

TARGETING TUMOUR VASCULATURE WITH ONCOLYTIC VIRUSES

By

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Abstract

Oncolytic viruses (OVs) have been engineered or selected for cancer cell-specific infection; however, we have found that following intravenous administration of vesicular stomatitis virus (VSV), tumour cell killing rapidly extends far beyond the initial sites of infection. This Bystander Effect is due to the virus' ability to specifically target tumour vasculature through tumour-specific infection of tumour endothelium and the induction of an inflammatory response resulting in tumour-restricted coagulation, acute vascular disruption, apoptosis and necrosis of the tumour core. VSV-infected tumours, reconstructed in three-dimensions from serial histological sections, revealed that the majority of the tumour mass lacks significant blood flow in contrast to uninfected tumours, which exhibit relatively uniform perfusion. VSV infection rapidly induced intravascular coagulation within 6 hours of intravenous administration. The induction of coagulation was dependent on neutrophils and could be prevented with inhibitors of the coagulation pathway. Normal vasculature was not infected by VSV and no increase in coagulation was observed. Vascular collapse was also observed with the oncolytic poxvirus, JX-594, in patients and preclinical models. Biopsies from patients enrolled in a dose escalation trial for JX-594 were immunoreactive for vaccinia antigens and transgene products in high dose cohorts. Tumour-associated vessels from patients treated with JX-594 were infected with JX-594 and expressed virally encoded transgenes. A decrease in blood flow was also observed 5 days post infection. Several viruses, VSV, JX-594, vvDD, Maraba, and Sindbis, were able to rapidly induce widespread bystander cell death in a subset of mouse models. Tumours responded to OV therapy in three

ways, and the type of response was determined by two factors - susceptibility to infection and the heterogeneity of the tumour microenvironment. Heterogeneity correlated with E-cadherin expression. Among tumours that supported viral replication, cancers with low E-cadherin expression were susceptible to vascular collapse. E-cadherin positive tumours were susceptible to infection and direct cell killing but resistant to vascular disruption or bystander cell death. If poorly-differentiated tumours were resistant to infection, no acute cell killing was observed. These histological subtypes provide a potential framework for the rational selection of patients, the integration of combination therapies and the creation of designer viruses to improve the success of OV therapy.

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List of Abbreviations

2D – two dimensional
3D – three dimensional
BrdU – bromodeoxyuridine
CAR – chimeric antigen receptor
CD – cluster of differentiation
CBP – CREB-binding protein
CEV – cell-associated enveloped virion
cRGD – cyclic Arginylglycylaspartic acid
CPA – cyclophosphamide
CTLA-4 – cytotoxic T-lymphocyte antigen 4
CYR61 – Cysteine-rich angiogenic inducer 61
DC – dendritic cell
DNA – deoxyribonucleic acid
ECM – extracellular matrix
EMT – epithelial to mesenchymal transition
eIF2 α – eukaryotic initiation factor 2 alpha
GFP – green fluorescent protein
GM-CSF - Granulocyte-macrophage colony-stimulating factor
H&E – haematoxylin and eosin
HCC – hepatocellular carcinoma
HDAC – histone deacetylase inhibitor
HER2 - human epidermal growth factor receptor 2
HIF-1 – hypoxia inducible factor 1
HSV-1 – herpes simplex virus 1
IEV – intracellular enveloped virion
IFN - interferon
IFN γ – interferon gamma
IHC - immunohistochemistry
IL-1 β – interleukin 1 beta
IL – interleukin
IMV – intracellular mature virion
IP-10 - interferon gamma-induced protein 10
IRF3 – interferon regulatory factor 3
IRF7 – interferon regulatory factor 7
ISG – interferon stimulated genes
IV – intravenous
JAK/STAT - Janus kinase/Signal Transducer and Activator of Transcription
LPS – lipopolysaccharide
MAGE-A3 - melanoma-associated antigen 3
MCP-1 – monocyte chemoattractant protein
MEF – mouse embryonic fibroblast

MMP – matrix metalloproteinase
mRNA – messenger ribonucleic acid
NK – natural killer
OV – oncolytic virus
PBS – phosphate buffered saline
PD-1 – Programmed cell death protein 1
PD-L1 - Programmed death-ligand 1
Pfu – plaque forming units
PKR – protein kinase r
PTEN - phosphatase and tensin homolog
qPCR – quantitative polymerase chain reaction
RAE1 - mRNA export factor
RNA – ribonucleic acid
STAT3 - *signal transducer and activator of transcription 3*
shRNA – short hairpin ribonucleic acid
TBK1 - TANK-binding kinase 1
TIL – tumour infiltrating lymphocytes
TIMP – tissue inhibitors of matrix metalloproteinase
TK – thymidine kinase
TME – tumour microenvironment
TNF α – tumour necrosis factor alpha
tPA – tissue plasminogen activator
VDA - vascular disrupting agent
VEGF – vascular endothelial growth factor
VEGFR1 – vascular endothelial growth factor receptor 1
Vstat120 - vasculostatin
VGF – vaccinia growth factor
VHL - Von Hippel–Lindau tumour suppressor
VSV – vesicular stomatitis virus
vvDD – vaccinia virus double deleted

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Chapter 1: Introduction

1.1. CANCER BIOLOGY

1.1.1 Cancer

Cancer describes a group of cells that are characterized by uncontrolled proliferation beyond their normal boundaries and by the potential to metastasize to other tissues and organs (1). It is the leading cause of death in Canada and accounts for approximately 30% of all deaths. An estimated 187,600 new cases of cancer and 75,500 deaths from cancer have occurred in Canada in 2013 (2). 95% of malignant tumours have been linked to somatic mutations rather than germline mutations. The etiology of cancer has been linked to several causes. 35% of malignancies have been linked to diet, 30% to tobacco use, 14-20% to obesity, 18% to infectious agents, and 7% to radiation of environmental pollutants (3). The biology of cancer is a multistep process described by hallmark capabilities; namely, sustained proliferative signaling, evasion of growth suppressors, resistance to cell death, replicative immortality, induction of angiogenesis, inflammation, reprogramming of energy metabolism and the ability to evade immune destruction (1).

1.1.2 Tumour Microenvironment

The growth of a tumour is a co-evolutionary process between neoplastic cells, extracellular matrix, tumour vasculature, immune cells, fibroblasts, and other cells of 'normal origin'. The interactive niche created by these cells establishes tumour microenvironments (4). Unlike normal tissue, the rapid, erratic growth of tumours results in a mass of cells that is characterized by high interstitial pressure, hypoxia, and restrictive extracellular matrix. The

tumour microenvironment can greatly influence the behavior of neoplastic cells. For example, tumour cells in hypoxic microenvironments are often more aggressive, resistant to apoptosis, and more metastatic (4). Tumour microenvironments influence tumour cell behavior not only within a solid malignancy, but also their propensity to metastasize to different organs. Paget's *seed and soil hypothesis* likens cancer cells to 'seeds' that grow best in certain 'soils' – organ environments that facilitate tumour cell growth through the right combination of homeostatic factors (5).

1.1.3 Tumour Vasculature

Vasculature is a network of vessels and capillaries that are part of the circulatory system whose job it is to transport nutrients, oxygen, cytokines, and red and white blood cells to tissues, while serving as a vehicle for the elimination of metabolic wastes (6). This network of capillaries and vessels is a transport system that is an absolute requirement to keep cells healthy when hydrostatic pressure and the extracellular matrix overcome the limits of passive diffusion (6). In the case of cells within solid tumour masses, vasculature is required to efficiently access nutrients and eliminate wastes in order to ensure survival. Most tumours begin as small, dormant, avascular nodules. The initiation of angiogenesis, often referred to as the 'angiogenic switch', ensures exponential growth of tumour cells (7). Tumours, where the switch is turned on, are highly vascularised, more invasive, and have poorer prognoses in patients, when compared to avascularised counterparts (8).

Vasculature is derived from two main processes: angiogenesis, the sprouting of new capillaries from existing local vessels, and vasculogenesis, the formation of vessels in situ from precursor cells, such as bone marrow-derived endothelial progenitor cells (6). Both

processes are highly dysregulated in tumours as vessel growth attempts to keep up with the uncontrolled proliferation of malignant cells. Furthermore, vessel development is controlled by a balance of positive regulators, such as VEGF, HIF-1, and STAT3, and by negative regulators, such as VHL and PTEN (6, 9). In cancer, endothelial cells rarely become quiescent as these factors are expressed to promote angiogenesis (5). For example, Ras, which is mutated in nearly 20% of all cancers, is a positive regulator of angiogenesis, while p53, which is downregulated in ½ of all cancers, is a negative regulator of angiogenesis (10, 11). The rapid, dysregulated growth of vasculature results in several characteristics of tumour vasculature that differentiate it from normal blood vessels (6). Structurally, tumour vasculature is erratic, tortuous and leaky (6, 12). Turbulent blood flow and abnormal architecture contribute to increased interstitial pressure, hypoxia, poor immune surveillance, and poor delivery of therapeutic agents (12).

1.1.4 Epithelial-to-mesenchymal transition (EMT)

EMT is a developmental regulatory program that has been implicated in the transition of low grade epithelial tumours to more aggressive, high grade malignancies. As tumours of epithelial origin become more malignant, certain pathological features are altered and malignant cells become less differentiated (2). Highly aggressive carcinomas exhibit increased invasion into surrounding tissues and increased metastases, often corresponding to higher pathological grades (1). Cancer cells acquire different morphological characteristics, becoming more mesenchymal in phenotype (Figure 1.1). Carcinoma cells lose their polarity, cell-cell contacts, acquire fibroblastic characteristics, and appear more spindle shaped in

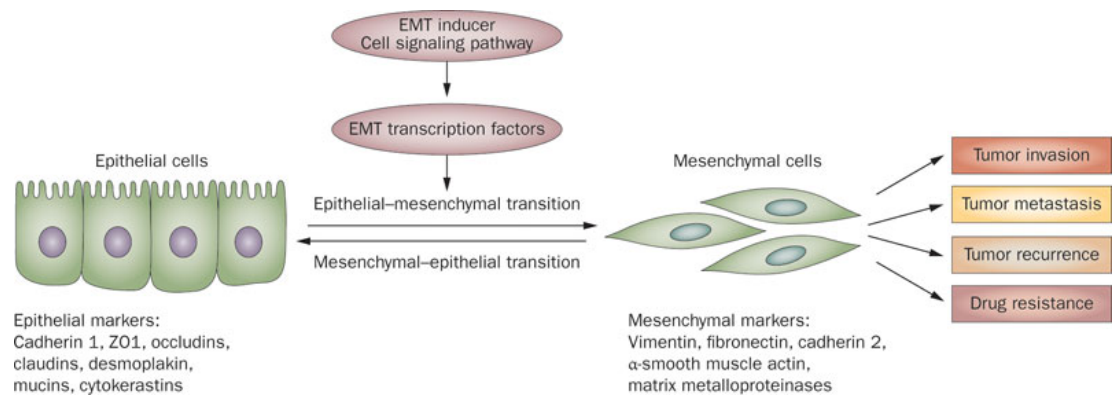


Figure 1.1. Epithelial cancers lose epithelial characteristics and gain mesenchymal features as tumours progress. These cells are more invasive, metastatic, have a higher likelihood of recurring, and are also drug resistant. EMT can be triggered by several signaling pathways. During the acquisition of mesenchymal characteristics epithelial cells lose cell–cell junctions and the actin cytoskeleton is reorganized, which decreases the expression of epithelial proteins such as cadherin 1. (from Wang et al. Nature Reviews Gastroenterology & Hepatology 8, 27-33 January 2011 (13)).

morphology (14, 15). The expression of E-cadherin, a cell adhesion molecule that helps tissues assemble in epithelial sheets, has been well established as a marker of low-grade tumours that are less invasive and metastatic; the loss of E-cadherin is a hallmark feature of EMT (1, 15). EMT has also been viewed as a process where epithelial cells acquire stem cell like features and/or increased cancer stem cell pools within a clonal population (16).

1.2 INFLAMMATION

1.2.1 Acute Inflammation

Under normal circumstances acute inflammation is a rapid reaction to a pathogen or physiological insult, clinically characterized by heat, pain, redness, and swelling. These symptoms are a consequence of cytokines and inflammatory cells that cause vasodilation, increased blood flow, and the infiltration of white blood cells. Within hours, tissue resident macrophages and dendritic cells facilitate the local release of cytokines that cause structural changes in vasculature and alter the adhesive properties of endothelial cells to circulating leukocytes. As leukocytes follow chemotactic gradients to the site of infection or injury, neutrophils and macrophages are often the first responders, with neutrophils being the most abundant in number. If inflammation continues, eosinophils later arrive to destroy invading pathogens (17).

1.2.2 Chronic Inflammation as a driver of cancer progression

Chronic inflammation is a prolonged inflammatory reaction where immune cells infiltrating a tissue are often chronically activated, resulting in functional differences when compared to the

same cells acting in an acute inflammatory event. (18). The dysregulation of inflammation contributes to several pathological conditions and also correlate with cancer (19). Patient's whose tumours have a high degree of infiltrating CD45+ leukocytes have been correlated with poor prognoses (20, 21). Several lines of evidence have established that chronic inflammation can promote the growth of malignant cells through secretion of growth signals that directly act on cancer cells as well as the manipulation of tumour stroma to facilitate tumour growth.

Innate immune cells have been shown in several preclinical models to promote tumour angiogenesis. For example, in a transgenic model of multistage pancreatic cancer, early stage tumour nodules were found to have a high degree of macrophage and neutrophil infiltration. Rather than exerting classical effector functions associated with these cell types, neutrophils promoted angiogenesis and tumour growth (22). In a transgenic model of breast cancer, tumour associated macrophages infiltrated primary tumours at the nonmalignant adenoma stage and were responsible for dramatically increasing the vascularization of tumours leading to malignant progression (23). Myeloid cells and VEGFR1+ bone marrow derived cells were also found to promote metastasis by preparing the "soil" for tumour cell colonization. The cells were found abundantly and were found to promote angiogenesis prior to cells even arriving at the site of tumour growth (24).

1.3 THE IMMUNE RESPONSE TO CANCER

1.3.1 Cancer Immunosurveillance

Burnett and Thomas postulated that 'cancer immunosurveillance' was responsible for preventing cancer development from occurring in immunocompetent hosts. The role of

adaptive immunity in this process was proven in experimental models of chemical carcinogenesis in immune competent and deficient hosts. Immunocompetent animals were far less likely to develop tumours, indicating that the adaptive immune system played a key role in controlling tumour growth (25). Subsequent studies have characterized that Patients are able to develop high levels of antibody and robust T cell responses against tumors antigens in some cases (26). Lymphocytes are able to recognize these antigens and often generate a response against them, leading to the elimination of the malignant cells. Innate immune cells (natural killer cells, macrophages, and dendritic cells) detect the transformed cell or related tissue damage and release proinflammatory cytokines that further transform the microenvironment into one that promotes the generation of tumour specific adaptive responses (26). Both innate and adaptive arms of the immune system are required for effective immunosurveillance. If this process leads to complete elimination of the transformed cells, tumours will not develop.

1.3.2 Cancer immunoediting

It is now known that immunity plays a dual role in cancer; the immune system doesn't just protect the host, but it also can 'edit' the immunogenicity of tumours. The immune system can target and destroy tumours leading to the generation of anti-tumour responses; however it can also promote tumour growth through the selection of cells that are able to evade immune responses and can create a tumour microenvironment that supports tumour progression and evasion of immunity (26). Cancer immunoediting describes the interplay between transformed cells, their immunogenicity and the immune system. The process is divided into three main phases: elimination, equilibrium and escape (26). Elimination is the ideal response where

innate and adaptive immunity work in concert to generate robust anti-tumour responses that lead to the complete elimination of the malignancy. Equilibrium occurs in dormant tumours when rare transformed cells survive the elimination phase. The adaptive immune system controls tumour outgrowth while simultaneously applying a selection pressure to 'edit' tumour cells. The escape phase occurs when the tumour has found ways to successfully evade recognition by the immune system. This can occur when the tumour cell population changes in response to editing functions, for example the loss of tumour antigen expression, or when the tumour microenvironment becomes immunosuppressive and affects host immunity (26). At this stage, tumours exhibit uncontrolled growth. The importance of generating appropriate immune responses against tumours is highlighted in several studies. For example, the spatial distribution of tumour infiltrating lymphocytes (TIL) was found to predict patient survival. Infiltration of CD8⁺ TILs into tumours, as opposed to CD8⁺ TILs accumulating at tumour margins, was found to be an indicator of positive clinical outcomes (27).

1.3.3 Chronic inflammation in the tumour microenvironment – sabotage for immunosurveillance

As previously described, chronic inflammation within the tumour microenvironment contributes to tumour growth through the promotion of angiogenesis and the secretion of growth factors that act on malignant cells. In addition, chronic inflammation has also been linked to the development of poorly immunogenic tumours, in part, because chronic inflammation can camouflage tumours and counteract acute inflammation which frequently precedes the generation of a robust adaptive anti-tumour response (28). Chronic inflammation and the production of reactive oxygen species, cause genotoxic stress, leading to the

generation of immune escape variants that lack tumour antigens. Innate inflammatory cells activated by tumour cytokines such as VEGF, become immunosuppressive (29). *Schrieber et al.* describe chronic inflammation and protective tumour immunity as they transit through cancer immunoediting (26).

1.4 CONVENTIONAL CANCER THERAPY

1.4.1 Surgery

Surgery, chemotherapy, and radiation are the mainstays of the current therapeutic arsenal against cancer. For solid malignancies, the primary, and often most effective, treatment is surgical resection of the tumour; however this strategy has certain limitations. The precise location of malignant tissue must be detectable and must be confined to an operable site. Surgeons often debulk any lesions fitting these criteria, and follow up with secondary treatments to target residual tumour tissue, pre-existing metastatic disease, and tumour cells that may be released while resecting the primary tumour (30).

1.4.2 Radiation

Radiation therapy is another regional therapy that induces DNA damage in cancer cells. DNA damage is mediated by free radicals derived from molecular oxygen. Modest specificity is achieved based on the fact that cancer cells are more susceptible to the effects of DNA damage and it is possible to direct radiation towards malignancies where the location is detectable. Because the tumour microenvironment is often hypoxic and deficient in oxygen, hypoxic tissues tend to be resistant to radiotherapy (31). Off target effects result in ‘radiation

sickness', characterized by nausea, loss of appetite, and vomiting, as well as bone marrow suppression, hair loss, and skin reactions (2).

1.4.3 Chemotherapy

Traditional chemotherapies are cytotoxic agents whose mode of action relies on targeting rapidly dividing cells. Common side effects resulting from chemotherapy are due to the fact that rapidly proliferating cells are not just cancer cells, but also normal cells including those of the bone marrow, digestive tract, and hair follicles. Consequently, many patients become immunosuppressed, lose their hair, and experience side effects relating to inflammation of the gastrointestinal tract (2, 30)).

1.5 BEYOND THE THERAPEUTIC TRIUMVIRATE - PROGRESS IN CANCER THERAPY

1.5.1 Coley's toxin – A pro-inflammatory vascular disrupting agent

In the late 1800s after learning of anecdotal evidence that some sarcoma patients experience regression of their disease following bacterial infections, William Coley started to deliberately treat patients with a mixture of killed bacteria (*Streptococcus pyogenes* and *Serratia marcescens*). Several components, such as LPS, were found to stimulate the production of inflammatory cytokines such as IL-1 β , TNF- α , IFN γ and IL-6 (32). In a series of case studies, Coley reported that the preparation was found to induce fever, inflammation, and lead to the cure of approximately 10% of in operable sarcomas. Tumours that responded well to this

agent exhibited rapid decreases in vessel density and increased acute necrosis. Subsequent small scale clinical trials had mixed results (32).

1.5.2 Clinical progress in vessel targeted therapies

Targeting vasculature is an attractive therapeutic target to control tumour growth. Two main classes of drugs are in clinical use and development (33). Antiangiogenic drugs such as bevacizumab, sorafenib, and sunitinib inhibit proangiogenic signaling to inhibit neovascularization to starve the tumour of nutrients (33, 34). Vascular disrupting agents, such as tubulin-binding combretastatins, aim to cause the rapid shutdown of established tumour vasculature (33). Although drugs targeting the VEGF pathway have slowed metastatic disease in some cancers, these drugs have generally failed clinical trials (35). In some indications, anti-angiogenic drugs are used to ‘normalize’ blood vessels by reducing pro-angiogenic signaling to levels that are similar to what would guide normal vessel development and maintenance (34). Normalizing vessels has been shown to improve the delivery of therapeutic agents and to improve radiotherapy in hypoxic tumours (34, 36). Targeting vasculature is an effective therapeutic strategy but treatment regimens that are more effective and can target a wider variety of cancers are needed.

1.5.3 Immunotherapy

Stemming from the observations that the immune system is responsible for eliminating tumours, and that tumours are able to counteract and evade the immune system through a multitude of mechanisms, scientists are developing methods to recalibrate a dysfunctional

anti-cancer immune response into one that will generate functional anti-tumour immune responses leading to the elimination of malignancies.

1.5.3.1 Classes of immunotherapy

Vaccines have been developed that target tumour antigens that exist on tumour cells but not on normal tissues. One example of this is the MAGE-A3 vaccine that targets the melanoma-associated antigen 3, which is specifically expressed on a variety of cancers and is associated with poor prognosis (37). Another approach is the adoptive transfer of lymphocytes. These are either expanded *in vitro*, or used in their original state, or even genetically engineered to target lymphocytes to tumours. Monoclonal antibodies that target proteins expressed on tumour cells are also in development. For instance, Herceptin is an antibody that is directed against HER2, a protein that is overexpressed on many breast cancer tumours. The most recent immunotherapeutic approach involves (38).

1.5.3.2. Emerging Immunotherapies

Although the impact of tumour immunology on cancer treatment in the clinic is still negligible, there is now a steady stream of preclinical and clinical evidence demonstrating its importance in cancer biology and cancer therapy (39). Several emerging therapies have found ways to harness the immune system to target malignancies. Chimeric antigen receptor therapy, where T cells are genetically modified to be directed specifically against tumours, has achieved some clinical success. For example chimeric antigen receptor (CAR) T cells directed against CD19, a protein expressed on B-cell malignancies, is in clinical development and has

led to the partial remission of a patient with advanced stage lymphoma (40). A growing body of literature has delineated several mechanisms by which cancer cells are able to evade immune recognition and destruction and these mechanisms are now being targeted. Ipilimumab, an antibody against cytotoxic T-lymphocyte antigen 4 (CTLA-4) has been used for the treatment of metastatic melanoma. CTLA-4 inhibits T-cell responses and by ‘taking the brakes off’ T cells, this strategy permits the generation of tumour specific T cell responses. Inhibiting the PD-1/PD-L1 axis has also proven to be an important target of T-cell immunosuppression. The two strategies have been used in combination and have led to rapid tumour regression in a third of melanoma patients enrolled on a trial run by Bristol-Myers Squibb (41). By targeting this aspect of cancer, immunotherapies have become a fourth weapon against cancer, in addition to conventional surgery, chemotherapy and radiation (42). Cancer immunotherapy is coming to the forefront of cancer research and the enthusiasm behind the concept of harnessing the immune system to target malignancies was hailed as *Science Magazine’s* “Breakthrough of the year” in 2013(41).

1.5.3.3 Pitfalls of current therapy

Conventional cancer therapies, namely surgery, radiotherapy, and chemotherapy, have the disadvantages of harming healthy tissues and causing serious side effects. Immunotherapy, one of the most recent and promising approaches to cancer treatment, also has its pitfalls.

Immunoediting can occur over the course of therapy and responses to immunotherapies can be heterogeneous. Some patients have therapeutic responses that follow the elimination phase of immunoediting. Tumours shrink, cells are destroyed, and tumour-specific T cells that exhibit classical effector functions infiltrate tumours. Other patients have responses where

tumour outgrowth of escape variants occur while others return to a dormant equilibrium phase (38). It is important to note that all three responses to immunotherapy can occur in the same patient, as was documented in a melanoma patient undergoing CTLA-4 blockade monotherapy (43). A vaccine targeting MAGE-A3 failed Phase III trials for both non-small cell lung cancer and melanoma (37). Patients also experience immune related adverse events including colitis, hepatitis, dermatitis, and endocrinopathies (44). While immunotherapies are surpassing milestones in the treatment of cancer, it is clear that these responses can be significantly improved and that these responses are heterogeneous within a patient and amongst cohorts of patients. Better therapies that can address the heterogeneity of cancers are required and we will likely need therapies that have multiple mechanisms of action that can be used in combination with existing therapies.

1.6 Oncolytic Viruses

1.6.1 General Properties of Oncolytic Viruses

Oncolytic viruses are able to selectively target, infect, and kill cancer cells by tumour-specific replication based on inherent differences between normal and cancer tissues. The promise of this therapy lies in the fact the oncolytic viruses possess several key advantages over conventional therapies. Oncolytic viruses are able to discriminate between normal and cancer cells due to the fact that a malignant phenotype is acquired through the mutation of several genes. These genetic mutations establish a tumour-specific susceptibility for viral replication. For example, some viruses favor replication in cells where mutations result in defective anti-

viral defense, over expressed signaling pathways, or membrane antigens that are unique or over expressed in malignant cells (45). Oncolytic viruses also have unique pharmacokinetic properties. Theoretically, viruses have the potential to replicate, lyse tumour cells, and then be cleared by the immune system once malignant cells have been eradicated. This property essentially creates a drug that can be customized to tumour burden. Viruses are also able to navigate through blood allowing for systemic delivery and targeting of undiagnosed or inoperable metastases. These viruses also have the potential to be improved by genome modification. Recombinant DNA technology allows for gene insertion that further promotes oncolysis and the rational creation of ‘designer viruses’ (45). Due to these distinct characteristics, oncolytic viruses have been likened to intelligent biological machines that can seek out, target, and kill cancer cells (45).

Oncolytic viruses are multi-mechanistic platforms. The three main mechanisms of action are direct infection, targeting tumour vasculature and inducing anti-tumour immunity. Direct infection occurs due to preferential delivery to leaky vasculature, infection and spread due to inherent properties of cancer cells that favour viral replication such as increased metabolic activity and dysregulated antiviral responses. Targeting tumour vasculature occurs in two ways – direct infection of tumour associated endothelial cells and the induction of an inflammatory response to which tumour vasculature is uniquely susceptible. The induction of anti-tumour immunity is thought to occur for several reasons such as expression of viral ‘danger signals’ in an immunosuppressive microenvironment, decreased regulatory T cells and increased infiltrating CD8⁺ effector T cells (46).

1.6.2 History of Oncolytic viruses

Interest in the field of oncolytic viruses has varied dramatically in the past century. Since the mid 1800s, there has been anecdotal evidence of viral infection coinciding with tumour regression and remission (47). Thirty-seven years before influenza was even known to be a viral infection, a myelogenous leukemia patient went into remission after an influenza infection (47). Regression was also reported in a leukemia patient infected with chicken pox (48). Promising anecdotal evidence led to a growing interest in the oncolytic property of viruses that peaked in the 1950s (49). Several *in vitro* and *in vivo* studies examining the effects of viruses, such as herpes, hepatitis, mumps, dengue, yellow fever, West Nile, vaccinia, and adenovirus (47), on several cancer types, were pioneering proof of principle experiments showing that certain viruses possessed anti-cancer properties. The results of several clinical trials were far less striking than results obtained from animal model studies; furthermore, the safety of non-attenuated pathogens was a concern that led to the near abandonment of the field in the 1970s (49). Moore demonstrated that a human virus could have oncolytic activity against a murine tumour (50), and this was followed by a shift in focus to look at viruses that are non-pathogenic, possess advantageous mutations, and/or have a non-human animal host. In the past two decades, new approaches, such as genetic engineering, and increased knowledge of the mechanisms of tumourigenesis, and promising clinical results have led to a resurgence of interest in the field (47). Consequentially in 2005, the first oncolytic virus, genetically modified adenovirus H101, was approved for marketing in China (51). A clinical trial of this virus had 3/46 complete tumour remissions and 11/46 partial responses (52). The combination of an increased understanding of cancer biology

coupled with the ability to rationally design viruses ushered in a new era of oncolytic virus research.

1.7 Vesicular Stomatitis Virus (VSV)

The oncolytic activity of VSV in mouse models was first investigated in the 1960s (47). VSV primarily infects rodents, cattle, swine, horses, and arthropods but is capable of infecting other species including humans. While infection in livestock is characterized by vesiculation and ulceration of the oral cavity, teats, and feet, human infection is largely asymptomatic and, at most, results in mild flu-like symptoms making VSV a good therapeutic candidate (53). Furthermore, the two most prevalent serotypes in the Americas, Indiana and New Jersey, have rare seropositivity in the general population (54). In addition to these epidemiological advantages, the biological characteristics of VSV provide key benefits. VSV is a 185nm x 65nm bullet shaped particle belonging to the genus Vesiculovirus and the family Rhabdoviridae (45, 55). VSV's 11-kilobase, non-segmented, negative sense RNA genome (53) means replication occurs exclusively in the cytoplasm, making the virus a safer therapeutic candidate than some DNA viruses that have the potential to transform host genomes. Furthermore, recombinant rhabdoviruses can be created and grown to large titres relatively rapidly.

VSV is also highly sensitive to the innate immune system, particularly interferon (IFN). Interferons are a class of cytokines comprised of the subtypes, IFN α , β , ω , κ , and δ , that bind to the IFN receptor and significantly perturb the spread of VSV. The virus and dsRNA is recognized by sensor proteins that translocate IRF3 to the nucleus through the action of TBK1

and IKKi kinases (56, 57). Through IRF3-CBP/p300 and IRF7, under negative translational control of 4E-BPs (58), and other transcription factors, NF κ B and ATF2/cJun, induce the translation of Type I IFN genes. One of these genes, IFN β is secreted and binds to the IFN receptor of other cells, which results in the transcriptional activation of hundreds of interferon stimulated genes (ISGs), such as PKR, mainly through the activation of the JAK/STAT signaling pathway. ISGs have effects of immune modulation, growth, and apoptosis. For example, PKR phosphorylates eIF2 α , leading to the inhibition of translation (59). ISGs are upregulated in waves of IRF3 and IRF7 control, resulting in a positive feedback model that can be likened to a cell sending a warning signal for adjacent cells to enter a protective anti-viral state.

Many cancer cells are resistant to interferon as mutations that minimize this anti-proliferative pathway enable uncontrolled proliferation. Previous studies in the Bell lab have shown that IFN signaling is defective in 42 out of 52 human cancer cell lines obtained from the National Cancer Institute's panel of 60 cell lines (60). The dysregulation of many ISGs, such as PKR, has been shown to have oncogenic properties (61).

Interest in VSV's oncolytic ability was hindered by safety concerns about its well known neurovirulence. Prior experiments in the Bell lab attempted to create an attenuated form of VSV that would retain the ability to infect IFN non-responsive cells. Δ 51-VSV, the strain used in this report, has a M51R mutation in the viral matrix protein. The wildtype M protein binds RAE1, preventing nuclear-cytoplasmic export of host mRNAs, including those for IFN

(62, 63). Therefore, while VSV normally is IFN non-inducing, this mutant form induces IFN production in cells where IFN signaling is normal.

VSV's sensitivity to interferon coupled with interferon resistance in cancer cells lays the foundation for tumour-specific infection. Previous research indicated that PKR plays a crucial role in protection against VSV; furthermore, PKR^{-/-} knockout mice were found to be incredibly sensitive to infection even in the presence of exogenously administered IFN (64). Another experiment showed durable cures in 5 of 8 Balb/c mice bearing CT-26 subcutaneous tumours when treated with VSV (60).

1.8 Vaccinia Virus Biology – a virus with natural oncolytic properties

Vaccinia virus belongs to the family *Poxviridae*, and the genus *Orthopoxvirus*. The family is a group of enveloped DNA viruses that replicate in the cytoplasm of cells (65). Poxviruses have several features that make them suited for use as an oncolytic. Vaccinia's linear, double-stranded DNA genome is approximately 190kbp, encoding approximately 250 genes (65). The relatively large genome permits the insertion of multiple transgenes for the purposes of imaging or enhancing the potency of the virus. Cytoplasmic replication is an important oncolytic property as there is no chance for genetic integration into the host genome. Poxviruses are known to traffic well through blood, making them suitable for systemic delivery to metastatic tumours in distant and unknown sites. Vaccinia virus is produced in four forms that are thought to aid spread of the virus via different routes: the extracellular enveloped virion (EEV) is stable in blood can spread systemically; the intracellular mature virion (IMV) is thought to cause spread from one host to another and remains intracellular; the intracellular enveloped virion (IEV) efficiently disseminates to the cell surface, the cell-

associated enveloped virions (CEV) plays a role in cell to cell spread (65, 66). The four forms create a coordinated strategy to disseminate virions throughout a host; when considering this virus as an oncolytic, these forms are proposed to mediate efficient delivery and spread within tumours and to distant metastases.

1.9 Oncolytic viruses and their interactions with the tumour microenvironment

(adapted from De Silva et al. Cytokine Growth Factor Reviews, 2010 with permission)

1.9.1 Targeting the interplay between the tumour microenvironment and oncolytic viruses

Originally, OV_s were designed to target malignancies by taking advantage of the defects of cancer cells observed *in vitro*. Subsequent analysis of virus delivery and spread *in vivo* has demonstrated that the tumour microenvironment can impede the ability of OV_s to effectively infect and spread. Despite this limitation, it is becoming increasingly evident that OV_s are also able to take advantage of certain features of the tumour microenvironment. Currently, a growing body of literature is delineating the complex interaction between OV_s and the tumour microenvironment that results in an additional therapeutic activity; these viruses are able to target malignancies by rapidly altering the tumour microenvironment into a milieu that potentiates anticancer activity. Understanding the complex and dynamic interplay between OV_s and various components of the tumour microenvironment and therapeutic responses provides opportunities to optimize OV therapy. Several strategies have been developed to

capitalize on the multifaceted relationship between OV_s and host-tumour interactions that enhance the toxicity of OV_s to the tumour microenvironment.

1.9.2 OV_s are poorly delivered to tumours upon systemic administration but can specifically, rapidly expand within tumours

OV_s have been shown to rapidly proliferate and spread *in vitro*, when given access to susceptible cancer cells. Limited delivery is attributed to the treacherous path that OV_s must navigate in order to gain access to susceptible cancer cells. Firstly, upon systemic administration, OV_s may be exposed to complement, neutralizing antibodies, blood cells as well as specialized immune cells within the liver designed to neutralize and eliminate pathogens (67, 68).

Once virions bypass neutralization by blood components and reach the tumour site, OV_s must overcome several obstacles within the tumour microenvironment as well. For example, OV_s must be able to navigate through erratic tumour vasculature, permeate extracellular matrix, and evade an army of innate immune and inflammatory cells. Numerous strategies have been employed to manipulate components of the tumour microenvironment to improve virus infection and spread within tumours. Despite the challenges of getting OV_s to the tumour, once they arrive at their destination, OV_s are able to selectively and rapidly expand even when only a small proportion of input virus reaches the tumour.

1.9.3 Rapid expansion of OV's within tumours is limited by components of the tumour microenvironment

Despite the rapid expansion of OV's within the tumour microenvironment, there exist multiple barriers that inhibit optimal virus spread throughout the tumour, which may be especially problematic as rapid spread within the tumour microenvironment is desirable prior to onset of the neutralizing antibody response. The tumour does not consist exclusively of cancer cells; tumour resident normal cells are resistant to virus infection and constitute a barrier to virus spread. A significant proportion of the tumour mass consists of normal cells like fibroblasts and endothelial cells that support tumour growth (69, 70). Tumours are also associated with inflammatory cells, i.e. tumour-associated macrophages, as these cells have been shown to enhance tumour growth when chronically activated within the malignancy. These normal cells found within the tumour are resistant to OV infection and can contribute to the generation of anti-viral responses. Fibrous extracellular matrix (ECM) can be a physical barrier to virus spread (71). For example, tumour dissemination of myxoma virus was inhibited by extracellular matrix protein deposition (72). ECM also restricted adenovirus access to breast cancer metastases (73). It is not only normal cells that are inherently resistant to infection but tumour cells themselves are genetically heterogeneous and certain cell subpopulations may be able to mimic the phenotype of normal cells, including partial or complete resistance to OV infection. For example, melanoma cells that form vasculogenic mimicry patterns within 3D matrigel culture systems become resistant to oncolytic HSV-1 infection (74).

1.9.4 Strategies to manipulate tumour microenvironment and improve OV spread

1.9.4.1 Manipulation of ECM

A number of strategies have been employed to modify physical aspects of the tumour microenvironment in order to provide fertile ground for viral amplification (some reviewed in (75) and (71)). Several strategies to degrade components of the ECM have been investigated. Tumour cells expressing matrix metalloproteases (MMP-1 and -8), enzymes that degrade components of ECM, respond better to HSV treatment than controls (76). Increased virus spread was attributed to depletion of tumour-sulfated glycosaminoglycans, which increased the hydraulic conductivity of tumours. Co-injection of collagenase, a bacterial enzyme that degrades collagen, a major component of ECM, resulted in higher HSV titers and more uniform virus distribution in the tumour (77). Hyaluronan, a high molecular weight nonsulfated glycosaminoglycan polymer, is another ECM constituent that may inhibit OV dissemination throughout tumours. Co-injection of an oncolytic adenovirus and hyaluronidase, an enzyme that degrades the hyaluronan, improved antitumour activity and overall survival in tumour bearing mice (78). An adenovirus expressing relaxin, another cell matrix degrading enzyme, was able to grow to increased virus titers within tumours and resulted in increased survival of mice (79). Furthermore, Nagano *et al* demonstrated that pre-treatment with a chemotherapeutic that induces apoptosis of tumour cells can increase virus spread throughout tumours due to formation of gaps between cancer cells that allow for better migration of HSV (80, 81).

1.9.4.2 Manipulation of tumour vasculature

Another strategy to improve OV delivery and spread has been to modify the tumour vasculature. For instance, antiangiogenic treatments, originally intended to decrease tumour perfusion, may actually ‘normalize’ tumour vasculature to allow for improved delivery of large therapeutics (81). Libertini *et al* successfully combined adenovirus with bevacizumab (Avastin) in order to achieve better delivery and dissemination of virus (82). When combined with bevacizumab, adenovirus was able to grow more uniformly in tumours. Also, bevacizumab alone did not induce appreciable tumour regression, suggesting that its therapeutic benefit is due to enhancing oncolytic potency of the OV (82). Co-administration of oncolytic HSV-1 with the angiostatic cRGD peptide that antagonizes integrin signaling resulted in decreased vascular permeability and increased virus titers in a rat glioma model (83). Furthermore, increased vessel density was observed in gliomas that regrow after HSV-1 treatment. The expression of CYR61, a gene that encodes an ECM associated protein that promotes adhesion of endothelial cells, was increased in tumours following OV treatment and inhibition of CYR61-induced integrin activation suppressed the pro-angiogenic effect of OV therapy (83). Aghi *et al* demonstrated that co-administration of thrombospondin, an anti-angiogenic protein also inhibited HSV-induced angiogenesis (84).

Conversely, the Meruelo group has demonstrated that increasing vascular leakiness can improve efficacy by augmenting OV infection and spread. Increased permeability achieved by administration of VEGF or chemotherapy resulted in increased tumour transduction with an oncolytic Sindbis virus and improved survival of those animals treated in combination (85).

Other groups have investigated whether chronic inhibition of angiogenesis over the course of OV therapy enhances efficacy in preclinical models. Though inducing collagen degradation may improve virus spread throughout the tumour (as discussed above), other groups have suggested that inhibiting MMPs and matrix degradation may slow down the angiogenic process, thereby potentiating the antitumour effect. OVs producing tissue inhibitors of matrix metalloproteases (TIMPs) were cloned with the hope of limiting angiogenesis in OV treated tumours. Indeed, treatment of human xenografts in mice with herpes virus expressing TIMP-3 resulted in delayed tumour growth and reduced tumour vascular density (86). VEGF mRNA levels have been controlled by an adenovirus expressing a VEGF promoter-targeted transcriptional repressor (87) or a VEGF targeted short hairpin RNA (shRNA) (88) which resulted in reduction of tumour vessel density. Expression of endostatin, a potent anti-angiogenic protein, dominant-negative fibroblast growth factor or platelet factor 4 by OVs are other strategies to inhibit angiogenesis in the context of OV therapy(89). Furthermore, an oncolytic herpes virus expressing vasculostatin (Vstat120) inhibited intracranial and subcutaneous growth of glioma tumours in mice. Tumours exhibited decreased vascular volume fraction and microvessel density (90). Finally, an oncolytic vaccinia virus expressing a single-chain anti-VEGF antibody exhibited greater antitumour efficacy than the corresponding vector not expressing the anti-angiogenic antibody (91).

1.9.5 Tumour infiltrating innate immune cells as barriers of OV infection and spread

The innate immune response to OV infection has been shown to inhibit spread and persistence of viruses within tumours. Neutrophils have been shown to inhibit VSV persistence within tumours (92). Other immune cells, in particular tissue resident macrophages, have been

implicated in virus clearance and therefore inhibition of efficient virus spread throughout tumours. Systemic depletion of macrophages using clodronate liposomes, lipid vesicles that deliver a toxic payload to phagocytic cells, resulted in decreased macrophages in herpes virus treated tumours and a 5-fold increase in virus titers (93). Similarly, depletion of microglia, brain resident phagocytes from *ex vivo* cultured brain slices resulted in a 10-fold increase in herpes virus titer in brain tumours (93). Shashkova *et al* undertook liver resident macrophage (Kupffer cell) depletion by pre-treatment with adenovirus and warfarin (94). Systemic adenovirus infusion was shown to deplete Kupffer cells but was associated with toxicity due to hepatocyte transduction (95). Co-treatment with the anti-coagulant warfarin detargeted infection of hepatocytes and exclusively resulted in Kupffer cell depletion. Upon subsequent treatment with oncolytic adenovirus, increased transduction of tumours was detected. However, a limitation of this approach may be that the use of an initial dose of virus to deplete macrophages can affect innate and adaptive immunity to the subsequent therapeutic virus dose. Besides tumour infiltrating macrophages, other innate immune cell types have also been implicated in restricting virus spread throughout tumours. Two VSV vectors expressing proteins that inhibit natural killer cell (NK cell) function and recruitment (herpes virus-1 glycoprotein G or cytomegalovirus protein UL141) were able to grow to increased virus titers in tumours in an orthotopic hepatocellular carcinoma (HCC) rat model. The decreased inflammatory response resulted in increased necrosis due to better virus spread and also resulted in enhanced survival of the animals (96, 97). Expression of these transgenes also resulted in decreased T cell infiltration to tumours. UL141 did not significantly affect neutrophil or macrophage infiltration to tumours, though there was a trend towards decreased macrophage recruitment (97).

1.9.6 Manipulating the innate response to OV infection to enhance OV efficacy

General immunosuppression has been investigated by many groups as a mechanism of increasing virus titers within tumours. Cyclophosphamide (CPA), whose metabolite can damage DNA, has been used clinically as an anticancer agent as well as an immunosuppressant. Due to this dual activity, CPA represents a rational combination therapeutic. Fulci *et al* demonstrated that CPA enhances HSV replication and oncolysis in a rat glioma model by reducing infiltration of tumour-associated phagocytic cells (98). Peripheral blood mononuclear cells produce anti-viral cytokines, such as type I interferons (IFNs), IFN- γ , TNF- α , IL-15 and IL-18 that inhibit virus proliferation in tumours (99). Furthermore, a high dose of CPA was shown to ablate development of neutralizing antibodies to reovirus, resulting in replication in normal tissue and severe toxicity. Careful administration of lower doses of CPA resulted in increased intratumour virus titers with few side-effects (100). This study highlights the importance of cautious administration of immune suppressants with replicating cancer therapeutics. Though able to enhance virus replication in tumours (a beneficial effect of immunosuppression), this strategy may also result in systemic spread of the virus (a potential detrimental side-effect). Subsequent strategies aim to provide a local immunosuppressive effect within the tumour microenvironment, e.g. by engineering an OV that is able to locally produce the drug. The CYP2B1 enzyme, which converts inactive prodrug to the active compound, has been cloned into herpes virus to allow for local activation of the immunosuppressant (101).

1.9.7 Targeting the tumour microenvironment to enhance antitumour activity of OV_s

The innate inflammatory response can play a beneficial role in the context of OV infection. Injection of HSV in a syngeneic model of ovarian carcinoma in mice resulted in induction of IFN- γ , monocyte chemoattractant protein (MCP-1) and IP-10 cytokines and recruitment of NK cells and CD8⁺ T cells which is thought to contribute to tumour rejection (102). Pro-inflammatory cytokines and chemokines induced by reovirus infection of melanoma cells caused bystander toxicity to virus-resistant tumour cells *in vitro* and activated dendritic cells (103). Though OV_s have been engineered to express immunomodulatory genes that aim to enhance the adaptive anti-tumour immune response, e.g. IL-4 (104), IL-12 (105-109) or GM-CSF(110), enhanced tumour regression due to these modified viruses has been partially attributed to activation of the inflammatory response.

For instance, tumour rejection following administration of measles virus expressing GM-CSF is attributed to enhanced neutrophil infiltration to tumours (111). GM-CSF produced by a recombinant Newcastle Disease Virus (NDV) was shown to activate monocytes and dendritic cells *in vitro* (112). In an *ex vivo* system, dendritic cells loaded with reovirus infected melanoma cells were able to activate NK cells which then also resulted in DC maturation (113). The authors suggest these DC-NK cell interactions may be important in mediating tumour regression.

Combination therapy with histone deacetylase (HDAC) inhibitors is a promising approach to sensitizing resistant cancer cells to OV therapy. Treatment of cancer cells with HDAC inhibitors in combination with VSV resulted in decreased interferon production as compared

to treatment with VSV alone. HDAC inhibitor treatment resulted in increased infection of resistant cancer cell lines *in vitro*, as well as human tumour specimens *ex vivo* and human tumour xenografts in immunocompromised mice (114). Interestingly, enhanced infection of tumours in the presence of HDAC inhibitors resulted in a reduction of tumour perfusion. This suggests that tumours may be sensitized to vascular disruption if virus infection can be sufficiently enhanced thereby likely triggering more inflammation. Concurrent treatment of trichostatin A (an HDAC inhibitor) with HSV resulted in decreased production of VEGF. Therefore, the authors suggest that HDAC inhibitors may also possess an antiangiogenic effect when combined with OV infection (115).

1.10 Current Clinical development

The safety of oncolytic viruses in clinical studies has been very convincing. Over 1000 patients have been treated in clinical trials for various oncolytic viruses in many cancer types with no treatment-related deaths observed (116). Several oncolytic viruses have entered clinical trials including VSV, reovirus, parvovirus, herpes simplex virus, measles, and vaccinia. JX-594 (pexastimogene devacirepvec) has been shown to be safe in patients and to also induce tumour necrosis in several solid tumour types (117). A Phase II study of JX-594 in HCC patients demonstrated survival improvement in patients receiving intratumoural injections (118). The following randomized Phase IIb study in second-line HCC patients did not meet its primary endpoint of survival improvement but the patient population was not optimal for successful OV therapy as those enrolled on this trial had end-stage disease with significant comorbidities, such as liver cirrhosis. JX-594 is planned to enter Phase 3 trials in hepatocellular carcinoma (HCC) patients and will continue Phase 2 testing in renal cancer and

colorectal cancer (119). Talimogene laherparepvec (Amgen), a replication competent herpes simplex virus (HSV), HSV-1 containing the GM-CSF transgene, showed positive results in its Phase III clinical trial. The multinational trial evaluated talimogene laherparepvec in the treatment of advanced melanoma compared to GM-CSF. Durable responses were demonstrated; however differences in overall survival were not significant, despite a strong trend in favor of talimogene (119).

1.11 RATIONALE, HYPOTHESIS AND OBJECTIVES

Rationale

Oncolytic viruses are therapeutics that have multi-faceted interactions with the tumour microenvironment. Oncolytic viruses are capable of transforming the tumour microenvironment into one that is cytotoxic to malignant cells. Limited infection can induce widespread death of uninfected tumour cells. This bystander effect is a significant contributor to the ultimate goal of tumour eradication. It has been established that VSV induces an inflammatory response that contributes to a reduction in tumour blood flow in response to virus infection. The exact mechanism by which this occurs remains to be determined. Although this effect is observed in preclinical models, the effect in patients or the ability of other viruses to induce this effect, remains to be determined. Therapeutic responses are often heterogeneous – a product of the agent's mechanism of action and tumour biology. Exploring how OV's interact with the tumour microenvironment is an opportunity to understand the

requirements for robust bystander killing so that we can use viruses, combination platforms, and patient selection, to improve the success of OV therapy.

Hypothesis

Multiple oncolytic viruses are able to target tumour vasculature, through the induction of tumour-specific inflammation, in a subset of mouse models and patients.

Objectives

1. Determine the mechanism by which VSV causes decreased blood flow and acute bystander killing.
2. Determine if OVs are able to affect tumour blood flow in patients.
3. Characterize factors that influence OV-mediated vessel targeting.

Chapter 2: Materials and Methods

Cell lines

The following cell lines were obtained from American Type Culture Collections: Vero, CT26.CL25 (CT26.lacZ), CT26 wildtype, HT29, U87MGvIIIΔEGFR, SW620, HCT116, B16. Bin67 cells were a gift of Dr. Barbara Vanderhyden. MEFs (mouse fibrosarcomas generated from oncogenically transformed fibroblasts by retroviral transduction with SV40 and HRAS) were a gift of Dr. Gillian Tozer. Cells were cultured in HyQ Dulbecco's Modified Eagle Medium (High Glucose) (HyClone) with 10% fetal calf serum (CanSera) and incubated at 37°C with 5% carbon dioxide.

Tumour-Bearing Mouse Models

Animal experiments were completed using Balb/C mice or CD1 nude mice obtained from Charles River Laboratories. Subcutaneous tumours were implanted by first growing cells in culture to subconfluency in HyQ Dulbecco's Modified Eagle Medium (High Glucose) (HyClone) with 10% fetal calf serum (CanSera). Cells were then trypsinized, pelleted, and resuspended in phosphate buffered saline (PBS) (Hyclone). Tumours were established by injection of cells suspended in 100µl of PBS into the right and left hind flanks of female, 6-8 week old Balb/c mice (Charles River Laboratories). Xenograft cell lines were injected into CD1 nude mice (Charles River Laboratories). MEFs were injected into SCID mice (Charles River Laboratories). For hind flank tumours experiments, cells and treatments were administered according to Table 2.1. Mice were sacrificed by cervical dislocation. All

experiments were conducted with the approval of the University of Ottawa Animal Care and Veterinary Service.

Table 2.1. Tumour model generation and treatment protocol. Cells were injected subcutaneously in hind flanks and mice were treated once tumours were palpable. The time of tumour growth to this size varied. Virus treatments were administered when tumours from different models were approximately the same size.

Cell line	Number of cells/injection	weeks until virus treatment
CT26lacZ	3×10^5	2.5
CT26 WT	3×10^5	2.5
MEF	1×10^6	2
U87MG	1×10^6	3
HT29	1×10^6	3
SW620	2×10^6	3
HCT116	1×10^7	3
Bin67	1×10^6	3

Virus Propagation and Treatments

VSV Δ 51

VSV Δ 51 was used throughout this study and is a recombinant interferon inducing mutant of the wild-type VSV Indiana serotype (60). In order to purify virions, Vero cells were

inoculated with VSV at an MOI of 0.01-0.001. 24 hours later, cell culture supernatants were filtered using 0.2µM Steritop filters (Millipore), and filtrate was centrifuged at 30 000 x g for 1.5 hours and resuspended in PBS (Hyclone). Mice were injected intravenously with 5×10^8 pfu of VSV unless otherwise indicated. Control animals were injected with PBS.

Vaccinia Virus

vvDD, denotes the Wyeth strain of vaccinia virus with a deletion in thymidine kinase (TK) and vaccinia growth factor (VGF) genes. JX-594 contains deletion of viral thymidine kinase and insertion of the transgene GMCSF. Viruses were propagated on HeLa cells and purified on a 36% sucrose cushion. Mice were treated with either 1×10^7 pfu or 1×10^8 pfu resuspended in PBS.

Tumour preparation for histopathology

Mice were euthanized and tumours were extracted in different ways to permit immunohistochemical analysis. The method employed depended on the type of analysis required and the antibodies' ability to stain tissues after different processing techniques.

Preparing Frozen Tissues for Immunohistochemistry Using the Peroxidase Chromogen System

Mice were euthanized at the indicated timepoints by cervical dislocation and one portion of tumour was frozen in Shandon Cryomatrix freezing medium (ThermoElectron, Waltham, MA) on dry ice. Five or ten µM sections were cut using a Microm HM500 OM cryostat (Microm, Walldorf, Germany). Another portion of tumour was placed in 10% formalin and embedded in paraffin. For some experiments, brain, lung, heart, and tumour adjacent

skeletal muscle tissue were also collected and paraffin embedded for histological analysis. Mice were not perfused before organ collection.

