are enriched in specific tissues. Additionally, transformed cells will upregulate certain miRNA sequences while down regulating others (290).

VSV constructs were created in our lab that encode copies of neuronal miRNAs expressed in normal neurons but not transformed neurons. In the brain, there are a number of upregulated miRNA sequences with each different subtype of cell having its own unique profile. Several of these sequences were engineered into the VSV genome either downstream of the M or L protein in the 3’ UTR region and oriented to target either the VSV genome or VSV mRNA transcripts. The efficacy of this strategy depended on both the sequence of miRNA inserted, its placement in the genome and its orientation. A VSV construct encoding multiple copies of miR-125 in the L gene 3’ UTR oriented to target the VSV mRNAs was the only construct to effectively attenuate VSV’s inherent neurotoxicity and still retain potent oncolytic potential (291). The reasoning behind the dependence on this specific design of a miRNA encoding VSV is unclear but the dependence of the L gene likely depends on the relatively low levels of the L gene expressed compared to the other genes. The other proteins are highly expressed in a gradient following relative expression levels of N>P>M>G>L. The lower levels of L gene mRNA may allow for a greater percentage of mRNA to be suppressed by the endogenous miR-125. Additionally, the dependence on the mRNA targeting over the genome targeting is likely due to the N protein protecting the VSV genome by encapsidating the genome and protecting it from nucleases and other insults such as miRNA targeting.
Pseudotyping VSV

A strategy for improving the efficacy of VSV as a clinical tool is pseudotyping the virus to suit a therapy. Pseudotyping is the process by which viral particles incorporate foreign envelope proteins with or without their own native envelope (292). VSV pseudotyped with a foreign viral glycoprotein can adopt certain characteristics of the virus from which the foreign gene is derived. This technique is commonly used with a VSV vector lacking its own envelope glycoprotein gene and is therefore dependent upon the pseudotyping for infection and replication. The foreign gene is commonly supplied either through coinfection of the virus from which the desired viral envelope protein is derived or supplied in trans through transfection with a plasmid expressing the desired pseudotyping protein. VSV is capable of budding from infected cells even if G is not expressed. The virions will bud off from the membrane and incorporate cellular proteins in the virus envelope. The particles are considered ‘naked’ virions as they lack the VSV-G protein. However, if a viral glycoprotein is being expressed on the cell surface and is compatible with the biology of VSV, the virions that bud and incorporate the foreign glycoprotein can adopt the tropism and infection mechanisms of the adopted viral protein. This makes pseudotyped VSV especially useful for infection studies and as vaccine vectors.

In addition to pseudotyping VSV particles through trans complementation of the viral glycoprotein, recombinant VSV’s that encode the heterologous viral envelope protein instead of the native VSV-G have been used. The creation of recombinant VSV particles is more difficult than the relatively simple process of pseudotyping but it produces several advantages over pseudotyping. Recombinant VSV particles are
replication competent as they express the foreign gene as part of their own lifecycle. This allows the study of multi cycle infections while pseudotyped VSV is limited to single cycle infections. Additionally, the mechanisms behind infection, assembly and budding using the foreign glycoprotein can be studied while pseudotyped virions only allow the study of infection. And finally, recombinant VSV can be produced in cells regardless of transfection efficiency while pseudotyped VSV require cells with high transfection efficiencies to allow sufficient expression of the foreign glycoprotein to be incorporated.

Pseudotyped or recombinant VSV vectors are also useful as oncolytic vectors to either reduce the potential neurotoxicity of VSV or to target VSV to a particular cell type. A VSV construct pseudotyped to contain the nonneurotopic envelope glycoprotein of the lymphocytic choriomeningitis virus was created (VSV-GP) as a potential therapy for brain cancer. It displays enhanced infectivity for brain cancer cells while simultaneously being attenuated for human and rat neurons in vitro and in vivo, respectively (293). The VSV-GP pseudotyped vector displayed promising results however as a replication defective variant, it is unlikely to produce a robust antitumor effect, however the results obtained showed proof of principle and a recombinant VSV-GP construct could be a promising strategy.

Additionally, a recombinant VSV construct expressing a modified gp gene from Sindbis Virus was created to specifically target breast cancer cells. The gp gene from Sindbis consists of an E1 fusion protein and an E2 binding protein. The E2 binding protein was modified to contain a single chain antibody domain (SCA) targeted to the Her2/neu receptor, erbb2, that is commonly over expressed on breast cancer cells while being absent on normal cells (294). This modification greatly increased the selectivity of
the VSV construct for erbb2 expressing cells and induced antitumor immunity in an immunocompetent breast cancer model that was CTL dependent.

**Experimental adaptation of VSV to cancer cells**

The replication of the VSV polymerase is error prone and a mutation is generated every $10^3$ to $10^4$ nucleotides (295). The relatively inaccurate replication of VSV allows for the rapid evolution of the virus to changing environments, namely the host cell from which it is being propagated. Serial passage of VSV expressing Sindbis gp on a mouse mammary carcinoma cell line, eventually produced a quasispecies of VSV-gp that was able to replicate far more effectively than the initial VSV-gp (296). The authors created a modified gp protein containing a SCA domain that binds to erbb2 allowing it to selectively infects Her2/neu breast cancer cells and inserted it in a VSV ΔG construct. However, the initial construct replicated poorly within D2F2/E2 breast cancer cells. To obtain clinical relevant titers the VSV-gp was subjected to natural selection to produce an improved VSV-gp against D2F2/E2 cells. After 15 cycles, a superior mutant of VSV-gp was produced and displaced the original virus. Sequencing of the viral genome revealed that only 2 permanent mutations were produced in the SCA segment of the gp gene. The mutations produced an additional N-glycosylation site and resulted in higher density of gp proteins in the mature virions. The adapted virus showed improved infectivity, more stability, higher replication kinetics, and less IFN induced.

**Using semi-replicative VSV**

The neurotoxicity inherent in VSV has severely limited its clinical development as an oncolytic vector. Recently, a semi replication-competent VSV (srVSV) vector
system was developed using a combination of deletion mutants. Deletion mutants lacking either the phosphoprotein P or the large polymerase L proteins (VSV ΔP or VSV ΔL) or G glycoprotein (VSVΔG), were created and used in combination to trans-complement gene expression and co-propagate within human glioblastoma (297). The essential genes for replication are package into 2 separate genomes and so only infectious progeny can be produced in doubly infected cells. This technique is possible since the VSV genome does not undergo any genetic reassortment or recombination, and so no replication-competent recombinant VSV will be produced.

Each combination of srVSV was studied and the VSVΔG/VSVΔL was the most potent in terms of both vector propagation and in vitro antitumor efficacy with it only being slightly attenuated compared to wild type VSV (VSV-WT). This is presumed to be partly due to the smaller genome of VSV ΔL allowing genome replication, packaging and viral budding to occur more rapidly. Additionally, the VSV ΔG genome provides the necessary genes for gene transcription and genome replication immediately upon coinfection. The study found that the VSVΔG/VSVΔL system mediated a significant antitumor response and prolonged survival of immunodeficient mice inoculated s.c. with human glioblastoma. More importantly, srVSV treated mice did not develop lethal neurotoxicity in either the tumor model or dose escalation experiments while VSV-WT induced rapid neurotoxic effects following inoculation.

Conclusions

Oncolytic virotherapy is a versatile therapy that has promise to develop into a leading treatment option for various malignancies. The wide variety of viruses shown to
actively induce oncolysis while assisting in mediating antitumor immunity affords clinicians the flexibility to direct a virotherapy best suited for each patient. The successful clinical trials and commercial approval of modified Adenovirus and HSV vectors is a strong indicator of the promise this field represents as a novel treatment modality (115, 298). Recombinant VSV vectors are an additional option in the developing field. VSV expressing hIFNβ is already in clinical trials for the treatment of liver cancer and numerous other VSV vectors are being studied as additional treatment options for cancer. The variety of rVSV currently under review is a testament to the versatility of this viral vector.

Oncolytic rVSV can preferentially replicate and kill most forms of malignancies due to mutations and defects that are often produced during the transformation process. A cancer cell’s ability to resist inhibitory signals in translation regulation as well as general defects in their IFN signaling pathways are key determinants in VSV oncoselectivity. Cell factors such as PKR, RIG-I, eIF2B and various ISGs have been implicated as key factors in VSV susceptibility. Despite its relative harmless phenotype in an immunocompetent setting, neurotoxicity can be observed in an immunocompromised host. Fortunately, VSV’s genetic malleability has allowed researchers to design numerous VSV strains designed to circumvent possible neurotoxicity while still retaining potent oncolytic potential.