Preparing Frozen Tissues for Immunofluorescent Staining of 80 μ m mouse cryostat sections

Mice were perfused with fixative infused under physiological pressure. Trans-cardiac perfusions were completed using a smoothed, blunt-ended cannula inserted in the proximal part of the ascending aorta of the heart. 500 ml perfusion bottles filled with 1% paraformaldehyde in phosphate buffered saline were used. The bottle had an outlet at the bottom for connection of Silastic tubing. The bottle was pressurized by affixing a rubber stopper attached to a manometer. The pressure bulb was used throughout perfusions to maintain pressure similar to systolic blood pressure. After fixation by vascular perfusion, organs or tumours were removed, rinsed several times with PBS, infiltrated with 30% sucrose overnight, and then frozen in OCT compound. Cryostat sections were cut 80 μ m in thickness. Sections were dried on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) for 12 to 15 hr for 80 μ m sections.

Immunofluorescent Staining of 80 μ m mouse cryostat sections

Specimens were permeabilized with PBS containing 0.3% Triton X-100 (Lab Chem Inc., Pittsburgh, PA) and incubated in 5% normal serum (Jackson ImmunoResearch) in PBS+ (PBS containing 0.3% Triton X-100, 0.2% bovine serum albumin (Sigma), and 0.01% thimerosal (Sigma)) for 1 hr at room temperature to block non-specific antibody binding. Sections were incubated with primary antibodies diluted in 5% normal serum in PBS+ for 12-15 hr at room temperature. Control specimens for immunofluorescence intensity analysis were incubated in 5% normal serum instead of primary antibody for the same period. After rinsing with PBS containing 0.3% Triton X-100, specimens were incubated for 4-6 hr for 80

µm sections at room temperature with fluorophore-conjugated (FITC, Cy3 or Cy5) secondary antibodies diluted in 5% normal serum in PBS+. Specimens are rinsed with PBS containing 0.3% Triton X-100, fixed in 4% paraformaldehyde, rinsed again with PBS, and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Hamster anti-mouse CD31 clone 2H8 diluted 1:1000 (Endogen, Woburn, MA or Chemicon, Temecula, CA) was used to stain mouse endothelial cells. Blocking serum and FITC, Cy3, or Cy5-conjugated fluorescent secondary antibodies were from Jackson ImmunoResearch, West Grove, PA.

Antibodies used for peroxidase immunohistochemical analysis

Immunohistochemistry detecting fibrinogen (1:500; DAKO, Glostrup, Denmark) was performed on paraffin-embedded sections using the Vectastain ABC kit for rabbit primary antibodies (Vector Labs, Burlingame, CA), according to instructions provided. The antigen retrieval step was omitted. For Ki67 detection, paraffin-embedded sections were boiled in 10 mmol/l citrate buffer, pH 6. Primary anti-Ki67 antibody (1:25 dilution; DAKO) was applied overnight and detected using anti-rat antibody detection system (DAKO). BrdU was detected using the Vectastain ABC kit for rat primary antibodies (Vector Labs), on paraffin-embedded sections treated with 4 N HCl. Primary anti-BrdU (1:100 dilution; Abcam, Cambridge, MA) was applied for an hour. Horseradish peroxidase activity was visualized with a Diaminobenzidine-Horseradish peroxidase kit (KPL Biosciences, Guelph, Canada). Apoptotic cells were detected with a rabbit primary antibody against the active form of Caspase 3 (BD Biosciences, Mississauga, Ontario, Canada) at a dilution of 1:500 using the Vectastain system. Rabbit polyclonal VSV serum was used at 1:5000 on frozen sections and 1:2000 on paraffin sections. VSV antibody was a kind gift of Earl Brown. Rabbit anti

vaccinia antibody (Quartett) and a secondary antibody kit (Vectastain, Vector Laboratories) were used to detect JX-594 and vvDD vaccinia virus. The vaccinia antibody was used at 1:5000 on frozen sections and 1:2000 on paraffin embedded sections. For detection of β -galactosidase by immunohistochemistry, an anti- β -galactosidase polyclonal antibody (Abcam) was used. Negative controls were run without primary antibody and tumours from mice treated with virus were included as positive controls. Nuclei were counterstained using hematoxylin. For assessment of cell morphology, sections were stained with hematoxylin and eosin according to standard protocols. Images were digitized using Aperio ScanScope (Axiovision Technologies, Toronto, Ontario, Canada) and analyzed using Aperio ImageScope software. Blood clots in normal tissues of Figure 3.5d were quantified on hematoxylin & eosin stained sections. Thirty vessels were counted per section.

Analysis of tumour perfusion

Mice were injected intravenously with 100 μ l of a 50% solution of 100 nm diameter orange fluorescent microspheres (Molecular Probes, Burlington, Ontario, Canada). Five minutes later, animals were euthanized and tumours immediately snap frozen as previously described. Tumour perfusion was analyzed by visualizing fluorescent microspheres in the vasculature of 10 μ m unfixed frozen sections using a ScanArray Express microarray scanner with a standard Cy3 laser (Packard Bioscience, Meriden, CT).

3D modeling of tumour vascularity during VSV therapy

CT-26 tumour-bearing mice were treated with VSV or PBS and euthanized 24 hours later. Microspheres were injected as described above and the tumour was collected and frozen.

The VSV treated tumour was cut into 1,085 6- μ m tissue sections and mounted on slides. The PBS treated tumour was cut into 952 sections. Every fifth section was scanned for microspheres and immunohistochemically stained for VSV as described above. The sections were stained for VSV using the Autostainer Plus (DakoCytomation, Burlington, Ontario, Canada). HTK Histology Toolkit software (Robarts Imaging Institute, University of Western Ontario, London, Ontario, Canada) was used to convert 2D images into 3D models. Volume reconstruction was completed using alignment and segmentation contouring algorithms, which oriented each tissue section on top of one another. Each tissue section image, once oriented, was then converted from 2D pixels into 3D voxels. These 3D stacks were then rendered to generate the reconstructed tumour. 2D images of microspheres were used to generate a model of perfusion, while 2D images of VSV staining were used to generate a model of infection. An overlay model was generated from superimposed images of microspheres and VSV. Regions of infection were highlighted in red to aid in visualization (Adobe Photoshop CS2).

3D reconstruction of a patient biopsy treated with JX-594.

An excisional biopsy was obtained from a patient on day 10 post infusion and processed for immunohistochemical detection of vaccinia virus. 126 serial sections were cut and every other section was stained for virus. Two-dimensional pictures of the sections were then converted into a three-dimensional volume using HTK histology toolkit software (Robarts Imaging Institute). Volume reconstruction was completed using alignment and segmentation-contouring algorithms that oriented each tissue section on top of one another as was done for the mouse tumours, three-dimensional stacks were then rendered to

generate the reconstructed tumour. Regions of infection were highlighted in green to aid visualization, and in another representation in which the model can be viewed in orthogonal planes, image contrast was adjusted to 50 (Adobe Photoshop CS2, Adobe). Scaling in the three-dimensional model is derived from scaling from two-dimensional images.

Assessment of Proliferation Over the Course of Oncolytic Virus Therapy

Tumour-bearing mice were treated with BrdU 24 hours prior to treatment with VSV. Mice were sacrificed 24 hours after administration of virus. Tumours were extracted, and paraffin-embedded histological sections were stained for BrdU, VSV, and Ki-67 to determine pre-treatment proliferation, virus, and post-treatment proliferation respectively.

BrdU pulse.

Tumour-bearing mice were treated with 100mg/kg BrdU (Sigma, Oakville, ON) intraperitoneally 24 hours before, or 22 hours after, intravenous injection of VSV or PBS. Twenty-four hours following virus challenge, three mice per group were euthanized and tumours removed, paraffin-embedded and analyzed by immunohistochemistry as described above.

In vivo neutrophil depletion.

Mice were injected intraperitoneally with 150µg purified RB6 8C5 rat monoclonal antibody, clone RB6-8C5 (BD Pharmingen, Rockville, MD) in order to systemically deplete granulocytes. One hundred and fifty micro liter of nonimmune rat serum was used as a negative control. Twenty-four hours later, mice were treated intravenously with 5×10^8

plaque-forming unit $\Delta 51$ VSV-GFP, perfused with fluorescent microspheres 24 hours later and sacrificed by cervical dislocation and tissues collected and stained as described above.

Manipulation of blood clot formation.

Tumour-bearing mice were treated with VSV or PBS intravenously and 200 U/kg heparin [dalteparin sodium injection (Fragmin), Pfizer, New York, NY], intraperitoneally four times over the course of 24 hours starting at the time of VSV injection and ending at 22 hours when the mouse was euthanized. Bothroaltein (Cedarlane, Burlington, ON) or tissue plasminogen activator (Activase rt-PA; Roche, Basel, Switzerland) were injected at 20 U/kg and 4 mg/kg, respectively, intraperitoneally 1 hour before intravenous VSV or PBS infusion and another three times over the course of 21 hours before euthanizing the mice. Effects of coagulation on tumour viability were similarly analyzed 10 hours after virus treatment with administration of 100 U heparin (unfractionated heparin 1,000 USP U/ml; Pharmaceutical Partners of Canada, Richmond Hill, Ontario, Canada) every 2 hours subcutaneously during treatment. In all experiments, mice were injected intravenously with fluorescent microspheres before sacrifice in order to visualize tumour perfusion.

Statistical analysis.

All statistical analysis was performed using Graphpad Prism 3.0 software. Data are represented as a mean $\pm$ SE. Analysis of variance was performed with Tukey's post-hoc test.

Chapter 3: Targeting tumour vasculature with an oncolytic virus

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Contribution: I generated the 3D model with help from Laura Evgin for the PBS control. Usaf Aladl designed the software and taught me how to use it. I designed and performed the BrdU pulse experiment in Figures 2a-c. Staining in generated Figure 3, 4c, 5b,c, 6, and 8 and wrote portions of the manuscript. Caroline Breitbach generated the remaining figures with the exception of 4a which was generated by Jennifer Paterson. All staining was verified by Manijeh Daneshmand.

ABSTRACT

Oncolytic viruses (OVs) have been engineered or selected for cancer cell-specific infection however, we have found that following intravenous administration of vesicular stomatitis virus (VSV), tumour cell killing rapidly extends far beyond the initial sites of infection. We show here for the first time that VSV directly infects and destroys tumour vasculature in vivo but leaves normal vasculature intact. Three-dimensional (3D) reconstruction of infected tumours revealed that the majority of the tumour mass lacks significant blood flow in contrast to uninfected tumours, which exhibit relatively uniform perfusion. VSV replication in tumour neovasculature and spread within the tumour mass, initiates an inflammatory reaction including a neutrophil-dependent initiation of microclots within tumour blood vessels. Within 6 hours of intravenous administration of VSV and continuing for at least 24 hours, we observed the initiation of blood clots within the tumour vasculature whereas normal vasculature remained clot free. Blocking blood clot formation with thrombin inhibitors prevented tumour vascular collapse. Our results demonstrate that the therapeutic activity of an OV can go far beyond simple infection and lysis of malignant cells.

INTRODUCTION

The idea of using viruses to attack and destroy cancer cells is gaining momentum as clinical support for the concept continues to mount.^{1,2} A variety of clever engineering strategies that lead to selective replication of oncolytic viruses (OVs) in cancer cells have created a remarkably safe therapeutic platform.³ Although the mechanisms behind restricted virus replication in malignant cells are well established, the complexities of the interplay between the therapeutic virus and the host are still incompletely understood.^{4,5} In particular

it appears that multiple interactions of the virus with the patient's immune system, blood components, reticuloendothelial system, and the tumour microenvironment all can augment or mitigate the therapeutic efficacy of a particular virus platform.⁶ Understanding the mechanism of action of OV_s in vivo is critical to the design and optimization of therapeutic regimens and combination therapies in future clinical trials as well as optimizing the therapeutic efficacy of the next generation viruses currently in development. Indeed, one key attribute of OV therapeutics is their potential to target the tumour via multiple mechanisms increasing malignant cell killing and decreasing the incidence of therapeutic resistance.⁷

We have been investigating the interaction of OV_s with tumour vasculature as this is the key entry point of any systemically administered therapeutic. Attacking the tumour vasculature with a therapeutic virus has some obvious potential advantages as this could lead to destruction of neovasculature, providing a beacon for recruiting the immune system to the infected tumour and of course be an entry point for the virus into the tumour mass.⁸ In earlier studies, we have shown that an engineered version of vesicular stomatitis virus (VSV), a prototype OV with activity in a large variety of mouse tumour models, causes catastrophic loss of blood flow in the tumour bed resulting in massive bystander killing of cancer cells following intravenous delivery.⁹ This phenomenon was also demonstrated with oncolytic vaccinia virus.^{9,10} Furthermore, infection of the tumour resulted in significant increases in the transcription of genes that encode proinflammatory molecules leading to the recruitment of neutrophils and other immune cells to the tumour bed.⁹ Here, we have examined the direct interaction of VSV with tumour blood vessels and show for the first time, that limited sites of virus infection of neovasculature correlate with massive cell death

within the tumour. We characterized the mechanism behind the massive bystander killing within the infected tumour and found that neutrophil-dependent initiation of microclots within blood vessels led to irreversible damage of tumour vasculature. We demonstrate that intravascular clot formation robustly potentiates the anticancer activity of VSV by reducing proliferation and inducing apoptosis of tumour cells. Most importantly, the infection of vasculature and subsequent initiation of fibrin deposition and clot formation is restricted to tumour beds. Our findings support the idea that OV infection of tumour vasculature and intravascular coagulation are important components of the antitumour activity of VSV.

RESULTS

3D rendering of images of tumour perfusion and virus infection reveals isolated areas of virus infection and a large reduction in tumour perfusion

We have previously shown that VSV infection of tumours causes a rapid reduction of tumour perfusion within 24 hours of treatment. Our initial findings were based upon immunohistochemical analysis of individual tumour sections (Figure 3.1a); however, these provided limited understanding of the virus interactions within the entire tumour. We therefore constructed 3D models of uninfected and VSV-infected CT-26 colon tumours from ~1,000 serial histological sections (example of individual section in Figure 3.1a) per tumour, in order to permit immunohistochemical analysis of virus infection and spread throughout the entire tumour. To gain an understanding of both sites of virus infection and tumour perfusion, fluorescent microspheres were infused intravenously 5 minutes before animal sacrifice and viewed in individual sections by scanning as described previously (Figure 3.1b infected and 3.1c uninfected).⁹ The immunohistochemically stained sections were digitally

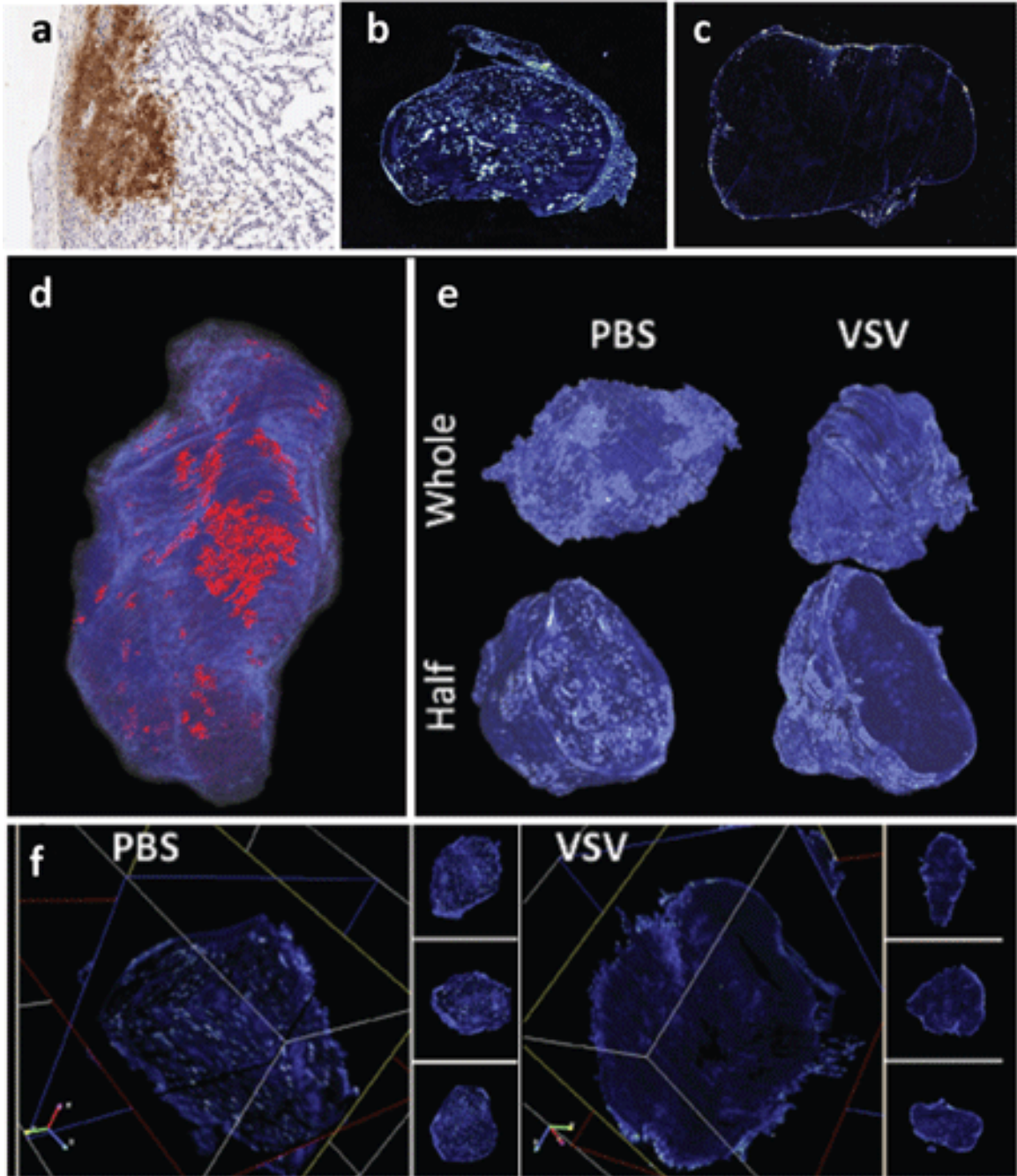


Figure 3.1. Vesicular stomatitis virus (VSV) infection and tumour perfusion are restricted to the tumour surface. A three-dimensional (3D) tumour model was reconstructed from two-dimensional (2D) digital images of serial histological sections. A CT-26 tumour-bearing BALB/c mouse was treated with VSV for 24 hours and perfused with fluorescent microspheres. **(a)** Serial sections from the excised tumour were stained with antibodies to VSV to visualize sites of virus infection. **(b)** Serial sections of an untreated tumour were then scanned for fluorescent microspheres in order to visualize perfusion. **(c)** A similar analysis of perfusion was generated from serial sections of a VSV-treated tumour. **(d)** Viewing the tumour in *HTK Histology Toolkit* reveals that infection (red) and perfusion (blue) are limited to the tumour surface. Areas of infection are localized to one region of the tumour surface. **(e)** 3D models of tumour perfusion of a VSV-treated tumour and a phosphate-buffered saline (PBS)-treated tumour were reconstructed from images of serial histological sections. Viewing perfusion of whole and half 3D models reveals that the surface of both VSV- and PBS-treated tumours are perfused. Half models show that the tumour core of the VSV-treated tumour is dramatically less perfused when compared to the PBS-treated tumour. **(f)** Using *HTK Histology Toolkit*, scanning through the VSV-treated tumour along x, y, and z axes consistently shows perfusion that is present in the tumour rim but absent from the core. In contrast, visualization of the PBS-treated tumour shows perfusion throughout the tumour.

scanned and combined with fluorescent images using the HTK Histology Toolkit to create a three-dimensional (3D) rendering of the infected tumour (see Materials and Methods section).

The 3D visualization of VSV 24 hours postintravenous infusion revealed that VSV (red) infects numerous areas of the tumour but is primarily limited to one major area of the tumour rim (Figure 3.1d) and in Supplementary Video S1. Tumour perfusion (light blue) is restricted exclusively to the tumour rim, as best visualized in a cross-section of the tumour (Figure 3.1e) and in Supplementary Video S2. Similar rendering of perfusion images of an untreated tumour reveals that CT-26 tumour perfusion is fairly uniform (Figure 3.1e and Supplementary Video S2) and provides a stark contrast to the reduction in perfusion triggered by virus infection. In another representation, cross-sections of the untreated and treated tumours in all three orthogonal planes revealed a complete loss of perfusion in the tumour core of the treated tumour with uniform perfusion in the untreated control (Figure 3.1f). By scanning through the entire tumour for each treatment (Supplementary Video S3), we observed that this loss of perfusion consistently extends throughout the entire tumour. The 3D rendering of virus infection and tumour perfusion reveals the full scope of the early events caused by OV infection and their impact on tumour physiology.

VSV treatment reduces proliferation of malignant cells within the tumour core

Given the drastic change in tumour perfusion observed over the course of OV therapy, we next investigated the effect of VSV infection on tumour cell proliferation. We monitored proliferation in the same tumour before (by BrdU incorporation) and after (by Ki67 staining) VSV treatment. Twenty-four hours before virus treatment, mice were pulsed with

5'-bromo-2'-deoxyuridine (BrdU), which is incorporated into DNA as it is being synthesized at the time of infusion. Mice were then challenged with VSV and euthanized the following day (Figure 3.2a). In histological sections of untreated tumours, regions of BrdU positive tumour cells (identified pathologically) correlate exactly with Ki67 staining, which marks cells proliferating at the time of mouse euthanasia. However, in tumours taken from mice treated with virus, while BrdU staining is uniform throughout the entire tumour, Ki67 staining is only detected in the tumour rim. Small areas of virus staining were also detected within the tumour rim (Figure 3.2b). Ki67 quantitation in treated versus untreated tumours demonstrated significantly less proliferation after OV therapy (Figure 3.2c). This experiment therefore demonstrates that the extent and distribution of tumour cell proliferation changes drastically over the course of VSV treatment. Decreased cell proliferation is maintained at late timepoints (22 days post-treatment) as observed in representative sections (Figure 3.2d). It is apparent that at both early and late timepoints, Ki67 staining is limited to the tumour rim, which is associated with perfused tissue (Figure 3.1f). These observations confirm that the reduction in cell proliferation, though caused by virus infection of tumours, is largely a result of uninfected tumour cell death, we believe triggered by a decrease in tumour perfusion.

OV therapy destroys tumour vasculature

Given the drastic change in tumour perfusion and proliferation observed over the course of OV therapy, we investigated the integrity of tumour vasculature after VSV treatment. Tumours removed from mice treated with replication incompetent UV-inactivated VSV were compared to tumours taken from mice treated with VSV for 15 hours. Sections were prepared

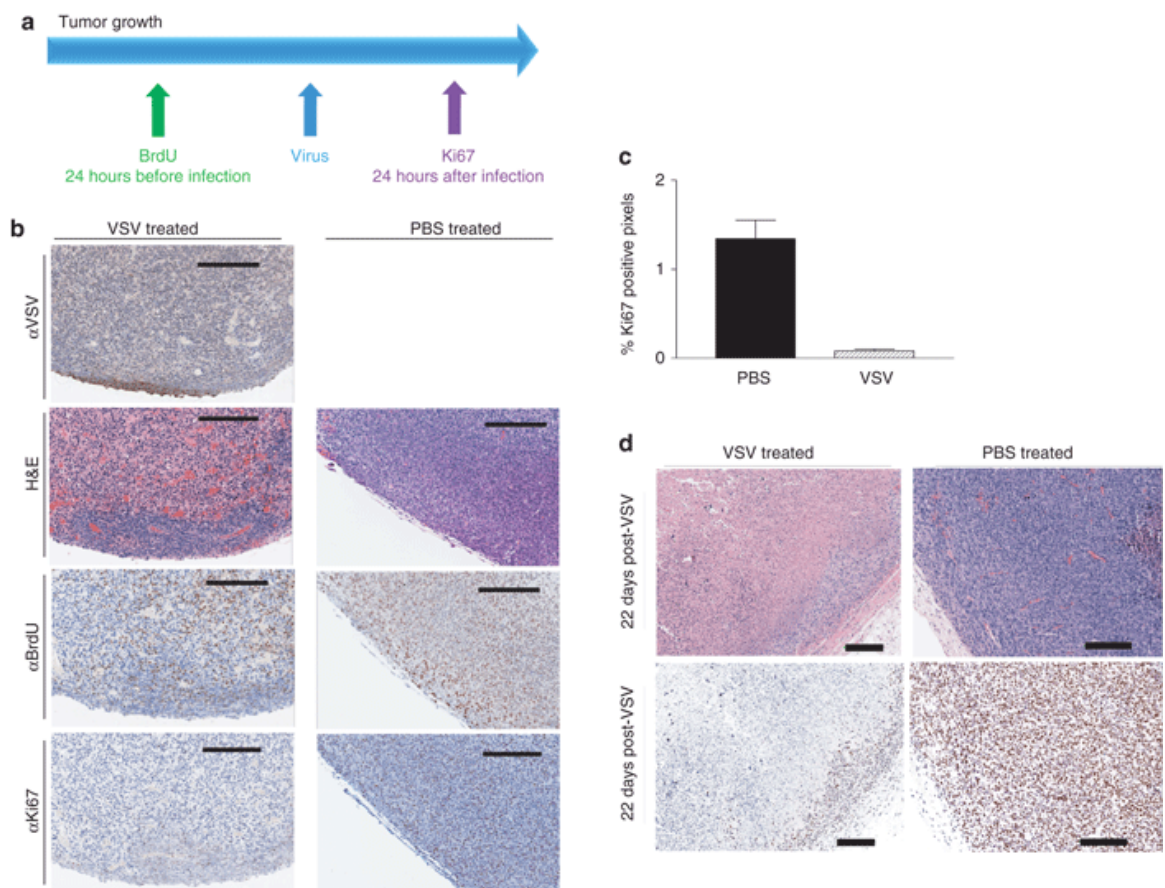


Figure 3.2. Decreased cell proliferation observed following vesicular stomatitis virus (VSV) treatment. (a) Experimental setup. BALB/c mice-bearing subcutaneous CT-26 tumours were pulsed with 5'-bromo-2'-deoxyuridine (BrdU) 24 hours before receiving VSV intravenously (or left untreated). Twenty-four hours following virus challenge, three mice per group were euthanized and tumours were embedded for sectioning. (b) Tumour sections were stained for immunohistochemical analysis to detect VSV (virus infection), Ki67 (cell proliferation), and BrdU (proliferating cells that incorporated bromodeoxyuridine). Serial sections were stained with hematoxylin & eosin to visualize tissue architecture. At 24 hours, there is a decreased visualization of Ki67 staining in the VSV-treated tumour. Bar = 200 μ m. (c) Quantification (mean + SD) of Ki67 staining in VSV-treated versus phosphate-buffered saline (PBS)-treated tumour at 24 hours post-treatment. Quantification of three mice per group was performed using Aperio software. (d) By 22 days postinfection, extensive necrosis is evident within the tumour core however, viable tumour cells persist within the tumour rim. It is apparent that Ki67 staining is limited to the tumour rim. Bar = 200 μ m. Image contrast was adjusted to 50 in all Ki67 stained images using Adobe Photoshop Version 8.

and stained with an anti-CD31 antibody to identify vascular endothelial cells. In sections from tumours infected with intravenously administered, UV inactivated VSV, vascular endothelial cells were uniformly distributed throughout the tumour (brown staining Figure 3.3). In contrast, tumours from mice treated with replication competent VSV had substantially fewer blood vessels and these were almost exclusively found in the tumour rim (Figure 3.3). We examined microscopically, five representative fields in both the rim and core of treated and untreated tumours to evaluate the number of CD31 positive vessels. We found a fourfold reduction of CD31 staining in the rim and 137-fold reduction in the core following VSV treatment. The observation that VSV-infected tumours lacked any intact blood vessels in the centre of the tumour but had apparently intact vessels at the rim is consistent with the tumour perfusion observed in our 3D model.

VSV directly infects tumour vasculature

The profound effect that VSV treatment had on both vascular endothelial cell numbers and tumour perfusion suggested that the virus was directly infecting neovasculature. Indeed, upon intravenous infusion of VSV, tumour-associated endothelial cells are likely the first cells within the tumour the virus comes in contact with. We therefore first evaluated whole mount tumours following intravenous infusion with a form of VSV that expresses green fluores-

cent protein (VSV-GFP) in infected cells. Mice were euthanized several hours post-treatment and tumours were evaluated under a fluorescent dissecting microscope. The expression of GFP within infected cells was clearly visible along the length of blood vessels on the surface of the tumour (Figure 3.4a). The GFP signal tracks into the tumour as the

virus apparently spreads from the site of initial infection into the tumour. Sections of infected

CD31

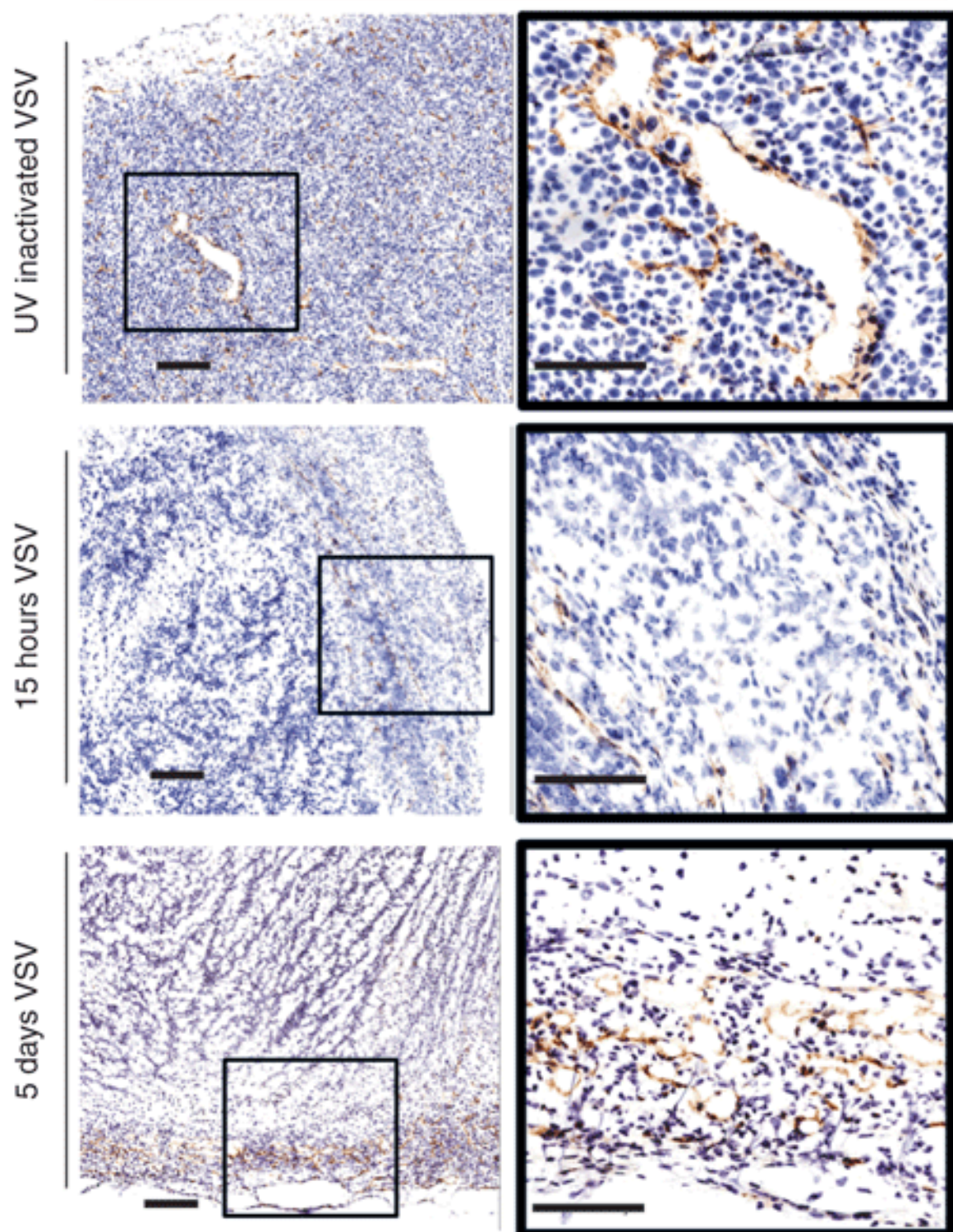


Figure 3.3. Vesicular stomatitis virus (VSV) treatment results in decreased tumour vasculature. BALB/c mice-bearing CT-26 tumours were treated intravenously with UV-inactivated VSV, or VSV intravenously. Five mice per treatment were euthanized at the indicated timepoints. Tumours were frozen for sectioning. Tumour sections were stained with an antibody detecting CD31, an endothelial cell marker. Endothelial cells are uniformly distributed in control tumours but are restricted to the tumour edge following VSV treatment. Bar = 200 μ m. Image contrast was adjusted to 50 in all images using Adobe Photoshop Version 8.

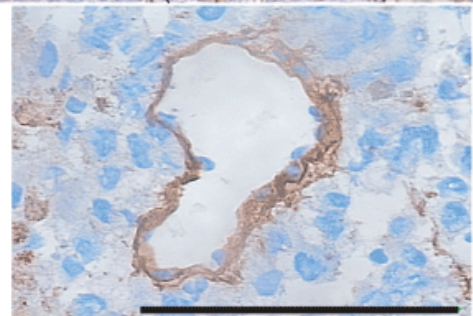
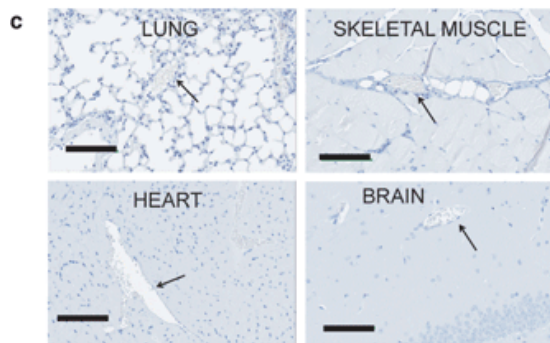
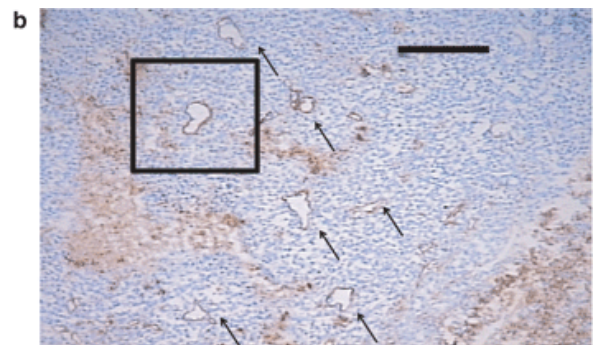
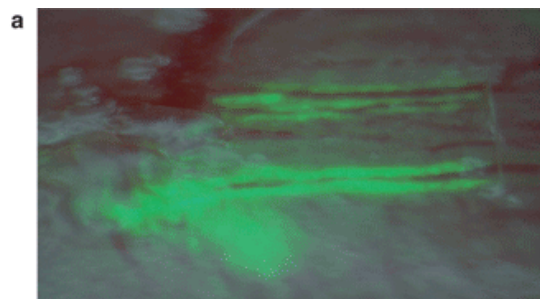


Figure 3.4. Vesicular stomatitis virus (VSV) infects tumour vasculature. (a) CT-26 subcutaneous tumour-bearing BALB/c mice were treated intravenously with VSV-expressing green fluorescent protein (GFP) and euthanized 24 hours later. Dissecting fluorescence microscopy was used to perform *in situ* imaging of tumour. Overlay of bright field and green fluorescence. VSV infection visualized along vessels. (b) BALB/c mice-bearing CT-26 tumours were treated intravenously with VSV-expressing GFP and sacrificed 24 hours later. Sections of tumours were stained by immunohistochemistry for VSV antigens. VSV infection is localized to tumour vasculature (black arrows) and surrounding tumour tissue. Bar = 100 μ m. (c) Brain, lung, heart, and tumour-adjacent skeletal muscle tissue were excised from five tumour-bearing BALB/c mice that were treated intravenously with VSV. No VSV antigen was detected in normal vasculature (arrows) and representative images are shown. Bar = 100 μ m.

tumours were prepared and examined microscopically. Immunohistochemical analysis of these sections confirmed that VSV is directly infecting tumour vasculature and then subsequently spreading into tumour tissue (Figure 3.4b).

We examined a variety of mouse tissues to determine whether VSV is capable of initiating infections in normal mature vasculature. In treated animals, we were unable to detect VSV-GFP expression in any normal organs (data not shown). We then prepared sections from a variety of normal tissues including skeletal muscle adjacent to the tumour, lung, heart, and brain. In none of these tissues nor the vasculature within them (Figure 3.4c) were we able to detect active VSV replication using immunohistochemical analysis. These results demonstrate that VSV appears unable to initiate infections in normal vascular endothelium but is capable of infecting tumour neovasculature and spreading from these initial sites of infection into the tumour proper.

OV therapy induces clot formation in tumour vasculature

Our 3D model clearly demonstrated that while there were limited sites of infection, the effects on tumour perfusion were quite profound (see Figure 3.1). This suggested that some limited infection of tumour vasculature and adjacent tumour cells leads to occlusion of blood vessels that resulted in a loss of blood flow throughout the tumour. One possible explanation is the virus damaged the neovasculature and this initiated the formation of blood clots

within infected vessels. We examined tumour sections using antibodies to fibrin since deposition of this protein is characteristic of clot formation.^{11,12} We found that infected

tumours had extensive fibrin staining consistent with the idea that VSV infection of CT-26 tumours triggers extensive blood clot formation in tumour blood vessels and areas of hemorrhage (Figure 3.5a). In contrast, uninfected tumours contain unclogged vessels with no detectable fibrin deposits.

As an impressive induction in intravascular blood clot formation was observed in tumours of VSV-treated mice, we wanted to determine whether this phenomenon was restricted to the infected tumour bed. We analyzed hematoxylin & eosin stained sections of brain, lung, heart, and tumour-adjacent skeletal muscle tissue and observed no increase in blood clot formation in these normal tissues taken from treated mice when compared to untreated controls (Figure 3.5d, quantification Figure 3.5e). These results demonstrate the normal tissue vasculature is resistant to VSV-induced clot formation.

Induction of clot formation is followed by decreased tumour proliferation

We observed that intravascular clot formation was absent in vessels located within the perfused, viable tumour rim (data not shown). Furthermore, intravascular clots were present throughout the tumour core and were associated with decreased tumour cell proliferation (Figure 3.2). A likely explanation is that occlusion of microcirculation by intravascular clots may deprive tumour cells of essential nutrients required to drive proliferation. Indeed, in mice treated with virus, we observe that decreased tumour cell proliferation occurs shortly after the induction of clot formation. In a detailed time-course experiment following fibrin clot formation and proliferation, increased blood clot formation was detected starting at 6

hours (Figure 3.5b), which preceded the decrease in proliferation observed as early as 9 hours (Figure 3.5c).

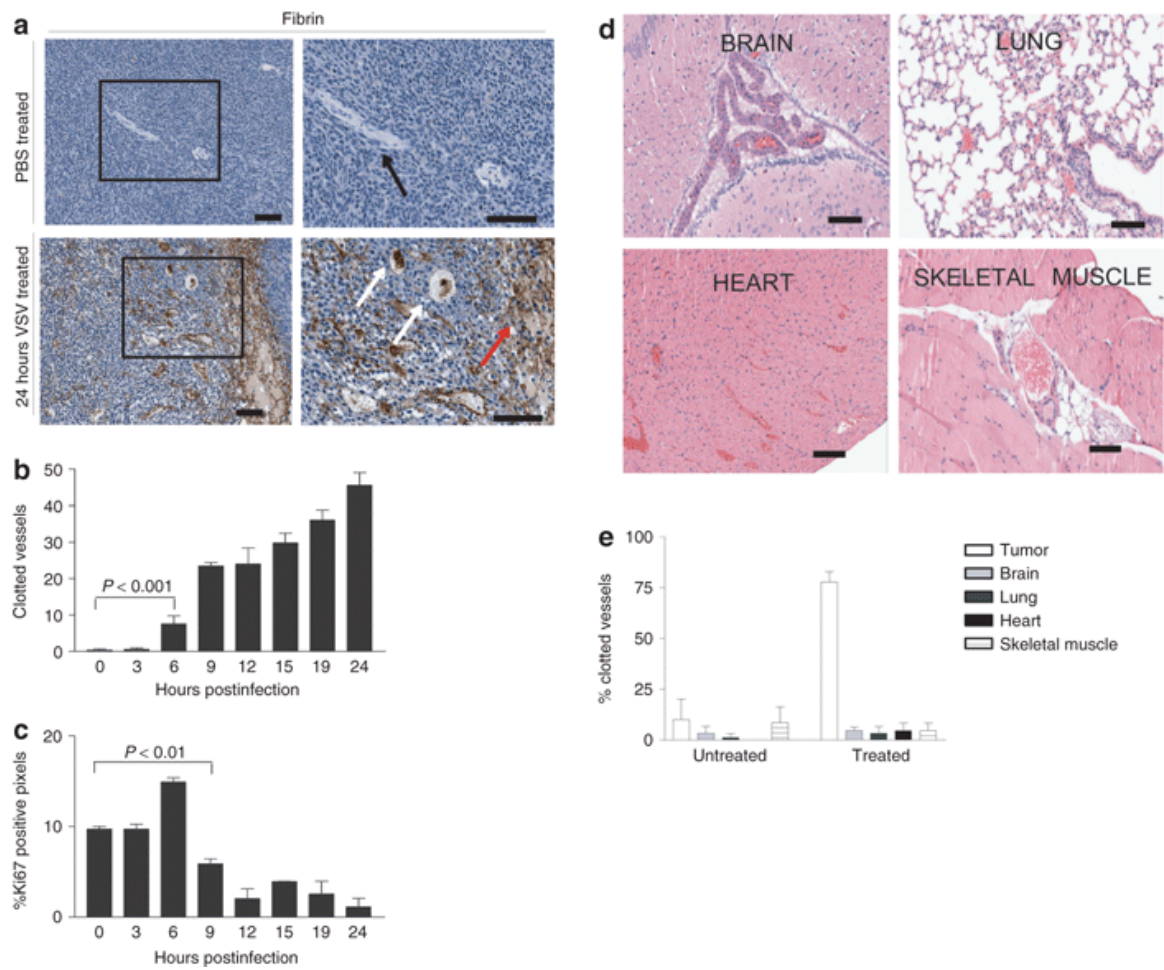


Figure 3.5. Oncolytic virus infection triggers coagulation within tumour vasculature and tumour cell proliferation decreases shortly after the onset of clot formation. (a) BALB/c mice with CT-26 tumours were treated intravenously with vesicular stomatitis virus- (VSV) expressing green fluorescent protein (GFP) and sacrificed at 3 hours postinfection (control) and 24 hours postinfection. Sections were stained with antibody that detects fibrin deposits. Note vessels (black arrows), clogged vessels (white arrows) and areas of hemorrhage (red arrows). Bar = 500 μ m, left images; bar = 200 μ m, right images. (b) Mice were treated with VSV and euthanized at various timepoints postinfection. Tumours were immunohistochemically stained to detect fibrin deposits. Clotted vessels of three tumours per timepoint were counted from five random fields of view per tumour (mean + SEM). Clot formation is initiated as early as 6 hours postinfection. *P* value determined by analysis of variance (ANOVA). (c) The same tumours were then stained for Ki67 and positive pixels were quantified and are expressed as percent positive pixels $\times 10$ (mean + SEM). Decreased proliferation is first detected 9 hours post-treatment. (d) Vessels associated with normal tissues do not exhibit increased coagulation. Brain, lung, heart, and tumour-adjacent skeletal muscle tissue were excised from tumour-bearing BALB/c mice that were treated intravenously with VSV. Hematoxylin & eosin stained sections of vessels associated with normal tissues are shown. Bar = 100 μ m. (e) Percentage of clotted vessels were quantified in tumour, brain, lung, heart, and tumour-adjacent skeletal muscle tissue from untreated mice and mice treated intravenously with VSV (mean + SD). Results obtained from five mice per treatment.

Intratumoural coagulation is dependent on neutrophils

In earlier studies, we demonstrated that virus mediated loss of tumour perfusion was dependent upon the presence of neutrophils.⁹ Others have demonstrated that neutrophil recruitment during natural pathological infections of normal tissues, leads to clot formation.¹³ In virus-treated tumours, we observed inflammatory cells, including neutrophils, at sites of thrombus formation (Figure 3.6a). Suspecting that neutrophils mediate clot formation, which would lead to decreased tumour perfusion, we compared and contrasted infected CT-26 tumours harvested from neutrophil intact or depleted mice for the presence of fibrin clots. To this end, mice were treated with GR-1, an antibody that can be used to deplete neutrophils.^{14,15} Under conditions where we observed dramatically reduced concentrations of neutrophils in the blood of treated mice (data not shown), we found that infected tumours had significantly reduced fibrin deposition (Figure 3.6b). These results coupled with our previous observations that neutrophils are required for virus-induced loss of tumour perfusion supports the idea that neutrophils are required for induction of coagulation in tumours treated with OV. These observations underscore the importance of an inflammatory reaction in triggering coagulation and loss of tumour perfusion during OV therapy.

OV therapy induces intratumoural coagulation triggering a reduction in tumour perfusion

To test whether the presence of fibrin clots is responsible for the loss of blood flow in OV-infected tumours, we attempted to inhibit clot formation during therapy using heparin or bothroaltnin, a thrombin inhibitor isolated from snake venom.¹⁶ Groups of three tumour-

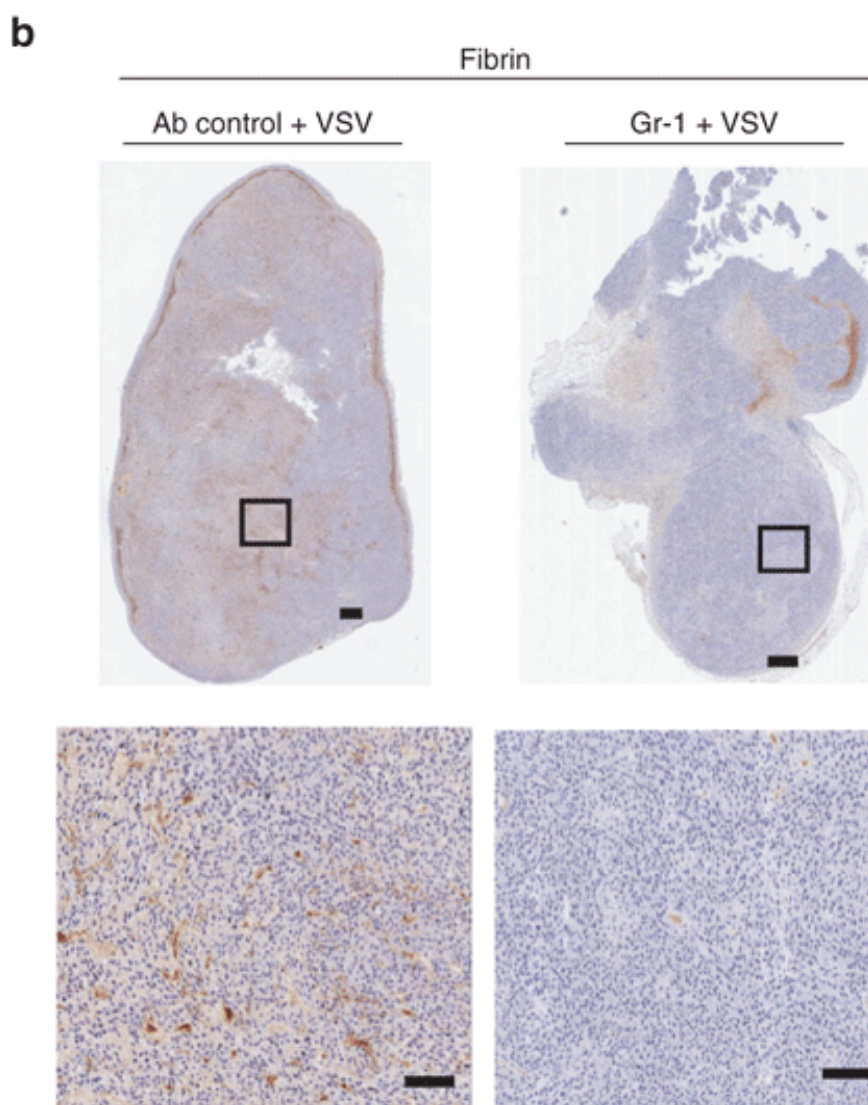
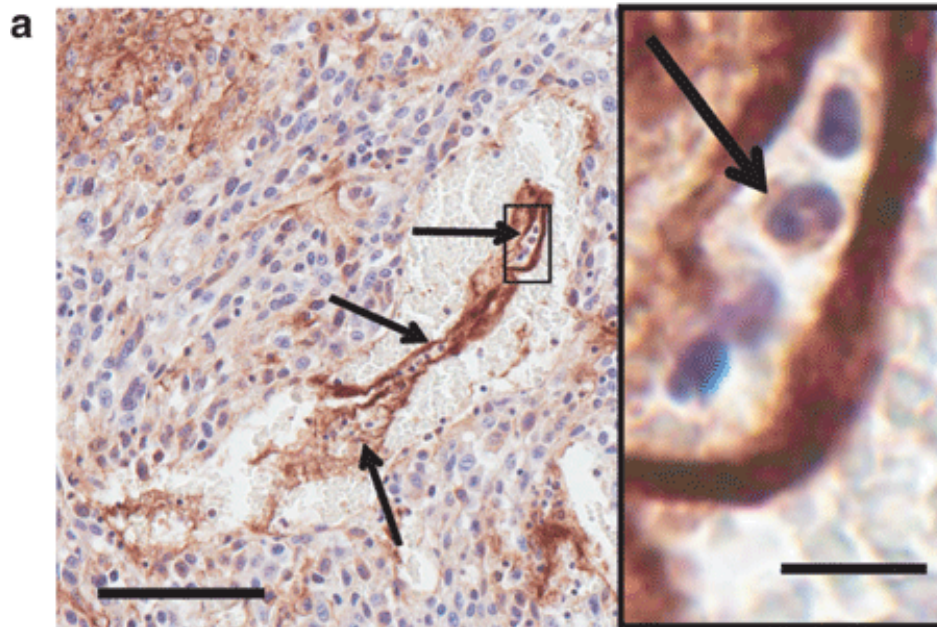


Figure 3.6. Decreased clot formation is observed in tumours from neutrophil-depleted mice. (a) A fibrin clot within a tumour vessel. Arrows indicate neutrophils. Bar = 100 μm , magnification: bar = 10 μm . (b) BALB/c mice-bearing CT-26 tumours were pretreated with RB6 8C5 antibody or 50:50 rat serum:phosphate-buffered saline intraperitoneally 24 hours before intravenous treatment with vesicular stomatitis virus (VSV). Twenty-four hours after virus treatment, mice were euthanized and tumours embedded for sectioning. Sections were stained to detect fibrin deposits. Decreased fibrin deposition is detected in tumours taken from neutrophil-depleted mice. Representative section of four mice shown. Bar = 100 μm .

bearing mice were continuously treated with either thrombin inhibitor in combination with VSV, and we found an even distribution of microspheres throughout the tumour (Figure 3.7) suggesting that formation of microclots within tumour vasculature is responsible for the loss of blood flow we observed during OV therapy. In contrast, mice treated with VSV in conjunction with tissue plasminogen activator, an enzyme that breaks down clots, we observed a loss of perfusion in the tumour core suggesting that once clots are initiated, irreversible vascular destruction ensues. These experiments demonstrate that inflammation triggered by OV infection within tumours activates blood clot formation in tumour microvasculature resulting in a loss of tumour perfusion. This effect can be blocked by continuous treatment with thrombin inhibitors but once initiated causes irreversible damage.

Virus initiated coagulation correlates with increased tumour cell killing

To determine the effect that virus-induced coagulation had on tumour physiology, we compared and contrasted VSV-treated tumour-bearing mice in the presence or absence of heparin. We found that heparin effectively reduced the formation of fibrin clots in response to VSV treatment (Figure 3.8). In heparin-treated tumours there was a coincident increase in Ki67 staining and a decrease in active caspase 3 staining (Figure 3.8). It is known that under some conditions, heparin can affect the ability of certain viruses to infect cells¹⁷ and therefore the reduction of fibrin clots could have simply been a consequence of reduced VSV infection. Upon examination of infected, heparin-treated tumours we found however no difference in the number of initial sites of VSV infection (identified by immunohistochemical staining). In addition, in vitro infections in the presence of heparin did not impact the ability of VSV to infect cells (data not shown). In these experiments,

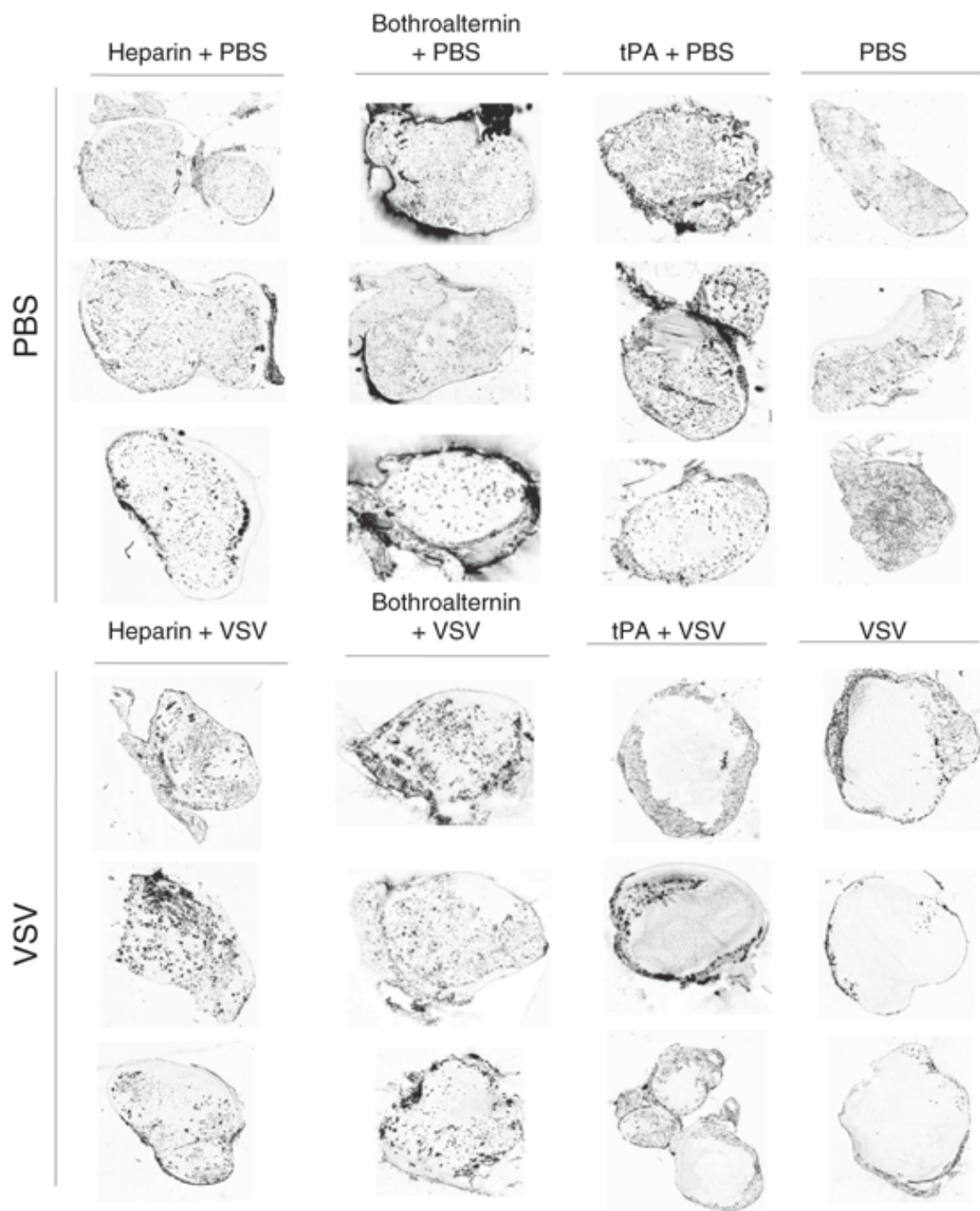


Figure 3.7. Inhibition of coagulation results in sustained perfusion of oncolytic virus-(OV) treated tumours. BALB/c mice with CT-26 tumours were treated intravenously with phosphate-buffered saline (PBS) or VSV as well as tissue plasminogen activator (tPA), bothroaltein or heparin intraperitoneally. Prior to being sacrificed, mice were perfused with fluorescent microspheres for 5 minutes. Tumour sections were prepared and analyzed for fluorescence using a microarray scanner. Black spots indicate perfused areas. Tumours excised from mice treated with PBS are uniformly perfused. Tumours taken from mice treated with VSV or VSV + tPA demonstrate a loss of tumour perfusion. In contrast, mice treated with thrombin inhibitors bothroaltein or heparin + VSV demonstrate uniform perfusion. Images representative of three mice per treatment are shown.

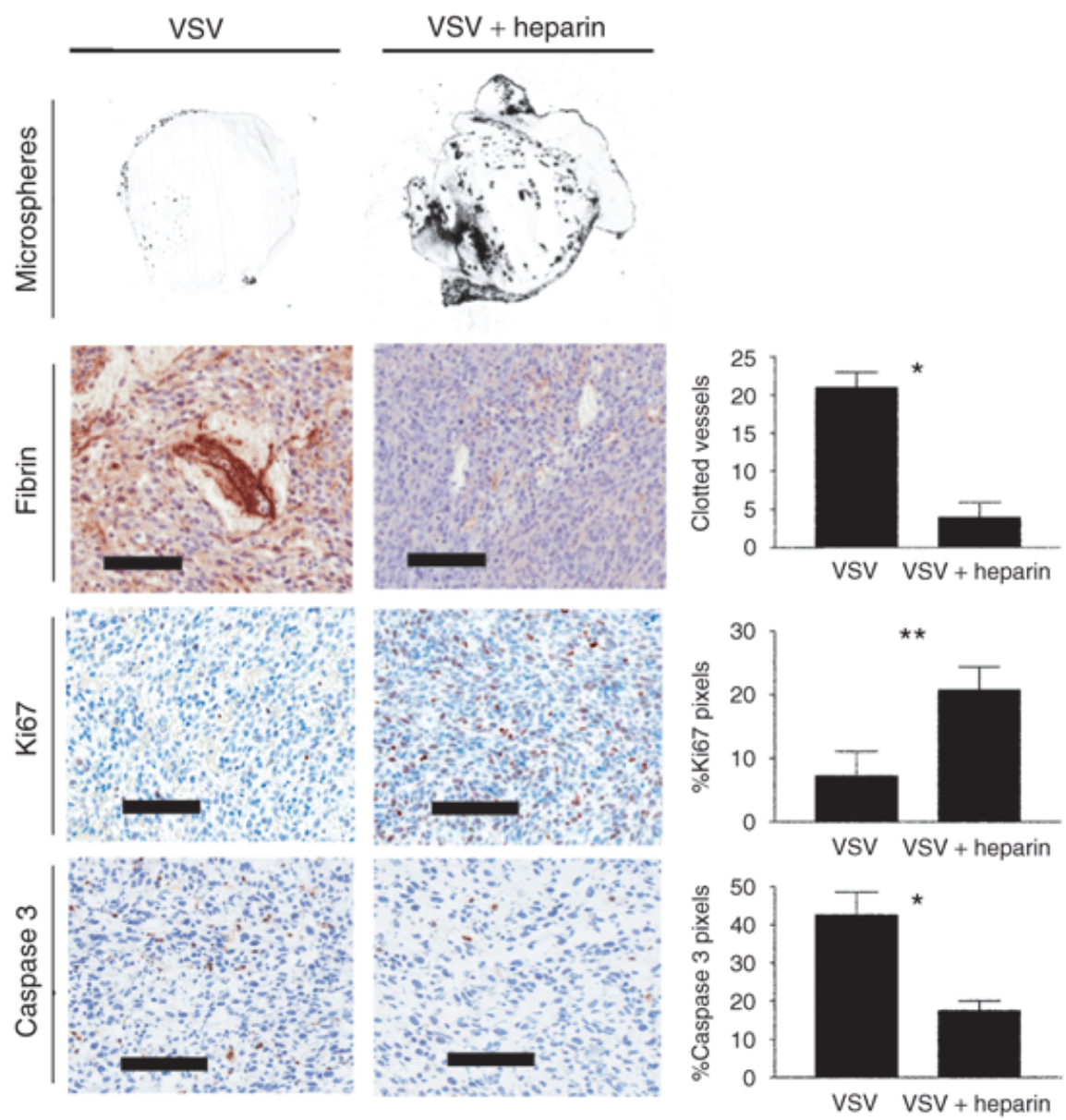


Figure 3.8. Clot formation results in reduced proliferation and increased apoptosis in oncolytic virus (OV)-treated tumours. BALB/c mice with CT-26 tumours were treated intravenously with either vesicular stomatitis virus (VSV) alone or with heparin in order to inhibit VSV-induced coagulation. Representative histological images are shown with corresponding quantification from four mice per group. Clot formation was quantified as described previously. The same tumours were stained for Ki67 and the active form of caspase 3, markers of proliferation, and apoptosis, respectively. Virus-treated, clotted tumours exhibited less proliferation and more apoptosis relative to tumours treated with VSV and heparin. Staining is expressed as percent positive pixels $\times 10$ (mean + SEM). $*P < 0.005$, $**P < 0.05$.

induction of clot formation correlated with potentiated tumour cell killing well beyond the limited sites of infection observed at 24 hours (Figure 3.1).

DISCUSSION

Herein, we describe for the first time, a natural tumour vasculature targeting ability of the OV VSV, which leads to tumour blood vessel coagulation and ultimately vascular collapse. Induction of blood clot formation over time correlated with decreased tumour cell proliferation. This phenomenon can be prevented by treatment with anticoagulants showing that blood clot formation in tumour microvasculature is critical for loss of tumour perfusion. Previously, we demonstrated a critical role for neutrophil recruitment to infected tumour beds that initiates a cascade of events leading to a profound loss of tumour perfusion.^{9,18} Our observation that neutrophils are also essential for the initiation of fibrin deposition and clot formation in infected tumours is consistent with a VSV-induced inflammatory reaction at the vascular endothelial surface that profoundly compromises tumour vasculature. As mentioned earlier, neutrophil-initiated coagulation is well known in pathological infections of normal tissues.¹⁹ In particular, a recent study has demonstrated that neutrophil elastase and cathepsin G promote coagulation and intravascular thrombus growth.²⁰ It appears that OV infection of tumour neovasculature simply initiates a preprogrammed host response to virus infection focusing the normal inflammatory response inside the tumour. This rapid innate host response, whose function in natural infections is to decrease blood flow preventing the spread of pathogens, instead potentiates the anticancer activity of VSV by targeting hypercoagulable tumour vasculature. Indeed our 3D modeling of tumour infection

demonstrates that VSV initiates profound tumour killing that would not be predicted from the modest level of tumour infection and virus spread within the tumour. These studies underline the importance of understanding the mechanism of tumour targeting of anticancer agents *in vivo*, in particular with proinflammatory agents like replicating viruses.

The observations presented here have significant implications for the OV field. While we do not currently know that all OVs can infect tumour vasculature, we have shown that this is also a property of oncolytic vaccinia virus.¹⁰ Targeting tumour vasculature has major advantages over only targeting tumour tissue; these include an ease of targeting with IV administration as well as a presumably greater genetic stability of tumour endothelial cells that should decrease the likelihood of evolved resistance to therapeutics that is characteristic of malignant cells. In addition, targeting tumour vasculature may result in “bigger bang for your buck” because as we show here virus targeting of a limited number of endothelial cells can cause broader vascular disruption and significant tumour cell killing. Clinically, the heterogeneity of cancer often poses barriers to direct infection and cell killing by OVs. For example, virus resistant subpopulations, stromal components, and extracellular matrix are able to prevent efficient infection and spread, thereby limiting the ability of OVs to directly infect and kill malignant cells. The ability of OV’s to target tumour vasculature provides a mechanism to achieve tumour cell killing while circumventing the need for efficient virus spread throughout the tumour mass.

We clearly demonstrate here for the first time that VSV is able to productively infect tumour neovasculature *in vivo*, but that vascular endothelium in normal tissues is resistant

to virus infection. The mechanism of VSV selectivity is not known but recently Vile and colleagues have shown that in vitro treatment of human vascular endothelial cells with vascular endothelial growth factor can transiently sensitize these normal cells to infection by reovirus and VSV.⁸ It may be that in the tumour microenvironment where blood vessels are being constantly remodeled as a consequence of stimulation by vascular endothelial growth factor and other proangiogenic factors, that vascular endothelium becomes prone to OV infection and destruction. Interestingly, Orf a parapoxvirus encodes a viral form of vascular endothelial growth factor that is known to enhance its pathogenenic effects.²¹ Perhaps vascular endothelial growth factor stimulation of vascular endothelial cells inhibits or dampens the innate antiviral programs of these cells making them transiently sensitive to virus infection.

Finally, our observation of tumour vascular shutdown in pre-clinical models led us to investigate changes in tumour perfusion in patients treated with OVs as part of clinical trials. A profound reduction in tumour perfusion was observed in some patients with advanced hepatocellular carcinoma following treatment with an oncolytic poxvirus, JX-594 in early phase trials.^{22,23} Clearly, acute vascular disruption of tumours causes decreased tumour cell viability and is therefore an additional mechanism of action of OVs.^{9,18} Understanding the mechanism by which OV infection targets tumour vasculature will allow for the development of strategies to exaggerate the effect and increase acute tumour cell killing.^{7,24}

SUPPLEMENTARY MATERIAL (available online)

Video S1. 3D model of VSV infection and perfusion.

Video S2. Cross-sections of perfusion models of VSV- and PBS-treated tumours.

Video S3. Scan throughs of VSV and PBS perfusion models.

ACKNOWLEDGEMENTS

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REFERENCES

1. Parato, KA, Senger, D, Forsyth, PA and Bell, JC (2005). Recent progress in the battle between oncolytic viruses and tumours. *Nat Rev Cancer* 5: 965–976.
2. Cattaneo, R, Miest, T, Shashkova, EV and Barry, MA (2008). Reprogrammed viruses as cancer therapeutics: targeted, armed and shielded. *Nat Rev Microbiol* 6: 529–540.
3. Bell, JC, Lichty, B and Stojdl, D (2003). Getting oncolytic virus therapies off the ground. *Cancer Cell* 4: 7–11.
4. Prestwich, RJ, Harrington, KJ, Pandha, HS, Vile, RG, Melcher, AA and Errington, F (2008). Oncolytic viruses: a novel form of immunotherapy. *Expert Rev Anticancer Ther* 8: 1581–1588.

5. Liu, TC, Galanis, E and Kirn, D (2007). Clinical trial results with oncolytic virotherapy: a century of promise, a decade of progress. *Nat Clin Pract Oncol* 4: 101–117.
6. De Silva, N, Atkins, H, Kirn, DH, Bell, JC and Breitbach, CJ (2010). Double trouble for tumours: exploiting the tumour microenvironment to enhance anticancer effect of oncolytic viruses. *Cytokine Growth Factor Rev* 21: 135–141.
7. Kirn, DH and Thorne, SH (2009). Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer. *Nat Rev Cancer* 9: 64–71.
8. Kottke, T, Hall, G, Pulido, J, Diaz, RM, Thompson, J, Chong, H et al. (2010). Antiangiogenic cancer therapy combined with oncolytic virotherapy leads to regression of established tumours in mice. *J Clin Invest* 120: 1551–1560.
9. Breitbach, CJ, Paterson, JM, Lemay, CG, Falls, TJ, McGuire, A, Parato, KA et al. (2007). Targeted inflammation during oncolytic virus therapy severely compromises tumour blood flow. *Mol Ther* 15: 1686–1693.
10. Kirn, DH, Wang, Y, Le Boeuf, F, Bell, J and Thorne, SH (2007). Targeting of interferon-beta to produce a specific, multi-mechanistic oncolytic vaccinia virus. *PLoS Med* 4: e353.
11. Corral, J, Yélamos, J, Hernández-Espinosa, D, Monreal, Y, Mota, R, Arcas, I et al. (2005). Role of lipopolysaccharide and cecal ligation and puncture on blood coagulation and inflammation in sensitive and resistant mice models. *Am J Pathol* 166: 1089–1098.
12. Chen, D, Giannopoulos, K, Shiels, PG, Webster, Z, McVey, JH, Kembell-Cook, G et al. (2004). Inhibition of intravascular thrombosis in murine endotoxemia by targeted expression of hirudin and tissue factor pathway inhibitor analogs to activated endothelium. *Blood* 104: 1344–1349.

13. Luscinskas FW (2001). Cytokines and endothelial cell activation. In: Ley, K (ed.). *Physiology of Inflammation*. Oxford University Press: New York, pp 80–89.
14. Maus, U, von Grote, K, Kuziel, WA, Mack, M, Miller, EJ, Cihak, J et al. (2002). The role of CC chemokine receptor 2 in alveolar monocyte and neutrophil immigration in intact mice. *Am J Respir Crit Care Med* 166: 268–273.
15. Tepper, RI, Coffman, RL and Leder, P (1992). An eosinophil-dependent mechanism for the antitumour effect of interleukin-4. *Science* 257: 548–551.
16. Castro, HC, Dutra, DL, Oliveira-Carvalho, AL and Zingali, RB (1998). Bothroaltein, a thrombin inhibitor from the venom of *Bothrops alternatus*. *Toxicon* 36: 1903 - 1912.
17. Baba, M, Snoeck, R, Pauwels, R and de Clercq, E (1988). Sulfated polysaccharides are potent and selective inhibitors of various enveloped viruses, including herpes simplex virus, cytomegalovirus, vesicular stomatitis virus, and human immunodeficiency virus. *Antimicrob Agents Chemother* 32: 1742–1745.
18. Varani, J and Ward, PA (1994). Mechanisms of endothelial cell injury in acute inflammation. *Shock* 2: 311–319.
19. Westlin, WF and Gimbrone, MA Jr (1993). Neutrophil-mediated damage to human vascular endothelium. Role of cytokine activation. *Am J Pathol* 142: 117–128.
20. Massberg, S, Grahlf, L, von Bruehl, ML, Manukyan, D, Pfeiler, S, Goosmann, C et al. (2010). Reciprocal coupling of coagulation and innate immunity via neutrophil serine proteases. *Nat Med* 16: 887–896.
21. Savory, LJ, Stacker, SA, Fleming, SB, Niven, BE and Mercer, AA (2000). Viral vascular endothelial growth factor plays a critical role in orf virus infection. *J Virol* 74: 10699–10706.

22. Liu, TC, Hwang, T, Park, BH, Bell, J and Kirn, DH (2008). The targeted oncolytic poxvirus JX-594 demonstrates antitumoural, antivascular, and anti-HBV activities in patients with hepatocellular carcinoma. *Mol Ther* 16: 1637–1642.
23. Park, BH, Hwang, T, Liu, TC, Sze, DY, Kim, JS, Kwon, HC et al. (2008). Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: a phase I trial. *Lancet Oncol* 9: 533–542.
24. Stanford, MM, Breitbach, CJ, Bell, JC and McFadden, G (2008). Innate immunity, tumour microenvironment and oncolytic virus therapy: friends or foes? *Curr Opin Mol Ther* 10: 32–37.

Chapter 4: Clinical development of the oncolytic poxvirus JX-594

INTRODUCTION

JX-594 – an oncolytic poxvirus in clinical development

JX-594 (*Pexa-Vec*) is an oncolytic poxvirus engineered from the Wyeth vaccinia strain. It has been engineered for tumour specificity by the deletion of thymidine kinase (TK), a virally encoded enzyme that would normally increase nucleotide pools, facilitating the production of viral progeny. Normally, cellular TK expression is restricted to the S phase of the cell cycle when nucleotides are incorporated into newly synthesized DNA (120). In cancer cells, TK is constitutively overexpressed, establishing a tumour-specific susceptibility to TK deficient viruses. JX-594 has also been engineered to express granulocyte-macrophage colony stimulating factor (GM-CSF) to enhance the immunostimulatory properties of JX-594 to promote anti-tumour immunity (118). JX-594 also encodes LacZ and its product, β -galactosidase, a foreign transgene not found in human tissues (121). The detection of β -galactosidase is a marker of virally produced transgenes.

Intravenously delivered agents to target metastatic disease

Metastatic disease is responsible for the vast majority of cancer related deaths. Detecting tumours within a patient isn't always possible due to the limitations in imaging capabilities. Micrometastatic disease often escapes primary surgical resection and detection (122).

The ability to administer therapeutic agents through the blood stream provides the advantage of promiscuous dissemination to reach undetectable or inoperable disease. Vaccinia biology has several features that make it amenable for systemic administration. It

has evolved mechanisms that increase stability in the blood, including the ability to evade antibody and complement-mediated neutralization (121).

Preliminary indications of vessel targeting with JX-594

The need for better antiangiogenic treatments has been discussed previously (Chapter 1). Following the observation that VSV could target tumour vasculature, induce tumour-targeted inflammation, coagulation, and bystander necrosis, we considered the possibility that a similar phenomenon could occur in patients enrolled in clinical trials for JX-594. Preclinically, a decrease in tumour blood flow was observed in response to vvDD, a Western Reserve vaccinia strain with attenuating deletions in TK and VGF (92). Several clinical observations suggested that vessel targeting could occur in patients treated with JX-594. In a small cohort of patients with advanced stage hepatocellular carcinoma (HCC) (123), treatment with GM-CSF expressing vaccinia virus resulted in increased circulating neutrophil counts in four out of six patients receiving virus at the maximum tolerated dose (MTD) (123). Furthermore, increased serum levels of pro-inflammatory cytokines were measured, including GM-CSF, TNF- α , IFN- γ , IL-4 and IL-6. In particular, TNF- α and IFN- γ have been shown to have anti-vascular effects (124). For these reasons, changes in tumour perfusion in the context of JX-594 therapy were closely monitored in an ongoing Phase II trial of JX-594 in patients with advanced HCC.

JX-594 trial design

A clinical trial was designed to test whether JX-594 could infect metastatic tumours after intravenous infusion and whether this infection had any effects on tumour vasculature (121). A Phase I dose-escalation trial was planned where patients received a single intravenous infusion of JX-594. Twenty-three patients, with advanced, treatment-refractory solid tumours (lung, colorectal, melanoma, thyroid, pancreatic, ovarian, gastric, and mesothelioma), were treated in one of six dose cohorts ranging from 1×10^5 to 3×10^7 pfu/kg. JX-594 delivery, gene expression, and replication in solid tumours were assessed through several parameters including IHC and detection of viral DNA through qPCR. Following the preclinical demonstration that OV6s could target tumour vasculature leading to durable necrotic responses, a phase II trial for JX-594 was designed to monitor for evidence of vascular disruption using dynamic contrast MRI. Patients had either liver-based tumours of HCC (n = 15) or colorectal carcinoma (n = 1) (125).

RESULTS

JX-594 is able to reach and amplify within tumour beds following systemic infusion in patients.

Twenty-three patients with advanced treatment-refractory solid tumours received a single intravenous infusion of escalating doses of JX-594 (121). Biopsy material was analyzed to determine whether JX-594 could infect metastatic tumours after intravenous infusion in

patients. Biopsies, taken 8-10 days after infusion, were stained using a polyclonal anti-vaccinia antibody for evidence of viral replication and β -galactosidase, a surrogate marker for virally-encoded transgene expression. Vaccinia immunoreactivity was detected in a colorectal tumour (Figure 4.1a). Negative controls where the primary antibody was omitted from the staining protocol, were included to ensure that the staining was specific to vaccinia. These samples were negative for all patients (Figure 4.1b). Pretreatment biopsies were negative for vaccinia virus indicating that vaccinia antigens were expressed in tumours following infusion of the virus (Figure 4.1c). The negative pretreatment biopsy staining and negative control results were consistent for all patients who tested positive (Figures 4.1d-f). Biopsies taken from lower dose cohorts were negative for anti-vaccinia immunoreactivity (Figure 4.1g). Positive staining in high dose patients ($\geq 3 \times 10^7$ pfu/kg) coincided with the detection of viral DNA by PCR (Figure 4.1h).

3D modeling of biopsy material from a patient treated with JX-594

To understand the extent and distribution of virus in patients treated with JX-594, we constructed a 3D model of a biopsy taken from a patient treated with 1.5×10^7 pfu/kg of JX-594, intravenously infused in 250 mL bicarbonate-buffered saline over 60 minutes. Ten days later, an excisional biopsy of the colorectal carcinoma was obtained. 126 serial sections were cut and every other section was stained for virus (Figure 4.2a). 2D pictures of the sections were then converted into a 3D volume using HTK Histology Toolkit software (Robarts Imaging Institute, University of Western Ontario). Regions of infection were highlighted to aid in visualization (Figure 4.2b). We analyzed the 3D volume in two ways. The first representation is of unadjusted histological staining for vaccinia virus (Figure 4.2c). 2D planes

on orthogonal axes can be moved through the reconstructed volume. We scanned the entire volume on x, y, and z axes. Malignant tissue was infected while intermixed normal tissues were not. This staining pattern was observed throughout the entire reconstruction of the biopsy. In another representation, a malignant glandular structure within the carcinoma was visualized in three dimensions, where positive viral staining is highlighted in green (Figures 4.2 d and e). The entire malignant structure was positive for virus while immunoreactivity was not detectable in luminal necrotic tissue or the surrounding normal tissues. These data indicate that JX-594 is able to extensively and consistently infect malignant cells while not infecting normal tissues.

JX-594 is capable of infecting tumour vasculature in mice and patients.

Having observed that VSV was able to directly infect and target tumour vasculature in mice, we tried to understand whether JX-594 was capable of directly infecting tumour endothelium in mouse models and patients. Mice bearing subcutaneous tumours were treated with JX-594, administered intravenously or injected intratumourally. Five days following the single treatment, IHC for vaccinia revealed that vascular structures were immunoreactive (Figure 4.3a-c). Vaccinia staining was observed specifically within the cytoplasm of endothelial cells (Figure 4.3c). These results demonstrate that vaccinia is capable of infecting tumour endothelium when injected via intratumoural or intravenous routes.

Biopsies from patients on a phase I dose-escalation trial of intravenous JX-594, who received approximately 3×10^7 to 3×10^9 pfu, were assessed for tumour-associated endothelial cell infection in visible vessels. Tumour cells and tumour-associated vessels had positive vaccinia staining (Figures 4.4a, d). Negative controls were included for all samples to validate that the

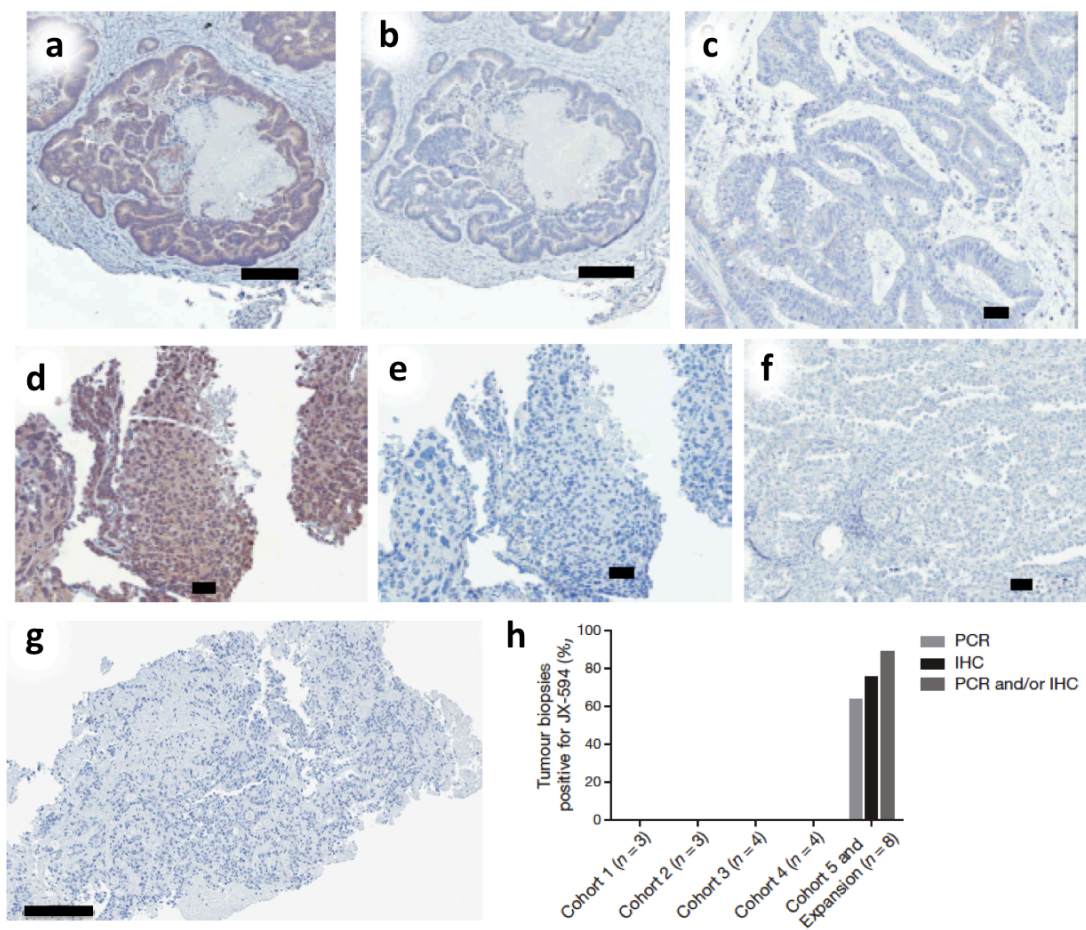


Figure 4.1. Tumours from patients infused with JX-594 virus are positive for vaccinia virus and β -galactosidase. Biospies were from patients obtained 8-10 days post infusion. Patients were given escalating doses of virus. Cohorts 1 and 2: 1×10^5 p.f.u/kg. Cohort 3: 3×10^5 p.f.u/kg. Cohort 4: 1×10^7 p.f.u/kg. Cohort 5 and expansion: 1.5×10^7 - 3×10^7 p.f.u/kg. a) Immunohistochemistry for vaccinia. Colorectal tumour, 10 days post infection. Scale bar 200 μ m. b) Immunohistochemistry with no primary antibody. Scale bar 200 μ m. c) Immunohistochemistry for vaccinia in a pretreatment biopsy from the same patient. Scale bar 50 μ m. d-f are the same conditions as a-c, but in a different patient. (adapted from Breitbach, C. et al. Nature, 2011) g) Patient from low dose cohort treated with 3×10^6 pfu of JX-594 was negative for anti-vaccinia immunoreactivity. h) qPCR for viral DNA and vaccinia immunoreactivity were positive in the high dose cohort.

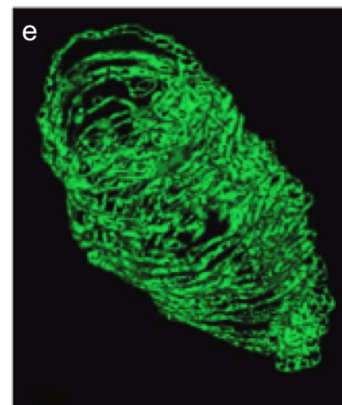
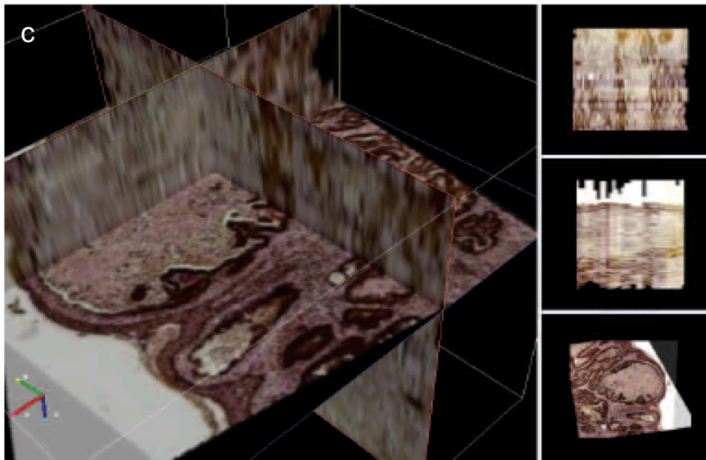
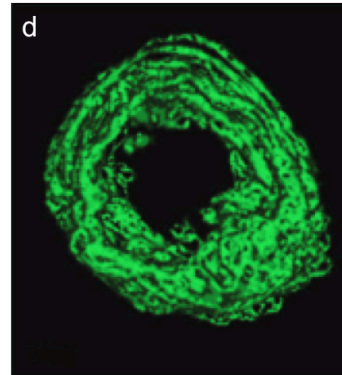
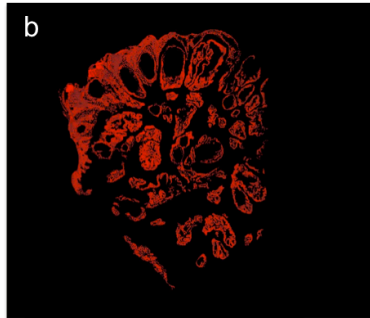
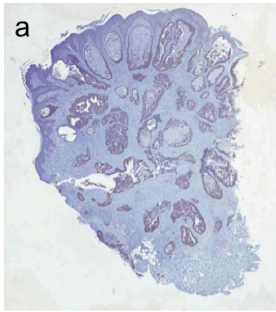
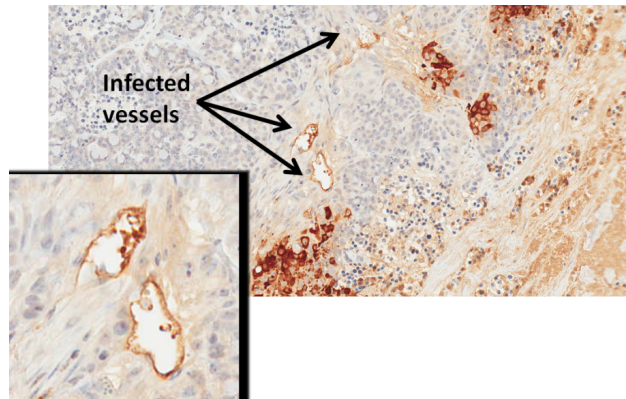
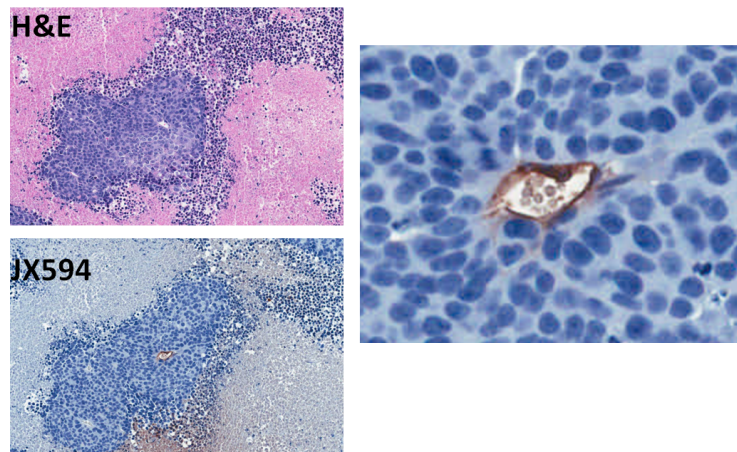


Figure 4.2. JX-594 achieves widespread infection in a 3D reconstruction of a patient biopsy. An excisional biopsy from a colon cancer patient was obtained 10 days post infusion of JX-594. a) A representative section of 126 sections that were stained for vaccinia virus proteins. b) 2D digital images of each slide were adjusted to highlight positive staining in red (Photoshop CS2). c) Extensive infection of cancer cells and vessels was observed by scanning orthogonal planes. d) 3D rendering of a single glandular structure where infection (green) was present throughout cancerous cells. e) The same glandular structure viewed from another angle.

a)



b)



c)

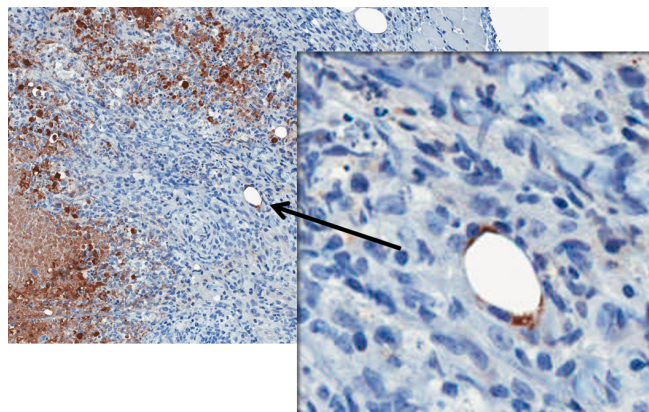
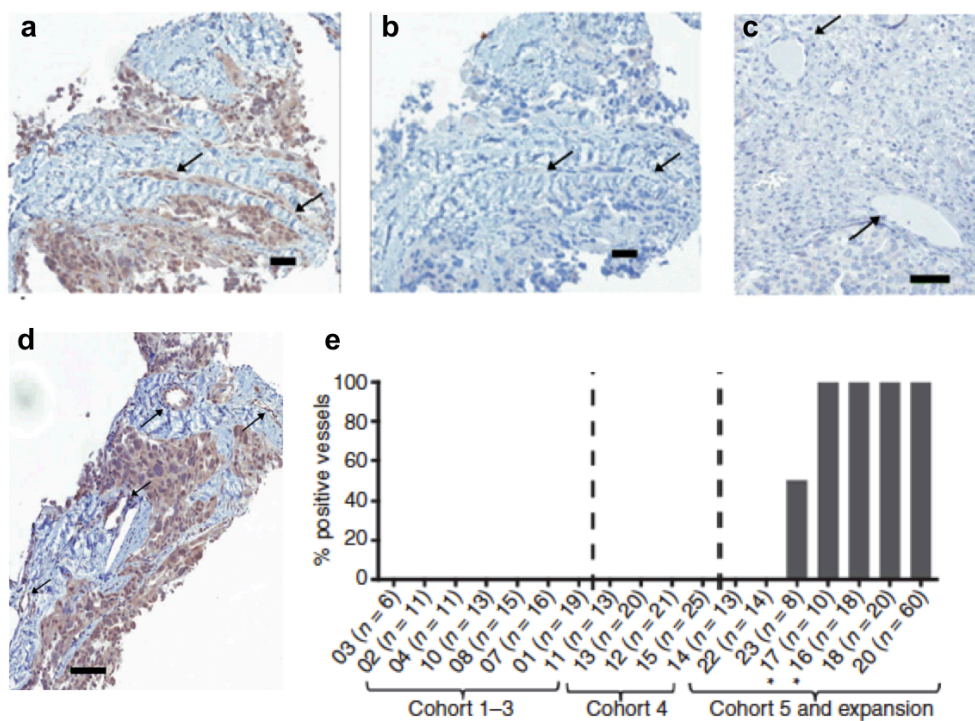


Figure 4.3. JX-594 is able to infect tumour endothelium in mice by systemic and intratumoural injection routes. a) HT29 tumours were injected intratumourally with a single injection 1×10^7 pfu of JX-594 for 5 days. b) SW620 tumours were injected with JX-594 for 5 days. Serial H&E staining shows a vessel within an island of tumour cells. The vessel was positive for vaccinia antigens. c) A vessel from an SW620 was also positive for virus. Vaccinia staining is restricted to the cytoplasm of endothelial cells.



f)

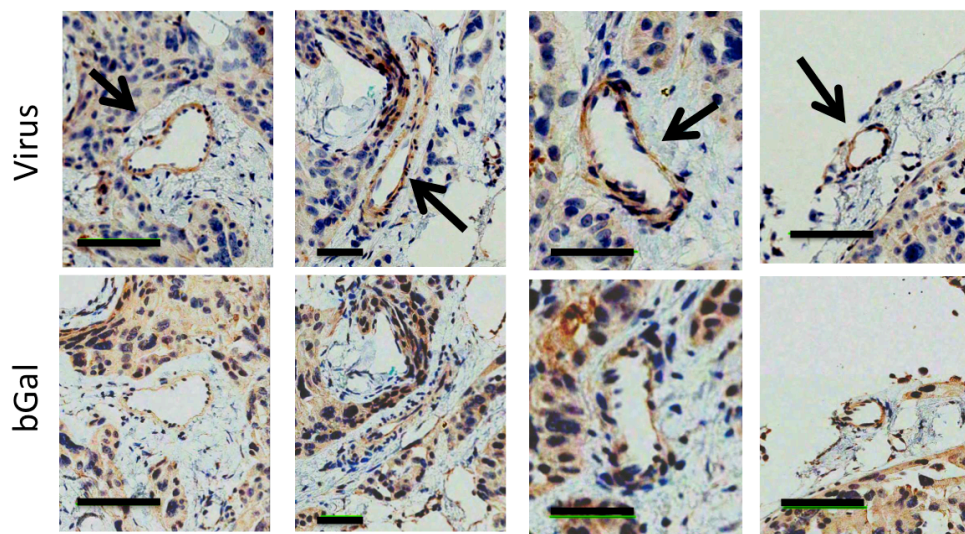


Figure 4.4. JX-594 is able to infect tumour endothelium in patients a) JX-594 infection of tumour-associated endothelial cells in a lymph node biopsy from a metastatic ovarian cancer patient (black arrows) Scale bar 50 μm . b) a serial section of the same antibody stained with no primary antibody. Scale bar 50 μm . c) Biopsy from the same patient obtained prior to treatment did not stain for virus. Scale bar 50 μm . d) Ovarian cancer stains positive for JX-594 in tumour cells and endothelial cells. Scale bar 100 μm . e) patients in escalating dose cohorts on a phase I dose-escalation trial of intravenous JX-594 received approximately 3×10^7 to 3×10^9 pfu and 7 days later tumour biopsies were collected and assessed for tumour-associated endothelial cell infection in visible vessels. Percentage of vessels positive for vaccinia IHC in tumour by patient and dose cohort. (from Brietbach et al. Cancer Res, 2013) f) representative images of vessels stained for vaccinia virus (top row) and serial sections stained for β -galactosidase (bottom row). Linear adjustments were made to brightness and contrast. Scale bars 100 μm .

staining was specific to vaccinia antigens and not background staining (Figure 4.4b). Pretreatment biopsies were also negative for vaccinia antigens (Figure 4.4c). Patients from escalating dose cohorts were assessed for their percentage of vaccinia-positive vessels by patient and dose cohort (reproduced from Brietbach et al. Cancer Res, 2013 (125). To assess whether JX-594 was capable of actively replicating and expressing transgenes within tumour-associated endothelial cells, serial sections were stained for vaccinia virus (Figure 4.4f, top row) and β -galactosidase (Figure 4.4f, bottom row). These data indicated that JX-594 is capable of infecting tumour endothelium in patients.

DISCUSSION

Intravenous administration of JX-594 can reach distant tumour beds and is specifically amplified.

This is the first clinical demonstration of an oncolytic virus being able to infect tumours after systemic administration. β -galactosidase expression validates the ability of JX-594 to deliver transgenes to tumour cells after systemic infusion. JX-594 provides a platform that could improve the efficacy and safety of biological peptides and siRNAs by acting as an agent that will selectively amplify these drugs within malignant tissue, and minimize the dose to normal tissues. Multiple gene products could also be expressed, allowing for specific and synergistic effects with biological gene products. The ability to express multiple gene products also opens up the possibility of expressing genes that have biological activity while simultaneously expressing genes for imaging purposes.

JX-594 specifically targets vasculature.

The observation that viruses can express transgenes in tumour and endothelial cells highlights the potential of JX-594 to express biologically active molecules at high concentrations specifically within malignant tissue. This is especially important considering that several antiangiogenic agents have effects on normal quiescent endothelium, stemming from the fact that many targets are also involved in vessel maintenance of normal vasculature. Multi tyrosine kinase inhibitors, sunitinib and sorafenib, target tumour vasculature but also have off target effects leading to side effects such as gastrointestinal bleeding, necessitating ‘drug holidays’ (126). In bevacizumab trials, patients in treatment arms for several trials had bleeding complications, pulmonary hemorrhage, and gastrointestinal bleeding (126). Oncolytic JX-594 has the ability to specifically express antiangiogenic transgenes to improve the specificity of antiangiogenic therapy. No evidence of toxicity to normal vessels or wound healing was observed on the trial, suggesting that JX-594 has tumour specific anti-vascular activity. JX-594 not only relies on signaling pathways that target proliferating endothelium but also discrepancies in antiviral responses, providing an added layer of discrimination between normal and healthy tissues (Rozanne Arulanandam, unpublished data).

Chapter 5:

The anti-vascular effects of oncolytic viruses are influenced by tumour heterogeneity and susceptibility to infection

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ABSTRACT

Oncolytic viruses (OVs) are multimodal platforms that target malignancies through direct infection of tumour cells, disruption of tumour vasculature causing bystander cell death, and the induction of anti-tumour immune responses. Several oncolytic viruses, VSV, JX-594, vvDD, Maraba, and Sindbis, were able to rapidly induce widespread bystander cell death. This impressive response only occurred in a subset of tumours. We observed that tumours responded to OV therapy in three ways, and the type of response was determined by two factors: susceptibility to infection and the heterogeneity of the tumour microenvironment. Among tumours that supported viral replication, cancers of mesenchymal origin, or poorly-differentiated tumours of epithelial origin had low E-cadherin expression and were susceptible to vascular disruption. Well-differentiated, E-cadherin positive tumours were susceptible to infection and direct cell killing but resistant to vascular disruption or bystander cell death. If poorly-differentiated tumours were resistant to infection, no acute cell killing was observed. Infection was required to elicit either form of cell death. Models that were susceptible to

bystander cell killing with one virus also demonstrated this effect with other viruses, suggesting that the observed response is dictated by tumour characteristics, rather than by virus type. These histological subtypes provide a potential framework for the rational selection of patients, the integration of combination therapies and the creation of designer viruses to optimize clinical outcomes.

INTRODUCTION

We have previously reported that vesicular stomatitis virus (VSV) is able to indirectly kill uninfected tumour cells by targeting tumour vasculature. Vascular disruption is achieved through direct infection of tumour endothelium and the induction of an inflammatory response leading to acute vascular disruption and bystander killing of uninfected tumour cells (31). Following the preclinical demonstration that OVVs could target tumour vasculature leading to durable necrotic responses, patients enrolled in phase II trial for the oncolytic poxvirus JX-594 were monitored for evidence of vascular disruption using dynamic contrast MRI. In a phase II trial of JX-594, patients with liver-based tumours of either HCC (n=15) or colorectal carcinoma (n=1) histology showed decreases in tumour perfusion 5 days post treatment (31). This effect was observed in both directly injected and uninjected metastases, and durable necrosis was observed 8 weeks post treatment. In tumours directly injected with JX-594, 33% of tumours showed a 15% or greater decrease in perfusion at 5 days post-treatment (125). It is unclear which factors contribute to discrepancies in tumour response and why only a subset of patients showed signs of vascular disruption.

Heterogeneous responses to therapy are common across several cancer types and therapeutic classes, based on the interaction between the mechanism(s) of action, tumour biology and host immunity. Understanding what factors predict tumour response and how to monitor for clinical indications of various responses, will facilitate the selection of platforms that give most “bang for their buck”.

Mounting evidence indicates that heterogeneity in the tumour microenvironment presents a barrier to several cancer therapies. Heterologous cell types (i.e clonal cancer cells, endothelial cells, pericytes, fibroblasts, immune cells, extracellular matrix) are able to influence therapeutic response (127). In the case of anti-angiogenic therapies, macrophages can counteract therapy by promoting the repair of damaged vessels, neo-angiogenesis and vessel maturation (128). Vasculature with more abundant pericyte coverage has also been implicated in the resistance to combretastatin, a vascular disrupting agent (129). The increase in tumour hypoxia following therapy can lead to cancer stem cell pool expansion, increased invasion, and metastasis (130). Stromal elements are also able to impede oncolytic virus growth in tumours. Tumour-associated macrophages can induce an antiviral state, shielding tumours from viral attack (131).

Epithelial to mesenchymal transition (EMT) is a developmental regulatory program that has been implicated in the transition of low-grade epithelial tumours to more aggressive, high-grade malignancies. As tumours of epithelial origin become more malignant, certain pathological features are altered and malignant cells become less differentiated, summarized in Table 5.1 (2). Highly aggressive carcinomas exhibit increased invasion into surrounding

Table 5.1. General Characteristics of Tumours with Epithelial or Mesenchymal Histology.

EPITHELIAL	MESENCHYMAL
Low-intermediate grade carcinoma Tumours of epithelial origin that are well-differentiated	High-grade carcinoma Tumours of epithelial origin that are poorly differentiated OR Tumours of mesenchymal origin (i.e. sarcoma, glioblastoma, neuroendocrine) OR Cancer stem cells
Low metabolic activity, slow growing, indolent, better prognosis	High metabolic activity, invasive, metastatic
Heterogeneous microenvironment with stromal elements	Homogenous histology with few distinguishable stromal elements
Mature vasculature, good pericyte coverage	Immature vasculature, poor pericyte coverage
High E-cadherin expression	Low E-cadherin expression

tissues and increased metastases, and altered stromal composition which often corresponds to higher pathological grades (1). Cancer cells acquire different morphological characteristics, becoming more mesenchymal in phenotype as EMT progresses. Carcinoma cells lose their polarity, cell-to-cell contacts, acquire fibroblastic characteristics, and appear more spindle-shaped in morphology (14, 15). EMT has also been viewed as a process where epithelial cells acquire stem cell-like features and/or increased cancer stem cell pools within a clonal population (16). The expression of epithelial markers generally decreases while mesenchymal gene expression increases. E-cadherin, a cell adhesion molecule that helps tissues assemble in epithelial sheets, has been well established as a marker of indolent, low-grade tumours that are less invasive and metastatic; the loss of E-cadherin is a hallmark feature of EMT (1, 15).

In some cases, OV infection is able to target tumour vasculature, effectively transforming the tumour microenvironment into a milieu that is lethal to malignant cells (132). To understand the heterogeneity of tumour response, we aimed to understand the factors governing acute responses across different tumour types. We hypothesized that vascular disruption and the downstream killing of uninfected tumour cells, was the result of a two-step process – (1) infection and (2) induction of a robust acute tumour response that causes widespread bystander killing. We further hypothesized that tumours deviating from this optimal permutation would lead to alternate types of OV responses.

RESULTS

OVs rapidly target vasculature causing widespread apoptosis

We have previously reported that VSV and Vaccinia viruses are able to target tumour vasculature through direct infection of tumour endothelium, induction of tumour targeted inflammation, and intravascular coagulation (31, 125). Targeting tumour vasculature abrogates blood flow, causing widespread apoptosis and necrosis of uninfected tumour cells within 24 hours (31). To better understand the kinetics of this phenomenon, we analyzed tumours treated with VSV at various time points (Figure 5.1a). We found that as early as three hours post-infection, vessels were more dilated and more blood was present in the tumour. At 6 hours, greater vasodilation was more pronounced, coincident with areas of hemorrhage, and some vessels containing blood clots (Figure 5.1a and b). Widespread necrosis and a decrease in proliferating tumour cells was first detected at 9 hours post-treatment (Figure 5.1c). These results support our previous findings and highlight how rapidly VSV can initiate vascular collapse and widespread apoptosis.

We observed a similar response when U87 glioblastoma tumours were treated with JX-594. JX-594 infected limited regions of U87 tumours (Figure 5.2a). Despite this limited infection, JX-594 was able to initiate alterations to tumour vasculature as early as 24 hours after infection. Infection caused vascular basement membranes to become irregular and dilated in regions of U87 tumours (Figure 5.2b). To assess vessel architecture in further detail, tumours were stained with a CD31 antibody that was specific to cell junctions of tumour endothelium. In control tumours, CD31 cell junctions formed a mesh-like pattern along vasculature.