Specific aims

We believe that the replacement of VSV-G with a fusion protein consisting of HIV gp160 and VSV-G, namely gp160G, will redirect VSV tropism to a CD4/CXCR4+
expression profile. This new strain, VSV-gp160G has potential to act as an oncolytic agent in a highly specific manner against CD4+ positive tumors such as Adult T-cell Leukemia and that the deletion of the G protein negates any possible neurotoxicity due to off target infection. VSV-gp160G will be tested and characterized using both \textit{in vitro} and \textit{in vivo} studies with the following aims:

\textbf{AIM I: Characterize the recombinant VSV-gp160G \textit{in vitro}.}

a) The replacement of VSV-G with gp160G will drastically affect the lifecycle. The tropism, growth kinetics, oncolytic potential of VSV-gp160G will need to be examined to determine if this is a viable modification to VSV.

b) Determine the effect VSV-gp160G will have on primary lymphocytes, namely CD4 T cells and whether the modification increases the risk of CD4 T lymphocyte depletion due to off target infection.

c) Characterize VSV-gp160G on a panel of ATL cells examining growth kinetics, oncolysis, and specificity.

\textbf{AIM II: Determine recombinant VSV-gp160G therapeutic potential against ATL \textit{in vivo}.}

a) Determine if VSV-gp160G retains neurotoxic potential inherent in VSV through survival studies and analyzing various organ tissue for presence of infectious VSV.

b) Establish an ATL tumor model and determine the therapeutic effect of VSV-gp160G
Chapter 4: Retargeting oncolytic vesicular stomatitis virus to human T-cell lymphotropic virus type 1-associated adult T-cell leukemia

Adult T cell leukemia (ATL) is a highly aggressive malignancy of activated mature CD4/CD25+ T lymphocytes that has been linked etiologically to human T-cell lymphotropic virus type 1 (HTLV-1) infection (299). A small percentage (2-6%) of the 15-20 million infected develop malignant disease after a latency period for 20-80 years (300), although the reason for the low penetrance is unknown. HTLV-I is mostly endemic to southern Japan, the Caribbean, Central and South America, intertropical Africa, and northern Iran (75, 301-303). ATL is generally classified into four clinical subtypes: acute, lymphoma, chronic, and smoldering (72), with the median survival of patients in the acute phase being only 6 to 9 months (304).

Complications in ATL patients include hypercalcemia, immune suppression, and organ failure arising from infiltrating leukemic cells (305, 306). ATL patients have impaired dendritic cell function with reduced interferon alpha (IFN-α) production and reduced capacity to mature into antigen-presenting cells (303, 307). Natural killer cells have significantly decreased cytotoxic activity, preventing NK mediated removal of infected CD4+ T lymphocytes (308). In addition, several reports have demonstrated that HTLV-1-infected cells have a blunted type I IFN response, thereby inhibiting the induction of antiviral genes following viral infection (309). HTLV-1 proteins Tax and HBZ are mechanistically responsible for suppressing the IFN signaling pathway(76, 93, 102, 310). HTLV-1 infection also induces the expression of miR-155 and miR-146a, which downregulate components of IRF3 and TLR and RLR signaling, respectively (311-315). Collectively, HTLV-1 infection disrupts multiple levels of host immunity, allowing opportunistic infections and leukemogenesis.
HTLV-1’s Tax protein affects multiple pathways in the host cell and is likely responsible for the transformation of CD4+ T cells through the activation of growth regulatory pathways like NF-κB, as well as repression of several tumor suppressor genes like pRB and p53 (316). The NF-kB pathway induces the expression of progrowth and prosurvival lymphokines such as IL-6, GM-CSF, TGFβ, IL-2Rα, c-fos, c-egr, and c-jun (317-322). Tax promotes T cell survival, proliferation, and overrides cell senescence, leading to immortalization, and ultimately, the transformation of human primary CD4+ T cells (316, 322, 323). Tax also mediates the accumulation of genetic changes through interfering with p53 function and the formation of the mitotic spindle apparatus, these genetic changes can lead to cellular transformation allowing for Tax independent proliferation and escape from cytotoxic T-lymphocyte (CTL) targeted destruction, as Tax is a preferential target of the immune response against HTLV-I (324). Interestingly, most ATL patients are Tax negative, indicating that Tax is necessary for oncogenesis but not required for maintenance of the malignant phenotype (325).

Despite significant progress in cancer therapy since ATL’s discovery in 1977 (326), there is no effective treatment regimen for ATL. ATL is highly refractory to most treatment regimens and although survival benefit is obtained in the majority of patients using combination IFN-α and zidovudine or with allogeneic hematopoietic stem-cell transplant, ATL recurrence rates are high. There are a variety of clinical trials for ATL both ongoing and in preparation. However, the need for new therapies is dire as most patients succumb to the disease within 5 years after diagnosis (71).

Oncolytic virotherapy is emerging as a versatile treatment option for a variety of malignant diseases. By using natural or engineered viruses, clinicians devise treatment
strategies to take advantage of specific viral properties and target them to a specific cancer. As cancerous cells progress through the transformation process they accumulate defects in innate immunity and translational control mechanisms conferring them a growth and survival advantage. However, this same advantage typically leaves them highly susceptible to viral infection making oncolytic virotherapy an attractive treatment option (327).

VSV is a promising candidate for oncolytic virotherapy. It is an 11-kb negative-stranded enveloped RNA virus of the Rhabdoviridae family. Recombinant VSV has several properties that make it preferable as a therapy over other clinical viruses. For instance, VSV has a RNA genome that remains in the cytoplasm during its life cycle; with no known risk of integration into the host genome or any potential for host transformation. VSV has been incorporated into a DNA plasmid allowing researchers to manipulate the genome with relative ease, allowing new strains to be created to suit a particular need or treatment strategy. The seroprevalence of VSV in the general population is extremely low, reducing the likelihood of a preexisting immunity that would render the virus ineffective as an oncolytic vector (328). VSV has a wide tropism allowing it to be used against a variety of different malignancies and VSV’s oncoselectivity is determined by the infected cell’s ability to induce the IFN pathway. For instance, cells permissive to VSV will become highly resistant to the virus when transfected with various ISGs and murine models harboring a defective IFN system are highly sensitive to a normally innocuous dose of VSV (279, 329).

The defects in IFN signaling that are present in ATL cells should render them highly susceptible to VSV mediated oncolysis. Previous studies have shown VSV-HR
Indiana serotype able to induce apoptosis in *ex vivo* ATL cells, supporting the theory that ATL is a promising target for the development of new recombinant VSV oncolytic strains (324). However, due to the immunocompromised nature of ATL patients, VSV may prove to be pathogenic, as the VSV G protein is highly tropic for numerous cell types, and lethal encephalitis can be induced in laboratory animals if they are unable to mount an effective immune response when the virus enters the CNS (304, 328). In the present study, plausible pathogenicity associated with neurotoxicity were circumvented through the creation of a recombinant VSV strain that is specific for CD4+ T cells, thereby reducing the tropism of VSV substantially and limiting its ability to replicate within the CNS. This was achieved by replacing the VSV-G with gp160G, a fusion hybrid glycoprotein combining the gp120 and gp41 domains of human immunodeficiency virus 1 (HIV-1) gp160 with the cytoplasmic C-terminal domain of VSV-G (330, 331).

We demonstrate that VSV-gp160G is an effective oncolytic agent for treatment of ATL. VSV-gp160 is replication competent, has excellent specificity for CD4+ cells, remains attenuated against primary cell types, and retains potent oncolytic activity against transformed ATL cells. Furthermore, VSV-gp160G displayed no potential neurotoxicity when administered intranasally to NOD/SHI-scid IL-2Rγ (NSG) mice. NSG mice are immunocompromised and succumb rapidly to VSV infection, especially when administered intranasally, indicating that VSV-gp160G was successfully restricted to hCD4+ cells and has no neurotoxic potential. In addition, NSG mice are excellent recipients for transformed human cell lines, including ATL (332). Tumor models using ATL bearing NSG mice indicated that VSV-gp160G successfully exhibited direct
oncolytic activity in vivo and provided a significant survival benefit by delaying metastasis and impeding tumor growth. Collectively, our data demonstrate that VSV-gp160G is a promising agent for the treatment of CD4+ T cell-related malignancies.