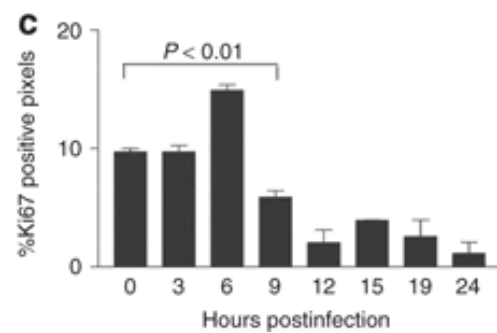
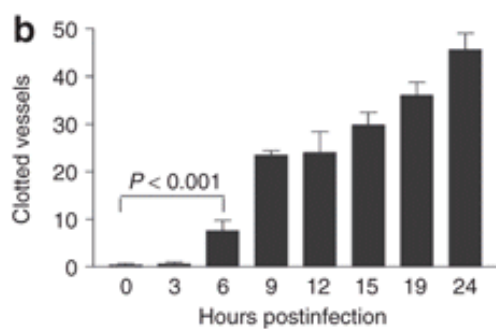
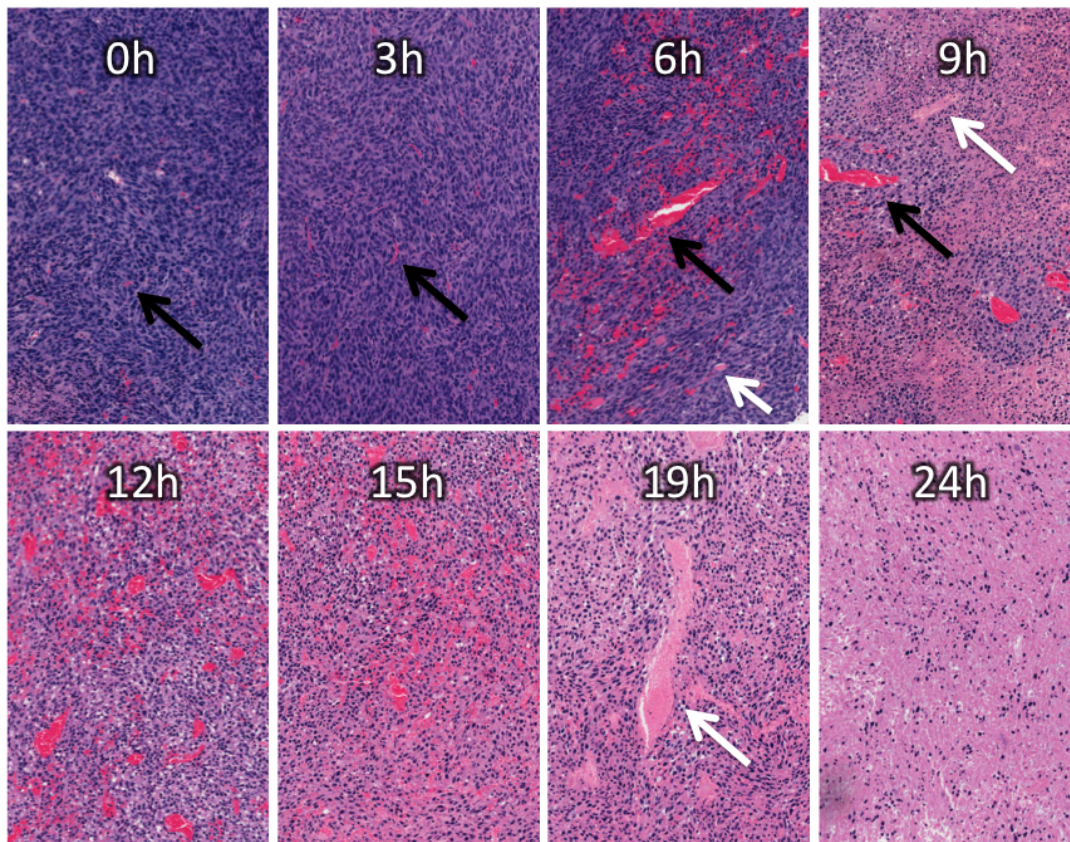


Figure 5.1. OV infection induces rapid changes in vasculature and bystander necrosis. a) CT26.lacZ tumours treated with VSV were analyzed and harvested over the course of 24hours. Representative H&Es show progressive changes to vasculature, intravascular coagulation, and necrosis. Black arrows point to patent blood vessels. White arrows point to clotted vessels. Three tumours per group were analysed. b) Clotted vessels appear as early 6 hours post treatment. c) Ki67 staining decreases as early as 9 hours post infection. (b and c from Breitbart C, De Silva N et al, 2011)

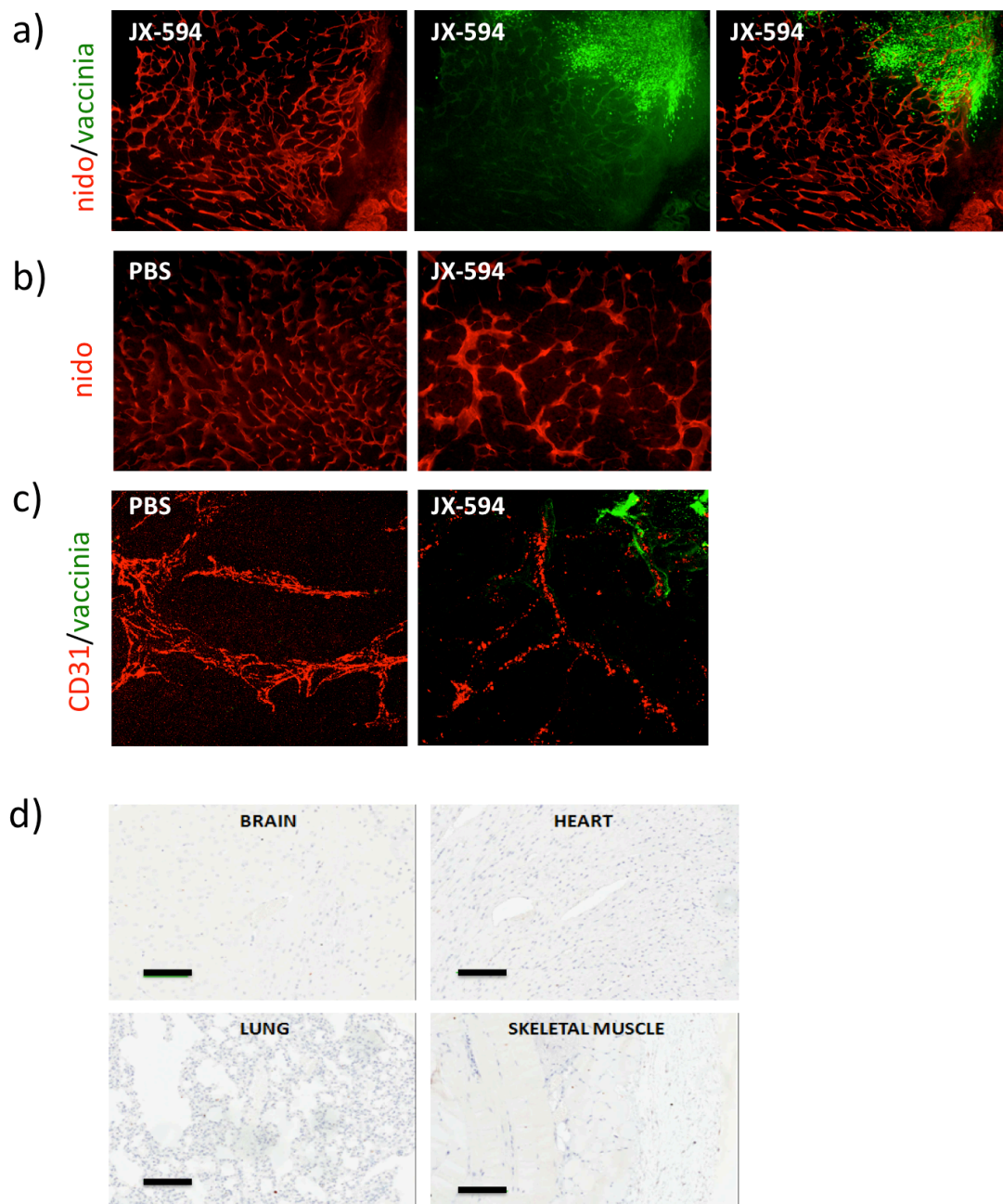


Figure 5.2. JX-594 treatment causes tumour specific destruction of vasculature. a) Mice bearing subcutaneous U87MG tumours were systemically treated with 1×10^7 pfu of JX-594 for 24 hours. Vaccinia staining (green) is restricted to limited regions of the tumour. Nidogen staining (red) is a component of vascular basement membrane. 5x magnification. b) At 24h post infusion, some areas exhibit alterations to vascular basement membrane when compared to PBS controls. 10x magnification. c) CD31 positive cell junctions are present along tumour endothelium prior to JX-594 treatment. 24h following JX-594 treatment, intact cell junctions begin to dissolve. 40x magnification. d) Normal vasculature is not affected by JX-594. Vessel abnormalities and virus staining were not detectable in brain, heart, lung, and skeletal muscle. Scale bars 100 μ m. Five mice per group were analyzed by immunohistochemistry.

Twenty-four hours post-infection, cell junctions were completely disrupted, indicating that JX-594 is able to cause acute alterations to tumour microvasculature (Figure 5.2c). Importantly, normal tissues had no observable vessel abnormalities or clot formation, and normal vessels were not infected (Figure 5.2d).

We then analyzed the kinetics of virally-induced necrosis over the course of 5 days of JX-594 treatment. At 24 hours, the disruption to vasculature coincided with increased tumour necrosis relative to control tumours (Figure 5.3a). The extent of necrosis gradually increased over the course of 5 days. Serial sections stained for virus revealed that infection occurred in limited foci and that necrosis occurred in uninfected regions of the tumour. We have previously reported that VSV infection is capable of initiating an inflammatory response that recruits neutrophils to tumour beds. These neutrophils induced fibrin deposition within tumour vasculature, effectively abrogating tumour blood flow (31).

Similarly, JX-594 was able to effectively target tumour vasculature and kill uninfected tumour cells (Figure 5.3b). Microsphere distribution was dramatically reduced, indicative of a reduction in tumour blood flow, and Gr1⁺ cells were recruited to virus-treated tumours, which coincided with an increase in tumour-restricted coagulation and the formation of intravascular clots. In the U87 model, one dose of JX-594 was sufficient to extend survival in 4/4 mice (Figure 5.4). It is important to note that tumours had average tumour dimensions that were much larger (10mm x 10mm) than the dimensions (5mm x 5mm) at which survival studies are usually commenced. In mouse survival studies, treating small tumours results in survival differences that are often lost when tumours are larger. The finding that one dose of virus can

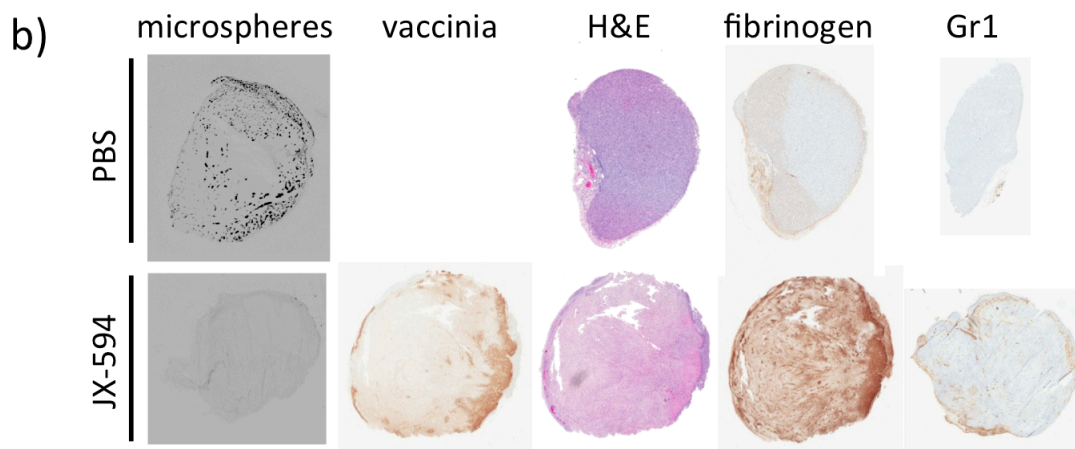
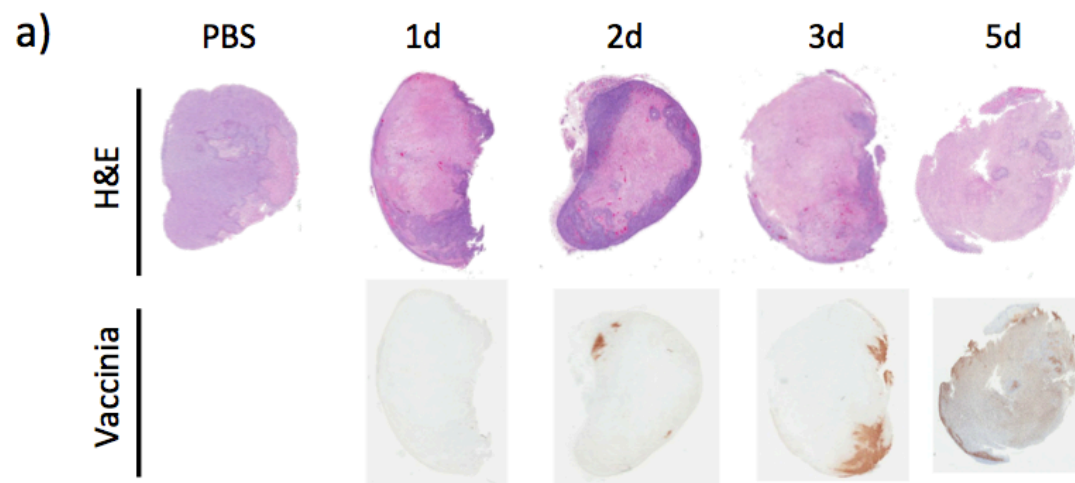


Figure 5.3. JX-594 causes bystander necrosis in U87MG glioblastoma tumours. a) U87 tumour bearing mice were treated for 0, 1, 2, 3, and 5 days. Virus staining is present in limited foci of tumours. Serial H&E sections reveal bystander cell killing of uninfected tumour cells occurs as early as 24 hours post infection and continues to increase until 5 days. Sections are representative of 4 tumours per time point. b) Mice bearing subcutaneous U87MG tumours were treated with a single intravenous infusion of 1×10^7 pfu of JX-594 for 5 days. Virus treated tumours exhibited a dramatic reduction in microsphere distribution, coincident with an increase in necrosis, fibrin deposition and Gr1 positive cell recruitment. Tumours are representative of 5 tumours per treatment.

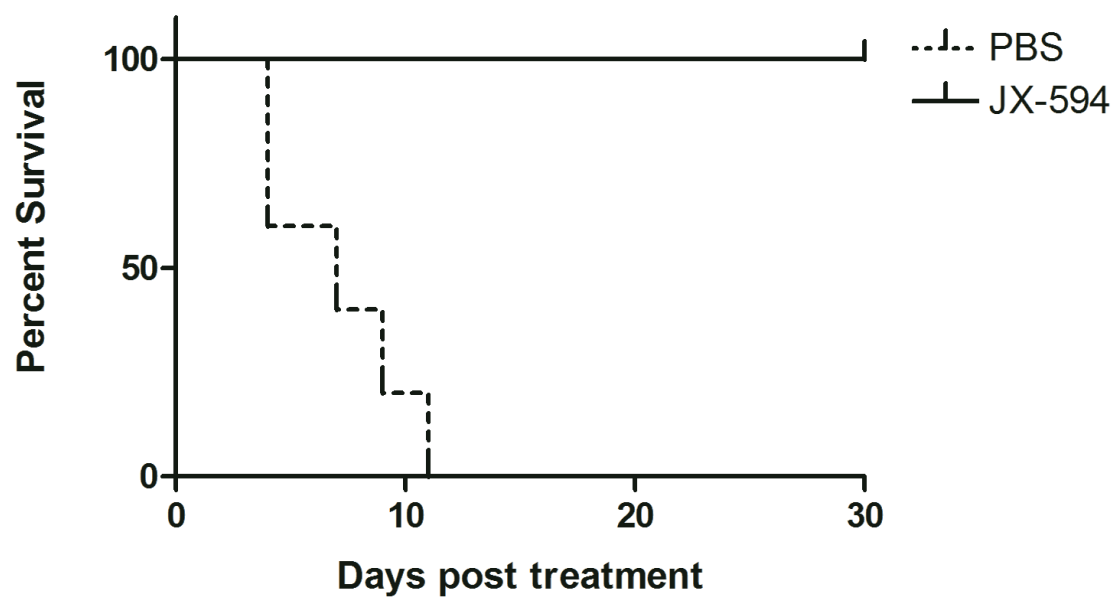


Figure 5.4. One dose of JX-594 increases survival in U87MGvIII tumour bearing mice. U87vIII tumour bearing mice received a single infusion of 1×10^7 pfu of JX-594 intravenously. Tumours had average dimensions of approximately 10mm x 10mm at the time of treatment (larger than the usual 5x5 volume for most efficacy studies). A single infusion of JX-594 was capable of significantly extending survival compare to PBS treated controls (Logrank Test: $P < 0.0045$). 5 mice per group were included in this study.

extend survival when advanced, large tumours are treated, demonstrates that JX-594 in this model has potent effects on tumour growth. As this finding was observed in nude mice, these effects can be attributed to innate immunity and vascular disruption. Stojdl et al. also demonstrated that VSV treatment of CT26.LacZ tumours was also able to cause impressive extensions in survival of 7/8 mice (60).

Two viruses, VSV and JX-594 are able to consistently target vasculature causing widespread bystander necrosis. In both cases, 10/10 tumours responded in this way (data not shown). Having observed that the clinical response rate of 33% was far from 100% observed in animal models, we sought to identify factors that could compromise the ability of viruses to target tumour vasculature. Having observed that the ability of OV_s to manipulate the tumour microenvironment was a key factor in achieving widespread killing of malignant cells, we hypothesized that microenvironmental factors could compromise this optimal response.

We tested a diverse panel of 13 models that varied in tumour type, histological grade, and species of origin (Table 5.2). We used five viruses (JX-594, vvDD, VSV, Maraba, and Sindbis) to treat colorectal carcinoma, glioblastoma, fibrosarcoma, ovarian, breast, and melanoma tumours. When examining acute responses to treatment we observed that tumours responded to OV therapy in three ways: OV_s caused widespread killing of infected and uninfected cells; OV_s caused direct killing of infected cells but uninfected tumour cells remained viable following therapy; or OV_s failed to cause significant cell death (Table 5.2). Amongst the tumours that supported virus replication (Table 5.2, Subtype I and II), we observed that these response classes were defined by differences in histological grade. Tumour cells that were susceptible to both virus infection and bystander cell death were found

CELL LINE	CANCER TYPE	BYSTANDER KILLING	REPLICATION IN VIVO (Viruses tested)	E-CADHERIN/ DIFFERENTIATION	EFFICACY
<i>Subtype I: OV's cause bystander killing</i>					
<i>Good Replication + Poorly Differentiated (N=4)</i>					
CT26.LacZ	Colon adenocarcinoma (murine)	Yes	Yes (VSV, vvDD, Maraba, Sindbis, NRPs*)	low E-cad/poor	Good
U87MGvIII	Glioblastoma (human)	Yes	Yes (VSV, JX-594)	low E-cad/poor	Good
MEF**	Fibrosarcoma (murine)	Yes	Yes (VSV)	low E-cad/poor	--***
Bin67	Ovarian, small cell (human)	Yes	Yes (VSV)	low E-cad/poor	--
<i>Subtype II: OV's cause direct killing, no bystander killing</i>					
<i>Good Replication + Poorly Differentiated (N=5)</i>					
HT29	Colon (human)	No	Yes (JX-594)	high E-cad/well	Moderate
SW620	Colon (human)	No	Yes (VSV, JX-594)	high E-cad/well	Moderate
HCT116	Colon (human)	No	Yes (JX-594)	high E-cad/well	Moderate
UACC-257	Melanoma (human)	No	Yes (VSV)	--/well	--
MDAMB435	Breast (human)	No	Yes (VSV)	--/well	--
<i>Subtype III: OV's are unable to cause significant cell death</i>					
<i>Poor Replication + Poorly Differentiated (N=4)</i>					
CT26.WT	Colon adenocarcinoma (murine)	No	No (VSV, vvDD)	low E-cad/poor	Poor
CT26.LacZ	Colon adenocarcinoma (murine)	No	No (JX-594)	low E-cad/poor	Poor
B16	Melanoma (murine)	No	No (VSV, vvDD)	low E-cad/poor	Poor
4T1	Breast (murine)	No	No (VSV, vvDD)	low E-cad/poor	Poor

*NRPs are infectious but non-replicating VSV particles

**Fibrosarcoma lines were generated from mouse embryonic fibroblasts transformed with HRAS and SV40 (129)

*** Data not available

Table 5.2. Acute responses to OV Therapy. Tumours respond to OV treatment in three ways based on differences in OV infection and histological subtype. Subtype I tumours exhibit the most extensive cell death. Tumours were positive for virus, poorly differentiated, and E-cadherin low. Subtype II tumours become necrotic in areas of direct infection. Virally induced necrosis was not observed in Subtype III tumours. Replication in vivo was assessed by the presence or absence of positive immunohistochemistry for viral antigens. Efficacy was qualitatively assessed from independent survival studies. All tumours were implanted subcutaneously and mice were given one dose of virus systemically. Tumours were analyzed 5 days after Vaccinia virus infection and 24 hours after all other viruses.

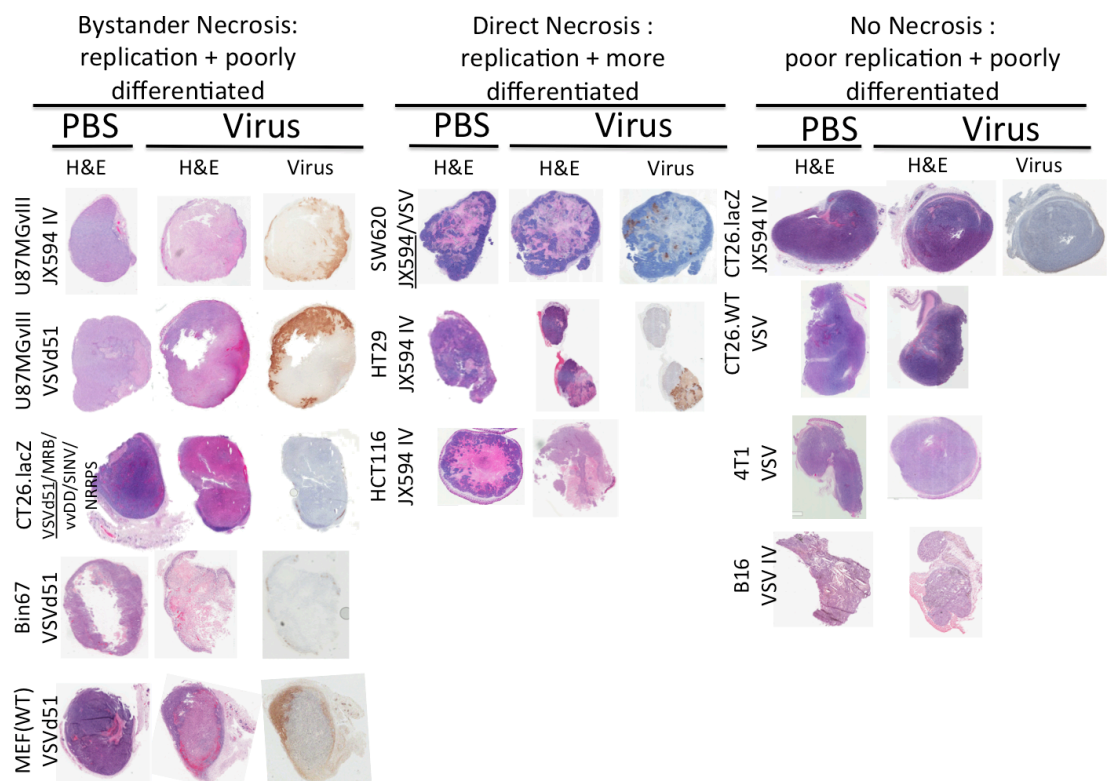


Figure 5.5. Tumours respond to OV treatment in three ways. Histological data from Table 5.2. Tumours respond to OV treatment in three ways based on differences in OV infection and histological subtype. Tumours susceptible to bystander necrosis exhibit the most extensive cell death. The second group of tumours permitted virus replication and tumours become necrotic in areas of direct infection. Viral replication and virally induced necrosis was not observed in the third group. Replication in vivo was assessed by the presence or absence of positive immunohistochemistry for viral antigens or by titering.

in poorly-differentiated and relatively homogenous microenvironments (Figure 5.6a). Tumour cells that could support virus replication, but were resistant to bystander cell death were found in well-differentiated tumours with more cellular heterogeneity.

We compared serial sections from Subtype I and II tumours for virus distribution, necrosis, and blood clot formation. Both types of microenvironments had limited areas of virus distribution, however, uninfected cells responded in dramatically different ways. In heterogeneous microenvironments, malignant cells were resistant to OV-mediated bystander cell killing while cells in homogenous microenvironments were susceptible. Necrosis coincided with the presence of intravascular clot formation and fibrin deposition. Coagulation within the vasculature of well-differentiated tumours was restricted to areas of direct infection.

We looked at E-cadherin, a marker of epithelial cell junctions, to assess whether heterogeneity could be related to the differentiation characteristics of cells. Tumours of epithelial origin (i.e. ovarian and colon carcinoma) lose epithelial markers and gain mesenchymal traits as they become more invasive. Tumours that were resistant to bystander necrosis were heterogeneous and expressed E-cadherin while a loss of E-cadherin expression was observed in poorly-differentiated, homogenous microenvironments (Figure 5.7).

Following the observation that the most responsive tumours consisted of poorly-differentiated, mesenchymal-like cells in vivo, we wanted to assess whether the mesenchymal

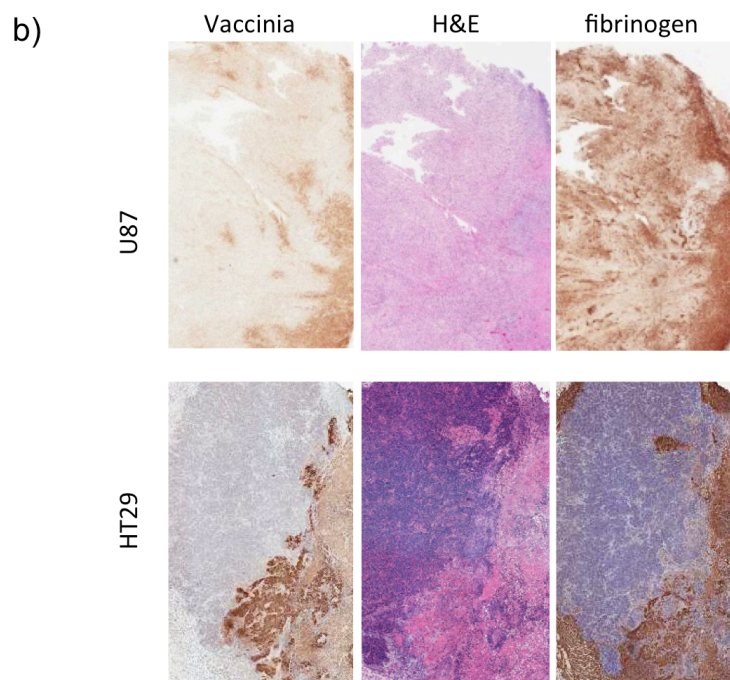
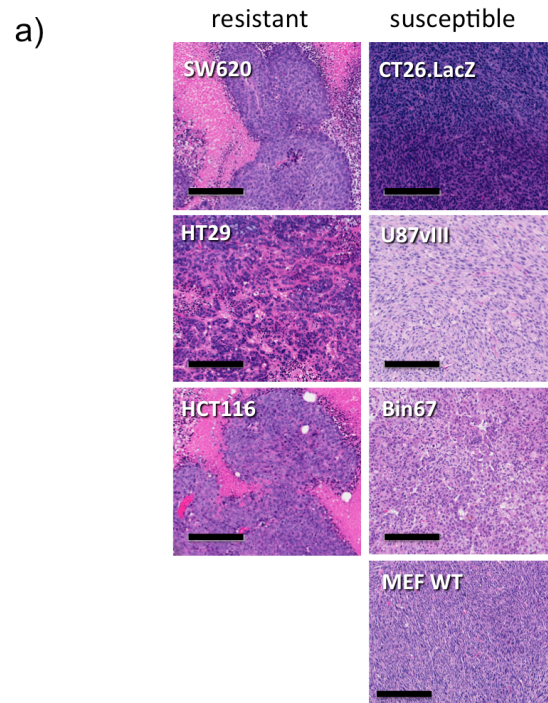
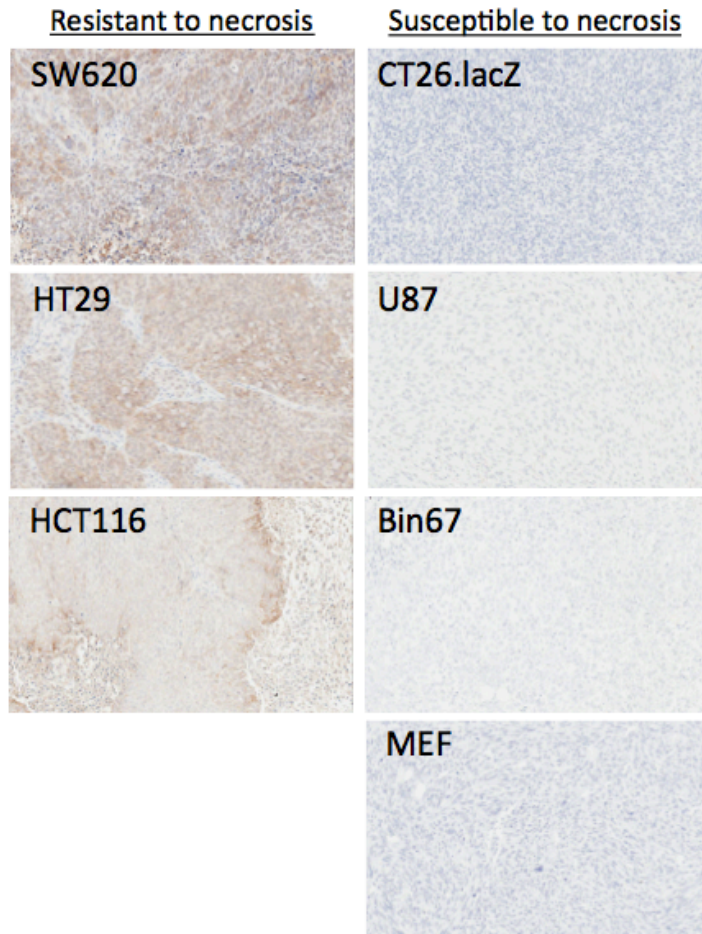


Figure 5.6. Tumour heterogeneity influences susceptibility to OV-mediated bystander cell death. Representative tumours were treated with JX-594 for 5 days. a) Amongst tumours that support virus infection, tumours that are resistant to bystander cell killing are more heterogeneous prior to treatment. b) Malignant cells in well-differentiated tumour microenvironments are resistant to OV mediated bystander cell killing while cells in poorly differentiated microenvironments are susceptible. H&E of U87 tumours shows widespread necrosis. Fibrin deposition occurs in areas of direct infection and in uninfected regions. In HT29 tumours, areas of vaccinia staining correlate with areas of necrosis. No intravascular coagulation is observed in uninfected regions. Scale bars 200 μm .

a)



b)

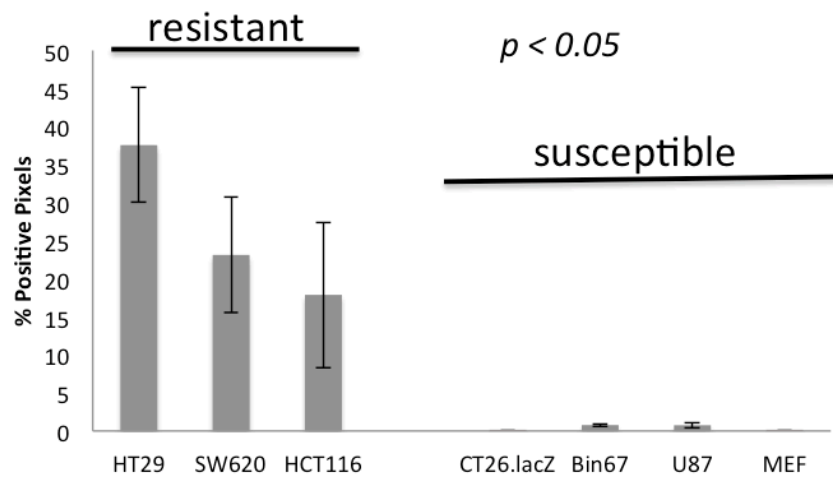


Figure 5.7. E-cadherin expression correlates with resistance to OV induced bystander necrosis. Tumours that support virus growth were stained for E-cadherin expression. Tumours that support virus replication, causing direct oncolysis only, express higher levels of E-cadherin. Tumours that support virus replication and are susceptible to bystander cell death have low levels of E-cadherin expression. a) Immunohistochemical detection of E-cadherin. b) Quantification of immunohistochemical staining. Five tumours from each tumour type were quantified using five random fields of view. E-cadherin expression was determined to be significantly different $p < 0.05$, Mann-Whitney test.

properties of cancer cells were relevant to their susceptibility to virus-induced cell death. Representative cell lines from poor and well-differentiated tumours were tested for their ability to produce virus. Poorly-differentiated CT26.lacZ cells produced more than 15-fold more virus than well-differentiated SW620 cells, raising the possibility that bystander necrosis could be independent of microenvironmental factors and could simply be due to increased virus production (Figure 5.8a). To determine if differentiation aspects correlated more broadly in other cell lines, the NCI-60 cell panel was infected with VSV to determine EC50 values for various human cancer lines (Figure 5.8b). The responsiveness to interferon was assessed by determining EC50 values of cells pretreated with interferon (60). The NCI-60 panel contains cell lines derived from tumours of different organs and from different histological grades. Kohn et al recently assessed cell migration characteristics of the NCI60 cell panel through expression profiling of genes related to EMT (30). By ranking EC50s according to how mesenchymal their gene expression was, we determined that OV cytotoxicity, *in vitro*, did not correlate with EMT characteristics.

Infection is required to initiate bystander responses in poorly-differentiated tumour microenvironments.

Noticing that bystander killing occurred only in poorly-differentiated tumour microenvironments, we looked at several poorly-differentiated mouse models. Within this subset, Subtype I models exhibited bystander cell death in response to treatment, while Subtype II did not (Table 5.1, Figure 5.5). Amongst the non-responsive tumours, we were unable to detect virus replication by immunohistochemistry, suggesting that viral replication

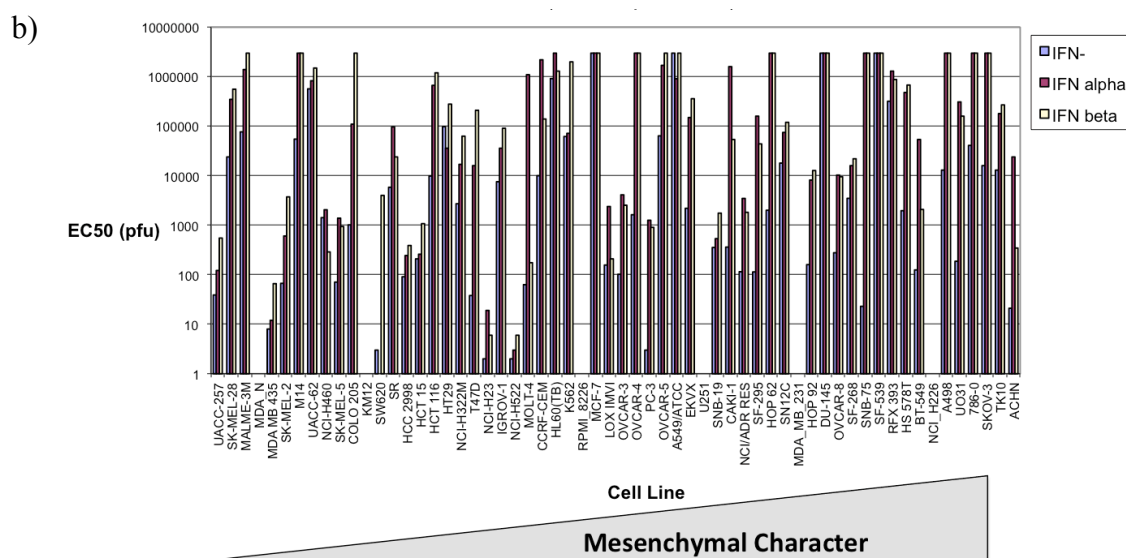
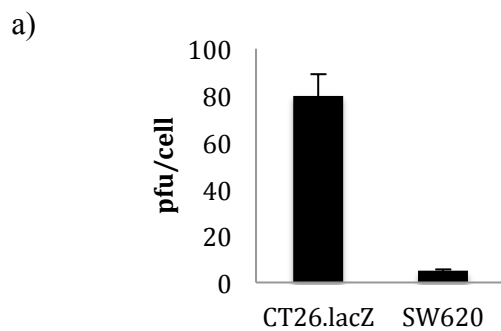


Figure 5.8. The susceptibility to infection and response to interferon does not correlate with epithelial/mesenchymal character. a) CT26.lacZ and SW620 cells were infected in vitro for 48 hrs with VSV at an MOI of 0.001 in 5% oxygen. Supernatants were harvested and tittered for infectious VSV. CT26 cells produced more virus than SW620 cells. b) Cell lines from the NCI60 panel were infected with VSV +/- interferon. EC50 values from (Stojdl et al, 2003, (60)) are plotted in order of increasing mesenchymal character. Epithelial and mesenchymal properties were determined from gene expression profiling of the NCI60 panel as described by Kohn et al (30).

was a necessary step to induce bystander necrosis in this type of tumour microenvironment. To test this, we compared similar tumour microenvironments that differed in their susceptibility to OV infection. CT26lacZ and CT26WT are genetically similar clones that form poorly-differentiated tumours, identical in general histology (Figure 5.9a) and E-cadherin expression (Figure 5.9b). WT cells respond to interferon and are resistant to VSV infection while the LacZ clones are non-responsive and support VSV infection in vivo (unpublished data). Necrosis is observed when VSV is able to replicate in LacZ tumours; however, WT tumours, where virus was unable to replicate, showed no signs of bystander necrosis. We then looked at a second pair of tumours with similar microenvironments that differed in their susceptibility to Vaccinia virus infection (Figure 5.9c). The host tropism of the Wyeth strain (JX-594) is such that it is able to infect cells of human origin but not mouse. We compared the ability of JX-594 to induce bystander killing in human U87 xenografts and CT26.lacZ tumours. In U87 xenografts, virus replication induced bystander cell killing while JX-594 was unable to replicate in murine CT26.LacZ cells and did not cause any noticeable changes to tumour histology, despite being capable of responding to VSV and another strain of vaccinia (vvDD).

Noticing that infection was necessary for bystander cell killing, we then asked which aspects of infection were responsible for this effect, and whether this phenomenon extended to other OVs. We had previously observed that non-infectious, UV-inactivated VSV did not cause acute changes to vasculature (31). Replication competent VSV was able to induce very rapid changes to vasculature, preceding vascular shutdown and bystander necrosis. These events were initiated as early as 3 hours after VSV treatment (Fig. 5.1). VSV was able to induce bystander cell killing when non-replicating particles (NRPs) were permitted to enter cells,

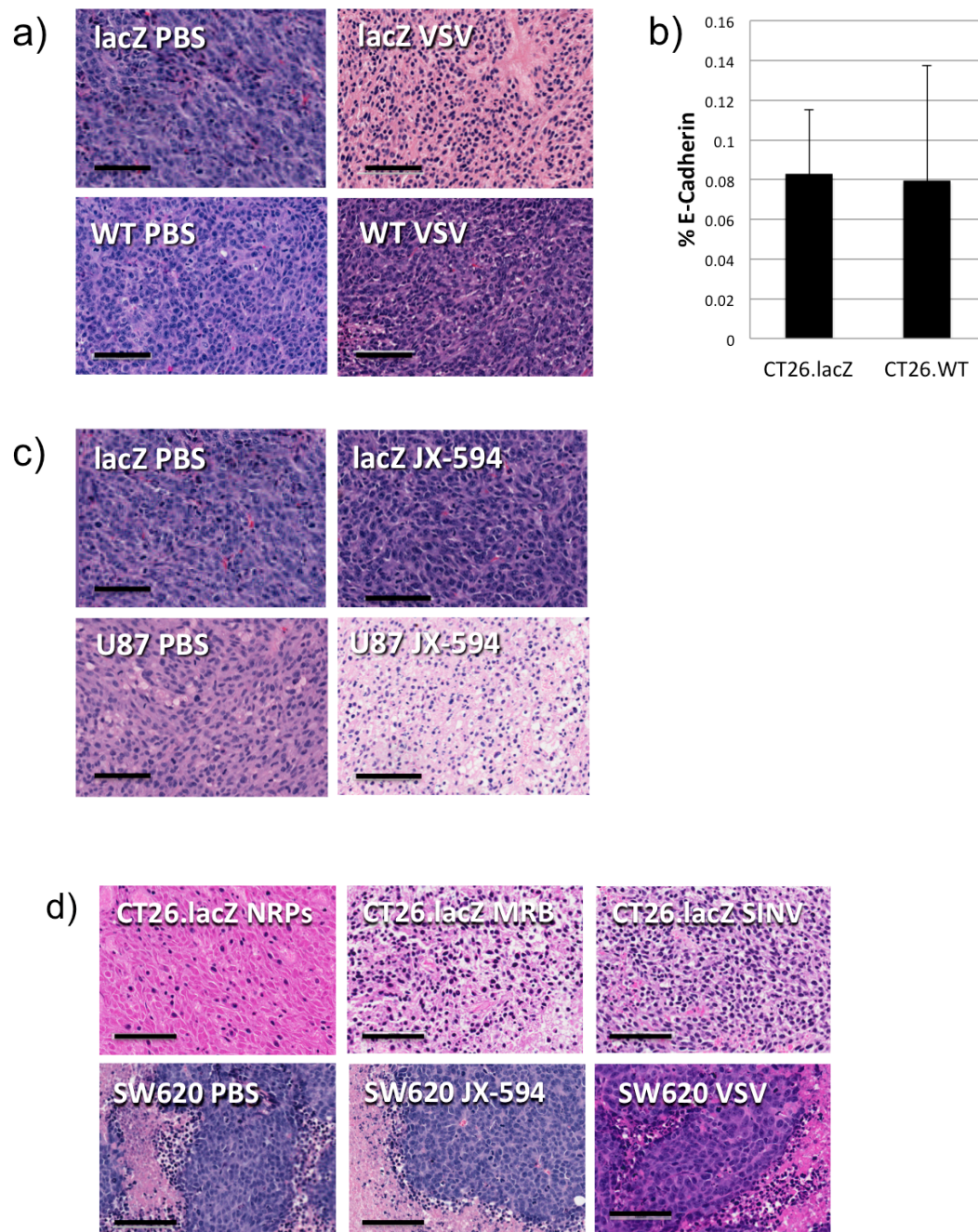


Figure 5.9. Infection is required to initiate bystander responses in poorly differentiated tumour microenvironments. a) VSV was delivered systemically to CT26.lacZ or CT26.WT tumours. Necrosis is observed when VSV is able to replicate in lacZ tumours that support virus replication. b) lacZ and WT clones form tumours with similar levels of E-caderin expression determined by quantification of immunohistochemistry. c) Virus replication is necessary in poorly differentiated models. A strain of Vaccinia (Wyeth, JX-594) is capable of replication and inducing necrosis in human U87 tumours but not murine CT26.lacZ tumours. d) Resistance or susceptibility to bystander cell death occurs in response to multiple viruses. Virus entry and gene transcription is sufficient to induce necrosis. Partially UV inactivated, non replicating virus particles (NRRPs) are able to cause bystander necrosis. LacZ tumours become necrotic after 24hrs of treatment with Maraba or Sindbis virus. SW620 tumours are resistant to JX-594 and VSV therapy. Scale bars 100 μm . VSV, Maraba, and Sindbis treatments were 5×10^8 pfu of virus injected systemically for 24h. 5×10^9 NRRPs were injected systemically for 24h. 1×10^8 pfu of JX-594 was injected systemically and mice were sacrificed 5 days later.

transcribe genes, but not form infectious progeny (Figure 5.9d). This finding suggests that the early events of infection and the associated inflammatory response are sufficient to achieve widespread cell death within this type of microenvironment.

Representative tumours of poor and well-differentiated microenvironments were tested for their response to multiple viruses. With all viruses tested, CT26.LacZ tumours became homogeneously necrotic while well-differentiated SW620 tumours had viable islands of tumour cells that were resistant to OV therapy (Fig. 5.9d). The finding that different tumour microenvironments respond in the same manner to several viruses, without exception, suggests that the tumour microenvironment dictates whether bystander necrosis will occur to OV therapy.

DISCUSSION

Infection is required to cause bystander necrosis.

The observation that infection must occur to cause bystander cell killing further supports the notion that infection and differentiation status are independent variables, influencing tumour response. In addition, these data highlight that poorly-differentiated tumour microenvironments permit bystander necrosis, but infection within this milieu is necessary for tumour response. Models that were susceptible to bystander cell killing with one virus also demonstrated this effect with other viruses, suggesting that this effect may be a common to several oncolytic viruses.

The amount of infection and response to therapy.

Amongst the tumours that support virus replication, it is possible that virus is able to replicate to higher titers in poorly-differentiated tumours, leading to more bystander cell killing. Although the current data do not exclude this possibility, it is unlikely for several reasons. Firstly, virus infection begins to disrupt vasculature at very early time points indicating that the virus exerts effects on vasculature through the rapid induction of inflammation rather than surpassing a theoretical threshold of virus growth required to cause bystander cell killing. In the case of VSV in CT26 tumours, acute changes to vasculature occur as early as 3 hours, a time at which differential amplification of virus is likely negligible. In further support of this assertion, partially inactivated VSV where virions can enter and transcribe genes but cannot produce progeny are also able to cause bystander necrosis. These data suggest that poorly-differentiated tumours may allow for more virus growth, but this difference does not contribute to the susceptibility of tumour vasculature to oncolytic viruses. The ability for virus to replicate to higher titers in poorly-differentiated tumours does raise the possibility that virally-expressed transgenes would be expressed to higher levels. If poorly-differentiated tumours permit more transgene expression, strategies that target barriers to replication in well-differentiated tumours represent a potential therapeutic avenue to improve OV therapy in this subtype. Delineating exactly which strategies could overcome barriers in well-differentiated tumours, will require an understanding of mechanisms governing virus infection in these subtypes.

Susceptibility of infection and EMT gene expression

Epithelial tumours progress and become more invasive and less differentiated. Advanced stage cancer cells generally have higher metabolic activity and develop more mutations due to

increased genetic instability, driver mutations, and passenger mutations. The increased probability of mutations in cell lines from advanced cancers could theoretically increase the probability that anti-viral or cell death pathways could be defective. However, the in vitro screening of cancer cell lines suggests that mesenchymal properties are not the major factor affecting a cell's susceptibility to OV_s (Fig. 5.8). These data suggest that in vitro, OV mediated cytotoxicity is independent of differentiation status. It is conceivable that anti-viral signaling is the major determinant of infectivity and that the mesenchymal properties of cells is a secondary factor contributing to increased infection in poorly-differentiated tumours. Therefore, differentiation status and susceptibility to infection are likely two independent variables that influence tumour response.

Variability of cancer type: experimental weakness or unifying trend?

The models tested vary in terms of species and cancer type. Comparing diverse tumour types in this manner risks drawing conclusions about samples that are widely different from one another. The proposed subtypes are observed across cancers derived from different organs, within different mouse backgrounds, in tumours derived from epithelial and mesenchymal origins, and in response to different viruses. If tumours of epithelial origin generally become more mesenchymal over the course of tumour progression, determining the response in mouse models where this phenomenon occurs would be an important step to eliminating some of the variables present in the existing data. It would be interesting to determine if these subtypes exist within a particular epithelial cancer type by comparing the response to oncolytic virus with respect to histological grade.

Considerations for preclinical development and testing of oncolytic viruses.

This work underscores the variability in responses to OV's based on tumour type in experimental models. The responses for different tumour types should be considered when designing experiments to test different oncolytic viruses. For example, viruses that are designed to boost antiangiogenic effects should be tested in well-differentiated models where vascular disruption could be improved, rather than models that are poorly-differentiated that already demonstrate robust decreases in vascularity in response to oncolytic virotherapy. It may be tempting to try candidate viruses in models that are known to have robust responses; however, it is conceivable that the effect of virally-expressed transgenes would be most apparent in models that have a less robust response. Well-differentiated tumours are also statistically more likely to be encountered in the clinic.

Rational Selection of Viruses

In the cohort of tumours that support viral replication, the type of response seems to be independent of which virus initiates inflammation, although the speed at which vasculature is disrupted varies depending on the virus. Measles virus is able to spread through nectin-4 in epithelial tumours. Cell-to-cell spread occurs with minimal cytopathic effects or recognition from the immune system, permitting the “free flow of virions throughout the tumour” (Roberto Cattaneo, Oncolytic Virus Conference 2014). Understanding how each virus interacts within the tumour microenvironment, and what types of immunological reactions will likely precipitate from this biology, will allow for the rational selection of viruses tailored to the patient's tumour and immune system to increase the probability of therapeutic success.

Clinical implications for the use of oncolytic viruses.

Future therapies might consider selecting patients depending on histological grade and the

susceptibility of biopsy material to infection. With these patient characteristics in mind, combination therapies could be rationally integrated into this framework to overcome predicted hurdles relating to infection and vascular disruption (Figure 5.10). Poorly-differentiated tumours are often the most invasive and metastatic. The observation that oncolytic viruses target vasculature and induce bystander necrosis in this histological subtype suggests the OV platform could target the most aggressive and metastatic cancers. Tumours that have mesenchymal features could be more responsive to the anti-vascular effects of OVs. In 90% of cancer deaths, it is metastasis that ultimately kills the patient and several inhibitors of EMT are under development for treating cancer (133). Oncolytic viruses are able to infect and kill metastatic cells through direct infection and lysis or through immune cell activation (134). The possibility of using OVs to target vasculature in mesenchymal tumours provides several advantages. Tumours that have acquired mesenchymal characteristics are the most aggressive primary tumours and targeting this type of malignancy could prevent colonization in distant tissues.

Further studies are required to understand what conditions are necessary to generate robust, durable adaptive responses against tumours. Is the disruption of vasculature an important initiating event that leads to the generation of a robust anti-tumour response and long-lasting efficacy? Anecdotally, the models where OVs are able to produce impressive extensions in survival are models where vascular disruption is observed. In the JX-594 Phase II trial, patients whose hypervascular HCC and colorectal tumours exhibited acute reductions in

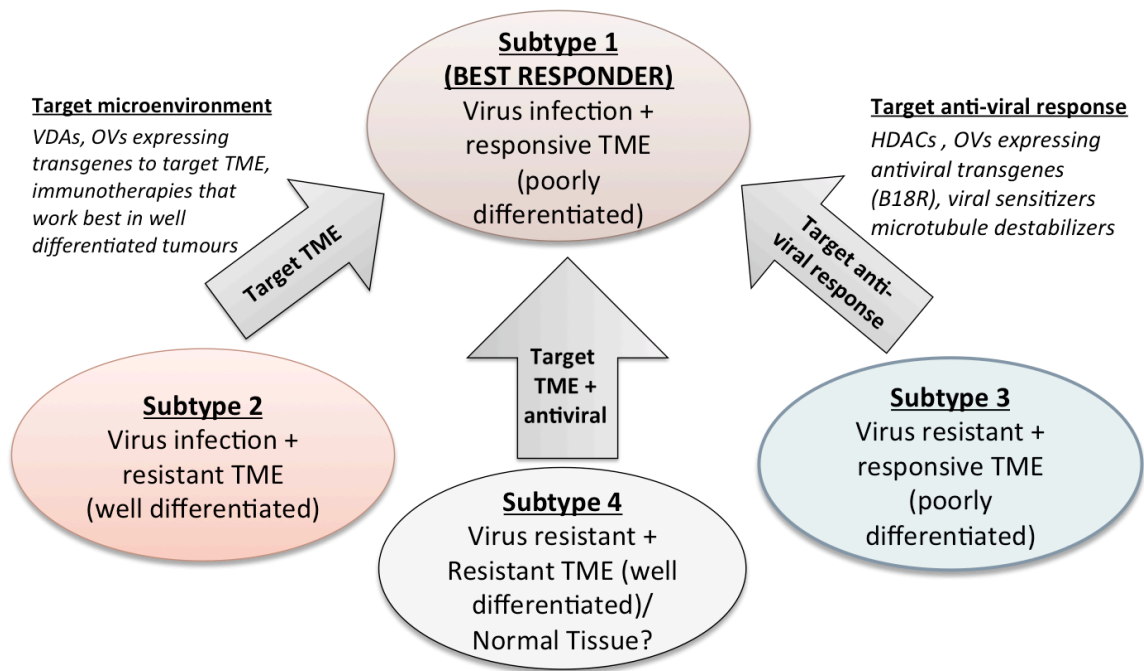


Figure 5.10. Conceptual framework for treating patients with oncolytic viruses.

Tumours acutely respond to virus in three ways. Subtype 1 supports virus replication and histology is poorly differentiated. The response to OV's in this class is widespread bystander cell death. Subtype 2 supports viral replication and histology is well differentiated. Cell death is restricted to areas of direct infection. In Subtype 3, tumours are resistant to virus infection, poorly differentiated, and resistant to cell death. The ideal response to therapy is Subtype 1 where both antiviral responses and the tumour microenvironment generate favourable responses to OV's. Oncolytic viruses should be modified and combined with therapies that target the microenvironment in subtype 2, or antiviral responses in subtype 3. Subtype 4 was not observed in the models tested and is a predicted subtype based on the TME and infection variables, this Subtype may also represent the normal tissue. VDA - vascular disrupting agent, HDAC – histone deacetylase inhibitor, TME – tumour microenvironment

perfusion contrast agent at day 5, continued to maintain reductions 8 weeks post infusion.

Therefore, acute reductions in perfusion could become a predictive marker to define the subset of patients who are ‘elite responders’ to OV therapy (66, 125).

Future controlled studies in animal models and patients are required to strengthen these findings. It will be important to understand which immune landscapes will lead to the generation of durable responses, how we can skew immune responses for therapeutic benefit, and when this should be integrated into multimodal treatment regimens. Is it possible to get the generation of robust anti-tumour responses in the absence of vessel targeting? Understanding how stromal components are altered throughout the course of OV therapy and how this can be best combined with other treatments will be important to optimize combination therapies involving oncolytic viruses. The histological subtypes observed in this study provide a potential framework for the rational selection of patients, the integration of combination therapies and the creation of designer viruses to optimize clinical outcomes.