**Results**

Recombinant VSV with its native glycoprotein has a diverse tropism and infects most cell types (212). VSV infection of neurons in the brain proves pathogenic to the host and in murine models, VSV in the CNS can lead to neurotoxicity causing lethal encephalitis (333). The research strategy presented here is to restrict the tropism of VSV using transductional targeting and use it as an oncolytic vector against CD4+ ATL. A rVSV was generated with the G protein substituted for a hybrid fusion protein containing gp120 extracellular and gp41 transmembrane domains from HIV1gp160 and the cytoplasmic C terminal region of VSV-G (figure 4.1A and B). HIV-1 utilizes its glycoprotein, gp160, to gain entry into CD4+ CXCR4+ cells through entry association with CD4 and subsequent association with CXCR4 (334). The gp160G is specific for hCD4 and so during the recovery of rVSV from the plasmid, VSV-G was transfected into the cells during the recovery protocol so that VSV-gp160G would be pseudotyped with VSV-G to enable it to infect additional cells and amplify. Then, after successful recovery, the cell milieu from VSV-gp160G infected cells was transferred onto HeLa CD4+ cells to amplify the progeny virions sans VSV-G. The virus was grown to a large scale and purified using ultracentrifugation.

After VSV-gp160G was successfully recovered we sought to determine if VSV-gp160G was successfully restricted to CD4+ cells. HeLa cells and HeLa cells that stably express hCD4 (HeLa CD4) were infected with either VSV-XN2 (that contain VSV-G) or
VSV-gp160G at an MOI of 0.01 and cells were analyzed approximately 24hpi. VSV-XN2 and VSV-gp160G each produced a distinctive CPE that was visible under bright-field microscopy within 24 hpi (Figure 4.1C). VSV-XN2 induced its standard cell rounding effecting both HeLa and HeLa CD4 cells. HeLa cells were resistant to VSV-gp160G and a CPE was not seen in these cells, whereas HeLa CD4 cells were highly susceptible and permissive to VSV-gp160G infection, producing syncytia that is characteristic of viral gp120 and cell surface CD4 interaction (335, 336). VSV infected cells were harvested for immunoblot analysis 24hpi using antibodies against HIV-gp120, gp41, VSV-G, and β-actin. Western blot analysis indicated that VSV-gp160G was only able to replicate and express viral gene products in the HeLa CD4+ cells as HIV gp120 and gp41 were only detected in HeLa CD4+ cells infected with VSV-gp160G (Figure 4.1D). The double band in the gp41 immunoblot indicates that there is cleavage of gp160G into two separate polypeptides after processing, consistent with typical gp160 processing (337). Growth kinetic assays were performed to verify, quantify, and compare the replicative abilities of VSV-XN2 and VSV-gp160G in both cell lines (Figure 4.1E). VSV-XN2 was fully capable of robust replication in a multicycle infection (MOI 0.001) in HeLa and HeLa CD4+ cells, whereas VSV-gp160G replication was restricted to HeLa CD4+ cells, with a significant attenuation in its replicative abilities.

To verify that VSV-gp160G retains its oncolytic potential, apoptosis was measured by Annexin V/PI staining with flow cytometry. HeLa and HeLa CD4+ cells were infected with either VSV-XN2 or VSV-gp160G at an MOI 0.1, and analyzed 24hpi. Cells were gated for Annexin V and PI staining and representative gating results at 24 hpi
are shown (Figure 4.2A). Additionally, cells and supernatant were collected and analyzed at several time points to establish the kinetics of VSV-mediated apoptosis for both HeLa and HeLa CD4 at MOI 0.1 (Figure 4.2B). Consistent with the immunoblot and growth
kinetics VSV-XN2 exerted oncolytic activity against both HeLa and HeLa CD4+ cells and induced robust apoptosis within 24 to 48hrs of infection. VSV-gp160G was completely inert in HeLa cells while being able to induce significant apoptosis in HeLa CD4+ cells within 48hrs. Even though VSV-gp160G displayed attenuated growth kinetics, its oncolytic ability did not appear to be adversely affected as it induced similar levels of apoptosis in HeLa CD4+ cells when compared to VSV-XN2. We confirmed VSV-gp160G specificity to CD4+ cells by performing infection assays in the presence of neutralizing antibody against either cellular hCD4 or viral HIV-gp120 (Figure 4.2C). VSV-XN2 was unaffected in the presence of either antibody inducing similar levels of apoptosis and CPE with the presence of either antibody. However, VSV-gp160G was drastically attenuated in the presence of the neutralizing antibodies. It was unable to mediate the formation of syncytia and levels of apoptosis were severely reduced (Figure 4.2C and D). Thus, VSV-gp160G depends on CD4 and gp120 interaction for infection and to mediate its cytopathic effects.

After characterizing VSV-gp160G within HeLa CD4+ cells, we sought to characterize VSV-gp160G within ATL lines. ATL lines MT-2, MT-4, and TLO-m1 and the CD4- Burkitt’s B cell lymphoma cell line BJAB were infected with either VSV-XN2 or VSV-gp160G at MOI 0.01 and analyzed for their respective CPEs 24 hpi (Figure 4.3A). VSV-gp160G was able effectively induce syncytium formation in the CD4+ ATL lines while the BJAB cell line was resistant to VSV-gp160G. The ATL lines and BJAB cells were analyzed by immunoblot 24hpi at MOI 0.01 to detect viral glycoprotein expression (Figure 4.3B). The HIV-1 glycoproteins gp120 and gp41 were expressed in the ATL cell lines infected with VSV-gp160G while BJAB cells did not express HIV-1
glycoproteins. The HIVgp41 antibody detected both the full length gp160G and gp41G subunit as indicated by the heavier band and the lighter band in the HIV-gp41 immunoblot. The gp41G fragment was not easily detected in MT-4 cells this may involve aberrant glycosylation events that could occur in these cells which may affect antibody recognition but was not explored further. The VSV-G antibody in VSV-gp160G-infected ATL cells also detected the gp160G glycoprotein under these conditions. The epitope for the VSV-G was included in the VSV-G cytoplasmic tail portion of the gp160G protein.
Next, the replicative capacity of VSV-gp160G was compared to VSV-XN2 in the ATL lines. The growth kinetic assays were performed in a multicycle infection (MOI of 0.001) and we observed that VSV-XN2 replicated in all cell lines, whereas VSV-gp160G was only able to effectively replicate within the CD4+ ATL cell lines (Figure 4.3C). The results indicate that VSV-gp160G is still growth attenuated in ATL cells compared to VSV-XN2 and was unable to replicate in the CD4- BJAB cells.

The oncolytic potential of VSV-gp160G was assessed using annexin V/PI staining and flow cytometry to measure apoptosis after infection. ATL cells were infected with either VSV-XN2 or VSV-gp160G at MOI 0.1, and representative gating 24 hpi are shown (Figure 4.4A). The apoptotic kinetics of VSV-mediated cell death in ATL cells was determined at 6, 12, 24, and 48 hpi (Figure 4.4B). MT-2 cells were the most susceptible to VSV-gp160G-mediated apoptosis, even significantly more than they were to VSV-XN2. VSV-gp160G induced extreme syncytia in the MT-2 cells and 48 hpi the syncytia formation likely interfered with the flow cytometry analysis, as the cells were completely infected and all in syncytia at this time point and weren’t expected to be viable. MT-4 cells were also very susceptible to both VSV-XN2 and VSV-gp160G with no significant difference between the relative levels of apoptosis. The TLO-m1 are an ATL patient derived cell line and was somewhat more resistant to VSV mediated oncolysis than the HTLV-I transformed lines MT-2 and MT-4. However, both constructs induced significant amounts of apoptosis within 48hrs with TLO-m1 cells being somewhat more resistant to VSV-gp160G than to VSV-XN2.

To verify that VSV-gp160G required gp120-CD4 interaction to induce apoptosis in ATL cells, infection assays in the presence of neutralizing antibody against either HIV-
gp120 or hCD4 were used (Figure 4.4C and D). Neutralizing antibody drastically

diminished the development of syncytia and reduce the levels of apoptosis in VSV-
gp160G infected ATL cells but did not affect VSV-XN2 infection.
The incorporation of gp160G successfully retargets VSV-gp160G to a CD4+ cell tropism, significantly reducing the risk of neurotoxicity through off target infection of...
neurons (338). Although it is well established that rVSV does not effectively replicate or induce apoptosis in primary cells, the consequences of infection upon primary CD4+ T cells, using VSV-gp160G, is unclear. It is known that a recombinant VSV vector expressing HIV 89.6 Env–VSV-G hybrid was nonpathogenic and did not affect primary CD4 T cell blood counts in rhesus monkeys (339). Furthermore, the VSV vector worked as a vaccine adjuvant and helped stimulate an antiviral response against SHIV, providing additional evidence that our VSV-gp160G should not be pathogenic to the host. VSV vectors induce antitumor immunity by producing tumor associated antigens through immunogenic cell death and VSV-gp160G should be especially effective in inducing antitumor immunity as syncytia formation is known to be an especially immunogenic form of cell death producing exosome like particles that contain tumor associated antigens and are readily endocytosed by antigen presenting cells (340).