Chapter 6: General Concluding Remarks

Although the current therapeutic artillery against cancer has dramatically increased rates of survival, high mortality rates and the disadvantages of conventional therapies have paved the way for the implementation of novel cancer therapeutics whose mode of action is dependent on not only the genetic discrimination between normal and cancerous cells but also, the features of the tumour microenvironment. Oncolytic viruses (OVs) are selected or designed to eliminate malignancies by direct infection and lysis of cancer cells. Though much is known about the interaction of viruses and the host in pathological situations, ‘artificial’ intravenous or intratumoural administration of OVs for the treatment of cancer likely does not mimic normal exposure to virus pathogens. Indeed, certain aspects of the tumour microenvironment can be detrimental to efficient virus delivery and spread throughout tumours. OV infection of tumours triggers a host response that is evolutionarily designed to inhibit spread of the invading virus. Conceptually, this will have detrimental effects on virus infection and spread within tumours. However, the increasing evidence from experiments using OVs in preclinical tumour models as well as clinical trials suggests that tumour targeted inflammation may contribute to tumour regression. Processes behind normal inflammation and wound healing are under strict spatial and temporal control. The lack of control establishes malignancies, likened to ‘wounds that do not heal’ (135), and this same lack of control provides a weakness for OVs to mediate catastrophic bystander cell killing. It is evident that OVs are able to target not only the weaknesses of cancer cells, but also to target the weaknesses of the tumour microenvironment. Since there are indications from clinical trials that JX-594 triggers a loss of tumour perfusion and infection of tumour associated vessels, it remains important to understand how virus infection of tumours is triggering this response.

Although oncolytic viruses are able to induce acute apoptosis and necrosis of tumour cells, it is unclear how the necrotic centre of virus treated tumours shapes tumour progression weeks and years after treatment. Necrotic tissue often leads to the recruitment of inflammatory cells that under normal circumstances function to remove cell debris and participate in wound healing. In the case of tumours, it has been demonstrated that inflammatory cells recruited in response to treatment may actually promote tumour growth. Necrotic cell death was originally viewed to be good for tumours, may actually do more harm than good by releasing protumour cytokines and recruiting tumour promoting inflammation (1).

Preclinical observations indicate that when OV's are able to target tumour vasculature, the vast majority of tumour cells are destroyed; however, a thin viable rim of tumour cells survives treatment. Understanding how cells within the tumour periphery are able to survive treatment will be important to optimizing therapy. In mouse models, we have demonstrated that adaptive immunity in immune competent mice can control, but not fully eradicate outgrowth of the tumour rim. In immunocompromised mice, this viable rim proliferates and expands (136). In the future, finding strategies to target the rim in tumours will be important, especially given that patients are often immunocompromised and may be unable to mount a robust adaptive response that controls outgrowth of the viable tumour rim (136).

The role of other innate immune cells in this effect will also be an important avenue to follow. Innate immune cells are not distinct effectors but rather, team players that act in a concerted effort through crosstalk to induce an inflammatory response. Understanding how innate

immune cells, other than neutrophils, are altered during bystander cell killing is important as these cells could be manipulated with viruses expressing transgenes to ameliorate this response for therapeutic benefit.

How these cell types allow for rim survival must also be understood. It is now clear that a repertoire of cell types originating in the bone marrow plays a crucial role in pathological angiogenesis (23). Macrophages, neutrophils, mast cells, and myeloid progenitors infiltrate premalignant lesions and assemble at the tumour rim; the peri-tumoural inflammatory cells help to sustain ongoing angiogenesis. In addition, they can help protect the vasculature from the effects of drugs targeting endothelial cell signaling (137).

Several studies have observed that it is the spatial distribution and the subtype of immune cell, rather than overall number of immune cells that are important for anti-tumour effects. CD8⁺ TILs accumulating at the rim of patient tumours had no effect on survival while infiltrating TILs correlated with better clinical outcomes (27). Perhaps OV_s are tools that can be employed to re-educate the phenotype of these cells to favour the generation of anti-tumour responses.

While much has been understood about oncolytic viruses in mice, we have only started to scratch the surface in the clinical setting. A key challenge is to understand and validate the mechanistic details of OV_s in patients by more detailed monitoring of patients, or the use of mouse models that accurately mimic a plethora of cancer types, likely to be encountered in the clinic. Understanding how several viruses work independently or on combination is an important area of study. The observation that VSV can induce bystander necrosis more

rapidly than vaccinia suggests differences between viruses, and perhaps the immunomodulatory genes that they encode, could be used to our advantage.

Current trials provide an opportunity to learn from failures in clinical and preclinical trials, and to study which patients are elite responders (66). Models that were susceptible to bystander cell killing with one virus also demonstrated this effect with other viruses without exception. Perhaps certain tumour microenvironments respond better to general inducers of inflammation and/or any virus. Creating a toolbox of viruses that can target different tumour types is an important therapeutic aim. Therefore, the interactions of various stromal elements need to be considered in the case of oncolytic virus treatment, not just before therapy, but also over the course of, and following treatments. Clinically, this is of even greater importance when multiple doses and combinations with existing treatments will likely be required to achieve therapeutic success.

The clinical demonstration that JX-594 can be delivered intravenously to patients combined with the observation that oncolytic viruses can rapidly target vasculature in the most metastatic cancers highlights the promise of oncolytic viruses to target metastatic disease. Solid tumours with distinct genetic anomalies (breast, lung, mesothelioma) have varied leukocyte complexity depending on the tissue or organ location and stage of malignancy, suggesting that immune-based therapies will need to reflect these nuances and be more personalized (138). The intratumoural variation of stromal composition and grade in animal models is relatively consistent; in patients the local microenvironment will vary from one lesion to another and within a given lesion. The advantage of oncolytic viruses is that if even

one lesion is responsive, it is conceivable that this could lead to the generation of an antitumour response that may target distant metastases with common antigens. Oncolytic viruses have the potential to target metastatic disease in multiple ways.

Another challenge of our field is to understand what therapies will work best in combination with varying tumour type and grade. A better understanding of how oncolytic viruses interact with the tumour microenvironment will facilitate the rational selection of combination therapies and patient selection. Understanding the mechanistic obstacles, present within well-differentiated tumours, will provide guidelines for selecting combination therapies and inserting transgenes that target this tumour type. The finding that E-cadherin expressing tumours tend to resist bystander cell death is one step towards achieving this goal. Understanding the clinical relevance of E-cadherin expression will test its validity as a prognostic marker, enabling patient selection based on differentiation status to improve the success rate of OV therapy.

Historically William Coley experimented with Coley's toxin, an inducer of inflammation, to treat a variety of patient tumours. Eventually, patients were deliberately selected, as it became obvious to Coley and his colleagues that patients with soft tissue sarcomas responded far more frequently. T.W. Harmer, independently stated that the therapy "should be instituted in no case unless proven microscopically to be sarcoma" (139). Like Coley, we must continue to monitor how patients respond to OV therapy; only when we ultimately understand how OVs interact with the tumour in patients can we design next generation viruses that may effectively enhance antitumour effect and evade barriers to OV efficacy.

References

- 1.Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74. Epub 2011/03/08.
- 2.Society CC. Cancer Statistics at a Glance. 2013 [2014 March 24]; Available from: <http://www.cancer.ca/en/cancer-information/cancer-101/cancer-statistics-at-a-glance/?region=en>.
- 3.Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA: a cancer journal for clinicians. 2010;60(5):277-300. Epub 2010/07/09.
- 4.Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. Nature reviews Cancer. 2009;9(4):239-52. Epub 2009/03/13.
- 5.Fidler IJ, Poste G. The "seed and soil" hypothesis revisited. The Lancet Oncology. 2008;9(8):808. Epub 2008/08/02.
- 6.Carmeliet P, Jain RK. Molecular mechanisms and clinical applications of angiogenesis. Nature. 2011;473(7347):298-307. Epub 2011/05/20.
- 7.Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. Nature reviews Cancer. 2003;3(6):401-10. Epub 2003/06/05.
- 8.Weidner N. Tumoural vascularity as a prognostic factor in cancer patients: the evidence continues to grow. The Journal of pathology. 1998;184(2):119-22. Epub 1998/05/29.
- 9.Rak J, Filmus J, Finkenzeller G, Grugel S, Marme D, Kerbel RS. Oncogenes as inducers of tumor angiogenesis. Cancer metastasis reviews. 1995;14(4):263-77. Epub 1995/12/01.

10. Kalas W, Yu JL, Milsom C, Rosenfeld J, Benezra R, Bornstein P, et al. Oncogenes and Angiogenesis: down-regulation of thrombospondin-1 in normal fibroblasts exposed to factors from cancer cells harboring mutant ras. *Cancer research*. 2005;65(19):8878-86. Epub 2005/10/06.
11. Downward J. Targeting RAS signalling pathways in cancer therapy. *Nature reviews Cancer*. 2003;3(1):11-22. Epub 2003/01/02.
12. Falcon BL, Pietras K, Chou J, Chen D, Sennino B, Hanahan D, et al. Increased vascular delivery and efficacy of chemotherapy after inhibition of platelet-derived growth factor-B. *The American journal of pathology*. 2011;178(6):2920-30. Epub 2011/06/07.
13. Wang Z, Li Y, Ahmad A, Banerjee S, Azmi AS, Kong D, et al. Pancreatic cancer: understanding and overcoming chemoresistance. *Nature reviews Gastroenterology & hepatology*. 2011;8(1):27-33. Epub 2010/11/26.
14. Ikushima H, Miyazono K. TGFbeta signalling: a complex web in cancer progression. *Nature reviews Cancer*. 2010;10(6):415-24. Epub 2010/05/25.
15. Batlle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, et al. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nature cell biology*. 2000;2(2):84-9. Epub 2000/02/03.
16. Singh A, Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene*. 2010;29(34):4741-51. Epub 2010/06/10.
17. Janeway CA. *Immunobiology: The immune system in health and disease*. 6th ed. New York, USA: Garland Science Publishing; 2005.
18. Nathan C, Ding A. Nonresolving inflammation. *Cell*. 2010;140(6):871-82. Epub 2010/03/23.
19. Mantovani A. Cancer: inflammation by remote control. *Nature*. 2005;435(7043):752-3. Epub 2005/06/10.

20. Ruffell B, Au A, Rugo HS, Esserman LJ, Hwang ES, Coussens LM. Leukocyte composition of human breast cancer. *Proceedings of the National Academy of Sciences of the United States of America*. 2012;109(8):2796-801. Epub 2011/08/10.
21. DeNardo DG, Brennan DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer discovery*. 2011;1(1):54-67. Epub 2011/11/01.
22. Nozawa H, Chiu C, Hanahan D. Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103(33):12493-8. Epub 2006/08/08.
23. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell*. 2010;141(1):39-51. Epub 2010/04/08.
24. Psaila B, Lyden D. The metastatic niche: adapting the foreign soil. *Nature reviews Cancer*. 2009;9(4):285-93. Epub 2009/03/25.
25. Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. *Immunology*. 2007;121(1):1-14. Epub 2007/03/28.
26. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nature immunology*. 2002;3(11):991-8. Epub 2002/10/31.
27. Naito Y, Saito K, Shiiba K, Ohuchi A, Saigenji K, Nagura H, et al. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer research*. 1998;58(16):3491-4. Epub 1998/08/29.
28. Vesely MD, Schreiber RD. Cancer immunoediting: antigens, mechanisms, and implications to cancer immunotherapy. *Annals of the New York Academy of Sciences*. 2013;1284:1-5. Epub 2013/05/09.

- 29.Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell*. 2010;140(6):883-99. Epub 2010/03/23.
- 30.Kohn KW, Zeeberg BR, Reinhold WC, Sunshine M, Luna A, Pommier Y. Gene expression profiles of the NCI-60 human tumor cell lines define molecular interaction networks governing cell migration processes. *PloS one*. 2012;7(5):e35716. Epub 2012/05/10.
- 31.Breitbach CJ, De Silva NS, Falls TJ, Aladl U, Evgin L, Paterson J, et al. Targeting tumor vasculature with an oncolytic virus. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2011;19(5):886-94. Epub 2011/03/03.
- 32.Karbach J, Neumann A, Brand K, Wahle C, Siegel E, Maeurer M, et al. Phase I clinical trial of mixed bacterial vaccine (Coley's toxins) in patients with NY-ESO-1 expressing cancers: immunological effects and clinical activity. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2012;18(19):5449-59. Epub 2012/08/01.
- 33.Tozer GM, Kanthou C, Baguley BC. Disrupting tumour blood vessels. *Nature reviews Cancer*. 2005;5(6):423-35. Epub 2005/06/02.
- 34.Goel S, Duda DG, Xu L, Munn LL, Boucher Y, Fukumura D, et al. Normalization of the vasculature for treatment of cancer and other diseases. *Physiological reviews*. 2011;91(3):1071-121. Epub 2011/07/12.
- 35.Ebos JM, Kerbel RS. Antiangiogenic therapy: impact on invasion, disease progression, and metastasis. *Nature reviews Clinical oncology*. 2011;8(4):210-21. Epub 2011/03/03.
- 36.Qayum N, Muschel RJ, Im JH, Balathasan L, Koch CJ, Patel S, et al. Tumor vascular changes mediated by inhibition of oncogenic signaling. *Cancer research*. 2009;69(15):6347-54. Epub 2009/07/23.
- 37.GlaxoSmithKline. Update on phase II clinical trial of investigational MAGE-A3 antigen-specific cancer immunotherapeutic in non-small cell lung cancer. London, UK [May 2nd, 2014];

Available from: <http://www.gsk.com/media/%5Bpress-releases/2014/update-on-phase-III-clinical-trial-of-investigational-MAGE-A3-antigen-specific-cancer-immunotherapeutic-in-non-small-cell-lung-cancer.html>.

38.Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science*. 2011;331(6024):1565-70. Epub 2011/03/26.

39.Van Belle E, Bauters C, McFadden E, Bertrand ME. Nature of coronary disease in patients with insulin resistance and its impact on revascularization strategies. *Coronary artery disease*. 2005;16(8):481-7. Epub 2005/12/02.

40.Kochenderfer JN, Rosenberg SA. Chimeric antigen receptor-modified T cells in CLL. *The New England journal of medicine*. 2011;365(20):1937-8; author reply 8. Epub 2011/11/18.

41.Couzin-Frankel J. Breakthrough of the year 2013. Cancer immunotherapy. *Science*. 2013;342(6165):1432-3. Epub 2013/12/21.

42.Junttila TT, Akita RW, Parsons K, Fields C, Lewis Phillips GD, Friedman LS, et al. Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. *Cancer cell*. 2009;15(5):429-40. Epub 2009/05/05.

43.Grosso JF, Jure-Kunkel MN. CTLA-4 blockade in tumor models: an overview of preclinical and translational research. *Cancer immunity*. 2013;13:5. Epub 2013/02/08.

44.Di Giacomo AM, Biagioli M, Maio M. The emerging toxicity profiles of anti-CTLA-4 antibodies across clinical indications. *Seminars in oncology*. 2010;37(5):499-507. Epub 2010/11/16.

45.Parato KA, Senger D, Forsyth PA, Bell JC. Recent progress in the battle between oncolytic viruses and tumours. *Nature reviews Cancer*. 2005;5(12):965-76. Epub 2005/11/19.

46.Kaufman HL, Kim DW, DeRaffele G, Mitcham J, Coffin RS, Kim-Schulze S. Local and distant immunity induced by intralesional vaccination with an oncolytic herpes virus encoding

- GM-CSF in patients with stage IIIc and IV melanoma. *Annals of surgical oncology*. 2010;17(3):718-30. Epub 2009/11/17.
- 47.Kelly E, Russell SJ. History of oncolytic viruses: genesis to genetic engineering. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2007;15(4):651-9. Epub 2007/02/15.
- 48.Bierman HR, Crile DM, Dod KS, Kelly KH, Petrakis NL, White LP, et al. Remissions in leukemia of childhood following acute infectious disease: staphylococcus and streptococcus, varicella, and feline panleukopenia. *Cancer*. 1953;6(3):591-605. Epub 1953/05/01.
- 49.Russell SJ, Peng KW. Viruses as anticancer drugs. *Trends in pharmacological sciences*. 2007;28(7):326-33. Epub 2007/06/19.
- 50.Moore AE. Inhibition of growth of five transplantable mouse tumors by the virus of Russian Far East encephalitis. *Cancer*. 1951;4(2):375-82. Epub 1951/03/01.
- 51.Garber K. China approves world's first oncolytic virus therapy for cancer treatment. *Journal of the National Cancer Institute*. 2006;98(5):298-300. Epub 2006/03/02.
- 52.Cervantes-Garcia D, Ortiz-Lopez R, Mayek-Perez N, Rojas-Martinez A. Oncolytic virotherapy. *Annals of hepatology*. 2008;7(1):34-45. Epub 2008/04/01.
- 53.Lichty BD, Power AT, Stojdl DF, Bell JC. Vesicular stomatitis virus: re-inventing the bullet. *Trends in molecular medicine*. 2004;10(5):210-6. Epub 2004/05/04.
- 54.Roberts A, Buonocore L, Price R, Forman J, Rose JK. Attenuated vesicular stomatitis viruses as vaccine vectors. *Journal of virology*. 1999;73(5):3723-32. Epub 1999/04/10.
- 55.Flint SJ E, LW, Racaniello, VR, Skalka, AM. *Principles of Virology*. 2nd ed. Washington DC, USA:ASM Press2004.

- 56.Sato M, Suemori H, Hata N, Asagiri M, Ogasawara K, Nakao K, et al. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. *Immunity*. 2000;13(4):539-48. Epub 2000/11/09.
- 57.Randall RE, Goodbourn S. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *The Journal of general virology*. 2008;89(Pt 1):1-47. Epub 2007/12/20.
- 58.Colina R, Costa-Mattioli M, Dowling RJ, Jaramillo M, Tai LH, Breitbach CJ, et al. Translational control of the innate immune response through IRF-7. *Nature*. 2008;452(7185):323-8. Epub 2008/02/15.
- 59.Gainey MD, Dillon PJ, Clark KM, Manuse MJ, Parks GD. Paramyxovirus-induced shutoff of host and viral protein synthesis: role of the P and V proteins in limiting PKR activation. *Journal of virology*. 2008;82(2):828-39. Epub 2007/11/06.
- 60.Stojdl DF, Lichty BD, tenOever BR, Paterson JM, Power AT, Knowles S, et al. VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer cell*. 2003;4(4):263-75. Epub 2003/10/31.
- 61.Hii SI, Hardy L, Crough T, Payne EJ, Grimmett K, Gill D, et al. Loss of PKR activity in chronic lymphocytic leukemia. *International journal of cancer Journal international du cancer*. 2004;109(3):329-35. Epub 2004/02/13.
- 62.von Kobbe C, van Deursen JM, Rodrigues JP, Sitterlin D, Bachi A, Wu X, et al. Vesicular stomatitis virus matrix protein inhibits host cell gene expression by targeting the nucleoporin Nup98. *Molecular cell*. 2000;6(5):1243-52. Epub 2000/12/07.
- 63.Faria PA, Chakraborty P, Levay A, Barber GN, Ezelle HJ, Enninga J, et al. VSV disrupts the Rae1/mrnp41 mRNA nuclear export pathway. *Molecular cell*. 2005;17(1):93-102. Epub 2005/01/05.

- 64.Stojdl DF, Abraham N, Knowles S, Marius R, Brasey A, Lichty BD, et al. The murine double-stranded RNA-dependent protein kinase PKR is required for resistance to vesicular stomatitis virus. *Journal of virology*. 2000;74(20):9580-5. Epub 2000/09/23.
- 65.Nirula R, Millar D, Greene T, McFadden M, Shah L, Scalea TM, et al. Decompressive craniectomy or medical management for refractory intracranial hypertension: an AAST-MIT propensity score analysis. *The journal of trauma and acute care surgery*. 2014;76(4):944-52; discussion 52-5. Epub 2014/03/26.
- 66.Bell J, McFadden G. Viruses for tumor therapy. *Cell host & microbe*. 2014;15(3):260-5. Epub 2014/03/19.
- 67.Stanford MM, Breitbach CJ, Bell JC, McFadden G. Innate immunity, tumor microenvironment and oncolytic virus therapy: friends or foes? *Current opinion in molecular therapeutics*. 2008;10(1):32-7. Epub 2008/01/30.
- 68.Power AT, Bell JC. Cell-based delivery of oncolytic viruses: a new strategic alliance for a biological strike against cancer. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2007;15(4):660-5. Epub 2007/02/01.
- 69.Dvorak HF. Rous-Whipple Award Lecture. How tumors make bad blood vessels and stroma. *The American journal of pathology*. 2003;162(6):1747-57. Epub 2003/05/22.
- 70.Jain RK, Munn LL, Fukumura D. Dissecting tumour pathophysiology using intravital microscopy. *Nature reviews Cancer*. 2002;2(4):266-76. Epub 2002/05/11.
- 71.Vaha-Koskela MJ, Heikkila JE, Hinkkanen AE. Oncolytic viruses in cancer therapy. *Cancer letters*. 2007;254(2):178-216. Epub 2007/03/27.
- 72.Stanford MM, Shaban M, Barrett JW, Werden SJ, Gilbert PA, Bondy-Denomy J, et al. Myxoma virus oncolysis of primary and metastatic B16F10 mouse tumors in vivo. *Molecular*

therapy : the journal of the American Society of Gene Therapy. 2008;16(1):52-9. Epub 2007/11/14.

73.Shayakhmetov DM, Li ZY, Ni S, Lieber A. Targeting of adenovirus vectors to tumor cells does not enable efficient transduction of breast cancer metastases. Cancer research. 2002;62(4):1063-8. Epub 2002/02/28.

74.Valyi-Nagy K, Dosa S, Kovacs SK, Bacsa S, Voros A, Shukla D, et al. Identification of virus resistant tumor cell subpopulations in three-dimensional uveal melanoma cultures. Cancer gene therapy. 2010;17(4):223-34. Epub 2009/11/07.

75.Kaur B, Cripe TP, Chiocca EA. "Buy one get one free": armed viruses for the treatment of cancer cells and their microenvironment. Current gene therapy. 2009;9(5):341-55. Epub 2009/10/29.

76.Mok W, Boucher Y, Jain RK. Matrix metalloproteinases-1 and -8 improve the distribution and efficacy of an oncolytic virus. Cancer research. 2007;67(22):10664-8. Epub 2007/11/17.

77.McKee TD, Grandi P, Mok W, Alexandrakis G, Insin N, Zimmer JP, et al. Degradation of fibrillar collagen in a human melanoma xenograft improves the efficacy of an oncolytic herpes simplex virus vector. Cancer research. 2006;66(5):2509-13. Epub 2006/03/03.

78.Ganesh S, Gonzalez-Edick M, Gibbons D, Van Roey M, Jooss K. Intratumoral coadministration of hyaluronidase enzyme and oncolytic adenoviruses enhances virus potency in metastatic tumor models. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008;14(12):3933-41. Epub 2008/06/19.

79.Kim JH, Lee YS, Kim H, Huang JH, Yoon AR, Yun CO. Relaxin expression from tumor-targeting adenoviruses and its intratumoral spread, apoptosis induction, and efficacy. Journal of the National Cancer Institute. 2006;98(20):1482-93. Epub 2006/10/19.

- 80.Nagano S, Perentes JY, Jain RK, Boucher Y. Cancer cell death enhances the penetration and efficacy of oncolytic herpes simplex virus in tumors. *Cancer research*. 2008;68(10):3795-802. Epub 2008/05/17.
- 81.Tong RT, Boucher Y, Kozin SV, Winkler F, Hicklin DJ, Jain RK. Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. *Cancer research*. 2004;64(11):3731-6. Epub 2004/06/03.
- 82.Libertini S, Iacuzzo I, Perruolo G, Scala S, Ierano C, Franco R, et al. Bevacizumab increases viral distribution in human anaplastic thyroid carcinoma xenografts and enhances the effects of E1A-defective adenovirus dl922-947. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2008;14(20):6505-14. Epub 2008/10/18.
- 83.Kurozumi K, Hardcastle J, Thakur R, Yang M, Christoforidis G, Fulci G, et al. Effect of tumor microenvironment modulation on the efficacy of oncolytic virus therapy. *Journal of the National Cancer Institute*. 2007;99(23):1768-81. Epub 2007/11/29.
- 84.Aghi M, Rabkin SD, Martuza RL. Angiogenic response caused by oncolytic herpes simplex virus-induced reduced thrombospondin expression can be prevented by specific viral mutations or by administering a thrombospondin-derived peptide. *Cancer research*. 2007;67(2):440-4. Epub 2007/01/20.
- 85.Tseng JC, Granot T, DiGiacomo V, Levin B, Meruelo D. Enhanced specific delivery and targeting of oncolytic Sindbis viral vectors by modulating vascular leakiness in tumor. *Cancer gene therapy*. 2010;17(4):244-55. Epub 2009/10/03.
- 86.Mahller YY, Vaikunth SS, Ripberger MC, Baird WH, Saeki Y, Cancelas JA, et al. Tissue inhibitor of metalloproteinase-3 via oncolytic herpesvirus inhibits tumor growth and vascular progenitors. *Cancer research*. 2008;68(4):1170-9. Epub 2008/02/19.

- 87.Kang YA, Shin HC, Yoo JY, Kim JH, Kim JS, Yun CO. Novel cancer antiangiotherapy using the VEGF promoter-targeted artificial zinc-finger protein and oncolytic adenovirus. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2008;16(6):1033-40. Epub 2008/04/10.
- 88.Yoo JY, Kim JH, Kwon YG, Kim EC, Kim NK, Choi HJ, et al. VEGF-specific short hairpin RNA-expressing oncolytic adenovirus elicits potent inhibition of angiogenesis and tumor growth. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2007;15(2):295-302. Epub 2007/01/20.
- 89.Su C, Na M, Chen J, Wang X, Liu Y, Wang W, et al. Gene-viral cancer therapy using dual-regulated oncolytic adenovirus with antiangiogenesis gene for increased efficacy. *Molecular cancer research : MCR*. 2008;6(4):568-75. Epub 2008/03/18.
- 90.Hardcastle J, Kurozumi K, Dmitrieva N, Sayers MP, Ahmad S, Waterman P, et al. Enhanced antitumor efficacy of vasculostatin (Vstat120) expressing oncolytic HSV-1. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2010;18(2):285-94. Epub 2009/10/22.
- 91.Frentzen A, Yu YA, Chen N, Zhang Q, Weibel S, Raab V, et al. Anti-VEGF single-chain antibody GLAF-1 encoded by oncolytic vaccinia virus significantly enhances antitumor therapy. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106(31):12915-20. Epub 2009/07/21.
- 92.Breitbach CJ, Paterson JM, Lemay CG, Falls TJ, McGuire A, Parato KA, et al. Targeted inflammation during oncolytic virus therapy severely compromises tumor blood flow. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2007;15(9):1686-93. Epub 2007/06/21.

- 93.Fulci G, Dmitrieva N, Gianni D, Fontana EJ, Pan X, Lu Y, et al. Depletion of peripheral macrophages and brain microglia increases brain tumor titers of oncolytic viruses. *Cancer research*. 2007;67(19):9398-406. Epub 2007/10/03.
- 94.Shashkova EV, Doronin K, Senac JS, Barry MA. Macrophage depletion combined with anticoagulant therapy increases therapeutic window of systemic treatment with oncolytic adenovirus. *Cancer research*. 2008;68(14):5896-904. Epub 2008/07/18.
- 95.Tao N, Gao GP, Parr M, Johnston J, Baradet T, Wilson JM, et al. Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2001;3(1):28-35. Epub 2001/02/13.
- 96.Altomonte J, Wu L, Chen L, Meseck M, Ebert O, Garcia-Sastre A, et al. Exponential enhancement of oncolytic vesicular stomatitis virus potency by vector-mediated suppression of inflammatory responses in vivo. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2008;16(1):146-53. Epub 2007/12/12.
- 97.Altomonte J, Wu L, Meseck M, Chen L, Ebert O, Garcia-Sastre A, et al. Enhanced oncolytic potency of vesicular stomatitis virus through vector-mediated inhibition of NK and NKT cells. *Cancer gene therapy*. 2009;16(3):266-78. Epub 2008/10/11.
- 98.Fulci G, Breyman L, Gianni D, Kurozumi K, Rhee SS, Yu J, et al. Cyclophosphamide enhances glioma virotherapy by inhibiting innate immune responses. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103(34):12873-8. Epub 2006/08/16.
- 99.Wakimoto H, Fulci G, Tyminski E, Chiocca EA. Altered expression of antiviral cytokine mRNAs associated with cyclophosphamide's enhancement of viral oncolysis. *Gene therapy*. 2004;11(2):214-23. Epub 2004/01/09.

- 100.Qiao J, Wang H, Kottke T, White C, Twigger K, Diaz RM, et al. Cyclophosphamide facilitates antitumor efficacy against subcutaneous tumors following intravenous delivery of reovirus. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2008;14(1):259-69. Epub 2008/01/04.
- 101.Currier MA, Gillespie RA, Sawtell NM, Mahller YY, Stroup G, Collins MH, et al. Efficacy and safety of the oncolytic herpes simplex virus rRp450 alone and combined with cyclophosphamide. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2008;16(5):879-85. Epub 2008/04/05.
- 102.Benencia F, Courreges MC, Conejo-Garcia JR, Mohamed-Hadley A, Zhang L, Buckanovich RJ, et al. HSV oncolytic therapy upregulates interferon-inducible chemokines and recruits immune effector cells in ovarian cancer. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2005;12(5):789-802. Epub 2005/06/01.
- 103.Errington F, White CL, Twigger KR, Rose A, Scott K, Steele L, et al. Inflammatory tumour cell killing by oncolytic reovirus for the treatment of melanoma. *Gene therapy*. 2008;15(18):1257-70. Epub 2008/04/11.
- 104.Post DE, Sandberg EM, Kyle MM, Devi NS, Brat DJ, Xu Z, et al. Targeted cancer gene therapy using a hypoxia inducible factor dependent oncolytic adenovirus armed with interleukin-4. *Cancer research*. 2007;67(14):6872-81. Epub 2007/07/20.
- 105.Shin EJ, Wana GB, Choi B, Aguila D, 3rd, Ebert O, Genden EM, et al. Interleukin-12 expression enhances vesicular stomatitis virus oncolytic therapy in murine squamous cell carcinoma. *The Laryngoscope*. 2007;117(2):210-4. Epub 2007/01/06.
- 106.Stanford MM, Barrett JW, Gilbert PA, Bankert R, McFadden G. Myxoma virus expressing human interleukin-12 does not induce myxomatosis in European rabbits. *Journal of virology*. 2007;81(22):12704-8. Epub 2007/08/31.

- 107.Varghese S, Rabkin SD, Nielsen PG, Wang W, Martuza RL. Systemic oncolytic herpes virus therapy of poorly immunogenic prostate cancer metastatic to lung. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2006;12(9):2919-27. Epub 2006/05/06.
- 108.Wong RJ, Chan MK, Yu Z, Ghossein RA, Ngai I, Adusumilli PS, et al. Angiogenesis inhibition by an oncolytic herpes virus expressing interleukin 12. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2004;10(13):4509-16. Epub 2004/07/09.
- 109.Varghese S, Rabkin SD, Nielsen GP, MacGarvey U, Liu R, Martuza RL. Systemic therapy of spontaneous prostate cancer in transgenic mice with oncolytic herpes simplex viruses. *Cancer research*. 2007;67(19):9371-9. Epub 2007/10/03.
- 110.Ramesh N, Ge Y, Ennist DL, Zhu M, Mina M, Ganesh S, et al. CG0070, a conditionally replicating granulocyte macrophage colony-stimulating factor--armed oncolytic adenovirus for the treatment of bladder cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2006;12(1):305-13. Epub 2006/01/07.
- 111.Grote D, Cattaneo R, Fielding AK. Neutrophils contribute to the measles virus-induced antitumor effect: enhancement by granulocyte macrophage colony-stimulating factor expression. *Cancer research*. 2003;63(19):6463-8. Epub 2003/10/16.
- 112.Janke M, Peeters B, de Leeuw O, Moorman R, Arnold A, Fournier P, et al. Recombinant Newcastle disease virus (NDV) with inserted gene coding for GM-CSF as a new vector for cancer immunogene therapy. *Gene therapy*. 2007;14(23):1639-49. Epub 2007/10/05.
- 113.Prestwich RJ, Errington F, Steele LP, Ilett EJ, Morgan RS, Harrington KJ, et al. Reciprocal human dendritic cell-natural killer cell interactions induce antitumor activity following tumor cell infection by oncolytic reovirus. *J Immunol*. 2009;183(7):4312-21. Epub 2009/09/08.

114. Nguyen TL, Abdelbary H, Arguello M, Breitbach C, Leveille S, Diallo JS, et al. Chemical targeting of the innate antiviral response by histone deacetylase inhibitors renders refractory cancers sensitive to viral oncolysis. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105(39):14981-6. Epub 2008/09/26.
115. Liu TC, Castelo-Branco P, Rabkin SD, Martuza RL. Trichostatin A and oncolytic HSV combination therapy shows enhanced antitumoral and antiangiogenic effects. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2008;16(6):1041-7. Epub 2008/04/05.
116. Liu TC, Hwang TH, Bell JC, Kirn DH. Development of targeted oncolytic virotherapeutics through translational research. *Expert opinion on biological therapy*. 2008;8(9):1381-91. Epub 2008/08/13.
117. Parato KA, Breitbach CJ, Le Boeuf F, Wang J, Storbeck C, Ilkow C, et al. The oncolytic poxvirus JX-594 selectively replicates in and destroys cancer cells driven by genetic pathways commonly activated in cancers. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2012;20(4):749-58. Epub 2011/12/22.
118. Heo J, Reid T, Ruo L, Breitbach CJ, Rose S, Bloomston M, et al. Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia JX-594 in liver cancer. *Nature medicine*. 2013;19(3):329-36. Epub 2013/02/12.
119. Moehler M, Goepfert K, Heinrich B, Breitbach CJ, Delic M, Galle PR, et al. Oncolytic virotherapy as emerging immunotherapeutic modality: potential of parvovirus h-1. *Frontiers in oncology*. 2014;4:92. Epub 2014/05/14.
120. Bello LJ. Regulation of thymidine kinase synthesis in human cells. *Experimental cell research*. 1974;89(2):263-74. Epub 1974/12/01.

121. Breitbach CJ, Burke J, Jonker D, Stephenson J, Haas AR, Chow LQ, et al. Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. *Nature*. 2011;477(7362):99-102. Epub 2011/09/03.
122. Spring BQ, Abu-Yousif AO, Palanisami A, Rizvi I, Zheng X, Mai Z, et al. Selective treatment and monitoring of disseminated cancer micrometastases in vivo using dual-function, activatable immunoconjugates. *Proceedings of the National Academy of Sciences of the United States of America*. 2014;111(10):E933-42. Epub 2014/02/28.
123. Park BH, Hwang T, Liu TC, Sze DY, Kim JS, Kwon HC, et al. Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: a phase I trial. *The Lancet Oncology*. 2008;9(6):533-42. Epub 2008/05/23.
124. Ruegg C, Yilmaz A, Bieler G, Bamat J, Chaubert P, Lejeune FJ. Evidence for the involvement of endothelial cell integrin $\alpha V\beta 3$ in the disruption of the tumor vasculature induced by TNF and IFN- γ . *Nature medicine*. 1998;4(4):408-14. Epub 1998/04/18.
125. Breitbach CJ, Arulanandam R, De Silva N, Thorne SH, Patt R, Daneshmand M, et al. Oncolytic vaccinia virus disrupts tumor-associated vasculature in humans. *Cancer research*. 2013;73(4):1265-75. Epub 2013/02/09.
126. Elice F, Rodeghiero F. Side effects of anti-angiogenic drugs. *Thrombosis research*. 2012;129 Suppl 1:S50-3. Epub 2012/06/15.
127. Junttila MR, de Sauvage FJ. Influence of tumour micro-environment heterogeneity on therapeutic response. *Nature*. 2013;501(7467):346-54. Epub 2013/09/21.
128. Welford AF, Biziato D, Coffelt SB, Nucera S, Fisher M, Pucci F, et al. TIE2-expressing macrophages limit the therapeutic efficacy of the vascular-disrupting agent combretastatin A4 phosphate in mice. *The Journal of clinical investigation*. 2011;121(5):1969-73. Epub 2011/04/15.

- 129.Tozer GM, Akerman S, Cross NA, Barber PR, Bjorndahl MA, Greco O, et al. Blood vessel maturation and response to vascular-disrupting therapy in single vascular endothelial growth factor-A isoform-producing tumors. *Cancer research*. 2008;68(7):2301-11. Epub 2008/04/03.
- 130.Gilkes DM, Semenza GL, Wirtz D. Hypoxia and the extracellular matrix: drivers of tumour metastasis. *Nature reviews Cancer*. 2014;14(6):430-9. Epub 2014/05/16.
- 131.Liu YP, Suksanpaisan L, Steele MB, Russell SJ, Peng KW. Induction of antiviral genes by the tumor microenvironment confers resistance to virotherapy. *Scientific reports*. 2013;3:2375. Epub 2013/08/08.
- 132.De Silva N, Atkins H, Kirn DH, Bell JC, Breitbach CJ. Double trouble for tumours: exploiting the tumour microenvironment to enhance anticancer effect of oncolytic viruses. *Cytokine & growth factor reviews*. 2010;21(2-3):135-41. Epub 2010/03/27.
133. Ledford H. Cancer theory faces doubts. *Nature*. 2011;472(7343):273. Epub 2011/04/23.
- 134.Rintoul JL, Lemay CG, Tai LH, Stanford MM, Falls TJ, de Souza CT, et al. ORFV: a novel oncolytic and immune stimulating parapoxvirus therapeutic. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2012;20(6):1148-57. Epub 2012/01/26.
- 135.Pan M, Cong P, Wang Y, Lin C, Yuan Y, Dong J, et al. Novel LOVD databases for hereditary breast cancer and colorectal cancer genes in the Chinese population. *Human mutation*. 2011;32(12):1335-40. Epub 2011/09/09.
- 136.Lemay CG, Rintoul JL, Kus A, Paterson JM, Garcia V, Falls TJ, et al. Harnessing oncolytic virus-mediated antitumor immunity in an infected cell vaccine. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2012;20(9):1791-9. Epub 2012/07/05.
- 137.Ferrara N. Pathways mediating VEGF-independent tumor angiogenesis. *Cytokine & growth factor reviews*. 2010;21(1):21-6. Epub 2009/12/17.

- 138.Coussens LM, Zitvogel L, Palucka AK. Neutralizing tumor-promoting chronic inflammation: a magic bullet? *Science*. 2013;339(6117):286-91. Epub 2013/01/19.
- 139.McCarthy EF. The toxins of William B. Coley and the treatment of bone and soft-tissue sarcomas. *The Iowa orthopaedic journal*. 2006;26:154-8. Epub 2006/06/23.

Contributions of Collaborators

Chapter 3: I generated the 3D model with help from Laura Evgin for the PBS control. Usaf Aladl designed the software and taught me how to use it. I designed and performed the BrdU pulse experiment in Figures 2a-c. Staining in generated Figure 3, 4c, 5b,c, 6, and 8 and wrote portions of the manuscript. Caroline Breitbach generated the remaining figures with the exception of 4a which was generated by Jennifer Paterson. All staining was verified by Manijeh Daneshmand.

Chapter 4: I created the 3D model with help from Sara Cvancic. I stained an estimated 75% of treatment biopsies and developed an SOP for staining vaccinia and bGal. Caroline Breitbach generated Figure 4.4e.

Chapter 5: I generated figure 5.1. 5.2 a, b, c were stained by me in the lab of Dr. Donald McDonald. Barbara Sennino helped me image these tumours. Samples were prepared in Ottawa by Dominic Roy, Michelle Becker and Theresa Falls or myself. In Figure 5.8 Shane Knowles generated the EC50 data. David Conrad and Cory Batenchuk prepared in NRPs.

All histological stains were verified by Manijeh. Mouse work was done with the assistance of Theresa Falls and Lisa Ferreira. All experiments and manuscript preparation received input from John Bell, Harold Atkins, Jean Simon Diallo, and Caroline Breitbach.

Appendices

APPENDIX I: ONCOLYTIC VACCINIA VIRUS DISRUPTS TUMOUR-ASSOCIATED VASCULATURE IN HUMANS.

Breitbach CJ, Arulanandam R, De Silva N, Thorne SH, Patt R, Daneshmand M, Moon A, Ilkow C, Burke J, Hwang TH, Heo J, Cho M, Chen H, Angarita FA, Addison C, McCart JA, Bell JC, Kirn DH.

Contribution: I developed standard operating procedures for staining patient tumours for vaccinia and β -galactosidase. I stained patient biopsies and analyzed them with Caroline Breitbach and Manijeh Daneshmand

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Oncolytic Vaccinia Virus Disrupts Tumor-Associated Vasculature in Humans

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Abstract

Efforts to selectively target and disrupt established tumor vasculature have largely failed to date. We hypothesized that a vaccinia virus engineered to target cells with activation of the ras/MAPK signaling pathway (JX-594) could specifically infect and express transgenes (hGM-CSF, β -galactosidase) in tumor-associated vascular endothelial cells in humans. Efficient replication and transgene expression in normal human endothelial cells *in vitro* required either VEGF or FGF-2 stimulation. Intravenous infusion in mice resulted in virus replication in tumor-associated endothelial cells, disruption of tumor blood flow, and hypoxia within 48 hours; massive tumor necrosis ensued within 5 days. Normal vessels were not affected. In patients treated with intravenous JX-594 in a phase I clinical trial, we showed dose-dependent endothelial cell infection and transgene expression in tumor biopsies of diverse histologies. Finally, patients with advanced hepatocellular carcinoma, a hypervascular and VEGF-rich tumor type, were treated with JX-594 on phase II clinical trials. JX-594 treatment caused disruption of tumor perfusion as early as 5 days in both VEGF receptor inhibitor-naïve and -refractory patients. Toxicities to normal blood vessels or to wound healing were not evident clinically or on MRI scans. This platform technology opens up the possibility of multifunctional engineered vaccinia products that selectively target and infect tumor-associated endothelial cells, as well as cancer cells, resulting in transgene expression, vasculature disruption, and tumor destruction in humans systemically. *Cancer Res*; 73(4): 1265–75. ©2012 AACR.

Introduction

Solid tumors must develop a blood supply to grow and metastasize (1). Growth factors including VEGF and fibroblast growth factor (FGF) play critical roles in promoting tumor angiogenesis (2–4). Both angiogenesis and the established tumor vasculature are promising therapeutic targets. VEGF/VEGF receptor (VEGFR)-targeted angiogenesis inhibitors have resulted in transient clinical benefit, although resistance eventually develops through diverse mechanisms including upregulation of alternate proangiogenic pathways (e.g., FGF-2) and “evasive resistance” through development of a more invasive or

metastatic tumor phenotype (3, 5). Small-molecule vascular disrupting agents (VDA) are designed to collapse established tumor vasculature, but despite reductions in tumor perfusion, VDAs have not improved survival significantly to date (6, 7). Therefore, novel approaches to targeting tumor angiogenesis and ablating established tumor vasculature are needed, both in antiangiogenic treatment-naïve and -refractory patients.

JX-594 is a Wyeth vaccinia vaccine-derived oncolytic virus engineered for viral thymidine kinase gene inactivation, and expression of the human granulocyte-monocyte colony stimulating factor (hGM-CSF) and β -galactosidase (β -gal) transgenes for immune stimulation and replication assessment, respectively (8, 9). In phase I trials, JX-594 was well tolerated and associated with replication in cancer cells, expression of its 2 transgenes, and tumor necrosis after intravenous or intratumoral injection (8, 10, 11). Vaccinia has evolved to exploit cellular signal transduction pathway activation for its replication and spread. Specifically, activation of the EGFR pathway has been shown to drive vaccinia replication, as has activation of other pathways (e.g., Abl kinase; refs. 12–15). In addition, JX-594 specifically requires high cellular thymidine kinase levels to replicate. We therefore hypothesized that endothelial cell-specific growth factor signal transduction pathway stimulation would create a cellular milieu supportive of JX-594 replication, including cellular thymidine kinase induction. We hypothesized that JX-594 could infect and disrupt tumor-associated vasculature selectively in humans. First, replication in cancer

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cells is driven by EGFR/Ras pathway signaling and cellular thymidine kinase levels (13, 16–18), and VEGF activates many of the same downstream mediators in endothelial cells (19). Second, we previously made anecdotal observations with oncolytic vaccinia from individual mice and humans that were consistent with this hypothesis (20, 21). Finally, with RNA-based oncolytic virus therapy, decreased tumor perfusion in mice (22, 23) and infection of tumor-associated endothelial cells following anti-VEGFR therapy were reported (24). Nevertheless, definitive clinical and laboratory mechanistic data were lacking.

To determine whether selective tumor vascular infection and disruption could be achieved by JX-594 in patients, we conducted detailed laboratory and clinical investigations. First, we evaluated whether JX-594 could infect and lyse normal human endothelial cells under VEGF and bFGF-2 stimulation *in vitro*. Next, we assessed vascular disruption in a murine tumor model. In humans, we evaluated human tumor biopsies for endothelial cell infection and transgene expression after intravenous administration of JX-594 in a phase I clinical trial, and we prospectively designed an analysis of tumor perfusion over time after JX-594 treatment in phase II trials of patients with advanced hepatocellular carcinoma (HCC), a hypervascular and VEGF-rich tumor type (25). Full safety and toxicity evaluations were conducted, including assessments for toxicity to normal blood vessels and to healing wounds.

Materials and Methods

Virus

JX-594 is a Wyeth vaccinia virus vaccine-derived oncolytic virus with disruption of the viral thymidine kinase gene and expression of the hGM-CSF and β -gal transgenes under control of the synthetic early-late and p7.5 promoters, respectively (8). A version of JX-594 was generated to monitor replication *in vitro*. JX-594-GFP+/ β -gal- expresses GFP instead of β -gal under the synthetic early/late promoter pSE/L in the vaccinia thymidine kinase gene locus. Viruses were propagated in HeLa cells. The vaccinia virus Wyeth strain was obtained from American Type Culture Collection (ATCC; distributed by Cedarlane Laboratories, Burlington, Ontario, Canada) and was propagated on U2OS cells. For mouse tumor model studies, a WR-TK(-) vaccinia was used as a mouse-adapted surrogate of JX-594; WR is a Wyeth strain vaccinia that was isolated through serial passage in mice.

In vitro infection of endothelial cells, Western blotting, and immunofluorescence staining

Human umbilical vein endothelial cell (HUVEC) or human dermal microvascular endothelial cells were subcultured in Endothelial or Microvascular Endothelial Cell Growth Medium-2 (Lonza). Endothelial cells used in experiments were not passaged more than 3 times since resuscitation. For Western blotting, cells were plated in 6-well dishes and stimulated with 50 ng/mL VEGF165 (R&D Systems/Cedarlane) or 50 ng/mL EGF (Sigma). Detergent cell extracts from triplicate wells were pooled and prepared as described (26). Following protein determination by Bradford assay (BioRad Protein Assay Solution), 20 μ g of clarified cell extract were

electrophoresed using the NuPAGE SDS-PAGE Gel System (Invitrogen). Blots were cut into strips and probed with antibodies specific for tyrosine-1175 phosphorylated VEGFR2 (Cell Signaling NEB), total VEGFR2 (Cell Signaling), thymidine kinase-1 (Abcam), or α -tubulin (Santa Cruz), followed by horseradish peroxidase-conjugated mouse or rabbit secondary antibodies (The Jackson Laboratory, Bar Harbor, Maine). Bands were visualized using Supersignal West Pico Chemiluminescent substrate (ThermoScientific Pierce). To assess the effect of endothelial growth factors on vaccinia replication, cells were plated in MCDB131 medium (Gibco/Invitrogen) in 24-well dishes and pulsed with 30 ng/mL VEGF₁₆₅ or 5 ng/mL bFGF-2 (R&D Systems) in the presence of JX-594-GFP+/ β -gal- or wild-type Wyeth control at multiplicity of infection (MOI) 0.01. Fluorescent images were taken 72 hours postinfection with an Axiovert S100 Fluorescence microscope (Carl Zeiss Ltd). Relative fluorescence units for GFP were obtained using the Synergy Mx microplate reader and Gen5 1.10 software (Biotek). Cells and supernatant were collected 48 to 72 hours postinfection and JX-594-GFP+/ β -gal- and Wyeth vaccinia were titrated from cell lysates by plaque assay on U2OS. Virus output was normalized to cell numbers following growth factor stimulation. Viable cells were counted by Trypan blue exclusion assay. For *in vitro* wound healing assays, HUVECs were grown on glass coverslips until confluent, treated with 30 ng/mL VEGF₁₆₅, scratched with a micropipette tip, and infected with JX-594-GFP+/ β -gal- at MOI 0.01 for 24 hours. Coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked using 5% goat serum with 1% BSA in PBS, and incubated with mouse monoclonal anti-CDI 05 (Dako) or rabbit monoclonal EPR3193 thymidine kinase 1 antibody (Abcam) overnight at 4°C. For detection, coverslips were incubated with Alexa Fluor 555-conjugated goat anti-mouse or rabbit secondary antibodies (Molecular Probes, Invitrogen) and mounted and sealed with Dako Prolong anti-fade (Dako) with 4', 6-diamidino-2-phenylindole (DAPI). Images were collected using the Zeiss Imager M1 microscope equipped with AxioCam HRm camera (Carl Zeiss Ltd). For microtubule disruption assays, endothelial cells cultured on glass coverslips were infected with JX-594 (MOI = 0.05) or mock infected. After 36 hours postinfection, cells were fixed as above and incubated with goat anti-tubulin antibody (Abcam) followed by Alexa Fluor 594 donkey anti-goat (Molecular Probes, Invitrogen) and visualized as above.

Mouse treatments

BALB/c mice (female, 8 to 10 weeks) were implanted with 1×10^5 4T1 cells (ATCC) subcutaneously on the hind limb. Tumors were allowed to form (7 to 10 days until tumors were approximately 100 mm³). Mice were then treated intravenously with 1×10^8 plaque-forming units (pfu) of WR-TK- (Vaccinia strain WR with a deletion in the viral thymidine kinase gene; strain WR was used in these experiments as it is better able to replicate in mouse cell lines relative to Wyeth; $n = 3$ per group, experiment repeated 3 times).

All animal work was conducted with the approval from the Institutional Animal Care and Use Committee.

Patients

Infection of tumor-associated vasculature was assessed in patients treated on a phase I dose-escalation trial of JX-594 (ClinicalTrials.gov number NCT00625456; ref 11; $n = 23$). Patients had treatment-refractory, histologically confirmed advanced/metastatic solid tumors. Patients were treated at doses ranging from 1×10^5 to 3×10^7 pfu per kg; a sixth cohort was added at 1×10^8 pfu (fixed dose). Acute effects of JX-594 on tumor perfusion were evaluated in patients with advanced HCC on randomized phase II trials of JX-594 (ClinicalTrials.gov number NCT00554372 and NCT01171651). On one randomized study, patients were treated intratumorally with a single dose of JX-594 at a dose level of 1×10^8 or 1×10^9 pfu before the evaluation of tumor perfusion on day 5 (dynamic MRI). One additional patient with metastatic rectal adenocarcinoma was treated intratumorally in liver metastases at a dose of 1×10^8 pfu. Patients with HCC on a second phase II study were treated with a single intravenous infusion of JX-594 at a dose of 1×10^8 pfu before the assessment of effects on tumor perfusion on day 6.

All patients gave written informed consent according to guidelines on good clinical practice. Protocol and consent forms were approved by the United States Food and Drug Administration, Health Canada, the Korea Food and Drug Administration, as well as the Institutional Review and Infection Control Committees at each hospital.

Patient biopsy analysis

Biopsies (excisional, core needle, or fine-needle aspirate) were obtained from all subjects 7 days (± 1) after iv. phase I treatment and formalin-fixed/paraffin-embedded. Hematoxylin and eosin (H&E)-stained sections were evaluated for the presence of tumor tissue, normal tissue, and vascular structures. Additional sections were used to detect vaccinia and β -gal by immunohistochemistry (IHC). IHC for vaccinia was conducted as previously described. For immunohistochemical detection of β -gal, an anti- β -gal polyclonal antibody (Abcam) was used. Negative controls were run without primary antibody, and tumors from mice treated with JX-594 were included as positive controls. The proportion of vessels infected by JX-594 was determined by a Board-certified human pathologist on vaccinia-stained sections; if vessels were too numerous to count, 5 random fields at $\times 20$ magnification were chosen for quantification. Tumor neovascularity was identified by 2 independent pathologists.

Imaging

Dynamic contrast-enhanced MRI imaging was conducted 5 days after intratumoral injection of JX-594 into primary liver tumors or liver metastases or 6 days after intravenous infusion of JX-594; this hyperacute MRI scan was conducted to evaluate early changes in tumor vascularity, and to determine whether early MRI responses correlated with longer term clinical endpoints. Imaging was conducted using an extracellular gadolinium contrast agent and a three-dimensional spoiled gradient echo pulse sequence with fat suppression. Lesion signal intensity measurements were used to calculate changes in contrast enhancement, a reflection of tumor perfusion.

Clinical/laboratory analysis

Serum chemistry, including creatinine and alanine aminotransferase levels, were evaluated by routine laboratory testing at screening, predose day 1, 5, 15, and 22 after JX-594 injection. Similarly, coagulation parameters, including prothrombin time (PT), activated partial thromboplastin time (aPTT), and international normalized ratio (INR) were evaluated at these time points. Blood pressure measurements were conducted at screening, predose day 1, and day 15.