In order to evaluate the potential effect VSV-gp160G may have on primary CD4 T lymphocytes and any corresponding pathogenicity of our vector, human primary CD4 and CD8 T lymphocytes were isolated from the peripheral blood of a healthy donor and expanded using TCR stimulation in the presence of IL-2. The isolated CD4 and CD8 T cells were confirmed to be a pure population using flow cytometry and antibodies to CD4, CD8, and CD3 (Figure 4.5A.). T cells were activated and induced to expand using TCR stimulation in the presence of IL-2. The expression of CD25 was used to determine activation status of CD4 T cells and T cells were considered activated if the expression of CD25 was positive for over 90% of the population. The resting CD4 T cell population was removed from TCR stimulation prior to plating and allowed to revert to a resting
state. The cells were considered resting if <50% of the population expressed CD25 (Figure 4.5B).

Activated T cells are more permissive to wild-type VSV (Indiana serotype) infection and cell death than resting primary T cells (324). T cell activation leads to the activation of ERK, JNK, and AKT pathways, which drive cell cycle transition from G0 to G1 phase. The activation of these pathways is crucial for VSV replication (341). Recombinant VSV clones are less pathogenic than wild-type VSV for unknown reasons but the altered genome is believed to be somewhat less effective in replication allowing the primary cell to properly activate its antiviral state before viral gene expression can override the host response (342). The recombinant strains VSV-XN2 and VSV-gp160G were used to infect activated or resting primary T lymphocytes and the effect of each VSV strain was determined. Cells were infected at MOI 0.01 and 1.0 and levels of apoptosis were measured 24 hpi using flow cytometry with fixable viability dye. (Figure 4.5C). As expected, none of the primary cell populations were significantly affected by either VSV-XN2 or VSV-gp160G exposure at either MOI while VSV induced significantly high levels of apoptosis in the ATL lines at MOI 1.0.

The relative cytotoxicity of wild-type VSV Indiana serotype was compared to our recombinant VSV-XN2 and VSV-gp160G in primary activated CD4 T cells. Wild-type VSV induces significant levels of apoptosis at MOI 0.01 by 24 hpi (Figure 4.5D). Neither the VSV-XN2 or VSV-gp160G induced significant levels of apoptosis under the same MOI and time point as the VSV-Indiana serotype. These results indicate that primary CD4 T cells are not adversely affected by the administration of VSV-gp160G and that ATL cells are far more susceptible to infection and apoptosis.
The neurotoxic potential of VSV is readily apparent when administered to immunocompromised hosts such as NSG (NOD/Shi-scid, IL-2Rγ-c-null) mice. NSG mice are severely immunocompromised, lacking mature T cells, B cells, and functional NK cells. They are also deficient in cytokine signaling. All of which makes them both highly susceptible to VSV infection and excellent recipients for engraftment of human cells as they lack the capability to reject the nascent tumor. We evaluated if VSV-gp160G retained any neurotoxic potential by administering VSV-gp160G to NSG mice and monitoring survival. The NSG mice were inoculated intranasally with either 3x10^5 PFU of VSV-gp160G or with 1x10^3 or 3x10^5 PFU of VSV-XN2 in 10µl of PBS (Figure 4.6A).
The NSG mice inoculated with VSV-XN2 at 3x10^5 PFU/mouse succumbed rapidly to neurotoxicity with 100% mortality within 2 weeks, while the lower dose resulted in approximately 60% survival. In contrast, mice inoculated with VSV-gp160G were unaffected with no obvious symptoms and no mortalities.

After determining that VSV-gp160G does not induce neurotoxicity in immunocompromised hosts, we sought to evaluate whether VSV-gp160G can relieve ATL tumor burden within a living host. TLO-m1-luc, a patient-derived ATL line that expresses the luciferase gene was used in an ATL tumor model. The expression of luciferase allows a non-invasive monitoring of tumor growth using IVIS. Luciferase activity can be measured and quantitated and used to assess relative tumor size and its dissemination throughout the host. Through this technology, we tracked tumor progression throughout the disease course and detected when metastatic lesions developed.

In our model, female NSG mice (n=7) were injected with 4x10^5 of TLO-m1-luc i.p. on day 0. The ATL tumor bearing mice received two different VSV-gp160G treatments of 2x10^6 PFU (day 3) and 1x10^7 PFU (day 18), while the control group received PBS injections. Tumor growth was monitored weekly using IVIS to assess progression and for the formation of metastatic lesions (Figure 4.6B). We observed that mice treated with VSV-gp160G survived for longer than the PBS control-treated mice (P=0.039). The luciferase activity measured at the injection site (flux (p/s)) increased at a much lower rate in the VSV-gp160G-treated mice and was significantly less at 30 days post inoculation (P=0.014) ((Figure 4.6C and D). VSV-gp160G treated mice also experienced a delay in the development of metastatic lesions, with fewer than 50% of the
VSV-gp160G-treated mice showing detectable metastasis after 22 days. In contrast, 100% of PBS-treated mice had detectable metastatic lesions by day 15 (Figure 4.6E and
F). Together, these results provide evidence that VSV-gp160G mediates tumor burden relief in an *in vivo* model.

VSV with endogenous G is capable of replicating in many different cell types while VSV-gp160G is restricted to a hCD4 tropism. VSV-XN2 replicates extremely well in neurons and induces lethal encephalitis if it enters the CNS. Here, we examine the biodistribution of both VSV-XN2 and VSV-gp160G in a naïve NSG mouse model. Mice were inoculated with $10^6$ PFU of either virus into the peritoneal cavity on day 0, and then the mice (n=4) were euthanized on days 1, 7, 14, and 21 post infection (Figure 4.7A). The presence of VSV in brain, lung, liver, and kidney was determined by plaque assay. Residual VSV-gp160G was only detectable in the kidney 1 day after inoculation. In contrast, VSV-XN2 grew to a considerable titer in the brain and kidneys of the mouse sacrificed on day 14, and the sacrificed mouse was displaying symptoms consistent with VSV-mediated neurotoxicity. The mouse sacrifice on day 21 did not appear to be experiencing encephalitis, likely due to VSV-XN2 not yet entering the CNS.

VSV-gp160G expression in tumor cells resulted in significant amounts of syncytia forming. To examine the nature of VSV-gp160G-mediated survival benefit and to determine if syncytia was occurring within a tumor mass, TLO-m1 luc bearing mice were treated with VSV-gp160G and was sacrificed 76 days post inoculation, and its organs were taken for histological analysis (Figure 4.7B). Syncytium formation inside the tumor lesions in both metastatic sites on the liver and the primary tumor were detected, indicating that the virus is successfully replicating and causing tumor destruction. Additionally, organ sections from the tumor bearing mouse were homogenized, and the presence of VSV- gp160G was detected by standard plaque assay (Figure 4.7C). Our data
thus indicates that VSV-gp160G does vacate the host if ATL is not present but is able to continually target ATL cells \textit{in vivo} and mediate tumor burden relief.

**Discussion**

Oncolytic viruses offer an intelligent targeted therapy to be used in the fight against cancer. The present study demonstrates that VSV can be retargeted to ATL cells
through their transformed phenotype and CD4 expression while remaining replication
competent and potently oncolytic. This was achieved by substituting the VSV-G for an
HIV-1 gp160–VSV-G hybrid fusion protein to restrict the virus’s tropism to CD4
expressing cells. HIV-gp160 is the surface glycoprotein of HIV and is responsible for the
binding and fusion of the virion to the target cell. It is translated as a precursor and
cleaved at the C5 domain by the convertases, kexin and furin, to yield external viral
gp120 and transmembrane gp41. Cleavage of gp160 is highly conserved among HIV
strains at the Arg-508-Gln-Lys-Arg-511 site. This tryptic cleavage site in gp160 has been
shown to be essential for the fusion process and infectivity since site-directed
mutagenesis at this site abolishes gp160’s ability to induce fusion (337) (343).
Accordingly, our data indicated that the gp160 portion expressed by VSV-gp160G was
efficiently cleaved in the cell lines examined, as indicated by the two bands in the gp120
and gp41 immunoblots.

We observed that the VSV-gp160G construct induced syncytia in CD4+
transformed cells characteristic of the parental strain of HIV-1 from which the gp160 is
derived. Syncytium formation is the fusion of cell membranes through the interaction of
the glycoprotein which it’s substrate, in this instance gp120 and CD4. This method
allows viruses to mediate cell-to-cell spread without having to bud from the host cell. By
fusing membranes together, creating giant multinucleated cells, viral particles spread
without neutralizing antibody significantly affecting viral spread within the tumor mass
(344). In our VSV neutralization assay we observed that either gp120 or CD4 antibody
was extremely effective at neutralizing VSV-gp160G in the HeLa CD4 cells. However, in
the ATL cell lines, we observed that although gp120 and CD4 antibody significantly
attenuated the virus, it was not as effective as in the HeLa CD4 cells. The most likely explanation for this discrepancy is how the gp160G fusion glycoprotein mediates viral spread.

The formation of syncytia does not require virions to bud off the cell and reattach but instead a multimolecular structure known as a virological synapse forms to transfer virions between cells (345). This drastically reduces the distance viral particles must travel to infect neighboring cells and may account for the reduction in the ability of the neutralizing antibody (Nab) to block viral infection within the ATL cells in suspension. The HeLa CD4 cells are plated onto a flat surface, so there is less range of movement, and thus the virus travels further to reach other cells and is more likely to encounter NAbs. Meanwhile, ATL cells tend to form aggregate masses, and this may prevent NAbs from associating with their targets, allowing the virions to spread within these cellular masses without encountering NAbs. The formation of syncytia in immunocompetent hosts may greatly increase the efficacy of a fusogenic rVSV versus a nonfusogenic rVSV since the efficacy of NAbs will be reduced in neutralizing viral spread. Deep within a tumor mass, viral spread is limited for a variety of factors, including intratumoral pressure gradients, limited extravasation from blood vessels, and interference from the extracellular matrix (ECM) (346). The formation of syncytia maybe able to circumvent some of these barriers, and it is possible that that the fusogenic properties of VSV-gp160G could aid in viral spread within the tumor mass since our histology analysis reveals syncytium formation deep within the tumor and since others have reported an increase in viral spread when incorporating fusogenic membrane glycoproteins (FMGs) into their viral constructs (347-349).
In addition, cancer cell death through syncytium formation is a necrotic form of cell death, which is a highly efficient immune activator (350). During syncytium formation, large numbers of tumor-derived, exosome-like vesicles are produced. These vesicles carry tumor-associated antigens that are efficiently picked up by immature dendritic cells and presented to T cells to generate systemic antitumor immunity (351). The bystander killing effect of FMGs on oncolytic viruses has been reported as being 10 times higher than the effect of expressed suicide genes such as cytosine deaminase or thymidine kinase (349, 352). This should enhance the antitumor effects of VSV within a tumor mass since the cell death induced by viral gene expression can act synergistically with the syncytium formation to mediate a significant oncolytic effect. This effect is likely the cause of the comparable levels of apoptosis between the constructs despite VSV-gp160G having significantly reduced growth kinetics (348).

Uncontrolled syncytium formation is a potential safety concern (340). However, in VSV, expression of FMGs depends directly on viral RNA replication. This effectively limits the fusogenic potential in normal cells since VSV-gp160G was still attenuated in normal cells. In addition, no syncytia was produced in normal tissue as shown in the histology data and were limited to the ATL metastatic foci in organ tissue. Previous studies also report no significant damage to normal tissue using a fusogenic oncolytic vector in both a VSV and HSV model (347, 348).

VSV-induced mortality of NSG mice is due to neurotoxicity (337). During infection, wild-type VSV can travel to the central nervous system (CNS) via the olfactory bulb. VSV then spreads trans-synaptically using both anterograde and retrograde transport and through the cerebrospinal fluid (353). The lethal dose of VSV is
significantly lower via intranasal inoculation compared to i.p. or i.v. injection since the former provides direct access to the CNS. VSV-XN2 was rarely detected and only in trace amounts in the kidney organ homogenates directly after i.p. injection, most likely because of residual VSV from the injection and not a product of robust viral replication. It was not until the mouse exhibited signs of neurotoxicity that high titers of VSV-XN2 could be detected in the brain and kidney, although it still was not present in other organs examined. The presence of VSV-XN2 in the kidney was somewhat surprising considering that it was primary tissue and VSV-XN2 should have minimal expression within primary cells. A possible explanation is that the proximity of the organ to the injection site or an accidental puncture to the organ resulted in the kidney being exposed to a higher titer of VSV-XN2 and the virus was therefore able to overwhelm the natural innate antiviral defenses of the kidney epithelial cells. Many transformed kidney epithelial cells are excellent producers of infectious VSV, and if a viable infection is established then, theoretically, VSV would be able to grow within the kidney. In addition, since these animals are severely immunodeficient, there is no adaptive immunity to effectively neutralize the virus or CTL response to kill infected cells. Regardless, VSV-gp160G was barely detectable immediately after inoculation and then quickly dropped to undetectable levels in all organs. The initial reading of VSV-gp160G in the kidney was likely due to residual VSV-gp160G being present and not indicative of replication. This data indicates that VSV-gp160G was unable to replicate within a non-tumor-bearing host and was rapidly cleared from the host.

*Ex vivo* analysis indicated that normal human CD4+ or CD8+ T cells were not destroyed by VSV-gp160G, suggesting that VSV gp160G should be a safe therapeutic
option when used in human patients. VSV-XN2 infects many types of cells, including T cells, although there has been no indication that T-cell repertoires are targeted or significantly depleted by VSV in experimental conditions. In a similar study to ours, the more virulent VSV-HR (Indiana serotype) induced marginal apoptosis in primary activated T cells but not in resting T cells (324, 354). Here, we demonstrate that wild-type VSV Indiana serotype induced significant levels of apoptosis in activated CD4 T cells, whereas recombinant VSV-XN2 and our novel VSV-gp160G did not affect the viability of activated primary CD4 T cells. The recombinant VSV-XN2 is more attenuated compared to the wild-type VSV strain. The reasons for this are not entirely clear, but it can probably be attributed to spontaneous mutations generated during the reverse genetics process or other sequence differences (212, 342).

The ability of VSV to effectively replicate and destroy transformed cells and not normal cells is due to defective antiviral innate immune mechanisms being prevalent in the latter population (242, 279). VSV has repeatedly been shown to be highly sensitive to the actions of the IFN pathway, and IFN deficiencies in ATL cells render them highly susceptible to VSV mediated oncolysis (324). Type I IFN defects are a common occurrence in ATL cells, and it has been demonstrated that other viruses take advantage of this phenotype (355). Thus, VSV-gp160G may provide a safe, effective means for the eradication of transformed CD4+ ATL cells.

We observed that VSV-gp160G could be detected for extended periods (76 days) within an ATL bearing host but was unable to fully clear the tumor from the system. We believe that the lack of complete tumor clearance while VSV-gp160G is present within the host indicates that the ATL tumor mass is so dense within this model that it prevented
the efficient spread of the virus throughout the mass and VSV-gp160G was unable to access the ATL cells. Considering that ATL is a blood cancer, this observation is likely to be an artifact of our chosen model, and in human patients this scenario is not likely to be encountered. In addition, tumor masses retrieved from ATL-bearing mice remained highly sensitive to VSV-gp160G and when extracted from the mouse and placed in suspension were readily infected with VSV-gp160G, which indicates that the tumor did not become resistant to the virus but rather was inaccessible to the virus.

The issue of safety that comes from using an HIV-1 fusogenic glycoprotein to target CD4+ T cells is a valid concern, but we believe that the properties of VSV will override the cytopathic potential of HIV-1 gp160. VSV-gp160G possesses far slower growth kinetics than its parental VSV-XN2, and our ex vivo studies indicate that VSV-gp160G is still highly attenuated against primary cell types, including CD4+ T cells. In addition, the use of a similar VSV construct was previously reported in studies where it was used as an HIV vaccine and generated a robust humoral immune response while not adversely affecting CD4 T cell counts (331, 339). This is a strong indicator that our construct will be cleared from the host, since neutralizing antibody is highly effective against VSV and the spread outside of tumor masses should be highly inhibited (356). Also, it is unlikely that VSV-gp160G will behave similarly to HIV as it lacks any accessory proteins and only incorporates the glycoprotein to alter its tropism.