Statistical analyses

All statistical analyses were conducted using the GraphPad Prism 5.0 software (GraphPad Software). The unpaired *t* test was used to assess differences in virus output in human dermal microvascular endothelial cells (HDMEC) in the presence/absence of growth factors.

Description of methods for the rabbit GLP toxicology study is available in Supplementary Information.

Results

JX-594 infects VEGF- and FGF-2-activated human endothelial cells *in vitro*

The ability of JX-594 to infect and express transgenes in normal human endothelial cells (HUVECs and HDMECs) was assessed in the presence and absence of known endothelial cell growth factors; a GFP-expressing version of JX-594 was used (JX-594-GFP+/ β -gal-). We first confirmed that our endothelial cells expressed VEGFR, and that stimulation with VEGF₁₆₅ resulted in VEGFR phosphorylation and downstream cellular thymidine kinase expression (Fig. 1A). Next, VEGF₁₆₅ stimulation resulted in a significant increase (10-fold) in JX-594 output in HDMECs (72 hours postinfection; $P < 0.0001$; Fig. 1B). In addition, stimulation with FGF-2, an endothelial cell growth factor implicated in resistance to VEGFR-targeted therapy (27) and also resulted in stimulation of JX-594 replication in normal endothelial cells ($P = 0.002$; Fig. 1B). Virus production on a plaque-forming unit per cell basis was also significantly increased (approximately 15-fold with VEGF; Supplementary Fig. S1). Similar results were obtained with HUVECs. The dependence of JX-594 on VEGF or FGF-2 for replication was related to its viral thymidine kinase deletion, as wild-type vaccinia (TK+) replicated efficiently in their absence (Fig. 1B). Increasing VEGF₁₆₅ concentrations correlated with increasing JX-594-GFP+/ β -gal- transgene expression following infection (Fig. 1c and 1d). Of note, chronic exposure of HDMECs or HUVECs to VEGF during growth, passage, and infection resulted in equivalent stimulation of thymidine kinase levels, JX-594 transgene expression, and replication as compared with acute VEGF exposure (Supplementary Fig. S2). Finally, we confirmed that the anti-VEGF antibody bevacizumab (Avastin) could block the VEGF stimulatory effect, by reducing thymidine kinase levels, and that VEGF did not sensitize control normal VEGFR(-) cells to JX-594 replication (GM38 fibroblasts, Supplementary Fig. S3). We next investigated the ability of JX-594 to selectively infect rapidly proliferating and cellular thymidine kinase-overexpressing endothelial cells in the context of an *in vitro* wound healing assay. As shown in Fig. 1E, when stimulated with VEGF₁₆₅, the thymidine

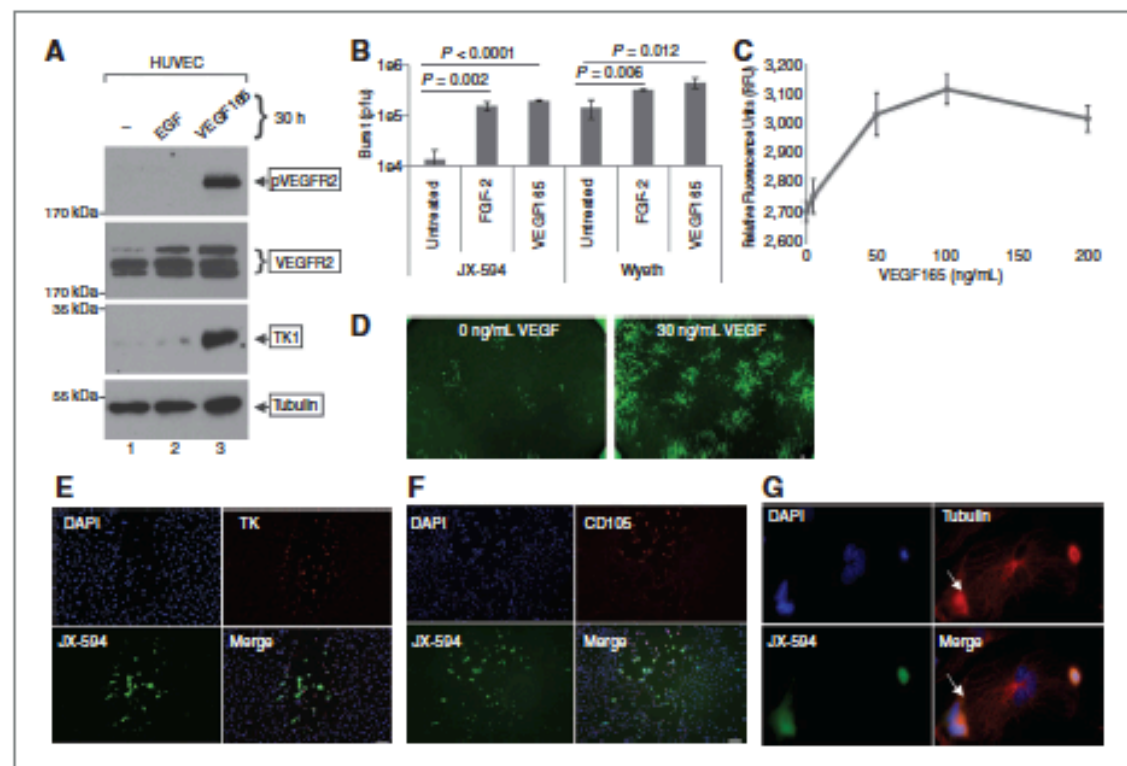


Figure 1. JX-594 expresses transgene and replicates in activated human endothelial cells *in vitro* following VEGF or FGF-2 stimulation. **A**, HUVECs were pulsed with 0.50 ng/mL EGF or 50 ng/mL VEGF165 for 30 hours and cell lysates were collected and resolved by gel electrophoresis. The blot was cut into strips and probed for pVEGFR2, VEGFR2, TK1, or tubulin as a loading control, as indicated. **B**, VEGF- and FGF-2-stimulated vaccinia replication: HDMECs were infected with JX-594-GFP+ β -gal- or wild-type Wyeth control at an MOI of 0.01 for 72 hours after pulsing with either 30 ng/mL VEGF₁₆₅, 5 ng/mL FGF-2 or neither. Cells and supernatant were collected and infectious virus titer was assayed on U2OS cells. **C**, HUVECs were pulsed with 0 to 400 ng/mL VEGF165 and infected with JX-594-GFP+ β -gal- for 48 hours. Mean fluorescence values for GFP (485 and 528 nm) were obtained and plotted as the average of quadruplicates. **D**, JX-594 transgene expression: HDMECs were pulsed with 0 or 30 ng/mL VEGF₁₆₅ and infected with JX-594-GFP+ β -gal- at MOI 0.01. Fluorescent images were taken after 72 hours. **E**, HUVECs grown in the presence of 25 ng/mL VEGF₁₆₅ infected with JX-594-GFP+ β -gal- at an MOI of 0.01 for 24 hours. Fixed cells were stained for TK1 and DAPI counterstain. Images were collected using a $\times 10$ objective. Scale bar, 100 μ m. **F**, HUVEC cells were treated with VEGF₁₆₅ as above and infected with JX-594-GFP+ β -gal- for 24 hours. Fixed cells were stained for CD105 (endothelial cell activation marker) and DAPI counterstain. Images were collected using a $\times 5$ objective. Scale bar, 100 μ m. **G**, Immunofluorescence staining for tubulin in HUVECs infected with JX-594-GFP+ β -gal- for 36 hours at an MOI of 0.05. White arrows indicate tubulin depolymerization in JX-594-GFP+ β -gal- infected cells. Images are representative of 3 independent experiments.

kinase-positive HUVECs at the leading edge of a scratch in the cell monolayer were preferentially infected. Furthermore, infection colocalized with the endothelial cell activation marker CD105 (Fig. 1F). We then sought to investigate *in vitro* whether JX-594 infection of endothelial cells resulted in microtubule depolymerization and cell rounding; these effects are associated with vascular disruption *in vivo*. This phenotype was confirmed following HUVEC infection with JX-594-GFP+ β -gal- (Fig. 1G). In summary, these data showed that JX-594 could selectively infect and express transgenes in VEGF- and FGF-2-activated human endothelial cells of 2 subtypes.

JX-594 infects and disrupts tumor vasculature in tumor models *in vivo*

We subsequently evaluated the ability of a mouse-adapted thymidine kinase gene-deleted vaccinia [WR-TK(-)] to infect

and disrupt tumor vasculature after intravenous administration in an immunocompetent BALB/c murine tumor model (4T1 breast cancer, subcutaneous hind limb location). Intravenous WR-TK(-) was previously shown to have significant efficacy in this model, and tumors contained high VEGF concentrations at baseline; VEGF concentrations in tumors were more than 10-fold higher than those in the liver and 100-fold higher than those in the spleen (data not shown). Tumors were harvested 48 hours after infusion of either WR-TK(-) (10^8 pfu) or PBS, and immunofluorescence staining was conducted detecting tumor vasculature (anti-CD31, endothelial cell marker), replicating vaccinia (anti-vaccinia polyclonal), and hypoxia (hypoxprobe detection). Endothelial cell infection was evident (Fig. 2A). In contrast, cancer cell infection was relatively limited at this early time point suggesting that endothelial cell infection can precede cancer cell infection (cancer cell

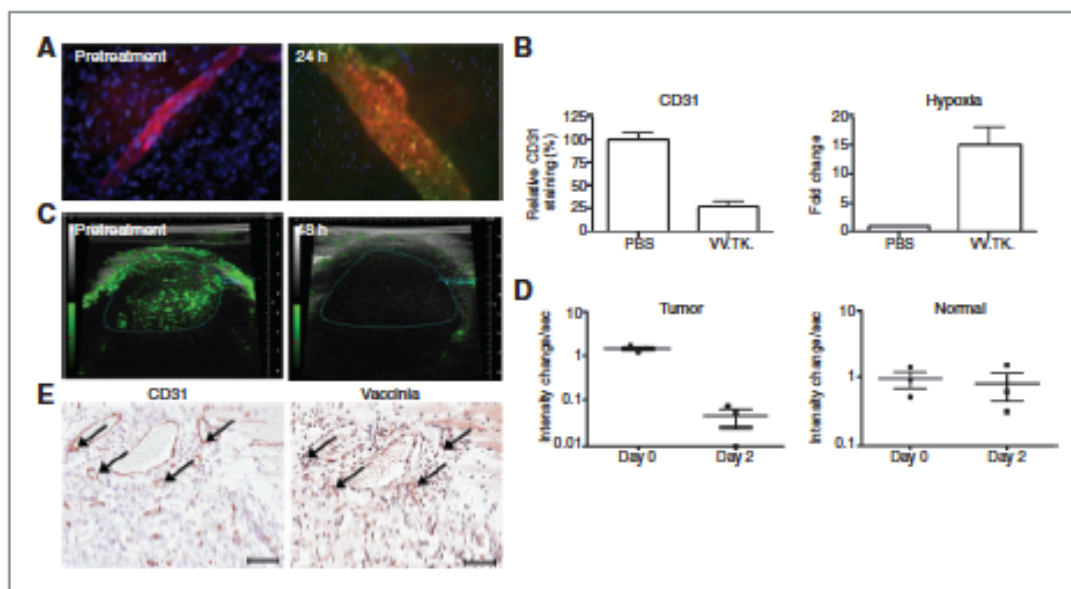


Figure 2. Intravenous JX-594 causes tumor-selective endothelial cell infection and acute vascular disruption. Immunocompetent Balb/C mice bearing subcutaneous 4T1 murine tumors were treated intravenously with 1×10^6 pfu of thymidine kinase (TK)-deleted vaccinia [WR-TK(-)] or PBS. Twenty-four to 48 hours later, tumors were assessed by ultrasound for blood flow and were subsequently harvested for immunohistochemical analysis. **A**, tumor sections were stained with conjugated antibodies targeting CD31 (red; endothelial cells) or vaccinia (green), showing diffuse endothelial cell infection. **B**, the relative density of CD31+ vessels in vaccinia-treated versus PBS-treated tumors collected at 48 hours. Immunohistochemical analysis comparing hypoxia at baseline and 48 hours following vaccinia or PBS treatment (mice were injected with hypoxyprobe 15 minutes before sacrifice). **C**, mice bearing 4T1 tumors were imaged using contrast-enhanced ultrasound during intravenous delivery of a nontargeted contrast agent to define vascular areas and to quantify perfusion rates within the tumor. Perfusion (rate of appearance of contrast agent in a defined region of interest, either tumor or surrounding tissue) was quantified, and representative vascular images are shown (areas of increased contrast are highlighted in green). **D**, box-whisker plots for experiment in **C** showing change in tumor perfusion ($P = 0.0006$) versus adjacent normal tissue ($P = 0.78$, not significant). **E**, C57BL/6 mice bearing MC38 murine colon tumors subcutaneously were injected with 10^6 pfu of TK-deleted vaccinia [WR-TK(-)] and harvested 72 hours later and stained with anti-CD31 and anti-VV by immunohistochemistry; representative images show sequential staining as shown. Arrows point to areas of colocalization (scale bar, 100 μ m). Arrows are pointing to areas of colocalization.

infection was extensive at later time points; data not shown). The density of CD31+ intratumor vessels had decreased significantly, and tumor hypoxia had increased significantly, at this time point in treated animals (Fig. 2B). Vessels in all normal tissues examined (liver, spleen, and lungs) and harvested at the same time point did not show infection as determined by immunofluorescence staining (not shown). Similar infection of tumor-associated vessels was shown by immunohistochemical analysis in a second strain of mice (C57BL/6) after intravenous infusion of WR-TK(-) vaccinia in immunocompetent animals bearing subcutaneous MC38 tumors (Fig. 2E).

We subsequently tested whether blood flow was selectively decreased to solid tumors within 48 hours after intravenous administration in the 4T1 model using contrast-enhanced ultrasound microbubble technology (28). Within 48 hours after intravenous vaccinia injection, the tumor perfusion was reduced by more than 90% from pretreatment ($P = 0.0006$, Student *t* test); in contrast, no change was noted in immediately adjacent normal tissue perfusion ($P = 0.78$; Fig. 2C and D). Therefore, TK(-) vaccinia induced tumor vessel-specific infection, blood flow disruption, and hypoxia within 48 hours in the 4T1 model. Importantly, these effects were evident before

widespread tumor cell infection and/or necrosis; these effects, therefore, preceded widespread cancer cell infection and necrosis shown at later time points.

To determine whether normal tissue vasculature would be damaged with high dose i.v. JX-594, rabbits (a species that is more sensitive to Wyeth vaccinia and human GM-CSF than mice or rats) were treated with 3 weekly doses of 3×10^7 pfu/kg (the highest dose per kg given to humans intravenously to date, and approximately twice the dose resulting in reproducible tumor infection); the study was conducted under Good Laboratory Practice guidelines by an independent contract toxicology group. Blood analyses showed no change in lab parameters, including those that would be consistent with disseminated vascular damage (changes in platelets, prothrombin time, PT/INR, creatinine), on days 3, 7, 17, 21, and 44. Detailed histopathology of solid organs revealed no vascular pathology in any tissues, including heart, liver, brain, and kidney (Supplementary Table S1).

JX-594 infects tumor-associated endothelial cells in patients after intravenous infusion

The distribution of JX-594 infection and transgene expression was evaluated within tumor biopsies collected

approximately 7 days after treatment from patients treated with a single intravenous infusion of JX-594 on a phase I dose-escalation trial ($n = 18$ patient tumor biopsy samples had evaluable vascular structures); we previously described dose-dependent delivery, replication, and transgene expression in cancer cells in these biopsy samples (11). Infection of tumor-associated vascular endothelial cells was shown in dose-related fashion: 0 of 10 low-dose ($\sim 1 \times 10^7$ – 3×10^8 pfu) versus 5 of 8

high-dose ($\geq 1 \times 10^9$ pfu) patients ($n = 18$ biopsies contained vascular structures). Positive immunohistochemical staining of endothelial cells was shown in patient tumor biopsies of diverse histologies including colorectal carcinoma, ovarian carcinoma (high-grade serous), and leiomyosarcoma (Fig. 3A, D–F). Endothelial cells in negative control sections (no primary antibody; Fig. 3B and G) as well as control tumors from the same patients collected before JX-594 treatment, were

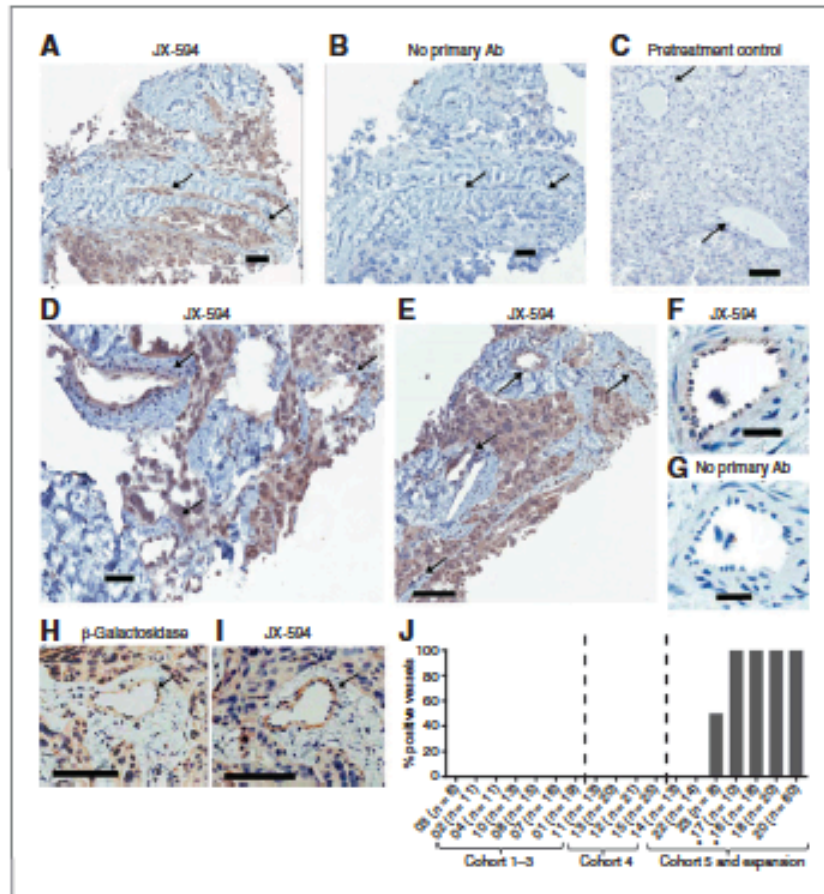
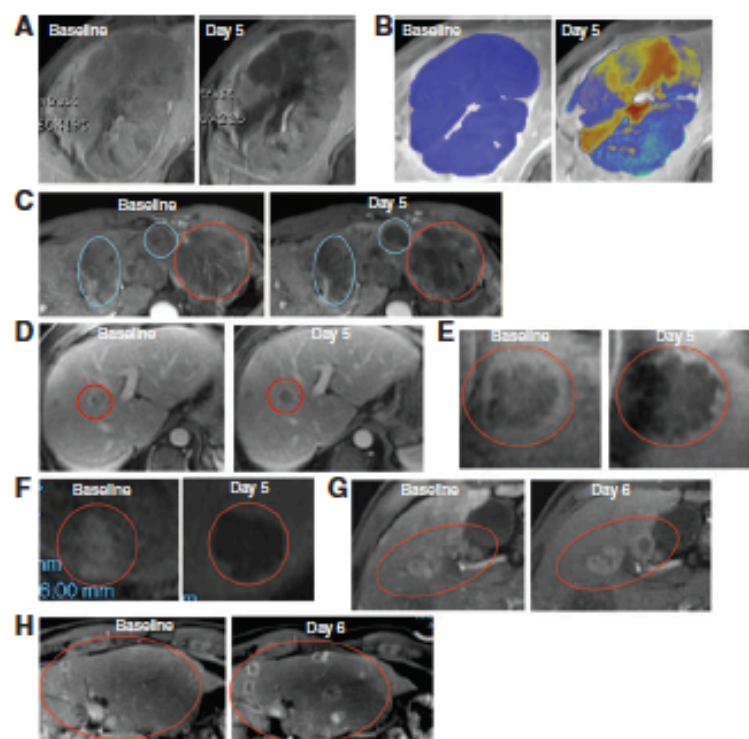


Figure 3. Patient tumor biopsies show dose-dependent JX-594 infection of tumor-associated endothelial cells after intravenous infusion. Patients in high dose cohorts on a phase I dose-escalation trial of Iv. JX-594 received 1 to 3×10^9 pfu, and 7 days later, tumor biopsies were conducted and samples were assessed for tumor-associated endothelial cell infection by IHC. **A**, JX-594 infection of tumor-associated endothelial cells (black arrows) in metastatic ovarian cancer biopsy (lymph node) visualized by immunohistochemical staining with polyclonal anti-vaccinia antibody (scale bar, 50 μ m). **B**, negative control: corresponding tissue to **A** stained with secondary antibody only (scale bar, 50 μ m). **C**, negative control: tumor biopsy from patient in **A**, **B**, **D**, **E** collected before JX-594 treatment stained with polyclonal anti-vaccinia antibody (scale bar, 50 μ m). **D**, Lower power magnification of sample in **A**. Black arrows indicate infected vessels (scale bar, 100 μ m). **E**, High magnification of JX-594 infection of additional tumor-associated vasculature in ovarian tumor (black arrows indicate infected vessels; scale bar, 50 μ m). **F**, JX-594 infection of tumor-associated vessels in tumor biopsy of patient with metastatic leiomyosarcoma visualized by immunohistochemical staining with polyclonal anti-vaccinia antibody (scale bar, 50 μ m). **G**, negative control: corresponding serial section to **F** stained with secondary antibody only (scale bar, 50 μ m). Linear adjustments to brightness and contrast made in **F** and **G**. **H**, immunohistochemical detection of β -gal in vessel of patient with ovarian cancer in **A**. Black arrow indicates vessel. **I**, serial section shows colocalized vaccinia staining (polyclonal anti-vaccinia antibody) in vessel positive for β -gal (linear adjustments to brightness and contrast made in **H** and **I**; scale bars, 100 μ m). **J**, patients in escalating dose cohorts on a phase I dose-escalation trial of intravenous JX-594 received approximately 3×10^7 to 3×10^9 pfu and 7 days later, tumor biopsies were collected and assessed for tumor-associated endothelial cell infection in visible vessels. Percentage of vessels positive for vaccinia IHC in tumor by patient and dose cohort. Number of vessels counted per patient biopsy indicated (all vessels in specimen or vessels in 5 random fields at $\times 20$ magnification). Asterisks indicate patients who have received prior antiangiogenic therapy.

Figure 4. Intravenous or intratumoral injection of JX-594 results in acute tumor vascular disruption in both injected and noninjected tumors of patients with advanced HCC and colorectal cancer. **A**, baseline and posttreatment day 5 dynamic MRI images of 10 cm maximum diameter HCC tumor directly injected with JX-594. **B**, segmentation analysis of images in **A** separating the poorly perfused (yellow) from well-perfused (blue) tumor. **C**, baseline and day 5 dynamic MRI images of JX-594 directly injected tumor (red circle) and noninjected tumors (blue circles) in a patient with advanced HCC. **D**, noninjected subclinical HCC tumor (<1 cm maximum diameter) at baseline and on day 5 after JX-594 treatment shows vascular disruption and edema in tumor. **E**, baseline and day 5 dynamic MRI images of metastatic colorectal cancer tumor directly injected with JX-594. **F**, baseline and day 5 dynamic MRI images of noninjected atypical hemangioma in patient with metastatic colorectal cancer as in **C**. **G**, noninjected subclinical HCC tumor (<1 cm maximum diameter) at baseline and on day 5 after JX-594 treatment show vascular disruption and edema in tumor. **H**, baseline and day 6 dynamic MRI images of sorafenib-refractory HCC patients following i.v. JX-594.



negative for endothelial cell staining (Fig. 3C). To confirm that immunohistochemical staining for vaccinia proteins was associated with productive infection of tumor-associated vasculature, sections were stained for the presence of the JX-594 marker transgene β -galactosidase (β -gal IHC); β -gal is not present in the product, therefore high-level expression requires JX-594 replication. Endothelial cells that were positive for vaccinia antigen staining by IHC also exhibited positivity for the β -gal transgene (Fig. 3H and I).

Finally, we quantified the total number of evaluable vascular structures, and the number staining positively for JX-594 replication, in all evaluable patient biopsies ($n = 18$). For all patients treated in lower dose cohorts ($n = 10$), no positive-staining vessels were identified in any biopsy (range = 6–25/sample). Of note, tumor cells were also negative at these dose levels. In contrast, at doses more than approximately 3×10^8 pfu, reproducible infection of blood vessels (as well as tumor cells) was observed (5/7 patients). The percentage of vascular structures staining positively per patient tumor varied from approximately 50% to 100% (Fig. 3J; range = 8 to 60 per sample). Importantly, extensive vessel infection was observed in 2 patients who had tumor progression on prior antiangiogenic therapy; these patients had non-small cell lung cancer (previous bevacizumab, anti-VEGF antibody) and leiomyosarcoma (previous α -IFN, a known antiangiogenic; ref. 29)).

JX-594 triggers acute tumor vascular disruption and decreased perfusion in patients with hypervascular liver tumors

Following the demonstration of tumor vascular endothelial cell infection after i.v. JX-594 in patients, we sought to evaluate acute changes in tumor perfusion within the same time period. Serial dynamic MRI scans were obtained (strict SOP and quality control) and read by expert central radiologists blinded to patient characteristics. Patients on a phase II trial of JX-594 had liver-based tumors of either HCC ($n = 15$) or colorectal carcinoma ($n = 1$) histology; of note, intratumoral injection resulted in intravenous dissemination acutely at high concentrations (data not shown). Tumor perfusion decreased significantly over time in both injected and noninjected tumors. In tumors directly injected with JX-594, (33% showed 15% or more decrease in perfusion at 5 days; range = –18% to –66%). For example, a patient with a single 10 cm HCC tumor received 1×10^9 pfu JX-594, and by day 5, the HCC perfusion was markedly decreased in large areas of the tumor mass, as evidenced by decreased contrast enhancement (Fig. 4A). Computer segmentation analysis was used to separate necrotic (yellow) from viable (blue) tumor (Fig. 4B); this analysis was also displayed in a three-dimensional tumor model (Supplementary Video S1 and S2). To evaluate whether vascular shutdown was the result of the intratumoral injection

procedure itself, perfusion of noninjected tumors was also monitored after intravenous dissemination following intratumoral injection. Similar vascular disruption was observed in the tumors of these patients. For example, vascular disruption was shown in 2 noninjected hypervascular infiltrative HCC tumors separated from the injected tumor by normal liver tissue in one patient (Fig. 4C; blue ovals). In addition, small hypervascular tumors that were distant from the injected tumor site, and therefore were not injected, underwent acute vascular disruption with acute swelling (likely edema) by day 5 (Fig. 4D, Supplementary Fig. S4). Tumor vascular disruption was also observed in a patient with metastatic colorectal cancer after intratumoral injection of 1×10^9 pfu (Fig. 4E). This patient also had a noninjected hemangioma with atypical features and an abnormal and hyperproliferative vascular tumor without associated malignant cancer cells (30), in another liver lobe that underwent acute vascular disruption (Fig. 4F); perfusion effects on typical nonmalignant hemangiomas were not assessed (not shown).

Finally, acute effects on tumor perfusion were also shown 6 days after i.v. JX-594 infusion in patients with advanced HCC who had failed prior treatment with sorafenib, a small-molecule VEGFR inhibitor (Fig. 4G and H). Following intravenous infusion, tumors became edematous, centrally hypointense, and thus more apparent.

Acute vascular disruption is maintained over at least eight weeks

In addition to acute tumor perfusion measurements, the durability of tumor responses was assessed by dynamic MRI imaging at week 8 in the phase II trials of patients with HCC. In patients exhibiting acute reductions in contrast enhancement at day 5 ($n = 5$), responses were maintained at week 8 (Fig. 5A and B). This indicates that early acute reductions in tumor perfusion can be durable and may predict for subsequent durable necrotic responses.

No clinical signs of damage to normal vasculature or to wound healing were observed

JX-594-related adverse events are typically flu-like symptoms lasting up to 24 hours (10, 11). For this study, to assess whether toxicity to normal vasculature occurred, we specifically assessed cardiovascular and coagulation end points over time following intravenous JX-594 administration on a phase I trial ($n = 23$); these included blood pressure, creatinine, prothrombin time (PT/INR), and platelet count. No significant changes were shown at doses associated with intravenous delivery and vascular disruption in tumors. Likewise, patients with liver cancer with documented acute (day 5) perfusion reduction in their tumor(s) following JX-594 treatment in a phase II trial had similar assessments; creatinine and transaminase concentrations, coagulation parameters, and changes in blood pressure were closely monitored for signs of damage to normal vasculature in patients exhibiting acute reduction in tumor perfusion ($n = 5$); no clinically significant changes were detected (Fig. 5C and D). Finally, we closely observed normal wound healing sites following JX-594 injection as endothelial cells are proliferating under growth factor stimulation in this

setting. Sites of large bore needle insertion (18 gauge, used for percutaneous intratumoral injections into liver tumors in phase II trials; $n = 33$ patients) induced a puncture wound in the skin and liver capsule; insertion site puncture wounds healed normally clinically with no bleeding, ulceration, or infection at injection sites reported as adverse events (Fig. 5E).

Discussion

We report here for the first time in humans that a biologic agent can be used to target and infect tumor-associated endothelial cells, resulting in transgene expression, vascular disruption, and subsequent tumor lysis. JX-594, an engineered transgene-expressing vaccinia virus oncolytic, achieved these effects in patients with diverse solid tumor histologies after either intravenous or intratumoral administration. *In vitro* studies confirmed that FGF-2, a mediator of VEGFR inhibitor resistance, stimulated JX-594 activation in endothelial cells. In an animal tumor model, tumor endothelial cell infection preceded widespread cancer cell infection and was associated with a significant reduction in perfusion and increased hypoxia. These acute effects on tumor vasculature in humans and animals were frequently followed by durable tumor necrosis. Toxicities related to adverse effects on normal blood vessels were not observed in humans or in a GLP toxicology study. Specifically, hypertension (toxicity commonly observed upon treatment with antiangiogenic agents; ref. 31) was not commonly observed upon treatment with JX-594. This platform opens up the possibility of treating highly vascular solid tumors with JX-594 and similar poxviruses, including hypervascular cancers such as hepatocellular and renal cell carcinomas. These findings describe an important mechanism by which JX-594 achieves efficient intravenous delivery and tumor infection; extravasation of this relatively large poxvirus through endothelial pores, a potentially limiting step, may not be necessary to achieve tumor infection.

We identified mechanisms by which JX-594 was able to specifically target and infect tumor-associated endothelial cells, but other mechanisms may also be operative. Our *in vitro* studies using primary human endothelial cells showed that VEGF and FGF-2 were able to stimulate JX-594 replication in these cells. Vile and colleagues have shown that oncolytic reovirus and VSV replication can also be induced in endothelial cells through modulation of the VEGFR (24). Pulses of VEGF or chemotherapy-induced damage of blood vessels increased the penetration of oncolytic reovirus into murine tumors (24). However, in these studies, there was no evidence that reovirus could inherently infect intact tumor neovasculature without exogenous VEGF treatment; the authors suggested that increased vascular permeability might allow tumor cell infection, rather than infection of the tumor vasculature itself (24). For JX-594, EGFR activation itself may play a role *in vivo*, as this pathway can be activated in tumor-associated endothelial cells in both VEGF inhibitor-naïve (32) and -refractory settings (33). Finally, efficient JX-594 replication was shown to be dependent on high cellular thymidine kinase concentrations due to a deletion of the viral thymidine kinase gene (34). Thymidine kinase has been shown to be a key enzyme in the modulation of

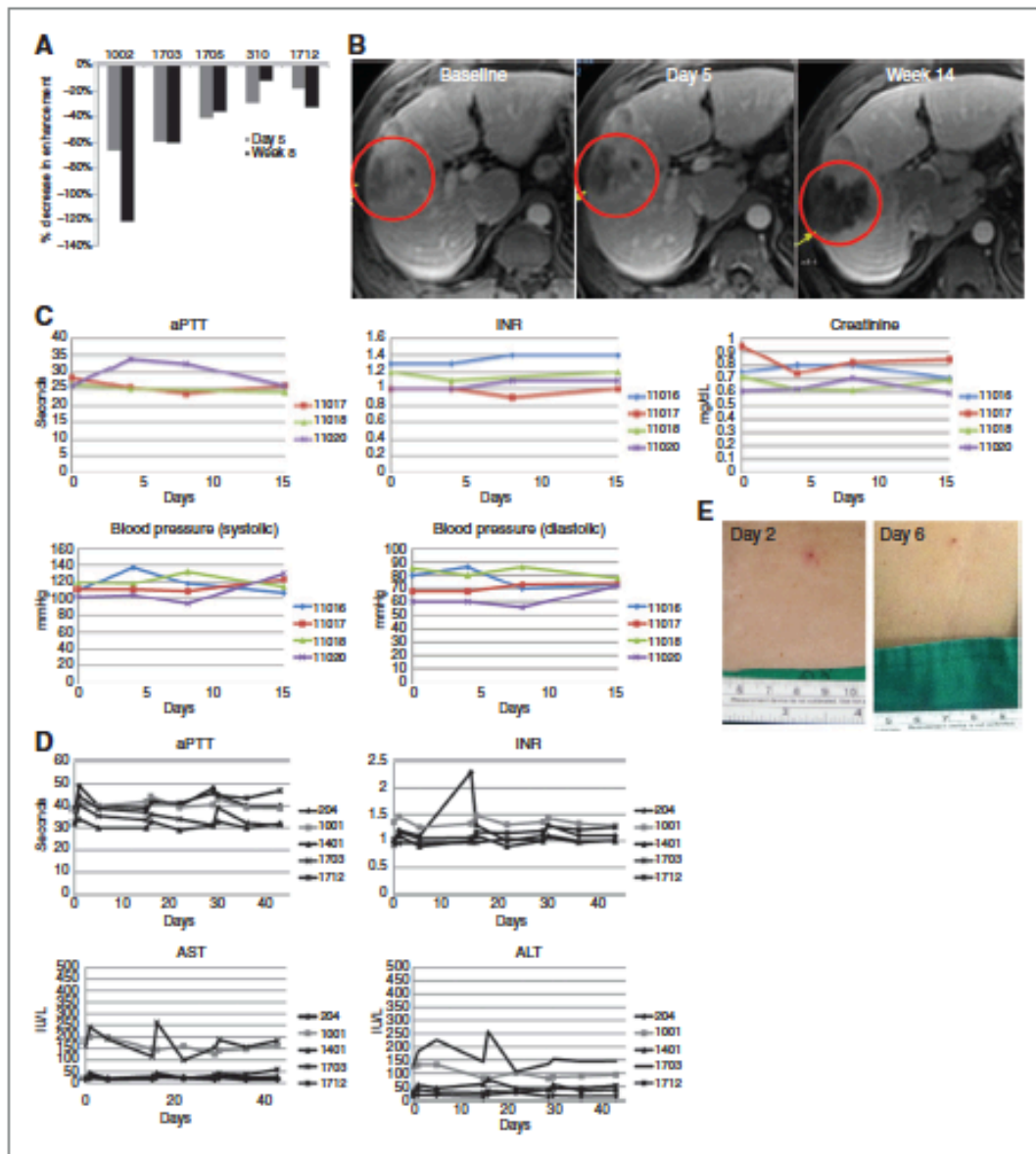


Figure 5. Acute vascular disruption with JX-594 is followed by durable, and in some cases progressive, tumor vascular disruption and necrosis over time. **A**, waterfall plot showing percent decrease in tumor enhancement (perfusion) from baseline over time (day 5, gray bar; Week 8, black bar) for all patients whose tumors showed significant vascular disruption on day 5 ($< 15\%$ reduced enhancement; $n = 5$). **B**, dynamic MRI images at baseline, day 5, and week 14 following intratumoral injection of JX-594 in HCC tumor. **C**, change in critical blood lab values and blood pressure over time in patients showing infection of tumor vasculature after intravenous JX-594 infusion ($n = 4$). **D**, change in critical blood lab values over time in patients showing tumor vascular disruption after intratumoral JX-594 injection ($n = 5$). **E**, wound healing over time following percutaneous insertion of large bore injection needle into liver for 1×10^6 pfu JX-594 injection.

DNA synthesis in proliferating endothelial cells (35); elevated thymidine kinase may therefore contribute to the enhanced replication of JX-594 in activated endothelial cells. Of note,

vaccinia vaccine replication in humans at sites of scarification (localized skin wounding) or eczema may involve proliferating endothelial cells. It is intriguing that JX-594 was able to cause

vascular disruption in an atypical hemangioma, an abnormal growth of vessels in which overproduction of VEGF is implicated (36); this clinical data, together with preclinical tumor model data, suggests that tumor cell proximity may not be necessary. Endothelial cells associated with a hemangioma reportedly secrete high levels of proangiogenic factors, including VEGF, when compared with normal endothelial cells from the same patient (37). Finally, we detected no change in normal liver perfusion or vascularity at similar time points by dynamic MRI.

Further research is necessary to confirm clinical safety in the setting of wound healing, and to elucidate additional mechanisms of vaccinia infection of endothelial cells and associated vascular disruption, as well as methods to augment this feature of JX-594. We are currently exploring several hypotheses to explain the tumor vascular disruption observed. First, many tumors reportedly exist in a hypercoagulable state (38), with tumor cells producing procoagulant factors (39). Proinflammatory cytokines (40) that are activated by vaccinia, as well as other viral infections (e.g., HSV; ref. 41), have been shown to induce tissue factor on endothelial cells. Therefore, vaccinia replication and cytolysis within the vascular endothelium presumably could result in tissue factor upregulation and subsequent blood clotting within infected vessels. In previous mouse studies, we showed that oncolytic VSV specifically induced clot formation within small intratumoral blood vessels (23), as well as neutrophil infiltration and proinflammatory gene expression (22). Inflammation-mediated blockage of blood flow to sites of infection is a known physiologic response to infection (42). In addition, both vascular disrupting agents (such as combretastatin and vinblastine) and vaccinia disrupt the normal cellular cytoskeleton leading to endothelial cell rounding and loss of normal cell-cell contacts; this phenomenon is believed to potentiate vascular collapse (6, 7). *In vitro* findings with JX-594 are consistent with this hypothesis.

While JX-594 has been generally well tolerated in more than 160 treated patients to date, more safety data is needed in relation to potential normal vessel and wound healing toxicities. For example, the safety of JX-594 should be assessed in patients with tumors lining the gastrointestinal or aerodigestive tracts whereas gastrointestinal bleeding can occur with bevacizumab (43); this complication has not been reported with JX-594 to date. In addition, further safety data are needed in the context of normal wound healing, including in the postoperative period. Importantly, impaired healing has not been reported to date at the site of core needle or excisional biopsies taken within 7 to 10 days after JX-594 treatment, or at large bore (18 gauge) needle insertion sites as described here. The risk of these theoretical complications will be assessed as more patients are treated over time.

These results also have implications for combination therapy with JX-594 and approved anticancer agents. For example, it may be important to avoid concomitant administration of VEGFR and/or RGF-2 inhibitors if the vascular targeting effect of JX-594 is dependent on signaling from this pathway. Sequential therapy, in contrast, may be highly

beneficial given the potential for combined antivascular and antiangiogenic effects or sensitization to antiangiogenic treatment due to growth factor rebound. Clinically, profound tumor vascular ablation and subsequent durable tumor necrosis and long-term survival have been induced by JX-594 followed by small-molecule inhibitors of VEGFR in hypervascular HCC and renal cell cancers (both VEGF-rich; ref. 44). Of note, this potential synergy was reported even in patients who had failed previous therapy with sorafenib. Randomized trials will be required to determine whether JX-594 followed by angiogenesis inhibitors is superior to either agent alone.

Future derivatives of JX-594 may be engineered to express multiple therapeutic proteins from tumor-associated blood vessels, including those with antitumor, antivascular, and/or antiangiogenic effects. This opens up the possibility of novel multifunctional products derived from JX-594 that can express high levels of multiple complementary therapeutic proteins in metastatic solid tumors and tumor vasculature in a highly selective fashion.

Disclosure of Potential Conflicts of Interest

C.J. Breitbach is employed (other than primary affiliation; e.g., consulting) as director of clinical research and has ownership interest (including patents) in Jemtex. S.H. Thorne has ownership interest (including patents) and is a consultant/advisory board member of Jemtex Inc. R. Patt is a consultant/advisory board member of RadMD. A. Moon is employed (other than primary affiliation; e.g., consulting) as VP product development and has ownership interest (including patents) in Jemtex Inc. J. Burke is employed (other than primary affiliation; e.g., consulting) as VP Clinical Research and has ownership interest (including patents) in Jemtex Inc. T.-H. Hwang is a consultant/advisory board member of SAR. J.C. Bell is employed (other than primary affiliation; e.g., consulting) as a chief scientific officer, has ownership interest (including patents), and is a consultant/advisory board member of Jemtex Biotechnology. D.H. Kim has a commercial research grant and ownership interest (including patents) from Jemtex Inc.

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References

- Folkman J. Angiogenesis in cancer, rheumatoid and other disease. *Nat Med* 1995;1:27-31.
- Kowanzet M, Ferrara N. Vascular endothelial growth factor signaling pathways: therapeutic perspective. *Clin Cancer Res* 2006;12:5018-22.
- Bergers G, Hanahan D. Modes of resistance to anti-angiogenic therapy. *Nat Rev Cancer* 2008;8:592-603.
- Yoshiji H, Harris SR, Thorgeirsson UP. Vascular endothelial growth factor is essential for initial but not continued *in vivo* growth of human breast carcinoma cells. *Cancer Res* 1997;57:3924-8.
- Ebos JM, Kerkel RL. Antiangiogenic therapy: impact on invasion, disease progression, and metastasis. *Nat Rev Clin Oncol* 2011;8:210-21.
- Tozer GM, Kanthou C, Baguley BC. Disrupting tumour blood vessels. *Nat Rev Cancer* 2005;5:423-35.
- Siemann DW, Chaplin DJ, Walicke PA. A review and update of the current status of the vasculature-disabling agent combretastatin A4 phosphate (CAAP). *Expert Opin Investig Drugs* 2009;18:189-97.
- Mazzone MJ, Maguire HC Jr, Eisenlohr LC, Laughlin CE, Monken CE, McCue PA, et al. Intratumoral recombinant GM-CSF-encoding virus as gene therapy in patients with cutaneous melanoma. *Cancer Gene Ther* 1999;6:409-22.
- Kim JH, Oh JY, Park BH, Lee DE, Kim JS, Park HE, et al. Systemic armed oncolytic and immunologic therapy for cancer with JX-594, a targeted poxvirus expressing GM-CSF. *Mol Ther* 2008;14:361-70.
- Park BH, Hwang T, Liu TC, Sze DY, Kim JS, Kwon HC, et al. Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: a phase I trial. *Lancet Oncol* 2008;9:533-42.
- Breitbach CJ, Burles J, Jonier D, Stephenson J, Haas AR, Chow LQ, et al. Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. *Nature* 2011;477:99-102.
- Katsanas GC, Moss B. Vaccinia virus intermediate stage transcription is complemented by Ras-GTPase-activating protein SH3 domain-binding protein (G3BP) and cytoplasmic activation/proliferation-associated protein (p137) individually or as a heterodimer. *J Biol Chem* 2004;279:52210-7.
- Yang H, Kim SK, Kim M, Rache PA, Morehead TJ, Damon IK, et al. Antiviral chemotherapy facilitates control of poxvirus infections through inhibition of cellular signal transduction. *J Clin Invest* 2005;115:379-87.
- Buller RM, Chakrabarti S, Moss B, Fredrickson T. Cell proliferative response to vaccinia virus is mediated by VGF. *Virology* 1988;164:182-92.
- Buller RM, Chakrabarti S, Cooper JA, Twardzik DR, Moss B. Deletion of the vaccinia virus growth factor gene reduces virus virulence. *J Virol* 1986;62:866-74.
- Kim DH, Thorne SH. Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer. *Nat Rev Cancer* 2009;9:64-71.
- Buller RM, Smith GL, Greiner K, Nodkins AL, Moss B. Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. *Nature* 1985;317:813-5.
- Alcami A, Symons JA, Smith GL. The vaccinia virus soluble alpha/beta interferon (IFN) receptor binds to the cell surface and protects cells from the antiviral effects of IFN. *J Virol* 2000;74:11230-9.
- Tortora G, Ciardiello F, Gasparini G. Combined targeting of EGFR-dependent and VEGF-dependent pathways: rationale, preclinical studies and clinical applications. *Nat Clin Pract Oncol* 2008;5:521-30.
- Kim DH, Wang Y, Le Boeuf F, Bell J, Thorne SH. Targeting of interferon-beta to produce a specific, multi-mechanistic oncolytic vaccinia virus. *PLoS Med* 2007;4:e853.
- Liu TC, Hwang T, Park BH, Bell J, Kim DH. The targeted oncolytic poxvirus JX-594 demonstrates antitumoral, antivascular, and anti-HBV activities in patients with hepatocellular carcinoma. *Mol Ther* 2008;16:1637-42.
- Breitbach CJ, Paterson JM, Lemay CG, Falls TJ, McGuire A, Parato KA, et al. Targeted inflammation during oncolytic virus therapy severely compromises tumor blood flow. *Mol Ther* 2007;15:1686-93.
- Breitbach CJ, De Silva NS, Falls TJ, Aladi U, Evgin I, Paterson J, et al. Targeting tumor vasculature with an oncolytic virus. *Mol Ther* 2011;19:886-94.
- Kottke T, Hall G, Pulido J, Diaz RM, Thompson J, Chong H, et al. Antiangiogenic cancer therapy combined with oncolytic virotherapy leads to regression of established tumors in mice. *J Clin Invest* 2010;120:1551-60.
- Yamaguchi R, Yano H, Iemura A, Ogasawara S, Hiramaki M, Kojima M. Expression of vascular endothelial growth factor in human hepatocellular carcinoma. *Hepatology* 1998;28:68-77.
- Vultur A, Cao J, Arulanandam R, Turkoen J, Jove R, Greer P, et al. Cell-to-cell adhesion modulates Stat3 activity in normal and breast carcinoma cells. *Oncogene* 2004;23:2800-16.
- Casanovas O, Hicklin DJ, Bergers G, Hanahan D. Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. *Cancer Cell* 2005;8:299-309.
- Lassau N, Kosdiny S, Opolon P, De Baere T, Peronneau P, Leclerc J, et al. Evaluation of contrast-enhanced color Doppler ultrasound for the quantification of angiogenesis *in vivo*. *Invest Radiol* 2001;36:50-5.
- Dvorak HF, Gresser I. Microvascular injury in pathogenesis of interferon-induced necrosis of subcutaneous tumors in mice. *J Natl Cancer Inst* 1989;81:497-502.
- Dinehart SM, Khannan J, Gronemus R. Hemangiomas: evaluation and treatment. *Dermatol Surg* 2001;27:475-85.
- Malland ML, Balers GL, Black HR, Chen HX, Durand JB, Elliott WJ, et al. Initial assessment, surveillance, and management of blood pressure in patients receiving vascular endothelial growth factor signaling pathway inhibitors. *J Natl Cancer Inst* 2010;102:596-604.
- Kim SJ, Uehara H, Karashima T, Shepherd DL, Killion JJ, Fidler IJ. Blockade of epidermal growth factor receptor signaling in tumor cells and tumor-associated endothelial cells for therapy of androgen-independent human prostate cancer growing in the bone of nude mice. *Clin Cancer Res* 2003;9:1200-10.
- Cascone T, Haryk MH, Xu L, Du Z, Kadara H, Nilsson MB, et al. Upregulated stromal EGFR and vascular remodeling in mouse xenograft models of angiogenesis inhibitor-resistant human lung adenocarcinoma. *J Clin Invest* 2011;121:1313-28.
- Parato KA, Breitbach CJ, Le Boeuf F, Wang J, Storbeck G, Ilow C, et al. The oncolytic poxvirus JX-594 selectively replicates in and destroys cancer cells driven by genetic pathways commonly activated in cancers. *Mol Ther* 2012;20:740-58.
- Junod AF, Petersen H, Jomot L. Thymidine kinase, thymidylate synthase, and endothelial cell growth under hypoxia. *J Appl Physiol* 1987;62:10-4.
- Carmeliet P. Angiogenesis in health and disease. *Nat Med* 2003;9:653-60.
- Zhang WJ, Ye LY, Wu LQ, Xin YL, Gu F, Niu JX, et al. Morphologic, phenotypic and functional characteristics of endothelial cells derived from human hepatic cavernous hemangioma. *J Vasc Res* 2006;43:522-32.
- Caine GJ, Stonelake PS, Lip GY, Kehoe ST. The hypercoagulable state of malignancy: pathogenesis and current debate. *Neoplasia* 2002;4:465-73.
- Dvorak HF, Rickles FR. Malignancy and hemostasis. In: Colman RW, Clowes AW, George JN, Goldhaber SZ, Marder VJ, editors. Hemostasis and thrombosis. 5th edition. Philadelphia, PA: Lippincott Williams & Wilkins; 2005. p. 851-73.
- Nawroth PP, Stern DM. Modulation of endothelial cell hemostatic properties by tumor necrosis factor. *J Exp Med* 1986;163:740-5.
- Key NS, Vercellotti GM, Winklerman JQ, Midow CF, Goodman JL, Emon NL, et al. Infection of vascular endothelial cells with herpes simplex virus enhances tissue factor activity and reduces thrombomodulin expression. *Proc Natl Acad Sci U S A* 1990;87:7095-9.
- Bryant AE, Bayer CR, Chen RY, Guth PH, Wallace RJ, Stevens DL. Vascular dysfunction and ischemic destruction of tissue in *Streptococcus pyogenes* infection: the role of streptolysin O-induced platelet/neutrophil complexes. *J Infect Dis* 2005;192:1014-22.
- Chen HX, Cleck JN. Adverse effects of anticancer agents that target the VEGF pathway. *Nat Rev Clin Oncol* 2008;4:665-77.
- Hao J, Breitbach CJ, Moon A, Kim CW, Platt R, Kim MK, et al. Sequential therapy with JX-594, a targeted oncolytic poxvirus, followed by sorafenib in hepatocellular carcinoma: preclinical and clinical demonstration of combination efficacy. *Mol Ther* 2011;19:1170-9.

APPENDIX II: RESISTANCE TO TWO HETEROLOGOUS NEUROTROPIC ONCOLYTIC VIRUSES, SEMLIKI FOREST VIRUS AND VACCINIA VIRUS, IN EXPERIMENTAL GLIOMA.

Vähä-Koskela MJ, Le Boeuf F, Lemay C, De Silva N, Diallo JS, Cox J, Becker M, Choi Y, Ananth A, Sellers C, Breton S, Roy D, Falls T, Brun J, Hemminki A, Hinkkanen A, Bell JC.

Contribution: I performed immunohistochemical analysis of brain tumours that had been treated with heterologous oncolytic viruses.

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Resistance to Two Heterologous Neurotropic Oncolytic Viruses, Semliki Forest Virus and Vaccinia Virus, in Experimental Glioma

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Attenuated Semliki Forest virus (SFV) may be suitable for targeting malignant glioma due to its natural neurotropism, but its replication in brain tumor cells may be restricted by innate antiviral defenses. We attempted to facilitate SFV replication in glioma cells by combining it with vaccinia virus, which is capable of antagonizing such defenses. Surprisingly, we found parenchymal mouse brain tumors to be refractory to both viruses. Also, vaccinia virus appears to be sensitive to SFV-induced antiviral interference.

Brain tumors are particularly life-threatening due to their sensitive anatomical location. Recently, temozolomide plus radiotherapy has provided a measurable survival benefit to a subset of patients (1), but more effective therapies are still needed. In this regard, oncolytic viruses (OVs) seem particularly promising, as they display higher tumor specificity and possibly fewer side effects than standard therapies (2). One of our OV candidates, attenuated Semliki Forest virus (SFV), was able to fully eradicate orthotopic U87 xenografts in 100% of treated nude mice following a single systemic injection (3). However, in other models, we and others have identified limitations to oncolytic virotherapy; in particular, innate antiviral defenses limit virus replication in tumor cells (4, 5).