VSV-gp160G is a prototype virus that specifically targets CD4+ ATL cells. By removing the glycoprotein and restricting the virus to a CD4+ tropism, we greatly reduced the possibility of neurotoxic side effects or off-target infection. We observed that the virus’s growth kinetics have been somewhat reduced, but it is still potently oncolytic.
against ATL cells. Logistically, this could be problematic for clinical use since the yield of VSV-gp160G is significantly lower than that of VSV-XN2. There are several ways to optimize virus production. First, we could investigate additional cell types for producing VSV-gp160G. It is known that BHK cells produce a far greater amount of infectious VSV per cell than HeLa cells, and so a logical first step would be to generate additional cell lines that could more efficiently produce VSV (245). An additional method would be to evolve the virus through serial passaging. VSV has a high mutation rate, averaging about $1 \times 10^{-4}$ to $4 \times 10^{-4}$ per base site, and typically exists as a mixture of genetic variants called quasispecies. Through natural selection, genetic mutants will compete during serial passaging until a superior mutant arises that will overgrow and displace the other mutants. This technique was performed previously using a VSV-expressing chimeric Sindbis virus glycoprotein, which included a single-chain antibody directed to the human Her2/neu receptor. The evolved VSV had a 10,000-fold improvement in viral yield while maintaining its specificity for Her2/neu-expressing cells (296). A similar protocol for VSV-gp160G is worth investigating in the future if clinically relevant concentrations of VSV-gp160G are needed.

Our results demonstrate that VSV-gp160G delivers a very real therapeutic benefit to immunocompromised ATL bearing NSG mice and maybe a promising new therapeutic option. In addition, VSV-gp160G still contains an open transgene site for the insertion of an additional gene to generate more potent strains of VSV-gp160G. The incorporation of a suicide gene such cytosine deaminase is already being considered and developed. We believe that CD has potential to greatly increase VSV oncolytic potential without compromising safety or the ability of the virus to replicate since we have already
incorporated CD into an earlier construct with wild-type VSV-G (357). Alternatively, the expression of matrix metalloproteinase 9 was used in an oncolytic HSV-1 vector in a glioblastoma model, and there was a significant increase in viral vector distribution (358). The use of a similar MMP could enhance the spread of VSV-gp160G by degrading the ECM and thus allowing improved distribution of the virus throughout the tumor mass. Also, there are a variety of treatment options that can be used alongside VSV-gp160G to improve its therapeutic effect. A promising option is radiation treatment, as numerous studies have shown oncolytic therapy is often synergistic with radiotherapy (359-364).

There is increasing appreciation for the use of oncolytic vectors as an immunotherapy. It was originally thought that the immune system was antagonistic to the outcome of virotherapy due to premature clearance of the oncolytic virus but recently studies have demonstrated the immunostimulatory properties of viral oncolysis can stimulate antitumor immunity, which will aid in tumor destruction and potentially prevent relapse (365). Tumor cell death from VSV-gp160G should be highly immunogenic due to the necrotic form of cell death induced by the fusogenic properties of gp160G, as discussed above. However, VSV-gp160G is highly specific for human CD4, and generating a syngeneic immunocompetent ATL tumor was beyond the scope of this study, as we were restricted to immunodeficient xenograft models. We believe that an active immune system will be beneficial to the overall survival of an ATL-bearing host. The fusogenic properties of VSV-gp160G enables the virus to spread without neutralizing antibodies significantly affecting intratumoral spread while inducing a known highly immunogenic form of cell death through syncytium formation. In addition, during tumorigenesis ATL cells generate massive amounts of mutations that form a wide
variety of tumor-associated antigens and should provide plenty of targets for the CTL response if the immunotolerance can be broken (366, 367). There is a humanized mouse ATL model that will be considered in future studies that provides an attractive target for immunocompetent testing of our construct in which the mice develop adaptive CTL responses (368).

The use of oncolytic viruses allows a new treatment option in the fight against cancer that is highly customizable and can target the tumor in a way that can act complementary to existing treatments, greatly improving the efficacy of either treatment alone. We believe that VSV-gp160G is an important platform from which we can develop new treatment regiments that can help alleviate the burden of ATL and extend the life span of patients.

**Materials and Methods**

**Cells**

BHK-21-WI cells (generously provided by M. A. Whitt) and HeLa cells were maintained in Dulbecco modified Eagle medium (DMEM; Gibco/Invitrogen) supplemented with 10% fetal bovine serum (FBS), 5% penicillin-streptomycin. HeLa CD4+ cells were maintained in DMEM supplemented with 15% FBS, 5% penicillin streptomycin, and 200µg of G418 sulfate (Calbiochem)/ml. The ATL cells, MT-2, MT-4, TLO-m1, and TLO-m1-luc (which were generously provided and developed by J. C. Ramos) and B cell line, BJAB, were maintained in RPMI 1640 Medium (Gibco/Invitrogen) supplemented with 10% FBS and 5% penicillin-streptomycin.
**Generation of rVSV expressing gp160G in lieu of VSV-G**

A fusion protein between HIV-gp160 and VSV-G was generated using overlap extension PCR with Pfx Super Mix (Invitrogen) and incorporated into the VSV construct (VSV-XN2) (45). To generate the HIV portion of the fusion protein, the first 750 amino acids of HIV gp160 were PCR amplified from the pNL4-3 HIV plasmid using the forward primer P1 (5'-CCGGACCGGTATGAGAGTGAAAGGAGAAAGTA-3'; the underlined region indicates the restriction site for MluI, and the boldfacing indicates the HIV portion) and the reverse primer P2 (5'-ATGGATACCAACTCGGGATC CGTTCACTAA-3'; the boldfacing indicates the HIV portion of the primer, and the rest is homologous to the VSV-G cytoplasmic tail region). The last 30 amino acids of VSV-G (containing the cytoplasmic tail region) were PCR amplified from pVSV-XN2 using the forward primer P3 (5'-TTAGTGAAACCGGATCCCGAGTTGTATCCAT-3'; the boldfacing indicates the VSV portion of the primer, and the rest is the HIV portion) and reverse primer P4 (5'-CCGGCTCGAGTTACTTTCCAAGTCGGTTC-3'; the underlined region indicates the restriction site for XhoI, and the boldfacing indicates the VSV-G portion). Next, both PCR fragments were mixed together and PCR amplified to generate the full-length fusion protein gp160G with MluI and XhoI restriction sites. The gp160G gene was then cloned into pVSV-XN2 after restriction enzyme digestion with MluI and XhoI (NEB) to remove VSV-G and create compatible ends to ligate gp160G into the VSV-XN2 cDNA plasmid using quick T4 DNA ligation (NEB).

**Recovery and purification of VSV-gp160G**

VSV-gp160G was recovered using a modified version of established VSV recovery
methods (46, 47). In brief, BHK-21-WI cells in six-well plates (~70% confluent) were infected with vTF7-3 (45) at MOI of 5 on day 0. After 45 min, vTF7-3-infected BHK-21-WI cells were washed with serum-free medium and transfected using Lipofectamine 2000 (Invitrogen) with 0.5µg of pBS-N, 0.83µg of pBS-P, 0.17µg of pBS-L, 2µg of pcDNA-G, and 5µg of pVSV-gp160G in DMEM with 5% low-IgG FBS (Life Technologies). After 3 to 5 hr of incubation, the cells were given fresh medium and then incubated at 37°C for 48 h. On day 1, a second six-well plate of BHK-21 cells was transfected with 2µg of pcDNA-G. After 48 hpi, the medium from the VTF7-3-infected cells was passed through a 0.2µm-pore-size syringe filter twice to remove the vTF7-3 vaccinia virus before being transferred to the BHK-21-G-transfected cells. If a cytopathic effect (CPE) was observed, indicating a viable VSV recovery, the medium was transferred to HeLa CD4+ cells for amplification of non-VSV-G- pseudotyped progeny. The virus was plaque purified and further amplified using HeLa CD4+ cells. Purification and concentration were achieved by pelleting the virus using ultracentrifugation with a low-density 10% OptiPrep cushion (Sigma). VSV was resuspended in phosphate-buffered saline (PBS) aliquots and kept at -80°C. Virus titers were determined by standard plaque assays using HeLa CD4+ cells in 24-well plates.