In order to probe further and overcome the mechanisms of glioma resistance to oncolytic SFV, we combined it with oncolytic vaccinia virus (VV), which itself has shown promise in glioma targeting and also has the capacity to facilitate replication of type I interferon (IFN)-sensitive OVs by antagonizing innate antiviral defenses (6–8). First, we demonstrate efficient killing of DBT mouse glioma cells *in vitro* but not *in vivo* by SFV alone, mirroring the results we observed in our previous rat model (4) (Fig. 1A and B). Lack of efficacy could not be explained simply by the immune competence of the animals, as SFV successfully eradicated another type of syngeneic tumor (CT26LacZ) at a similar dose (not shown). Next, we observed that SFV limits its own spread in DBT cells under spatially restrictive conditions (under agarose) and that this limitation could be lifted by coinfecting cells with VV or neutralizing type I IFN using polyclonal antibody (anti-beta interferon [IFN-β]) or recombinant vaccinia virus-encoded B18R protein (Fig. 1C). Facilitation of SFV spread in DBT cells by VV is dependent on B18R, as we did not see enhancement when B18R-deleted VV was used. While the spread of SFV under agarose was enhanced when SFV was combined with VV, replication of VV itself was strongly inhibited (Fig. 1C), which was also confirmed by quantifying virus output from coinfecting DBT cells in free culture (Fig. 1D). DBT cell killing by the virus combinations was synergistic, as calculated by the Chou-Talalay method (Fig. 1E). However, combination of SFV with VV in an orthotopic DBT model did not provide statistically significant improvement in

survival compared to the next best therapy, VV alone, by either systemic injection or direct intracranial administration or when viruses were coinjected or given 48 h apart ($P < 0.0729$, log rank test) (Fig. 1F and G). Moreover, systemic delivery of the viruses resulted in one mouse of five displaying hind leg paralysis in two separate experiments. Following intravenous administration, VV is known to reduce the ability of plasmacytoid dendritic cells to secrete type I interferon, thereby increasing systemic infection levels and pathogenesis of at least lymphocytic choriomeningitis virus and Pichinde virus (9, 10).

In order to understand whether the difference in treatment efficacy between coinjected and 48-h-sequenced treatment groups (VV-SFV versus VV-48-h-SFV, $P < 0.0037$, log rank test) (Fig. 1G) was due to inhibition of VV replication by SFV or due to enhancement of SFV by VV, we tracked virus replication in the brains of tumor-bearing animals using luciferase-encoding viruses. Correlating with survival data and arguing for both mechanisms, replication of intracranially injected SFV was enhanced by VV but only when VV was given 48 h before SFV, and, conversely, VV replication was dramatically inhibited only when VV was coinjected with SFV (Fig. 2A). However, since heterologous virus interference did not occur when VV was given 48 h prior to SFV, we still needed to find an explanation for the overall lack of therapeutic efficacy of the combination in the intracranial DBT model. Subsequently, upon immunohistochemical examination of the brains of tumor-bearing virus-treated mice using virus-specific polyclonal rabbit antibodies, we observed a lack of infection of intraparenchymal DBT tumors by either virus (Fig. 2B). This demonstrated that when growing in the brain, DBT cells acquire resistance to both type I IFN-sensitive and IFN-insensitive

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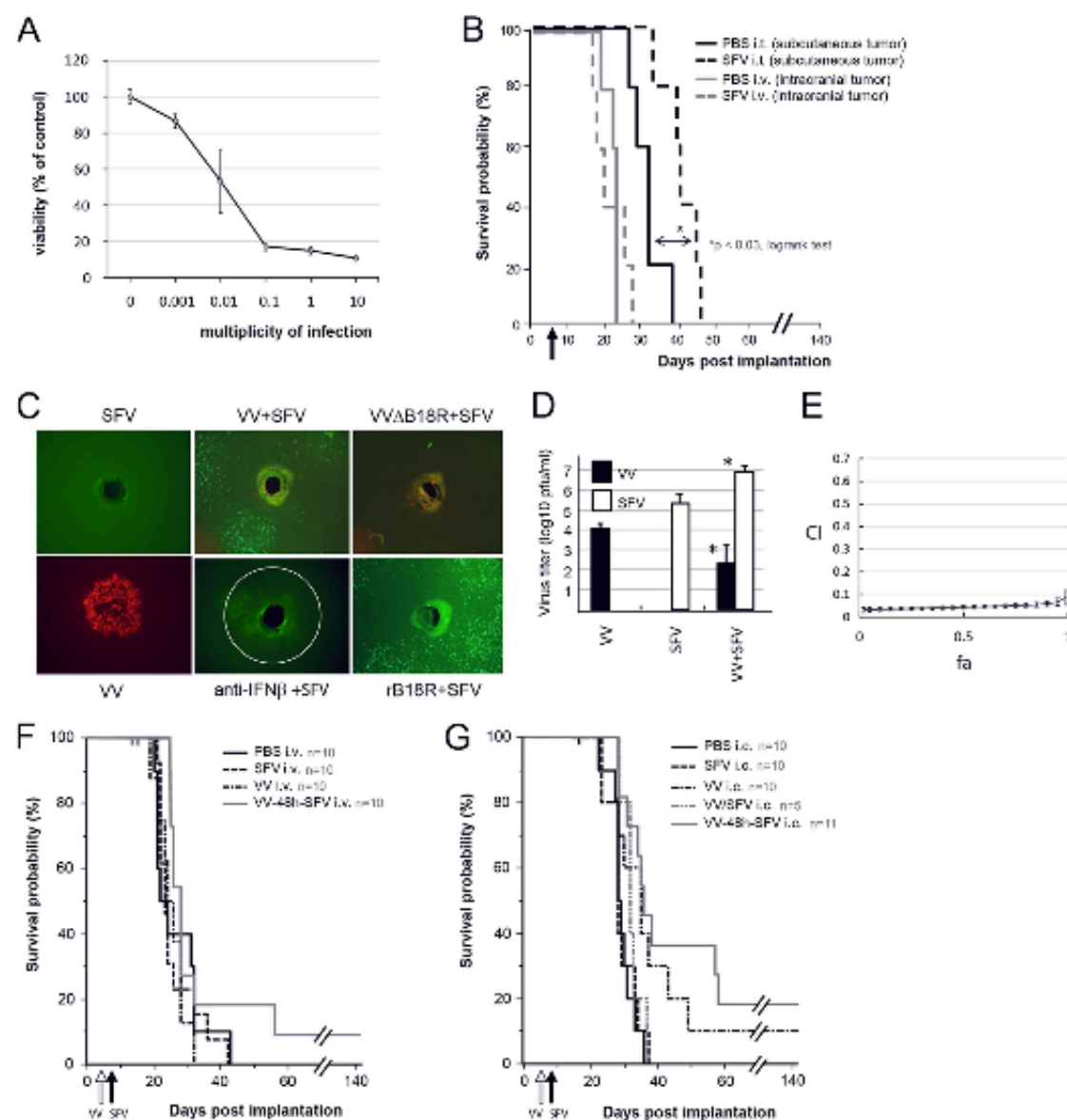


FIG 1 Vaccinia virus facilitates replication of Semliki Forest virus in mouse glioma cells in culture but not *in vivo*. (A) Dose-dependent killing of BALB/c mouse DBT glioma cells by SFV *in vitro*, assessed by alamarBlue measurement from triplicate wells 48 h postinfection. (B) Apart from a transient delay in subcutaneous DBT tumor growth upon a single intratumoral injection of 10^6 PFU SFV, efficacy of SFV in the DBT model is lacking. (C) Plaque expansion assay (20) in DBT cell monolayers in 6-well plates showed that while SFV is unable to spread on its own under agarose, VV coinfection as well as VV-encoded soluble type I interferon scavenger protein B18R (0.1 μ g per well; eBioscience, San Diego, CA) and neutralizing polyclonal IFN- β antibody (peripheral blood lymphocyte interferon source) can overcome this restriction, confirming that type I IFN may limit SFV replication in DBT cells. (D) Plaque assay of infected DBT cell culture supernatants (VV MOI, 0.1; SFV MOI, 0.01) shows that while SFV replication is enhanced in DBT cells when SFV is combined with VV at 48 h p.i., replication of VV is significantly inhibited in the presence of SFV (*, $P < 0.02$ [Mann-Whitney U test]). (E) SFV and VV synergize in cell killing, as measured 72 h postinfection in DBT cells infected with various ratios of VV to SFV. The plot represents the algebraic estimate of the combination index (CI) as a function of the fraction of cells affected (fa) $\pm$ the standard deviation, where a CI of < 0.7 is considered synergistic. (F) A single systemic injection of 10^6 PFU of VV followed 48 h later by SFV into mice harboring intracranial (day 6 postimplantation) DBT tumors showed a trend toward increased survival compared to the next treatment, VV alone ($P < 0.0729$ [log rank test]). One mouse in each of two experiments in the combination group displayed hind leg paralysis and was promptly sacrificed (censored). (G) Survival of mice treated intracranially with 10^6 PFU virus (sum of two experiments). While the results for the sequential (VV-48 h-SFV) combination were statistically different from those for the VV-SFV-coinjected group and the group receiving SFV alone ($P < 0.0132$ [log rank test]), they did not differ statistically from those for the group receiving VV alone, which in turn did not differ from those for any other group. Censored animals are shown as small vertical bars along the curves (F and G, top).

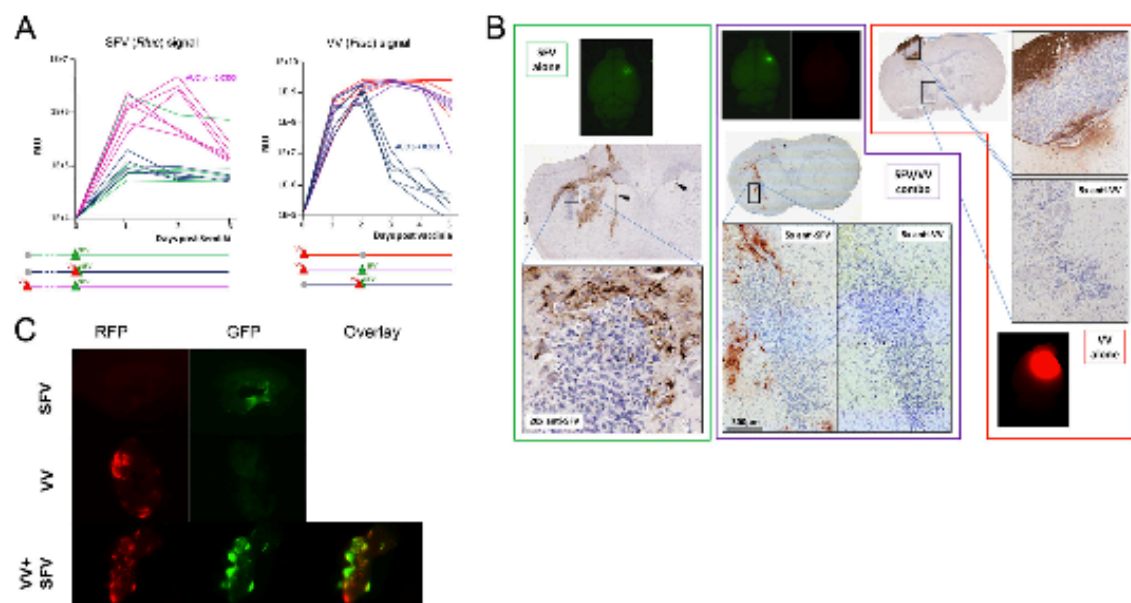


FIG 2 Mouse glioma growing in the brain is not permissive to oncolytic SFV or VV. (A) Day 6 DBT tumor-bearing mice were injected intracranially with *Renilla* luciferase-expressing SFV, with firefly luciferase-expressing VV, or with both viruses given either in the same injection or 48 h apart; virus replication was tracked by the IVIS *in vivo* imaging system over 5 days (72 h after the last administration of virus). Results show transient (up to 2 days) enhancement of SFV signal in mice that were sequentially treated (48 h apart) but not in those that were coinjected. Conversely, VV replication is strongly inhibited by simultaneous coadministration of SFV but not when SFV is given 48 h after VV. (B) Immunohistochemical analysis of brains sampled 24 h postinfection shows absence of infection of DBT tumor tissue deep in the brain by either SFV or VV or their mixture but prominent infection of healthy brain by SFV. VV is able to infect only DBT tumor tissue growing outside the brain parenchyma. (C) In contrast to the results obtained *in vivo*, SFV is able to infect DBT brain tumor explants and VV is able to enhance SFV replication in them. Tumor pieces were infected with 10^7 PFU of each virus immediately upon extraction from the brain and visualized 24 h postinfection.

viruses, suggesting broad-spectrum IFN-independent mechanisms of antiviral resistance. Our results also suggest that virus-expressed luminescence (Fig. 2A) originates from infected normal cells in the case of SFV and from tumor tissue growing outside the brain parenchyma in the case of VV (Fig. 2B).

As DBT cells are permissive to both SFV and VV *in vitro*, we wanted to explore the stability of the brain-dependent virus resistance; therefore, we extracted DBT tumor tissue from the brains of tumor-bearing mice and infected it *ex vivo*. We observed infection of explanted tumor tissue by each virus alone as well as facilitation of SFV replication when SFV was combined with VV (Fig. 2C), mirroring our findings in cultured DBT cells (Fig. 1C and D) and corroborating the idea that DBT cells acquire reversible and context-dependent antiviral resistance when implanted and growing in the brain. In line with our findings, a recent collaborative report showed that GL261 mouse glioma cells preinfected with SFV at very high MOI *in vitro* (and thus destined to succumb to the infection) formed intracranial tumors with an efficiency similar to that of uninfected glioma cells (5).

Both tumor stroma (11) and the brain microenvironment (12) significantly modulate global gene expression pathways in glioma cells, making them more resistant to a variety of challenges *in vivo* than *in vitro*. Such mechanisms may not only hinder oncolytic SFV or VV but also underlie the previously reported reduced replication of oncolytic adenovirus in intracranial gliomas compared to cultured glioma cells (13). While reducing type I IFN-mediated

antiviral defenses has been shown to enhance replication and oncolytic efficacy of several other OV in other tumor models (14–17), our paper underscores the fact that such strategies may not automatically translate into better therapy and may be hazardous if not controlled precisely. Fortunately, glioma resistance to OV may be context dependent and reversible, which opens the possibility of developing specific countermeasures. Also, combination therapy of cancer with heterologous viruses, even with SFV and VV, is still an appealing concept, as safety may be increased by using replication-defective vectors or vectors carrying microRNA targets or by employing prime-boost-type vaccination regimens where virus injections are temporally separated and do not allow uncontrolled virus enhancement (18, 19).

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John Bell is cofounder of Jennerex Biotherapeutics and sits on the board of directors. Akseli Hemminki is cofounder and shareholder of Oncos Therapeutics.

REFERENCES

1. Stupp R, Hegi M, Weller M. 2010. Neuro-oncology, a decade of temozolomide and beyond. *Expert. Rev. Anticancer Ther.* 10:1675–1677.
2. Guo ZS, Thorne SH, Bartlett DL. 2008. Oncolytic virotherapy: molecular targets in tumor-selective replication and carrier cell-mediated delivery of oncolytic viruses. *Biochim. Biophys. Acta* 1785:217–231.
3. Heikkilä JE, Vähä-Koskela MJ, Ruotsalainen JJ, Martikainen MW, Stanford MM, McCart JA, Bell JC, Hinkkanen AE. 2010. Intravenously administered alphavirus vector VA7 eradicates orthotopic human glioma xenografts in nude mice. *PLoS One* 5:e8603. doi:10.1371/journal.pone.0008603.
4. Mäntä AM, Liimatainen T, Wahlfors T, Wirth T, Vähä-Koskela M, Jansson L, Valonen P, Hakkinen K, Rautsi O, Pellinen R, Mäkinen K, Hakumäki J, Hinkkanen A, Wahlfors J. 2007. Evaluation of cancer virotherapy with attenuated replicative Semliki forest virus in different rodent tumor models. *Int. J. Cancer* 121:863–870.
5. Ruotsalainen J, Martikainen M, Niittykoski M, Huhtala T, Aaltonen T, Heikkilä J, Bell J, Vähä-Koskela M, Hinkkanen A. 2012. Interferon-beta sensitivity of tumor cells correlates with poor response to VA7 virotherapy in mouse glioma models. *Mol. Ther.* 20:1529–1539.
6. Le Boeuf F, Diallo JS, McCart JA, Thorne S, Falls T, Stanford M, Kanji F, Auer R, Brown CW, Lichty BD, Parato K, Atkins H, Kirn D, Bell JC. 2010. Synergistic interaction between oncolytic viruses augments tumor killing. *Mol. Ther.* 18:888–895.
7. Le Boeuf F, Bell JC. 2010. United virus: the oncolytic tag-team against cancer! *Cytokine Growth Factor Rev.* 21:205–211.
8. Lun XQ, Jang JH, Tang N, Deng H, Head R, Bell JC, Stojdl DF, Nutt CL, Senger DL, Forsyth PA, McCart JA. 2009. Efficacy of systemically administered oncolytic vaccinia virotherapy for malignant gliomas is enhanced by combination therapy with rapamycin or cyclophosphamide. *Clin. Cancer Res.* 15:2777–2788.
9. Welsh RM, Che JW, Brehm MA, Selin LK. 2010. Heterologous immunity between viruses. *Immunol. Rev.* 235:244–266.
10. Zuniga EI, Liou LY, Mack L, Mendoza M, Oldstone MB. 2008. Persistent virus infection inhibits type I interferon production by plasmacytoid dendritic cells to facilitate opportunistic infections. *Cell Host Microbe* 4:374–386.
11. McMillin DW, Delmore J, Weisberg E, Negri JM, Geer DC, Klippel S, Mitsiades N, Schlossman RL, Munshi NC, Kung AL, Griffin JD, Richardson PG, Anderson KC, Mitsiades CS. 2010. Tumor cell-specific bioluminescence platform to identify stroma-induced changes to anticancer drug activity. *Nat. Med.* 16:483–489.
12. Camphausen K, Puro B, Sproull M, Scott T, Ozawa T, Deen DF, Tofflon PJ. 2005. Influence of in vivo growth on human glioma cell line gene expression: convergent profiles under orthotopic conditions. *Proc. Natl. Acad. Sci. U. S. A.* 102:8287–8292.
13. Lamfers ML, Idema S, Bosscher I, Heukelom S, Moeniralm S, van der Meulen-Muileman III, Overmeer RM, van d van Beusechem VVW, Gerritsen WR, Vandertop WP, Dirven CM. 2007. Differential effects of combined Ad5- delta 24RGD and radiation therapy in in vitro versus in vivo models of malignant glioma. *Clin. Cancer Res.* 13:7451–7458.
14. Huang PY, Guo JH, Hwang LH. 2012. Oncolytic Sindbis virus targets tumors defective in the interferon response and induces significant bystander antitumor immunity in vivo. *Mol. Ther.* 20:298–305.
15. Krishnamurthy S, Takimoto T, Scroggs RA, Portner A. 2006. Differentially regulated interferon response determines the outcome of Newcastle disease virus infection in normal and tumor cell lines. *J. Virol.* 80:5145–5155.
16. Naik S, Russell SJ. 2009. Engineering oncolytic viruses to exploit tumor specific defects in innate immune signaling pathways. *Expert. Opin. Biol. Ther.* 9:1163–1176.
17. Paglino JC, van den Pol AN. 2011. Vesicular stomatitis virus has extensive oncolytic activity against human sarcomas: rare resistance is overcome by blocking interferon pathways. *J. Virol.* 85:9346–9358.
18. Näslund TI, Uytendhoeve C, Nordström EK, Colau D, Warnier G, Jondal M, Van den Eynde BJ, Liljestrom P. 2007. Comparative prime-boost vaccinations using Semliki Forest virus, adenovirus, and ALVAC vectors demonstrate differences in the generation of a protective central memory CTL response against the P815 tumor. *J. Immunol.* 178:6761–6769.
19. Zhang YQ, Tsai YC, Monie A, Wu TC, Hung CF. 2010. Enhancing the therapeutic effect against ovarian cancer through a combination of viral oncolysis and antigen-specific immunotherapy. *Mol. Ther.* 18:692–699.
20. Duca KA, Lam V, Keren I, Endler EE, Letchworth GJ, Novella IS, Yin J. 2001. Quantifying viral propagation in vitro: toward a method for characterization of complex phenotypes. *Biotechnol. Prog.* 17:1156–1165.

APPENDIX III: INTRAVENOUS DELIVERY OF A MULTI-MECHANISTIC CANCER-TARGETED ONCOLYTIC POXVIRUS IN HUMANS.

Breitbach CJ, Burke J, Jonker D, Stephenson J, Haas AR, Chow LQ, Nieva J, Hwang TH, Moon A, Patt R, Pelusio A, Le Boeuf F, Burns J, Evgin L, De Silva N, Cvancic S, Robertson T, Je JE, Lee YS, Parato K, Diallo JS, Fenster A, Daneshmand M, Bell JC, Kirn DH.

Contribution: I developed standard operating procedures for staining patient tumours for vaccinia and β -galactosidase. I stained patient biopsies and analyzed them with Caroline Breitbach and Manijeh Daneshmand. I generated a 3D model of patient biopsy with the help of Sara Cvancic.

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Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans

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The efficacy and safety of biological molecules in cancer therapy, such as peptides and small interfering RNAs (siRNAs), could be markedly increased if high concentrations could be achieved and amplified selectively in tumour tissues versus normal tissues after intravenous administration. This has not been achievable so far in humans. We hypothesized that a poxvirus, which evolved for blood-borne systemic spread in mammals, could be engineered for cancer-selective replication and used as a vehicle for the intravenous delivery and expression of transgenes in tumours. JX-594 is an oncolytic poxvirus engineered for replication, transgene expression and amplification in cancer cells harbouring activation of the epidermal growth factor receptor (EGFR)/Ras pathway, followed by cell lysis and anticancer immunity¹. Here we show in a clinical trial that JX-594 selectively infects, replicates and expresses transgene products in cancer tissue after intravenous infusion, in a dose-related fashion. Normal tissues were not affected clinically. This platform technology opens up the possibility of multifunctional products that selectively express high concentrations of several complementary therapeutic and imaging molecules in metastatic solid tumours in humans.

Despite recent advances in cancer treatment, truly innovative approaches are required to move beyond the modest benefits achieved to date. One novel strategy is the use of replication-competent oncolytic viruses that selectively infect tumours^{2,3}. Vaccinia and other poxviruses have several biological properties that make them ideally suited for intravenous delivery and subsequent amplification of transgenes within tumours¹. First, vaccinia has evolved mechanisms for intravenous stability and spread to distant tissues, including resistance to antibody- and complement-mediated neutralization in the blood^{4,5}. Second, vaccinia has also evolved for rapid and motile spread within tissues^{6,7}. Because of their relatively large size, vaccinia virions may preferentially deposit in tumours, where neovasculature has increased permeability. Finally, the replication of vaccinia virus is dependent on EGFR/Ras pathway signalling^{8,9} which is commonly activated in epithelial cancers¹⁰.

JX-594 is a Wyeth strain vaccinia-vaccine-derived oncolytic virus engineered for viral thymidine kinase gene inactivation, and expression of transgenes encoding human granulocyte-macrophage colony stimulating factor (GM-CSF, encoded by *CSF2*) and β -galactosidase (β -gal, encoded by *lacZ*) under control of the synthetic early-late and p7.5 promoters, respectively^{11,12}. Selective replication of the virus in cancer cells is driven by cellular EGFR/Ras pathway signalling, thymidine kinase elevation and type-1 interferon resistance^{13,14}. In a Phase 1 trial of intratumoural injection into liver tumours, JX-594 was well-tolerated and associated with replication, expression of biologically active hGM-CSF and tumour destruction¹⁵.

The clinical trial described herein was designed to test whether JX-594 could infect metastatic tumours after intravenous infusion in patients. First, we assessed JX-594 selectivity for tumour tissue after *ex vivo* infection of paired explants of viable tumour and adjacent normal tissue obtained from patients undergoing surgery. Within 24 h of exposure, JX-594 was able to infect tumour tissue selectively in seven of ten samples: most tumours had high-intensity staining whereas normal tissues did not (Fig. 1). Peripheral blood mononuclear cells were also highly resistant to infection (data not shown).

We subsequently performed a Phase 1 dose-escalation trial of a single intravenous infusion of JX-594 in 23 patients with advanced, treatment-refractory solid tumours (Table 1). Patients were treated in one of six dose cohorts (1×10^5 to 3×10^7 plaque-forming units (p.f.u.) kg^{-1}). JX-594 delivery, gene expression and replication in solid tumours were assessed. Safety (including determination of the maximum tolerated dose (MTD) or maximum feasible dose (MFD)), pharmacokinetics and antitumour activity were also evaluated.

For pharmacokinetic analyses, quantitative PCR (qPCR) was used to measure genome concentrations in blood during the 1 h of intravenous

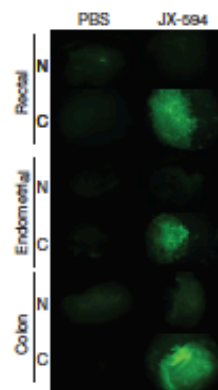


Figure 1 | *Ex vivo* infection of explants of tumour and normal tissue from patients reveals tumour-selective JX-594 gene expression. JX-594 expressing green fluorescent protein (GFP) (JX-594-GFP⁺/β-gal⁻) was used to infect primary live-tissue specimens from cancer patients undergoing surgical resection. Matched tumour and adjacent normal tissues were infected overnight to assess the selectivity of transgene expression and replication, or were treated with PBS as a negative control. GFP expression from JX-594-GFP⁺/β-gal⁻-infected cells was assessed using a fluorescence microscope. N = normal tissue, C = cancer tissue.

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Table 1 | Overview of patient characteristics, JX-594 delivery to tumours and antitumour activity

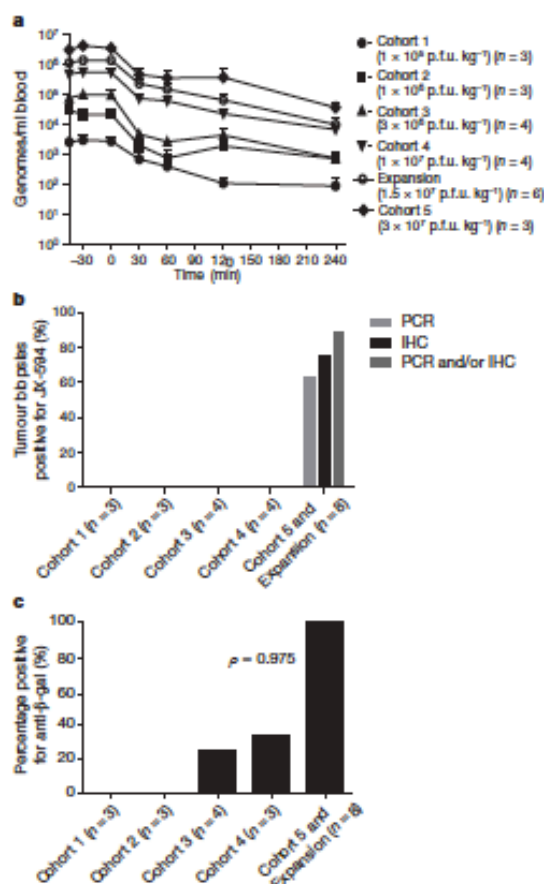
Pt	Tumour type	Patient characteristics						JX-594 delivery, replication				Anti-tumour activity		
		Dose (p.f.u. kg ⁻¹)	Age (yrs)	Gender	Previous lines of therapy	Metastatic tumour sites	Sum LD (cm)	BL NAb	Anti-β-gal (day 29)	Biopsy (day 8–10) IHC	Biopsy (day 8–10) PCR	DC* (RECIST)	DC Duration	Mod. Choi
01	Lung (NSCLC)	1 × 10 ⁵	64	F	2	6	16.2	NEG	NEG	NEG	NEG	No (PD)	NA	NR
02	Colorectal	1 × 10 ⁵	72	M	5	3	14.7	POS	NEG	NEG	NEG	No (PD)	NA	NR
03	Lung (NSCLC)	1 × 10 ⁵	59	F	5	4	3.1	NEG	NEG	NEG	NEG	Yes (SD)	>10 weeks	NR
04	Colorectal	1 × 10 ⁵	80	F	0	6	22.2	NEG	NEG	NEG	NEG	No (PD)	NA	NR
05	Melanoma	1 × 10 ⁵	76	M	1	9	26.5	NEG	NEG	NEG	NEG	No (PD)	NA	ND
06	Thyroid	1 × 10 ⁵	66	M	2	1	3.2	POS	NEG	NEG	NEG	Yes (SD)	>4 weeks	NR
07	Lung (NSCLC)	3 × 10 ⁵	67	F	4	6	10	POS	NEG	NEG	NEG	Yes (SD)	>4 weeks	NR
08	Pancreatic	3 × 10 ⁵	75	F	3	4	7.1	NEG	POS	NEG	NEG	No (PD)	NA	R
09	Melanoma	3 × 10 ⁵	52	M	3	5	13	POS	NEG	NEG	NEG	Yes (SD)	4 weeks	R
10	Colorectal	3 × 10 ⁵	64	M	2	1	5.4	NEG	NEG	NEG	NEG	Yes (SD)	>10 weeks	NR
11	Ovarian	1 × 10 ⁷	60	F	5	4	14.4	NEG	NEG	NEG	NEG	Yes (SD)	>4 weeks	NR
12	Melanoma	1 × 10 ⁷	60	M	4	3	37.1	NEG	ND	NEG	NEG	ND	ND	ND
13	Lung (NSCLC)	1 × 10 ⁷	67	F	3	6	3.4	POS	NEG	NEG	NEG	Yes (SD)	10 weeks	R
15	Gastric	1 × 10 ⁷	61	F	5	5	11	NEG	POS	NEG	NEG	No (PD)	NA	R
14	Melanoma	3 × 10 ⁷	79	F	3	6	15.1	NEG	POS	NEG	NEG	Yes (SD)	>4 weeks	ND
16	Lipomyosarcoma	3 × 10 ⁷	55	F	3	6	17	NEG	POS	POS	NEG	Yes (SD)	>16 weeks	NR
17	Lung (NSCLC)	3 × 10 ⁷	43	M	3	4	13.5	NEG	POS	POS	POS	No (PD)	NA	ND
18	Ovarian	1.5 × 10 ⁷ †	61	F	7	5	13	NEG	POS	POS	POS	Yes (SD)	>16 weeks	ND
20	Colorectal	1.5 × 10 ⁷	67	F	3	8	22	NEG	POS	POS	POS	No (PD)	NA	R
21	Colorectal	1.5 × 10 ⁷	57	F	2	7	17.8	NEG	POS	POS	POS	Yes (SD)	4 weeks	ND
22	Mesothelioma	1.5 × 10 ⁷	68	F	2	4	12	NEG	POS	NEG	POS	Yes (PR)†	>10 weeks	ND
23	Colorectal	1.5 × 10 ⁷	68	M	3	3	7.8	POS	POS	POS	NEG	Yes (SD)	4 weeks	ND

Patient 19 was not evaluable. Anti-β-gal, antibody development to the product of the β-galactosidase marker transgene between baseline and day 29; BL, baseline; DC, disease control; LD, longest diameter of tumour; Mod. Choi, modified Choi response; NA, not applicable; NAb, neutralizing antibody to JX-594 (due to previous vaccine vaccination); ND, not determined; NEG, negative; NR, no response; NSCLC, non-small-cell lung cancer; PD, progressive disease; POS, positive; PR, partial response; Pt, Patient number; R, response; SD, stable disease.

* Stable disease or partial response by RECIST criteria at week 4 and/or 10.

† Approximate dosage based on 65.7 kg weight (patients were treated at 1 × 10⁸ p.f.u. (fixed dose)).

‡ Partial response determined according to modified RECIST for mesothelioma²².



infusion and 4 h afterwards. The highest concentrations were detected during infusion. At doses $\geq 10^7$ p.f.u. kg⁻¹ ($\geq 5 \times 10^8$ p.f.u. dose⁻¹), genomes were still detectable in blood at 4 h (Fig. 2a). Peak concentrations and area-under-the-curve were both dose-related. JX-594 infectious units were also detected during intravenous infusion and at 4 h in high-dose patients (data not shown).

JX-594 was generally well tolerated. Dose escalation proceeded without dose-limiting toxicities. Therefore, more than 3×10^7 p.f.u. kg⁻¹ (approximately 2×10^9 p.f.u. dose⁻¹) was the MFD; a fixed dose of 1×10^9 p.f.u. was administered to patients in the expansion cohort ($n = 5$ evaluable patients). The most common treatment-related adverse events were grade 1–2 flu-like symptoms lasting up to 24 h: fever (78%), chills (56%), fatigue, headache, nausea, hypotension (22% each), vomiting (17%), tachycardia, hypertension, anorexia and myalgia (13% each). A single grade-1 skin pustule was noted in each of two patients one week after infusion and resolved without sequelae. Levels of interferon (IFN)- γ , tumour necrosis factor (TNF)- α and interleukin (IL)-6 increased acutely in a dose-dependent manner (peak, 8 h; resolution, day 4); levels of IL-10 increased on days 4–8. In contrast, IL-1 did not change significantly and IL-4 decreased transiently (data not shown). Neutralizing antibodies to vaccinia were detectable in six patients at baseline, and titres increased by day 15 in all high-dose patients. No correlation was demonstrated between antibody titres (baseline or induced) and JX-594 replication, safety or antitumour activity. Shedding to the environment was assessed (Supplementary Discussion).

Cancer-selective and dose-related JX-594 delivery and replication in tumours were demonstrated in biopsies obtained 8–10 days after infusion (Fig. 2b and Table 1). Of patients treated at $\geq 1.5 \times 10^7$ p.f.u. kg⁻¹

Figure 2 | JX-594 is selectively delivered to, and amplified within, tumours after intravenous infusion. **a**, Acute pharmacokinetics of JX-594 genomes (qPCR) after a single intravenous infusion, plotted by dose cohort. Error bars are s.e.m. **b**, Dose-dependent delivery of JX-594, as demonstrated by PCR and/or immunohistochemical (IHC) analysis of tumour biopsies collected 8–10 days after treatment. **c**, Dose-dependent induction of antibodies to β-galactosidase in patients evaluable for this endpoint. $p = 0.975$ Spearman's rank correlation coefficient. The number of patients evaluable for each group (n) is indicated.

($\geq 10^3$ p.f.u.dose $^{-1}$) and evaluable for biopsy analysis, 87% showed JX-594 positivity by qPCR and/or immunohistochemistry, whereas JX-594 was not detected in biopsies collected from subjects treated at lower doses. Of note, delivery and replication were demonstrated in a patient with baseline antibodies to JX-594/vaccinia (Supplementary Fig. 1). Infection resulted in granular cytoplasmic staining by immunohistochemistry, indicative of virus replication ("virus factories") (Fig. 3a, d and Supplementary Fig. 2). Diffuse infection and interspersed necrosis of malignant glandular structures were also evident. Adjacent and intermixed normal tissues were negative for replication by immunohistochemistry (Fig. 3i). In immediately adjacent normal squamous epithelium, low-level diffuse staining without cytopathogenicity indicated uptake without replication. Staining was absent in negative-control biopsy samples (pretreatment tumour from the same patient, Fig. 3c, f; no primary antibody, Fig. 3b, e). Three-dimensional visualization of JX-594 infection in a tumour gland revealed diffuse infection and spread (Fig. 3g, h and Supplementary Movie 1).

Immunohistochemical staining for β -galactosidase protein, the product of the *lacZ* transgene, confirmed expression in JX-594-infected tumour cells (Fig. 3j–l). In addition, the induction of antibodies to β -galactosidase was a surrogate marker for JX-594 replication and transgene expression (β -galactosidase is not present in the product; high-level expression requires replication). Induction of anti- β -galactosidase antibody was dose-related, occurring in 100% of high-dose patients (Spearman's rank correlation coefficient $\rho = 0.975$; $P = 0.005$) (Fig. 2c). Tumour-biopsy positivity correlated strongly with β -galactosidase antibody induction (Table 1). Finally, expression of the second JX-594 transgene (GM-CSF) was assessed. In a previous clinical

trial, we demonstrated that high-level replication could result in detectable GM-CSF in blood when other inflammatory cytokines had returned to baseline (days 4–15)¹⁴, despite the relatively short half-life of GM-CSF (< 2 h)¹⁵. Therefore, detection of GM-CSF in blood is indicative of high-level GM-CSF expression, and may be a specific but insensitive marker of transgene expression. Three patients had significant increases from baseline in plasma GM-CSF concentrations (days 4–15; all other inflammatory cytokines returned to baseline in < 24 h). All three had evidence of β -galactosidase expression, tumour infection and/or antitumour activity (modified Choi and/or fluorodeoxy-D-glucose positron emission tomography (FDG-PET) criteria). GM-CSF-protein-responsive subsets of white blood cells (neutrophils, eosinophils and monocytes) peaked on days 4–15 (Supplementary Fig. 3).

Dose-related antitumour activity was demonstrated (modified Choi¹⁶ or response evaluation criteria in solid tumours (RECIST)¹⁷ criteria), and correlated with delivery and replication of JX-594 (Table 1 and Supplementary Fig. 4). Furthermore, new tumour outgrowth in the time after treatment was less frequent in patients treated with high doses than low doses (Spearman's rank correlation coefficient $\rho = -0.872$; $P = 0.05$), indicating suppression of microscopic tumour foci. Two out of five high-dose patients had antitumour activity by FDG-PET (>25% decrease in standardized uptake value).

Here we report the first reproducible dose-related delivery, replication and transgene expression from a viral vector or oncolytic virus in metastatic solid tumours in humans after intravenous administration. Engineered oncolytic poxviruses such as JX-594 can express several complementary therapeutic proteins¹⁸ and/or siRNAs¹⁹ in metastatic

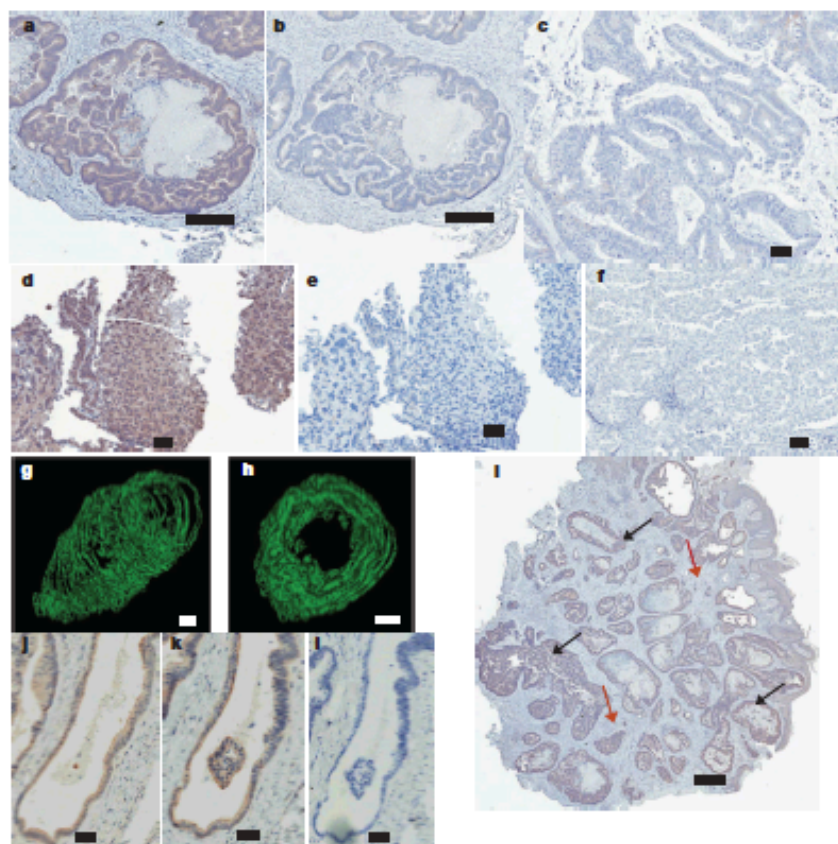


Figure 3 | Immunohistochemical staining reveals JX-594 infection and β -galactosidase expression in tumours. a, Immunohistochemistry for vaccinia (patient 20, 10 days after treatment). Scale bar, 200 μ m. b, Immunohistochemistry, no primary antibody. Scale bar, 200 μ m. c, Immunohistochemistry for vaccinia in pre-treatment biopsy. Scale bar, 50 μ m. d–f, As in a–c for patient 18, biopsy at 8 days after treatment. Scale bars, 50 μ m. g, h, Three-dimensional reconstruction of vaccinia (green) throughout tumour in patient 20. Scale bars 200 μ m. i, Immunohistochemistry for vaccinia at low magnification (patient 20). Scale bar, 500 μ m. Black arrows indicate tumour; red arrows indicate normal tissue. j, Immunohistochemistry for β -galactosidase (patient 20). Scale bar, 50 μ m. k, Immunohistochemistry for vaccinia. Scale bar, 50 μ m. l, Negative control. Scale bar, 50 μ m. Linear adjustment to brightness and contrast was applied to j–l.

tumours systemically and in a cancer-selective fashion; therapeutic concentrations in tumour tissues should therefore be markedly higher than in normal tissues. Incorporation of transgenes encoding marker proteins can facilitate the monitoring of product replication and transgene expression through blood²⁰ or radiographic assessments^{21,22}. Repeated intravenous dosing with JX-594 is currently being assessed and related oncolytic poxviruses should also be tested^{23,24}. Although anti-viral immunity may in theory decrease the efficiency of delivery, all patients on this trial had a history of vaccination with live vaccinia virus as children, and delivery was demonstrated in a patient with neutralizing antibodies present at baseline. In preclinical murine tumour models, intravenous vaccinia delivery and efficacy was feasible despite high-titre antibodies²⁵. Repeated intravenous delivery may be feasible because of the unique biology of vaccinia, including its ability to produce 'stealth' particles (extracellular enveloped virus) that can traffic in blood in the presence of neutralizing antibodies and complement^{4,5}. In addition, intravenous pharmacological dosing of JX-594 constitutes a route and dose that may transiently saturate native mechanisms of viral clearance. JX-594 and related poxvirus constructs thus represent a novel, systemic, multi-functional cancer-biotherapeutic platform.

METHODS SUMMARY

Patients. Twenty-three patients with treatment-refractory, histologically confirmed, advanced, metastatic solid tumours were enrolled and received a single intravenous infusion of JX-594 at one of six dose levels. This trial was registered with clinical trials registration number NCT00625456.

All patients gave written informed consent according to guidelines on good clinical practice. Protocol and consent forms were approved by the United States Food and Drug Administration and Health Canada, as well as the Institutional Review and Infection Control Committees at each hospital. An independent data-safety monitoring board reviewed the clinical safety data from each patient cohort before each of the four dose escalations.

Tumour biopsy analysis. Biopsies (excisional, core-needle or fine-needle aspirate) were obtained from all subjects 8–10 days after treatment, and were formalin-fixed and paraffin-embedded. Sections were subjected to haematoxylin and eosin staining, immunohistochemical staining for JX-594 proteins, and PCR for JX-594 genomes. Immunohistochemistry used anti-vaccinia polyclonal antibody (Quarterm) and a secondary antibody kit (Vectastain, Vector Laboratories). For detection of β -galactosidase by immunohistochemistry, an anti- β -galactosidase polyclonal antibody (Abcam) was used. Negative controls were run without primary antibody and tumours from mice treated with JX-594 were included as positive controls. For PCR, DNA was extracted from 5 $\times$ 10- μ m sections using Formapure kit (Agencourt) and amplified using primers corresponding to the vaccinia E3L gene, TCCGTGATGTCTACACAGG and ATGTATCCGGGAGAAATCA, designed using Primer3 software²⁶, using Quantitect SYBR Green PCR kit (Qiagen). Stained sections were digitized using the Aperio Scanscope (Aperio) and analysed using ImageScope software. Adobe Photoshop CS software (Adobe) was used to apply linear adjustments to brightness and contrast across all compared stains where indicated.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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- Kim, D. H. & Thorne, S. H. Targeted and armed oncolytic poxviruses: a novel mechanistic therapeutic class for cancer. *Nature Rev. Cancer* **9**, 64–71 (2009).
- Parato, K. A., Senger, D., Forsyth, P. A. & Bell, J. C. Recent progress in the battle between oncolytic viruses and tumours. *Nature Rev. Cancer* **5**, 965–976 (2005).
- Liu, T. C., Galanis, E. & Kim, D. Clinical trial results with oncolytic virotherapy: a century of promise, a decade of progress. *Nat. Clin. Pract. Oncol.* **4**, 101–117 (2007).
- Vanderplazschen, A., Hollinshead, M. & Smith, G. L. Antibodies against vaccinia virus do not neutralize extracellular enveloped virus but prevent virus release from infected cells and comet formation. *J. Gen. Virol.* **78**, 2041–2048 (1997).
- Vanderplazschen, A., Mathew, E., Hollinshead, M., Sim, R. B. & Smith, G. L. Extracellular enveloped vaccinia virus is resistant to complement because of incorporation of host complement control proteins into its envelope. *Proc. Natl. Acad. Sci. USA* **95**, 7544–7549 (1998).

- Wein, L. M., Wu, J. T. & Kim, D. H. Validation and analysis of a mathematical model of a replication-competent oncolytic virus for cancer treatment: implications for virus design and delivery. *Cancer Res.* **63**, 1317–1324 (2003).
- Smith, G. L., Murphy, B. J. & Law, M. Vaccinia virus motility. *Annu. Rev. Microbiol.* **57**, 323–342 (2003).
- Katsafanas, G. C. & Moss, B. Vaccinia virus intermediate stage transcription is complemented by Ras-GTPase-activating protein SH3 domain-binding protein (G3BP) and cytoplasmic activation/proliferation-associated protein (p137) individually or as a heterodimer. *J. Biol. Chem.* **279**, 52210–52217 (2004).
- Yang, H. et al. Antiviral chemotherapy facilitates control of poxvirus infections through inhibition of cellular signal transduction. *J. Clin. Invest.* **115**, 379–387 (2005).
- Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100**, 57–70 (2000).
- Mastrangelo, M. J. et al. Intratumoral recombinant GM-CSF-encoding virus as gene therapy in patients with cutaneous melanoma. *Cancer Gene Ther.* **6**, 409–422 (1999).
- Kim, J. H. et al. Systemic armed oncolytic and immunologic therapy for cancer with JX-594, a targeted poxvirus expressing GM-CSF. *Mol. Ther.* **14**, 361–370 (2006).
- Buller, R. M., Smith, G. L., Cremer, K., Notkins, A. L. & Moss, B. Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. *Nature* **317**, 813–815 (1985).
- Park, B. H. et al. Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: a phase I trial. *Lancet Oncol.* **9**, 533–542 (2008).
- Hovgaard, D., Mortensen, B. T., Schiffer, S. & Nissen, N. L. Clinical pharmacokinetic studies of a human haemopoietic growth factor, GM-CSF. *Eur. J. Clin. Invest.* **22**, 45–49 (1992).
- Choi, H. et al. Correlation of computed tomography and positron emission tomography in patients with metastatic gastrointestinal stromal tumour treated at a single institution with imatinib mesylate: proposal of new computed tomography response criteria. *J. Clin. Oncol.* **25**, 1753–1759 (2007).
- Byrne, M. J. & Nowak, A. K. Modified RECIST criteria for assessment of response in malignant pleural mesothelioma. *Ann. Oncol.* **15**, 257–260 (2004).
- Moss, B. Vaccinia virus: a tool for research and vaccine development. *Science* **252**, 1662–1667 (1991).
- Davis, M. E. et al. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature* **464**, 1067–1070 (2010).
- Peng, K. W. et al. Intraperitoneal therapy of ovarian cancer using an engineered measles virus. *Cancer Res.* **62**, 4656–4662 (2002).
- McCart, J. A. et al. Oncolytic vaccinia virus expressing the human somatostatin receptor SSTR2: molecular imaging after systemic delivery using ¹¹¹In-pentetate. *Mol. Ther.* **10**, 553–561 (2004).
- Msaouel, P. et al. Noninvasive imaging and radiotherapy of prostate cancer using an oncolytic measles virus expressing the sodium iodide symporter. *Mol. Ther.* **17**, 2041–2048 (2009).
- McCart, J. A. et al. Systemic cancer therapy with a tumor-selective vaccinia virus mutant lacking thymidine kinase and vaccinia growth factor genes. *Cancer Res.* **61**, 8751–8757 (2001).
- Thorne, S. H. et al. Rational strain selection and engineering creates a broad-spectrum, systemically effective oncolytic poxvirus, JX-63. *J. Clin. Invest.* **117**, 3350–3358 (2007).
- Thorne, S. H. et al. Targeting localized immune suppression within the tumor through repeat cycles of immune cell-oncolytic virus combination therapy. *Mol. Ther.* **18**, 1698–1705 (2010).
- Rosen, S. & Skolitsky, H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* **132**, 365–386 (2000).

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Author Contributions Study design: D.H.K. and J.C.B. Data analysis and study write-up: C.J.B., D.H.K., T.-H.H., A.M., R.P., A.P., T.R., J.C.B. and A.F. Enrolment and management of patients: J.B., D.J., J.S., A.R.H., L.Q.M.C. and J.N. Laboratory work: F.L.B., J.B., N.D.S., S.C., J.-E.J., L.E., Y.S.L., K.P., J.S.D., M.D. and J.-S.D. C.J.B. and D.H.K. had access to all the data in the trial. C.J.B. and D.H.K. took the final decision to submit for publication.

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METHODS

Ex vivo infection of tumour explants. Primary cancer and normal tissue specimens were obtained from consenting patients who underwent tumour resection. The institutional review board of Ottawa Hospital Research Institute has approved all human studies. A total of ten tumour samples and accompanying adjacent normal tissue samples from the affected organ were assayed for JX-594-GFP+/ β -gal⁻ sensitivity. Samples were received in cell culture medium and processed within 2–16 h. Samples were manually divided using a 15-mm scalpel blade into approximately 10-mm² pieces and placed on 12-well plates with minimum essential medium (α -MEM) containing 10% fetal bovine serum (FBS), under sterile techniques. Samples were inoculated with 1×10^7 p.f.u. JX-594 in 100 μ l of α -MEM serum-free medium. JX-594 was allowed to adsorb for 2 h at 37 °C, then 1.9 ml of α -MEM supplemented with 10% FBS was added. Infected tissue specimens were incubated in a humidified incubator at 37 °C for 24 h before imaging JX-594-GFP+/ β -gal⁻-driven GFP expression.

Patients. Between 21 July 2008 and 9 February 2010, 23 patients were enrolled. Patients had treatment-refractory, histologically-confirmed, advanced, metastatic solid tumours. At least one tumour mass had to be amenable to biopsy and/or fine-needle aspirate. Patients had adequate haematological function (leukocyte count $>3,500$ cells per mm³, CD4 count ≥ 200 per mm³, haemoglobin ≥ 0.1 g ml⁻¹, platelet count $\geq 100,000$ platelets per mm³) and adequate organ function (including aspartate aminotransferase/alanine aminotransferase $<2.5 \times$ upper normal limit, bilirubin $\leq 1.5 \times$ upper normal limit and serum chemistries within normal limits or Grade 1), a coagulation status of (International normalized ratio (INR) $\leq$ upper normal limit + 10%), and Karnofsky performance score of ≥ 70 .

Exclusion criteria included known central-nervous-system malignancy, clinically significant and/or rapidly accumulating ascites, pericardial and/or pleural effusions, unstable cardiac disease, increased risk of vaccination complications (exfoliative skin conditions such as eczema or ectopic dermatitis), clinically significant immunodeficiency, anticancer therapy within the preceding four weeks, and pulse oximetry O₂ saturation $<90\%$ at rest.

All patients gave written informed consent according to good clinical practice guidelines. Protocol and consent forms were approved by the United States Food and Drug Administration and Health Canada, as well as the Institutional Review and Infection Control committees at each hospital. An independent data-safety monitoring board reviewed the clinical safety data from each patient cohort before each of the four dose escalations.

Manufacturing, product characterization and release testing. Clinical trial material (CTM) lots ($n = 2$) that were used in this study were manufactured according to good manufacturing practice guidelines. Virus was grown in adherent mammalian cells and purified through sucrose-gradient centrifugation or by tangential flow filtration. *In vitro* and *in vivo* comparability testing demonstrated equivalence of the two lots. Quality control tests on the final product included assays for sterility and endotoxin, DNA, protein, p.f.u. and genome concentration in the CTM; functional assays included potency and GM-CSF production. CTM was formulated in either phosphate-buffered saline with 10% v/v glycerol (pH 7.1), or 30 mM Tris with 10% (w/v) sucrose (pH 7.7). Immediately before the intravenous infusion, JX-594 was diluted in bicarbonate-buffered saline in a total infusion volume of 250 ml.

Treatment. JX-594 was infused in 250 ml bicarbonate-buffered saline over 60 min. Patients in the dose-escalation portion of the trial received one of six dose levels (1×10^5 p.f.u. kg⁻¹, cohort 1; 1×10^6 p.f.u. kg⁻¹, cohort 2; 3×10^6 p.f.u. kg⁻¹, cohort 3; 1×10^7 p.f.u. kg⁻¹, cohort 4; 3×10^7 p.f.u. kg⁻¹, cohort 5; and 1×10^8 p.f.u. expansion cohort) in a group-sequential dose-escalation design (standard 3 $\times$ 3 design; 2–6 patients for each dose cohort) at one of four sites: the Billings Clinic, the Cancer Center of the Carolinas, the Ottawa Hospital Research Institute and the University of Pennsylvania. One additional patient could be treated in each dose cohort once that cohort was cleared for safety. An additional six patients were enrolled as part of an expansion cohort at the approximate midpoint between the cohort-4 and cohort-5 doses, a fixed dose of 1×10^9 p.f.u. (approximately 1.5×10^7 p.f.u. kg⁻¹, depending on patient weight). A starting dose of 1×10^5 p.f.u. kg⁻¹ was chosen on the basis of findings from a Good Laboratory Practice toxicology study, and demonstrated safe patient blood concentrations after intratumoural injection of JX-594 into liver-based tumours¹⁴. Dose-limiting toxicities were defined as any of the following treatment-related adverse events (through the 4-week evaluation period): 1) any grade-4 toxicity (except isolated grade-4 lymphopenia lasting ≤ 7 days); 2) grade-3 or -4 hypotension, disseminated intravascular coagulation or allergic reaction/hypersensitivity; 3) grade-3 non-haematological toxicity persisting >7 days (except if toxicity is transaminitis, which may last >7 days if total bilirubin is normal or grade-1, or flu-like symptoms that respond to standard treatments); and 4) grade-3 haematologic toxicity persisting for >7 days (except isolated lymphopenia). The MTD was defined as the dose immediately preceding that for which two or more dose-limiting

toxicities were recorded. If no MTD was defined, the highest dose was defined as the MTD.