**Virus infections**

Cells were seeded in 6- or 12-well plates. Adherent cells were grown to 80% confluence, while cells in suspension were immediately infected after plating with rVSV at the indicated MOI. Adherent cells were infected with rVSVs at the indicated MOI in a reduced volume of serum-free DMEM for 1 hr with agitation at 15-min intervals. The
cells were then washed with 1xPBS twice, and complete medium was added back to the cells. Cells in suspension were pelleted and resuspended in serum-free RPMI medium containing rVSV at the indicated MOI. After 1 hr of incubation with agitation at 15-min intervals, the cells were washed twice in 1xPBS, and resuspended in complete medium.

**Western blotting**

Infected cells were collected and incubated in lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 1x protease inhibitor cocktail (Sigma) (369) for 30 min at 4°C with gentle agitation. Cell debris was removed by centrifugation for 10 min at 15,000x g. Protein concentration was quantitated using Coomassie blue (Thermo Scientific), and the optical density was read at 595 nm. Equal amounts of protein were separated using SDS–10% PAGE and transferred to a polyvinylidene difluoride membrane (PVDF). Membranes were blocked with 5% milk powder in PBS–0.1% Tween 20 at room temperature and then probed with antibodies against HIV gp41 (ARRRP 2F5, 1:5,000; PBS-Tween, 0.1%), HIV gp120 (Santa Cruz, 1:5,000; PBS-Tween, 0.1%), VSV-G (Sigma, 1:10,000; 5% milk in 0.1% PBS-Tween), or β-actin (Sigma, 1:20,000; 5% milk in 0.1% PBS-Tween) overnight at 4°C with gentle rocking. The membranes were probed with horseradish peroxidase-conjugated anti-human, anti-goat, or anti-mouse antibody (Santa Cruz) diluted 1:10,000 in blocking buffer. Membranes were washed with PBS 0.1% Tween 20, and then the image was resolved using chemiluminescence (Thermo Scientific) and captured by autoradiography (Kodak Film).
Growth kinetic assays

For adherent cells, i.e., HeLa and HeLa CD4+ cells, a total of 10^5 cells/well were seeded in a six-well plate. Cells were infected with either VSV-XN2 or VSV-gp160G at an MOI of 0.001 in serum-free DMEM for 1 hr, with agitation every 15 min. Next, the cells were washed three times with 1xPBS, and 3 ml of complete medium was added to each well. The culture supernatants were harvested at the indicated times and kept at -80°C until virus titer was measured using a standard plaque assay with HeLa CD4+ cells. For cells in suspension (MT-2, MT-4, TLO-m1, and BJAB), 10^5 cells were collected and resuspended in rVSV inoculum with serum-free RPMI at an MOI of 0.001 for 1 hr, with agitation every 15 min. Next, cells were washed three times in 1x PBS and seeded in six-well plate with 3 ml of complete RPMI supplemented with 10% FBS. Then, a 100µl sample was clarified of cell debris by mild centrifugation, and the culture supernatant was collected and stored at -80°C until the virus titer was measured by a standard plaque assay using HeLa CD4+ cells. The remaining pelleted cells were resuspended in 100µl of RPMI–10% FBS and transferred back to the well.

Flow cytometry and viability assessment

Cells were seeded at 2x10^5/well in their respective media and infected with the indicated MOIs of either VSV-XN2 or VSV-gp160G. After incubation for the indicated times, the cells were collected, washed in PBS, and suspended in annexin V buffer (eBioscience). Annexin V (Southern Biotech) and propidium iodide (PI; BD Pharmingen) staining was performed per the protocol supplied by the manufacturer. Cell staining was analyzed
using flow cytometry (with an Aria IIu cell sorter). The cells were considered dead if they stained positive for annexin V.

**VSV neutralization assays**

VSV infections were performed in the presence of neutralizing antibody against either CD4 (BioLegend clone SK3) or HIV-gp120 (NIH AIDS Reagent Program, clone 2G12). Cells were seeded in a 12-well plate and then incubated with neutralizing antibody against CD4 (1 µg) for 1 hr at 37°C before VSV was added. The VSV inoculum was incubated with neutralizing antibody against gp120 (2 µg) for 1 hr at 37°C before being added to the cells. At 24hpi cells were analyzed by flow cytometry using annexin V-PI staining. Additional wells were photographed once significant syncytia occurred in the control wells (at 24 hpi for MT-2 and MT-4 and at 96 hpi for TLO-m1).

**Ex vivo testing on primary human lymphocytes**

*Ex vivo* purified primary CD4 and CD8 T cells were obtained from the blood of a healthy donor using Rosettesep CD4 and CD8 T-cell enrichment cocktails, respectively (Stemcell Technologies). Primary T lymphocytes were maintained in RPMI 1640 supplemented with 10% FBS and 30 U of rIL-2/ml. T cells were expanded using Dynabeads human T-activator CD3/CD28 (Life Technologies) immediately after isolation. The purity and activation status of CD4 and CD8 T cells were determined by using flow cytometry and antibodies against CD4 (BD Biosciences), CD8 (BioLegend, clone RPA-T8), CD3 (BD Pharmingen), and CD25 (BioLegend, clone BC96). The resting cell population was removed from T-cell receptor (TCR) stimulation 24 hr prior to the experiment, and the activation status was determined at the time of plating for the viability test. Viability was
determined using the fixable viability dye eFlour 660 (eBioscience) at 24 hpi with VSV-XN2 or VSV-gp160G at the indicated MOI. Activated primary CD4 T cells were also infected with wild-type VSV Indiana serotype at an MOI of 0.01 at 24 hpi and measured for viability compared to VSV-XN2 and VSV-gp160G. Prior to analysis, cells were fixed in 2% paraformaldehyde. Cells were analyzed using LSR-Fortessa-HTS.

**Mouse studies**

Male and female NOD/Shi-scid, IL-2Rγ-c-null (NSG) mice were purchased from Jackson Laboratory and used to establish a breeding colony in the University of Miami (UM) animal facilities. All mice were housed under pathogen-free conditions. Female mice 6 to 8 weeks old were used for all studies.

**Toxicity**

Female NSG mice (n=7) were inoculated intranasally under isoflurane anesthesia with rVSV in 20µl of PBS (10µl/nostril). The mice were monitored for survival. Mice were euthanized if they displayed gross morbidity, signs of neurotoxicity, or hind limb paralysis.

**Tumor studies**

Female NSG mice (n=7) were injected with 4x10⁵ TLO-m1-luc cells through intraperitoneal (i.p.) injection. After tumor inoculation, mice were injected with 2x10⁶ PFU of VSV-gp160G on day 3 and 1x10⁷ PFU on day 18. Mice were monitored for survival.
**In vivo imaging of mice**

Tumor-bearing NSG mice were transferred to the UM in vivo imaging system (IVIS) facility for imaging before and then weekly after VSV-gp160G inoculation (Caliper/Xenogen IVIS Spectrum). During imaging, mice were injected with luciferin (Caliper Life Science; 150 mg/kg diluted in PBS), anesthetized using isoflurane, and imaged 15 min after luciferin injection with the time post injection matching between groups. The measurement region of interest (ROI) was expressed as flux (p/s), and the dimensions of ROI were kept constant for each weekly measurement.

**Histology**

VSV-gp160G-treated TLO-m1-bearing NSG mice were sacrificed through cervical dislocation under anesthesia using an i.p. injection of ketamine-xylazine. Organs were explanted and fixed in 4% paraformaldehyde overnight. Samples were embedded in paraffin, sectioned, and stained with hematoxylin-eosin.

**VSV detection in organ homogenates**

VSV-treated NSG mice were sacrificed through cervical dislocation under anesthesia using an i.p. injection of ketamine-xylazine. Organs were explanted and stored at -80°C for later use. Organs were weighed and then resuspended in 1 ml of cold PBS. Organs were homogenized and passed through a 0.2-µm-pore-size mesh filter. Organ homogenates were then used in a standard plaque assay using HeLa CD4+ cells to determine virus titer.
Statistics

The data are presented as means +/- the standard deviations. The statistical significance was estimated with a Student t test or a log-ranked test. P values of <0.05 were considered statistically significant.
Chapter 5: Conclusion and future directions

The field of oncolytic virotherapy is growing rapidly. As a therapy, they are located at an interesting intersection between biologic therapy, gene therapy, and immunotherapy. The administration of a therapeutic virus can exert a direct oncolytic effect on the tumor through its replication, perform gene therapy by inserting human genes into the viral genome that will be expressed in the tumor through viral replication, and the effects of viral growth and immunogenic cell death creates a highly immunostimulatory environment that will attract immune cells to the site of viral replication prompting an immune response against the tumor. The idea of using a virus to treat cancer began over a 100 years ago but it wasn’t until the rise of genetic engineering in the 1990s that we could alter viral genomes to design recombinant viruses to be more selective and more oncolytic. With genetic engineering, researchers can now create specialized viruses that will produce a desired effect within a chosen malignancy allowing for a nearly unlimited amount of strategies to combat various types of cancer.

Although oncolytic viruses are engineered in various ways to display certain characteristics, there are several attributes that are considered necessary to make an ideal oncolytic virus. The virus must infect and replicate to destroy human tumor cells including the non-cycling cancer cells. But despite its aggressive phenotype towards cancerous cells, the virus needs to be relatively innocuous to normal tissue to not cause significant adverse effects. Ideally, the virus will be non-integrating as to avoid unpredictable events that would be cause by viral genomic insertion into host cells. Likewise, the ideal virus should be genetically stable to avoid detrimental mutations to
the virus from both a safety and an efficacy viewpoint. And logistically, the virus must be able to be produced under GMP guidelines and grow to clinically relevant titers (370).

VSV is an excellent oncolytic vector that has many of the prerequisites of an ‘ideal’ oncolytic virus. VSV’s RNA genome has no known integration capabilities and its genome is relatively stable for a virus. Additionally, VSV is inherently tumor selective due to vulnerabilities to innate immune pathways, specifically the IFN response (328). Upon administration VSV is capable of infecting both tumor and normal cells as VSV is believed to bind to the ubiquitous LDLR receptor (246). Cells with intact innate immunity will detect VSV infection through TLR proteins 3, 7, and 13 and RIG-I. After detecting VSV, these proteins will initiate a signaling cascade that produces type I IFN and pro-inflammatory cytokines. Type I IFN acts in both an autocrine and paracrine manner to upregulate ISGs that restrict VSV replication and infection directly (212).

Cancer cells commonly acquire defects in the IFN pathway as many of the ISGs restrict cell growth, reduce angiogenesis, or are pro-apoptotic (259). The inactivation of IFN pathway gives tumor cells a growth and survival advantage and helps them evade the immune system but it creates a vulnerability to viral infection that is the rationale behind oncolytic virotherapy as a therapeutic strategy. Despite their vulnerability to oncolytic viruses like VSV, there are still several issues that prevent oncolytic virotherapy from clearing tumor from the host.

Perhaps the biggest hurdle for oncolytic virus to be successful is the host immune system. Host immunity needs special consideration in the context of oncolytic virotherapy. The immune system can be utilized by a designer virus to create antitumor
immunity that will help clear the tumor from the host but it can also target the virus and prevent it from mediating a significant antitumor effect before being destroyed. Host immunity is a complex system in the host and blood cells, complement, antibodies, and antiviral cytokines all collaborate to remove invading pathogens such as oncolytic viruses. In addition to premature clearance from host immunity another issue is poor viral escape from the vascular compartment (371). Systemic delivery of a virus to a patient allows the agent to quickly circulate throughout the host potentially exposing distant metastatic sites to the virus as well as the primary tumor. Unfortunately, direct delivery to the vascular system requires the virus to extravasate passing through extracellular matrix and layers of structural tissue before encountering the tumor cells. In systemic delivery, much of the virus will be sequestered by tissues such as lung, liver, spleen reducing the amount of actual virus engaging the tumor as well as potentiating creating deleterious side effects. Intratumoral injection of the oncolytic virus bypasses these obstacles but dissemination of the virus throughout the tumor is difficult to achieve. The vasculature within solid tumors is chaotic and porous offering poor transportation for both blood and viruses alike. Other barriers like necrotic cells, non-tumor cells, and interstitial pressure also reduce viral penetration within the tumor mass (372).

Strategies to overcome these and other obstacles is a major focus of the field and drives the creation of new and improved viral constructs. The additional of transgenes is a major strategy used by researchers and has resulted in majorly successful oncolytic vectors. T-VEC, a modified herpes simplex virus-1 expressing GM-CSF was the first approved oncolytic virus in the U.S. in October 2015 for the treatment of advanced melanoma (298). The inclusion of GM-CSF serves to promote APC maturation and to
stimulate CTL response against tumors. Numerous other viral and cytokine combinations have also met with success (373).

Altering viral tropism is another strategy commonly employed. VSV is mostly harmless but within certain scenarios it can be fatally neurotoxic. Severely immunocompromised hosts can have difficulty clearing the virus and if the virus enters the CNS it can induce lethal encephalitis. The removal or alteration of VSV-G can remove this liability. A chimeric VSV-G containing a single-chain variable fragment (scFv) antibody was constructed to bind to the human MHC-I and HIV-1 particles pseudotyped with this construct displayed improved specificity (374). The removal of VSV-G and pseudotyping VSV particles with the envelope glycoprotein of lymphocytic choriomeningitis virus (LCMV) was also accomplished to reduce its neurotoxic potential (293).

The creation of VSV-gp160G is especially interesting for the treatment of CD4+ leukemia in that it is fully replication competent and has little potential for neurotoxicity. Even when administered intranasally to NSG mice, they tolerated the viral load with no visible adverse effects. This should allow a much higher titer of VSV to be tolerable to an immunocompromised ATL patient than rVSV expressing wild type VSV-G. Additionally, VSV-gp160G still has an open transcription site available for the insertion of a therapeutic transgene that would potentially improve its function as an oncolytic agent.

The commercial success of several oncolytic viruses has paved the way for new and improved oncolytic viruses. They represent a novel class of therapy in the fight
against cancer that can easily be incorporated into existing treatment modalities. The combination of a novel oncolytic virus alongside an established cancer treatment is arguably a more intelligent and efficient strategy to implement oncolytic viruses in the clinic. Cancer mutates quickly and the development of resistance to current therapies is commonly observed by clinicians. By adding in new and functionally diverse treatment options, the likelihood of a tumor having resistance to both types of therapy is drastically reduced.

Oncolytic viruses have been combined with radiation therapy, chemotherapies, immune modulatory agents like checkpoint inhibitors, antibodies, cytokines and have all displayed synergistic effects. The success of the field likely will depend on successfully combining these novel oncolytic viruses with established strategies.
References


17. **Sporn MB.** 1996. The war on cancer. Lancet **347:**1377-1381.


24. **Langley RR, Fidler IJ.** 2011. The seed and soil hypothesis revisited - the role of tumor-stroma interactions in metastasis to different organs. Int J Cancer **128:**2527-2535.


100. **Clerc I, Polakowski N, Andre-Arpin C, Cook P, Barbeau B, Mesnard JM, Lemasson I.** 2008. An interaction between the human T cell leukemia virus type 1 basic leucine zipper factor (HBZ) and the KIX domain of p300/CMED contributes to the down-regulation of tax-dependent viral transcription by HBZ. J Biol Chem 283:23903-23913.


112. **Dock G.** 1904. The Influence of Complicating Diseases Upon Leukaemia.*. The American Journal of the Medical Sciences **127**.


144. **Chou J, Roizman B.** 1994. Herpes simplex virus 1 gamma(1)34.5 gene function, which blocks the host response to infection, maps in the homologous domain of the genes expressed during growth arrest and DNA damage. Proc Natl Acad Sci U S A 91:5247-5251.


154. **Balzarini J, Bohman C, De Clercq E.** 1993. Differential mechanism of cytostatic effect of (E)-5-(2-bromovinyl)-2'-deoxyuridine, 9-(1,3-dihydroxy-2-propanoxymethyl)guanine, and other antiherpetic drugs on tumor cells transfected by the thymidine kinase gene of herpes simplex virus type 1 or type 2. J Biol Chem **268:**6332-6337.


221. **Barr J, Tang X, Hinzman E, Shen R, Wertz GW.** 2008. The VSV Polymerase can initiate at mRNA start sites located either up or downstream of a transcription termination signal but size of the intervening intergenic region affects efficiency of initiation. Virology **374:**361-370.


228. **Bowzard JB, Ranjan P, Sambhara S, Fujita T.** 2009. Antiviral defense: RIG-Ing the immune system to STING. Cytokine & Growth Factor Reviews **20:**1-5.


238. Reich NC. 2013. STATs get their move on, JAKSTAT, vol 2.


312. **Pichler K, Schneider G, Grassmann, Ralph.** 2008. MicroRNA miR-146a and further oncogenesis-related cellular microRNAs are dysregulated in HTLV-1-transformed T lymphocytes. Retrovirology **5**:100-100.