Starting with a subset of patients in cohort 4, patients were pre-medicated with acetaminophen (and also treated every 6 h as needed). Patients were advised to hydrate orally for 24 h before treatment (for example, ≥ 1 litre of fluid-containing fluids), and they received hydration through 24 h after treatment (for example, 1–2 litres of fluids, orally or intravenously). Patients were monitored after treatment for 24 h in the hospital and for at least 29 days as outpatients. A physical exam and an interval medical history were performed on each weekly study visit. Safety monitoring included adverse-event monitoring (National Cancer Institute common toxicity criteria, version 3.0) and standard laboratory toxicity grading for haematology, liver and renal function, coagulation studies, serum chemistry and urinalysis.

Pharmacokinetic measurements in blood. JX-594 pharmacokinetics as well as gene expression and replication in solid tumours were assessed. JX-594 genome concentration in blood was measured by qPCR as previously described^{14,27}. The pharmacokinetics of JX-594 during and immediately after administration were determined by qPCR. Whole EDTA-blood samples were taken before the start of infusion; 15, 30 and 60 min after the start of infusion; and 30, 60, 120 and 240 min after the end of infusion. Area under the curve (AUC), maximum concentration (C_{max}) and half-life (t_{1/2}) were calculated using WinNonLin, version 5.2 (WinNonLin copyright 2010, Pharsight Corporation). The AUC was calculated by the linear trapezoidal method and C_{max} was determined directly by inspection.

Antibody titres to the β -galactosidase marker transgene. Serum samples were obtained at baseline and on days 15 and 29. Human IgG antibodies to β -gal were measured by ELISA. Briefly, plates (NUNC MaxiSorp, Thermo Fisher Scientific) with wells containing β -galactosidase (Sigma) or bicarbonate/carbonate buffer only were incubated overnight at 4 °C and washed with PBS-Tween before incubation with blocking buffer (PBS with 1% bovine serum albumin (BSA), ELISA grade (Sigma)). Diluted serum (1:50, 1:100 or 1:200 in PBS with 0.05% Tween and 1% BSA) was added to β -galactosidase-coated and control wells in duplicate and incubated at 23 °C. Plates were washed and incubated with alkaline-phosphatase-labelled goat anti-human IgG (Abcam) diluted 1:2,000. After washing, the colorimetric substrate alkaline phosphatase yellow (pNPP, Sigma) was added, and NaOH was added to stop colour development after 10 min. Absorbance was read at 405 nm, and absorbance at 630 nm was subtracted. Control-well values were subtracted to account for non-specific binding, and titres values were calculated by comparison to a standard curve of positive sera, arbitrarily assigned a titre of 8,000.

Neutralizing antibody titres to vaccinia virus. This procedure is based on the ability of neutralizing antibodies in patient serum samples to reduce the cytopathic effect caused by live vaccinia virus. Serum samples obtained at baseline and on days 4, 8, 15, 22 and 29 were heat-inactivated, serially diluted in 96-well format (dilution factor 10–3,200,000) and incubated with vaccinia virus for 2 h before transfer of the mixture onto monolayers of A2780 cells. Cell viability was measured 3 days after inoculation by a colorimetric assay based on live-cell-mediated reduction of tetrazolium salt to formazan (Cell Counting Kit-8, Donjindo Laboratories). The neutralizing antibody titre was defined as the reciprocal of the highest dilution of serum that results in $\geq 50\%$ cell viability.

White blood cell induction. White blood cell count was performed by routine laboratory testing and was included in the safety assessment as defined in the protocol. White blood cell counts were assessed at baseline and on days 4, 8, 15, 22 and 29.

Cytokine measurements. The endogenous cytokines IL-1 β , IL-4, IL-6, IL-10, TNF- α and IFN- γ were measured in multiplex in plasma samples obtained at baseline, 3 h and 8 h after dosing, and on days 4 and 8 using a Milliplex kit as directed by the manufacturer (Millipore). GM-CSF concentrations in plasma were determined at baseline, 3 h and 8 h after dosing and on days 4, 8, 15, 22 and 29 using the Quantikine hGM-CSF sandwich ELISA kit as directed by the manufacturer (R&D Systems).

Tumour biopsy analysis. Biopsies (excisional, core-needle, or fine-needle aspirate) were obtained from all subjects 8–10 days after treatment, formalin-fixed and paraffin-embedded. Sections were subjected to haematoxylin and eosin staining, immunohistochemical staining for JX-594 proteins, and PCR for JX-594 genomes. Immunohistochemistry used anti-vaccinia polydonal antibody (Quartett) and secondary antibody kit (Vectastain, Vector Laboratories). For detection of β -galactosidase by immunohistochemistry, an anti- β -galactosidase polydonal antibody (Abcam) was used. Negative controls were run without primary antibody and tumours from mice treated with JX-594 were included as positive controls. For PCR, DNA was extracted from 5 $\times$ 10- μ m sections using Formapure kit (Agencourt) and amplified using primers corresponding to the vaccinia E3L gene, TCGGTGATGTCTACACAGG and ATGTATCCGGGAGAAATCA, designed using Primer3 software²⁸, using QuantiTect SYBR Green PCR kit (Qiagen).

Three-dimensional reconstruction. An excisional biopsy was obtained from patient 20 on day 10 and processed for immunohistochemical detection of vaccinia virus. Serial sections (126) were cut and every other section was stained for virus. Two-dimensional pictures of the sections were then converted into a three-dimensional volume using HTK histology toolkit software (Robarts Imaging Institute). Volume reconstruction was completed using alignment and segmentation-contouring algorithms that oriented each tissue section on top of one another. Each tissue-section image, once oriented, was then converted from two-dimensional pixels into three-dimensional voxels. These three-dimensional stacks were then rendered to generate the reconstructed tumour. Regions of infection were highlighted in green to aid visualization, and in another representation in which the model can be viewed in orthogonal planes, image contrast was adjusted to 50 (Adobe Photoshop CS2, Adobe). Scaling in the three-dimensional model is derived from scaling from two-dimensional images.

Tumour response analysis. Tumour response was assessed by contrast-enhanced computed tomography (CT) imaging on day 29 in all patients, and on week 10 in patients who remained in the study. Maximum tumour diameters and Hounsfield units (density estimate) were obtained at all time points. Patients in the expansion cohort had PET-CT scans done at the same time points; standard uptake values were determined from PET scans. RECIST¹⁸ and modified Choi criteria¹⁹ for response were employed in image evaluations. Patient 22 (metastatic mesothelioma) was evaluated by modified RECIST for mesothelioma²⁰. The PET response was also determined for PET-evaluable patients.

Equipment and settings. GFP expression from JX-594-GFP⁺/β-gal⁻ in human tissue explants was visualized using the Leica M205FA microscope and Leica microsystem LAS AR6000 acquisition software (Leica Microsystems) at ×2–4 magnification. The same exposure time was used across all samples. Images of tumour and normal-tissue samples for each patient were captured on the same day. Images were stored using Adobe Photoshop version 7.0 (Fig. 1).

Immunohistochemistry sections were digitized using the Aperio Scanscope (Aperio) and analysed using ImageScope v10.22.2319 software (Aperio). No image adjustments were applied to Fig. 3a–f and i. Adobe Photoshop CS software (Adobe) was used to apply linear adjustments to brightness and contrast across all compared stains where indicated (Fig. 3j–l).

Statistical analysis. The study sample size was set to assess safety issues. The primary objectives were to study the safety and to determine the MTD/MFD of JX-594 after intravenous infusion. Secondary objectives included pharmacokinetics and pharmacodynamics, immune responses (neutralizing antibodies, anti-β-galactosidase antibodies and cytokines) and delivery of JX-594 to solid tumours after intravenous infusion. The likelihood of dose escalation, given variation in true dose-limiting toxicities in the treated population, was calculated as per routine in Phase I dose-escalation trials. The expected sample size was 18–24 patients.

The Spearman's correlation coefficient between ranks was used to calculate statistical dependence between antibody induction to β-galactosidase and dose cohort (percentage of patients with antibody induction in cohorts 1–5), as well as appearance of new tumours and dose cohort (percentage of patients with new tumours in cohorts 1–5)¹⁰.

27. Kulesh, D. A. et al. Smallpox and pan-orthopox virus detection by real-time 3'-minor groove binder TaqMan assays on the Roche LightCycler and the Cepheid Smart Cycler platforms. *J. Clin. Microbiol.* **42**, 601–609 (2004).
28. Therasse, P. et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J. Natl. Cancer Inst.* **92**, 205–216 (2000).
29. Choi, H. et al. CT evaluation of the response of gastrointestinal stromal tumors after imatinib mesylate treatment: a quantitative analysis correlated with FDG PET findings. *Am. J. Roentgenol.* **183**, 1619–1628 (2004).
30. Myers, J. L. W. & Arnold, D. *Research Design and Statistical Analysis*. 2nd edn, 505–512 (Laurence Erlbaum, 2003).

APPENDIX IV: A MECHANISTIC PROOF-OF-CONCEPT CLINICAL TRIAL WITH JX-594, A TARGETED MULTI-MECHANISTIC ONCOLYTIC POXVIRUS IN PATIENTS WITH METASTATIC MELANOMA

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Contribution: I developed standard operating procedures for staining patient tumours for vaccinia in melanoma tissue using a red chromogen. I stained patient biopsies and analyzed them with Manijeh Daneshmand.

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A Mechanistic Proof-of-concept Clinical Trial With JX-594, a Targeted Multi-mechanistic Oncolytic Poxvirus, in Patients With Metastatic Melanoma

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JX-594 is a targeted and granulocyte macrophage-colony stimulating factor (GM-CSF)-expressing oncolytic poxvirus designed to selectively replicate in and destroy cancer cells through viral oncolysis and tumor-specific immunity. In order to study the mechanisms-of-action (MOA) of JX-594 in humans, a mechanistic proof-of-concept clinical trial was performed at a low dose equivalent to $\leq 10\%$ of the maximum-tolerated dose (MTD) in other clinical trials. Ten patients with previously treated stage IV melanoma were enrolled. Tumors were injected weekly for up to nine total treatments. Blood samples and tumor biopsies were analyzed for evidence of transgene activity, virus replication, and immune stimulation. The β -galactosidase (β -gal) transgene was expressed in all patients as evidenced by antibody induction. Six patients had significant induction of GM-CSF-responsive white blood cell (WBC) subsets such as neutrophils (25–300% increase). JX-594 replication and subsequent shedding into blood was detectable in five patients after cycles 1–9. Tumor biopsies demonstrated JX-594 replication, perivascular lymphocytic infiltration, and diffuse tumor necrosis. Mild flu-like symptoms were the most common adverse events. In sum, JX-594 replication, oncolysis, and expression of both transgenes were demonstrated; replication was still evident after multiple cycles. These findings have implications for further clinical development of JX-594 and other transgene-armed oncolytic viruses.

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INTRODUCTION

Targeted therapies for cancer with novel mechanisms-of-action (MOA) are needed. One strategy is the use of replication-competent

viruses which self-amplify within the tumor causing lysis of infected cancer cells^{1–3} and a number of agents have entered phase 1 and 2 clinical trials.^{4–6} Engineered oncolytic poxviruses can replicate selectively in cancer cells, resulting in virus progeny production, tumor cell necrosis, release and spread within tumor tissues.³ These virotherapies can also be engineered to express multiple therapeutic, marker, and noninvasive imaging transgenes. We hypothesized that the poxvirus pharmacophore would result in rapid replication and motile spread, activation by the epidermal growth factor receptor-tas pathway (pathway activated in the vast majority of solid cancers), and efficient systemic delivery to metastatic tumors, secondary to intratumoral (IT) administration or after direct intravenous (IV) infusion. Safety attributes include the fact that vaccinia was used in smallpox vaccination programs in tens of millions of humans globally, and specific antiviral agents are available (e.g., cidofovir⁷ and vaccinia immune globulin⁸).

JX-594 is a targeted and transgene-armed oncolytic poxvirus engineered from the Wyeth vaccine strain (Dryvax; Wyeth Laboratories, Marietta, PA).^{9–11} JX-594 has both a disruption of the thymidine kinase (TK) gene and expression of human CSF2 (granulocyte macrophage-colony stimulating factor (GM-CSF)) complementary DNA under the control of a synthetic (i.e., engineered) early-late promoter, and the *lac-Z* marker transgene-expressing β -galactosidase (β -gal) protein under control of the p7.5 late promoter.¹⁰ JX-594 is tumor-selective due to epidermal growth factor receptor-tas pathway and elevated cellular TK protein dependency^{12,13} and tumor-resistance to interferons.¹⁴ Cellular TK is driven to high levels in cancer by cell cycle abnormalities.¹⁵ GM-CSF is effective in augmenting the tumor-specific immunity induced by oncolytic vaccinia.^{16,17} JX-594 caused complete tumor responses and enhanced survival after IV and IT administration in immunocompetent rat and rabbit tumor models, and product MOA were demonstrated.¹⁰

In a pilot clinical study of JX-594, seven melanoma patients were first revaccinated with wild-type vaccinia in normal skin,

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and subsequently received escalating doses of JX-594 injected into superficial melanoma skin metastases.⁹ No maximum-tolerated dose (MTD) was reported [up to the relatively low dose of 8×10^7 plaque-forming units (pfu)]; regressions of small superficial tumors were documented. A phase 1 trial in patients with liver tumors was performed ($n = 14$ patients); whereas data on biological activity was reported, tumor histologies ($n = 7$) and doses (10^6 – 3×10^8 pfu) were heterogeneous.¹⁸ The MTD within the liver was 10^8 pfu. Biological effects following repeated dosing and biopsy data on injected tumors were not reported from this trial.

In order to confirm and expand upon the preliminary findings from these two previous phase 1 trials, we performed a low-dose MOA-driven clinical trial of JX-594 in patients with metastatic melanoma injected weekly for up to nine total doses. The objectives of this trial were to assess the multiple MOA of JX-594 after repeated weekly IT injections. The results reported here differ from previously published trials because this trial assessed biological activity as follows: (i) after six to nine cycles (including JX-594 replication and shedding into the blood), (ii) without immediately preceding prevaccination, (iii) in a homogeneous patient population, (iv) at a fixed dose, (v) with serial blood sample analysis for induction of white blood cells (WBC), and (vi) with detailed biopsy evaluation for replication, inflammation, and necrosis induction over time. The dose of 10^8 pfu was selected because this was similar to the highest dose in the melanoma pilot study and the lowest dose in the liver tumor phase 1 trial; this dose was 10% of the MTD in the liver. Detailed data were obtained to assess JX-594 replication, transgene expression (GM-CSF and β -gal) and pharmacodynamics (immune cell stimulation). Of note, expression of these transgenes from JX-594 is under the control of two distinct promoters; for both promoters, high transgene expression is dependent on virus replication.¹⁹ We believe the results reported herein have significant implications for future clinical trials with JX-594 and other products from this novel therapeutic class.

RESULTS

Patient demographics

Ten patients were enrolled and treated with JX-594; 8 subjects (80%) completed the study. Two patients did not complete study. One patient requested to withdraw (after three treatments) and a second withdrew (after two treatments) due to investigator request. Patient baseline characteristics are listed in Table 1. Mean age was 65.1 years (range: 46–81 years). Gender was evenly distributed. All subjects had metastatic disease; mean duration of melanoma was 44.6 months. Patient prognosis at screening was evaluated using the American Joint Committee on Cancer staging system. Seventy percentage of patients belonged to the M1c category (visceral metastasis to sites other than skin, lymph nodes and lung with normal lactate dehydrogenase or distant metastasis with elevated lactate dehydrogenase). Patients were heavily pretreated, having received a mean of 6.2 prior therapeutic interventions (median 6, range 1–10). These could include prior surgery, radiation therapy, chemotherapy, or biological therapy. Prior biological therapies included interferon, interleukin-2, dendritic cell vaccines, and anti-CTLA-4 antibody treatment. Supplementary Table S1 provides a listing of prior biological agents by patient. Five patients were known to be previously vaccinated with varicella; five patients' vaccination status was unknown.

Pharmacodynamics: induction of GM-CSF-responsive WBC subsets

Significant increases (>50% increase from baseline) in WBC subsets were detected in 60% of patients (Figure 1a). Changes in WBC were evaluated on days 5, 8, 12, 15, 22, 29, 36, 43, and 64. Since WBC generally peaked after the first injection, peak induction on days 5 or 8 was plotted in Figure 1a. Four subjects had an increase in neutrophils of $\geq 100\%$ from baseline (Figure 1b) and an absolute neutrophil count increase of $\geq 2,000$ cells/ μ l from baseline within 5–8 days of the first JX-594 treatment. Four subjects had significant increases in eosinophils (>100% increase from baseline). In a patient with detectable GM-CSF (9.4 pg/ml)

Table 1 Patient demographics

Patient ID	Age	Sex	Race/ethnicity	Vaccination status	Number of previous therapeutic interventions ^a	Tumor size (cm)	Tumor duration (months)	Cycles of JX-594 received	Tumor stage (AJCC)
05001	71	Male	White	Yes	10	21	18.9	6	M1b
05002	60	Female	White	Yes	3	16.3	20.2	6	M1b
05003	55	Female	White	Yes	9	18	80.9	9	M1c
05004	46	Male	Hispanic or Latino	Unknown	5	29.2	39.1	3	M1c
05005	81	Male	White	Yes	6	11.4	43.5	9	M1c
05006	72	Male	White	Unknown	10	6	114.6	6	M1c
05007	69	Female	White	Yes	1	19.8	22.6	6	Mx ^b
05008	53	Male	White	Unknown	4	23.2	37.4	6	M1c
05009	77	Female	White	Unknown	6	19.8	16.2	6	M1c
05010	67	Female	Hispanic or Latino	Unknown	8	18.9	52.9	2	M1c

Abbreviation: AJCC, American Joint Committee on Cancer.

^aIncludes surgery, radiation therapy, chemotherapy & biological therapy. Note: Multiple chemotherapeutics administered on the same day considered one therapeutic intervention. ^bSubstage not known.

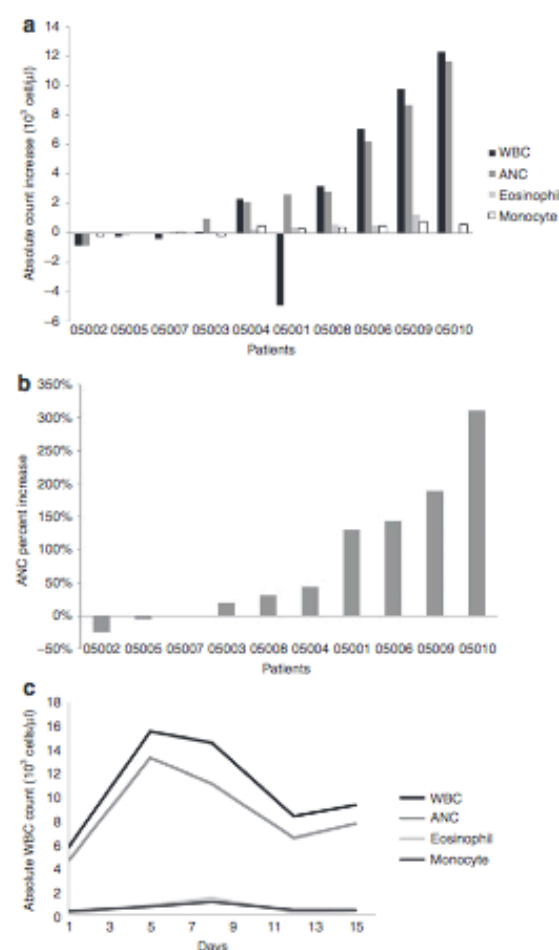


Figure 1 Induction of granulocyte macrophage-colony stimulating factor (GM-CSF)-responsive white blood cell (WBC) subsets by JX-594 treatment. (a) Absolute changes in WBC, neutrophil counts (ANC), eosinophils, and monocytes on day 5–8 post-JX-594. (b) Percentage changes from baseline in neutrophil counts (ANC), on day 5–8 post-JX-594. (c) Time course of WBC subset induction (absolute counts) during cycle 1 in patient 05009 (blood positive for detectable GM-CSF on day 5).

in blood on day 5 (patient 05009), the absolute neutrophil count increased by 189% (day 5) and eosinophils increased by 1,300% (from 100 at baseline to 1,400 cells/μl on day 8) (Figure 1c).

No significant change in hemoglobin/hematocrit was observed. No significant thrombocytopenia was detected. Three subjects had transient increases (>50% increase from baseline lasting <14 days) in platelet counts at various time points during the study (data not shown).

Pharmacokinetics of JX-594 genomes in blood: two phases

Blood samples were obtained from each subject before the first treatment, at 15 minutes, 3 hours after infection and at multiple

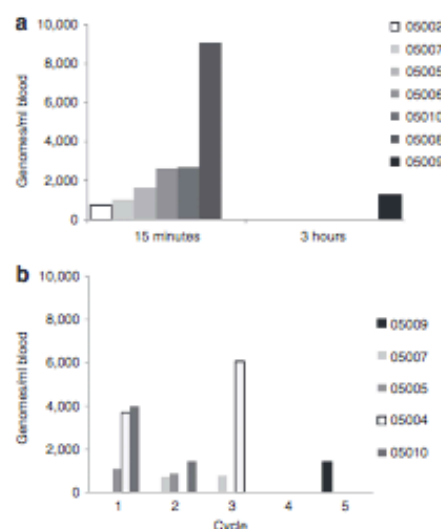


Figure 2 Pharmacokinetics of JX-594: acute clearance followed by delayed re-emergence associated with replication and shedding into the blood. (a) Acute genome concentrations in circulation at 15 minutes and 3 hours (patients with genomes below the limit-of-detection are not included). (b) Peak genome concentrations 5–7 days after treatments 1 through 5 (patients with no genomes detected during this time are not included). The delayed reappearance of JX-594 genomes provides evidence for replication and shedding into the blood.

time points between days 5 and 64 (Table 2). During the initial clearance phase, low but detectable concentrations of JX-594 genomes were present in the blood immediately (15 minutes) after injection of superficial tumors in six subjects (60%), but rarely 3 hours after injection (Figure 2a). After clearance (15 minutes to 3 hours), delayed re-emergence of circulating JX-594 was detected in a subset of subjects, consistent with replication and shedding into the blood. Five subjects (50%) had detectable genomes from 5 to 7 days after the preceding injection (i.e., the 5–7 days following injections 1, 2, 3, 4, and/or 5) (Figure 2b). Two subjects received 3 additional treatments, and one had detectable JX-594 genomes in blood collected during this period as well on day 71 (7 days after 7th injection) and on day 85 (7 days after 9th injection) (Table 2). In some cases, subjects received JX-594 injections concurrently with detection of replication peaks in the blood (replicating JX-594 from a previous injection); no significant toxicity was noted in these patients.

Tumor biopsies: Assessment of JX-594 replication, inflammatory infiltration, and cell death

Tumor biopsies were optional based on patient consent. Tumor biopsies were obtained post-treatment in five patients; biopsies contained viable tumor tissue in three cases, and contained completely necrotic tumor tissue in two patients (not interpretable for immunohistochemistry). In addition, one of these patients had evaluable tumor tissue at baseline, day 5 and day 43. At baseline, no necrosis or inflammation was noted; rare scattered apoptotic tumor cells were noted (Figure 3a). In marked contrast, the day 5 biopsy of a noninjected tumor showed vaccinia replication,

Table 2 Patient treatment and initial plus delayed pharmacokinetics of JX-594

Number of cycles	Patient ID	Tumor ID (sum LD cm)	Baseline NAb titer	Pre-JX-594	Acute dose 1		Day 12-15	Day 5-8	Day 22	Day 29	Day 36	Day 43	Predose 6	Predose 7	Predose 8	Predose 9
					Day 1 15 min	Day 1 3 hours										
2	10	Yes (19)	<10	<LOD	Det (2,687)	<LOD	Det (3,976)	<LOD	Det (1,461)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
3	04	Yes (29)	<10	<LOD	<LOD	<LOD	Det (3,703)	<LOD	Det (6,057)	0	0	0	0	0	0	0
6	01	No (9)	<10	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
6	02	No (3)	32	0	Det (735)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
6	06	No (6)	100	<LOD	Det (2,635)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
6	07	Yes (11)	32	<LOD	Det (1,031)	<LOD	<LOD	<LOD	Det (793)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
6	08	Yes (23)	10	0	Det (9,077)	0	<LOD	<LOD	<LOD	0	0	<LOD	<LOD	<LOD	<LOD	<LOD
6	09	Yes (20)	<10	<LOD	0	Det (1,270)	<LOD	<LOD	<LOD	<LOD	Det (1,431)	<LOD	<LOD	<LOD	<LOD	<LOD
9	03	No (6)	10	<LOD	0	0	0	0	<LOD	0	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
9	05	No (6)	<10	<LOD	Det (1,638)	<LOD	Det (1,108)	Det (893)	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	7,384

Det, detected (values in parentheses are in genomes/ml blood); LD, longest diameter; LOD, limit-of-detection.

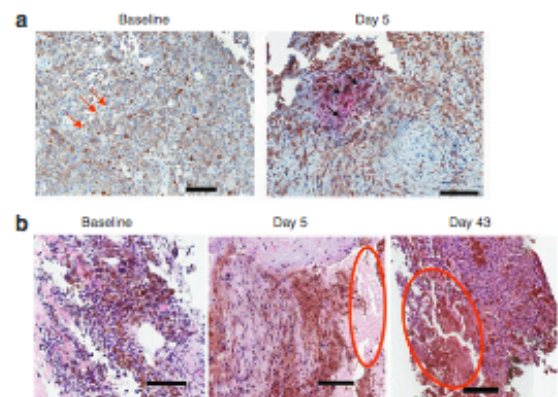


Figure 3 Biopsy evidence for JX-594 replication and necrosis induction. (a) JX-594 detection by immunohistochemistry (IHC) (red staining) in melanoma cells post-treatment (patient 05007): baseline (negative) and day 5 (positive). Examples of apoptotic and infected cells indicated with red and black arrows, respectively. (b) Hematoxylin and eosin (H&E) stain. Necrosis induction post-treatment (patient 05007): baseline (negative), day 5 (moderate), and day 43 (intense). Bar = 100 μ m. Areas of necrosis circled in red.

and day 5 and day 43 biopsies demonstrated progressive widespread necrosis from moderate (day 5) to intense (day 43) grade (Figure 3a,b). Similar necrosis was noted in tumor biopsies from two other patients on day 5 and day 12. Intense perivascular lymphocytic infiltration was noted on days 5–43 in two of three evaluable biopsies (Figure 4a–c).

Antibody development to JX-594 and to the β -gal marker transgene product

Neutralizing antibody (NAb) titers were assessed in serum samples taken at baseline, and at multiple time points between days 8 and 64. Vaccinia vaccination in childhood can result in residual antivaccinia antibodies in patients. At baseline, anti-JX-594 NAb titers were as follows: undetectable ($n = 3$), low (≤ 10 ; $n = 4$), or high (> 10 ; $n = 3$). NAb titers increased as early as day 8 in 7 of 10 subjects. By day 15 all patients showed high titers, and titers had peaked by day 21 despite continued treatment for 6 weeks (Figure 5a). As a group, patients showed statistically significant induction (P value of 0.0141 using paired Student's t -test comparing baseline to D43 titers, $n = 8$ patients reaching D43).

Immunoglobulin G antibodies to the β -gal transgene product were detected by enzyme-linked immunosorbent assay. Antibodies to β -gal were negative at baseline in all patients, except one patient with a very low titer at baseline. Antibodies to β -gal were evident in all patients post-treatment (Figure 5b). One patient had not developed antibodies on day 43, but showed induction on day 64 (data not shown). Multiple additional treatments and/or time may increase titers—the two patients who withdrew from study early had lower titers on day 12 and 22 compared to patients completing study and being tested on day 43 (Figure 5b). As a group, patients showed statistically significant induction (P value of 0.0211 using paired Student's t -test comparing baseline to D43 titers, $n = 8$ patients reaching D43). Significant expression of β -gal

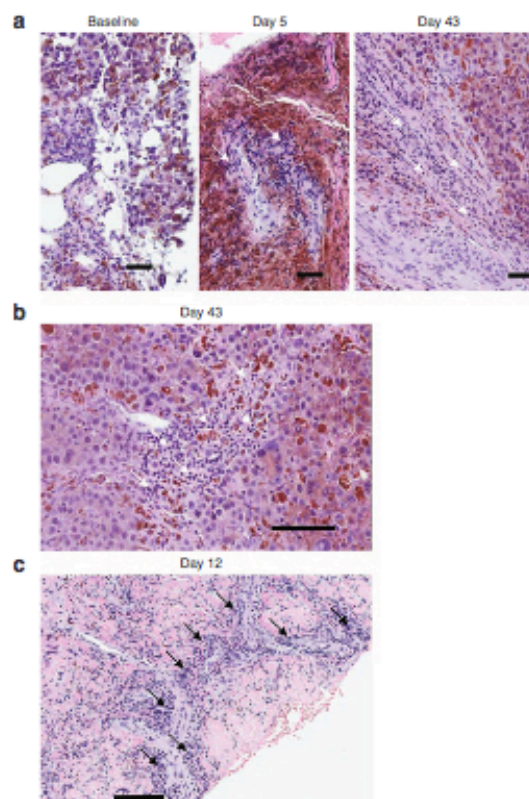


Figure 4 Biopsy evidence for JX-594-induced lymphocytic infiltration [hematoxylin and eosin (H&E) stain]. **(a)** Lymphocytic infiltration over time post-treatment (patient 05007): baseline (negative), day 5 (intense), day 43 (intense). Bar = 50 μ m. Examples of perivascular lymphocytes indicated with white arrows. **(b)** High magnification of perivascular lymphocyte infiltration on day 43 (intense—patient 05007). Bar = 100 μ m. Examples of perivascular lymphocytes indicated with white arrows. **(c)** High magnification of perivascular lymphocyte infiltration and day 12 (intense—patient 05004). Bar = 100 μ m. Examples of perivascular lymphocytes indicated with black arrows.

is dependent on viral replication,¹⁸ thus development of antibodies to β -gal is dependent on JX-594 replication; antibodies therefore are markers of previous JX-594 replication in the patient.

Radiographic tumor assessment and survival duration

Radiographic tumor assessment was not the focus of this low-dose trial analysis. Anecdotal data was reported. Five subjects were evaluable for radiographic tumor assessments; protocol-defined radiographic assessments were not evaluable in five patients. Reasons included patient refusal, patient withdrawal from study and protocol violation. These patients clinically deteriorated before obtaining repeat tumor measurements, and they died soon thereafter. All five evaluable subjects had stable disease of injected tumors by RECIST criteria ($n = 5$). Stable disease (including a mixed response) was noted in injected tumors at week 6 in all patients. Noninjected tumors were present in all patients. Three

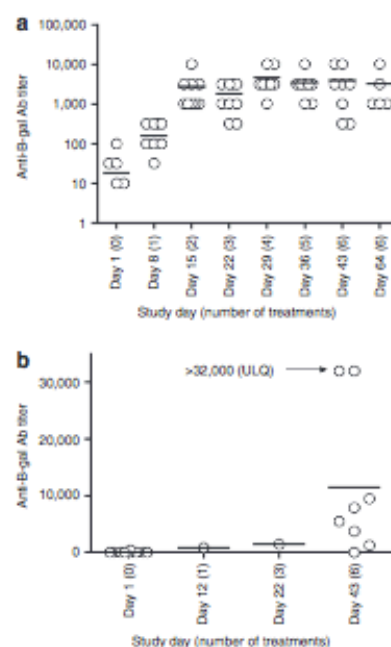


Figure 5 Antibody induction to JX-594 and to the β -gal marker transgene product. **(a)** Neutralizing antibody titers over time following treatment initiation; individual data points are circles and the line is the mean. **(b)** Anti- β -gal antibody titers over time; individual data points are circles and the line is the mean. All patients were tested on day 43 except two patients that withdrew early were tested on day 12 or day 22. Two patients had values above the upper limit of quantitation of 32,000—value is presented as 32,000.

patients had stable disease in noninjected tumors and two had progressive disease at week 6.

Durability of responses and progression-free survival were not assessable since most patients went off study within ≤ 6 weeks. Two patients (05003 and 05005) with stable disease on the day 43 efficacy read received an additional three JX-594 treatments according to protocol. One patient (05003) had stable disease of injected and noninjected tumors on day 43 and on day 77 (after nine total JX-594 treatments). One noninjected tumor had a partial response on day 77. The second (05005) patient had stable disease in their injected tumors on days 43 and 77, whereas non-injected tumors progressed between days 43 and 77. Eight patients survived for at least 4 months, and four patients survived more than 7 months; one patient survived >20 months. Mean survival was 7.1 months (range 2.2–20.5).

Adverse events

All 10 subjects reported at least one treatment-emergent AE. JX-594 was generally well-tolerated. The most common treatment-related adverse events included nausea, chills, fever, fatigue, pain, myalgia, anemia, and headache. Most events were grade 1 or 2. One case each of hypoglycemia, fever, and anemia were reported as severe (grade 3) and deemed possibly or probably related to JX-594 treatment; all were transient and resolved without sequelae. Seven

Table 3 Treatment-emergent adverse events possibly related to JX-594 by grade

	Grade 1–2	Grade 3	Grade 4 (5)	Total patients n = 10
<i>Blood and lymphatic system disorders</i>				
Anemia	3 (30.0%)	1 (10.0%)	0 (0.0%)	3 (30.0%)
<i>Cardiac disorders</i>				
Dyspnea	1 (10.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)
<i>Gastrointestinal disorders</i>				
Nausea	2 (20.0%)	0 (0.0%)	0 (0.0%)	2 (20.0%)
Dyspepsia	1 (10.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)
Vomiting	1 (10.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)
<i>General disorders and administration site conditions</i>				
Oedema	1 (10.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)
Fatigue	4 (40.0%)	0 (0.0%)	0 (0.0%)	4 (40.0%)
Pyrexia	3 (30.0%)	0 (0.0%)	0 (0.0%)	3 (30.0%)
Facial pain	1 (10.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)
Tenderness	1 (10.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)
Injection site pain	3 (30.0%)	0 (0.0%)	0 (0.0%)	3 (30.0%)
Injection site erythema	1 (10.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)
Injection site rash	1 (10.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)
Injection site pruritus	1 (10.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)
<i>Neoplasms benign, malignant and unspecified (incl. cysts and polyps)</i>				
Tumor pain	1 (10.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)
<i>Metabolism and nutrition disorders</i>				
Anorexia	1 (10.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)
Hypoglycemia	1 (10.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)
<i>Musculoskeletal and connective tissue disorders</i>				
Arthralgia	1 (10.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)
Chills	2 (20.0%)	0 (0.0%)	0 (0.0%)	2 (20.0%)
Pain in extremity	1 (10.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)
Myalgia	3 (30.0%)	0 (0.0%)	0 (0.0%)	3 (30.0%)
Musculoskeletal discomfort	1 (10.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)
<i>Nervous system disorders</i>				
Dysgeusia	1 (10.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)
Headache	2 (20.0%)	0 (0.0%)	0 (0.0%)	2 (20.0%)
<i>Skin and subcutaneous tissue disorders</i>				
Erythemas	1 (10.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)
Urticaria	1 (10.0%)	0 (0.0%)	0 (0.0%)	1 (10.0%)

subjects reported a total of 55 treatment-related AEs (Table 3). No grade 4 or grade 5 (death) treatment-related events were reported. No patients died during the active study period (day 1–day 64). Two subjects had a serious adverse event (acute esophagitis, spinal cord compression due to tumor progression); both resolved (Note: The spinal cord compression resolved following a course of palliative radiation therapy) and both serious adverse events were assessed as not related to treatment.

No clinically significant treatment-emergent changes were detected in any serum chemistries (including alkaline phosphatase, albumin, aspartate aminotransferase, alanine aminotransferase,

creatinine, potassium, sodium, total protein, calcium, bilirubin) or coagulation parameters (PT, PTT, or INR) while on study. Transient, treatment-emergent grade 1 hyperglycemia was noted in four subjects. Clinically significant cytopenias were not recorded (except a single transient case of anemia as described above).

DISCUSSION

In this pharmacokinetics- and pharmacodynamics-focused clinical trial, we have shown that the multiple proposed JX-594 MOA were active in cancer patients, even at a low dose of $\leq 10\%$ of the MTD in humans.¹⁸ JX-594 is a targeted, GM-CSF-expressing

oncolytic poxvirus designed to selectively replicate in and destroy cancer cells through virus replication-dependent cytolysis and induction of antitumoral immunity. JX-594 replication was demonstrated reproducibly by several methods, including both serial analyses of blood and of tumor biopsies. In addition, the expression and/or pharmacodynamic activity of the two transgene products (GM-CSF, β -gal) were confirmed reproducibly. GM-CSF-responsive WBC subsets (neutrophils, eosinophils, and monocytes) were induced ~5–7 days after treatment as predicted. In addition, immune stimulation was also shown through intense perivascular infiltration of tumors by lymphocytes. Finally, associated tumor necrosis was evident on tumor biopsies. These data expand our understanding of this emerging product class in several important ways. First, the ability of JX-594 to replicate in the face of neutralizing antibodies, after multiple cycles, was demonstrated for the first time. Second, replication of JX-594 and shedding into the blood following the first treatment was studied concurrently with WBC stimulation and GM-CSF concentrations in blood. Third, these findings could be compared with the detailed evaluation of injected and noninjected tumor biopsies for replication, inflammation and necrosis induction over time. Fourth, the patient population studied here was of uniform tumor type and received a uniform dosage. Finally, no immediate prevaccination was carried out on this trial, in contrast to a previous study.⁹

Oncolytic viruses such as JX-594 must by definition replicate in tumors in order to cause direct oncolysis. In addition, for transgene-expressing (a.k.a. “armed”) viruses such as JX-594, transgene expression is also closely tied to virus replication since the transgene expression is under control of promoters whose activation is markedly higher and more sustained in late stages of virus replication. The evaluation of virus replication in cancer patients is therefore critical for the rational development of these agents. Unlike animal tumor models, in humans it is not feasible to directly assay for the precise amount of virus infectious units or genomes in all tumor masses over time. Innovative methods are required to assess this critical feature. Tumor biopsies can give a snapshot at a single point in time as long as histological markers of replication are available, although the sensitivity of this approach is likely low with a standard fine needle aspiration or core biopsy. For biopsies of tumors that were injected directly, replication markers must rely on detection of viral products that are not in the input dose and/or should be linked to cytopathic changes unique to viral infection. Furthermore, as demonstrated on this trial, valuable information regarding JX-594 replication can be gleaned from biopsies of noninjected tumors. Detection of JX-594 at noninjected tumor sites is evidence of infection at the primary site, spread of JX-594 to distant sites of disease and subsequent productive infection at the noninjected site. Another method for evaluating virus replication is to follow virus genome concentrations in blood serially over time. A biphasic replication-dependent pharmacokinetic pattern was demonstrated. Of note, this assay does not delineate between virus-incorporated or free DNA; in either case, however, the secondary peak should be indicative of viral replication. An initial peak concentration was typically detectable 15 minutes after dosing, and by 3 hours JX-594 was nearly always undetectable. Between days 5 and 8 of each cycle, however, as a result of replication there was a re-emergence of the

agent into the blood. In order for JX-594 genomes to be detectable in blood at these late time points after injection, virus must replicate in cells and be released into the extracellular milieu, and subsequently gain access to the vasculature. Once in the blood, JX-594 is cleared with a half-life of ~30–60 minutes.¹⁸ Therefore, detection of JX-594 genomes in the blood 5–8 days after treatment appears to be a highly selective yet relatively insensitive marker for replication in humans. As predicted, in this trial JX-594 replication in tissues was demonstrated in biopsy material at the same time that a blood sample was negative for JX-594 (the converse did not occur). Another more durable and potentially more sensitive marker for JX-594 replication is the development of antibodies to β -gal protein; because β -gal protein expression only reaches significant levels during replication, antibody generation should also correlate with replication of JX-594 at some point in the past. Future products might express a secreted marker protein (e.g., CEA)²⁰ and/or a noninvasive imaging gene marker for real-time assessment and localization of replication^{21,22}.

The immune response will very likely play a critical role in the clinical activity of JX-594 and other oncolytic virus products. Antiviral immunity, including NAb development, may inhibit virus replication and induce clearance. However, vaccinia viruses like JX-594 express numerous immune avoidance gene products,²³ and the tumor milieu is often immunosuppressed²⁴ and resistant to penetration by antibodies. Therefore, the outcome of the virus-immune system-tumor interaction is critical to evaluate and understand in humans. In this trial we demonstrated for the first time that JX-594 replication can occur repeatedly over time, despite high NAb titers, after as many as nine cycles. At the same time, immunostimulatory oncolytic viruses such as JX-594 also rely on the anticancer immune response for enhanced and complementary efficacy. JX-594-associated WBC stimulation and lymphocytic infiltration of tumors were shown. GM-CSF transgene production was monitored by detecting GM-CSF in plasma of all patients. One patient exhibited detectable GM-CSF in the peripheral blood on this study. On a previous phase I trial studying IT injection of JX-594, in patients with evidence of high-level replication it was possible to occasionally detect GM-CSF in the systemic circulation at time points of peak replication (days 4–15),¹⁸ despite the short GM-CSF half-life in blood (<2 hours).²⁵ Therefore, GM-CSF detection in blood is indicative of high-level GM-CSF expression; detection between 4 and 15 days after treatment (when all other inflammatory cytokines have returned to baseline values) may be a specific but insensitive marker of transgene expression. In addition, induction of immunoglobulin G antibodies to the β -gal transgene indicate Immunoglobulin class switching (requiring T-helper cells) has occurred. This proof-of-concept is critical for understanding this novel therapeutic approach. Nevertheless, future trials should explore the induction of functional anticancer immunity in treated patients (e.g., induction of cell-mediated and antibody-mediated immunity against tumor cells). Based on the data presented here, it does appear feasible for JX-594 to simultaneously avoid rapid immune clearance and to stimulate immunity in a fashion that could benefit the patient. The optimization of these two immune aspects will require further study. IV administration in the context of immunity, while feasible in animal tumor models, should be explored in humans.

samples obtained every 7 days after cycles 1–6, in addition to days 4 after cycles 1 and 2.

NAb titers. NAb titers were determined by cytopathic effect inhibition assay. Heat-inactivated serum was serially diluted in media using half log dilutions. 50 µl samples were incubated with 1,000 pfu JX-594 for 2 hours, then inoculated onto A2780 cells. After 3 days, cell viability was determined using Cell Counting Kit-8 (Donjindo Laboratories, Kumamoto, Japan). NAb titer was defined as the reciprocal of the highest dilution of serum that resulted in ≥50% cell viability.

Antibody titers to the β-gal marker transgene. Human immunoglobulin G antibodies to β-gal were measured by enzyme-linked immunosorbent assay. Briefly, plates (NUNC MaxiSorp; Thermo Fisher Scientific, Waltham, MA) with wells containing β-gal (Sigma, St Louis, MO) or carbonate/carbonate buffer-only were incubated overnight at 4°C and washed with phosphate-buffered saline-Tween before incubation with blocking buffer [phosphate-buffered saline with 1% bovine serum albumin (ELIS A grade; Sigma)]. Diluted plasma (1:50, 1:100, 1:200, in phosphate-buffered saline + 0.05% Tween + 1% bovine serum albumin) was added to β-gal-coated and control wells in duplicate and incubated at room temperature. Plates were washed and incubated with alkaline phosphatase labeled goat antihuman immunoglobulin G (AbCam, Cambridge, MA) diluted 1:2,000. After washing, colorimetric substrate pNPP (Sigma) was added, and NaOH was added to stop color development after 10 minutes. Absorbance was read at 405 nm, and absorbance of 630 nm was subtracted. Control well values were subtracted to account for nonspecific binding, and titers values were calculated by comparison to a standard curve of positive sera arbitrarily assigned a titer of 8,000.

Quantitative-PCR for JX-594 in blood samples. Quantitative-PCR was used to measure JX-594 genomes in whole blood over time due to its reproducibility and ability to detect product regardless of antibody and/or complement neutralization. JX-594 DNA was purified from samples using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Quantitative-PCR was run as described previously.^{18,28} The lower limits of JX-594 detection ranged from 667 to 3,333 copies/ml blood.

GM-CSF blood assay. GM-CSF was measured in plasma of all patients at predose day 1 and days 5, 8, 12, 15, 22, 29, 36, and 43. GM-CSF was detected in plasma by enzyme-linked immunosorbent assay kit (BioSource International; Carlsbad, CA) following the instructions of the vendor.

Tumor biopsy processing and analysis. Core biopsies or excisional biopsies were obtained in several patients (optional, if patient consent obtained separately) to evaluate viral replication, inflammatory, and immune cell infiltration, necrosis, apoptosis at injected tumor sites pre- and postinjection. Biopsies were collected from injected or noninjected tumors and were obtained at baseline (pretreatment), day 5–8, day 12 and/or day 43. Formalin-fixed, paraffin-embedded biopsies were stained with hematoxylin and eosin for routine histology (including assessment of viable tumor and/or normal tissues), including assessment of necrosis (graded based on proportion of necrotic area, pattern of necrosis and its location), apoptosis and immune/inflammatory cell infiltration (graded for severity and prominent cell type(s)). Viral replication in tumor and adjacent normal tissues was assessed by immunohistochemistry for viral proteins on the paraffin-embedded biopsies (graded based on expression intensity and percentage of positive tumor cells).

For immunohistochemistry, an antivaccinia polyclonal antibody (Quartett, Berlin, Germany) was used, followed by incubation with LeVamizole block and permanent red chromagen secondary antibody kit (DAKO, Carpinteria, CA); negative controls were run without primary antibody and tumors from mice treated with JX-594 were included as positive controls. For immunohistochemistry detection of β-gal, an anti-β-gal polyclonal antibody (Abcam, Cambridge, MA) was used,

though sections were not evaluable due to intense melanin deposition in tumor cells.

Radiographic assessment. All measurements were performed by physical examination and/or computed tomography or magnetic resonance imaging at baseline (within 14 days before first treatment with JX-594) and 1 week after the 6th dose was administered (day 43). At baseline, tumor lesions were categorized as target (injected) or nontarget. Maximum tumor diameters were obtained. Tumor assessments were performed according to RECIST criteria.²⁹ For subjects who did not progress after discontinuing study drug, additional tumor assessments were to be performed approximately every 6 weeks until subject either met the criteria for progression or alternate therapy started. Nontarget (noninjected) tumor evaluation was the same as for target lesions.

Data quality assurance. Monitoring was conducted by the sponsor. The study was monitored at appropriate intervals to assure satisfactory enrollment rate, data recording, and protocol adherence. The frequency of monitoring varied depending on enrollment rate and the quality of the data collected.

Statistical issues. Study sample size was determined by safety and practical issues. The intent-to-treat population was assessed. This was a noncontrolled, nonrandomized clinical trial targeting a small number of subjects; no hypothesis testing was performed. Individual subject data listings as well as combined data are presented. The intent-to-treat population included all subjects who received at least one dose of JX-594. The evaluable population was the subset of the intent-to-treat population comprised of subjects who received at least one treatment and had undergone appropriate tumor measurement at protocol-specified time points.

Median NAb induction and antibody induction to β-gal was calculated using GraphPad Prism 5 software. Student's *t*-test was used to test the significance of induction of antibodies over time.

SUPPLEMENTARY MATERIAL

Table S1. Listing of prior therapy with biologic anticancer agents, by patient.

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REFERENCES

- Parato, KA, Senger, D, Forsyth, PA and Bell, JC (2005). Recent progress in the battle between oncolytic viruses and tumours. *Nat Rev Cancer* 5: 965–976.
- Castano, R, Miest, T, Shashikova, EV and Barry, MA (2006). Reprogrammed viruses as cancer therapeutics: targeted, armed and shielded. *Nat Rev Microbiol* 6: 529–540.
- Kim, DH and Thorne, SH (2009). Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer. *Nat Rev Cancer* 9: 64–71.
- Liu, TC, Galanis, E and Kim, D (2007). Clinical trial results with oncolytic virotherapy: a century of promise, a decade of progress. *Nat Clin Pract Oncol* 4: 101–117.
- Msaouel, P, Dispenzieri, A and Galanis, E (2009). Clinical testing of engineered oncolytic measles virus strains in the treatment of cancer: an overview. *Curr Opin Mol Ther* 11: 43–53.
- Senzee, NN, Kaufman, HL, Amatruda, T, Nemunaitis, M, Reid, T, Daniels, G et al. (2009). Phase II clinical trial of a granulocyte-macrophage colony-stimulating factor-encoding, second-generation oncolytic herpesvirus in patients with unresectable metastatic melanoma. *J Clin Oncol* 27: 5763–5771.
- De Clercq, E (2002). Cidofovir in the therapy and short-term prophylaxis of poxvirus infections. *Trends Pharmacol Sci* 23: 456–458.
- Witteck, R (2006). Vaccinia immune globulin: current policies, preparedness, and product safety and efficacy. *Int J Infect Dis* 10: 193–201.
- Mastrangelo, MJ, Maguire, HC Jr, Eisenlohr, LC, Laughlin, CE, Monken, CE, McCue, PA et al. (1999). Intratumoral recombinant GM-CSF-encoding virus as gene therapy in patients with cutaneous melanoma. *Cancer Gene Ther* 6: 409–422.
- Kim, JH, Oh, JY, Park, BH, Lee, DE, Kim, JS, Park, HE et al. (2006). Systemic armed oncolytic and immunologic therapy for cancer with JX-594, a targeted poxvirus expressing GM-CSF. *Mol Ther* 14: 361–370.

11. Merrick, AL, Ilett, LJ and Melcher, AA (2009). JX-594, a targeted oncolytic poxvirus for the treatment of cancer. *Curr Opin Invest Drugs* **10**: 1372–1382.
12. Grant, MF, Noll, LA, Irvine, KR, Puhmann, M, Terrell, RL, Alexander, HR Jr et al. (1999). Tumor-specific gene delivery using recombinant vaccinia virus in a rabbit model of liver metastases. *J Natl Cancer Inst* **91**: 1744–1750.
13. Buller, RM, Smith, GL, Cremer, K, Notkins, AL and Moss, B (1985). Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. *Nature* **317**: 813–815.
14. Stojdl, DF, Lichty, B, Knowles, S, Marini, R, Atkins, H, Sonenberg, N et al. (2000). Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nat Med* **6**: 821–825.
15. Hengstschläger, M, Pfeilstöcker, M and Wawra, E (1998). Thymidine kinase expression: A marker for malignant cells. *Adv Exp Med Biol* **431**: 455–460.
16. Thome, SH, Hwang, TH, O'Connor, WL, Bartlett, DL, Sel, S, Kang, F et al. (2007). Rational strain selection and engineering creates a broad-spectrum, systemically effective oncolytic poxvirus, JX-594. *J Clin Invest* **117**: 3350–3358.
17. Dranoff, G, Jaffee, L, Lazenby, A, Golumbek, P, Levitsky, H, Brose, K et al. (1993). Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc Natl Acad Sci USA* **90**: 3539–3543.
18. Park, BH, Hwang, T, Liu, TC, Sze, DY, Kim, JS, Kwon, HC et al. (2008). Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: a phase I trial. *Lancet Oncol* **9**: 533–542.
19. Hammond, JM, Oke, PG and Coupar, BE (1997). A synthetic vaccinia virus promoter with enhanced early and late activity. *J Virol Methods* **66**: 135–138.
20. Peng, KW, TenEyck, CJ, Galanis, E, Kall, KR, Hartmann, LC and Russell, SJ (2002). Intraperitoneal therapy of ovarian cancer using an engineered measles virus. *Cancer Res* **62**: 4656–4662.
21. McCart, JA, Mehta, N, Scollard, D, Reilly, RM, Camasquillo, JA, Tang, N et al. (2004). Oncolytic vaccinia virus expressing the human somatostatin receptor SSTR2: molecular imaging after systemic delivery using ¹¹¹In-pentetreotide. *Mol Ther* **10**: 553–561.
22. Msaouel, P, Iankov, ID, Allen, C, Aderca, I, Federspiel, MJ, Tindall, DJ et al. (2009). Noninvasive imaging and radiotherapy of prostate cancer using an oncolytic measles virus expressing the sodium iodide symporter. *Mol Ther* **17**: 2041–2048.
23. Smith, GL, Symons, JA, Khanna, A, Vanderplasse, A and Alcamí, A (1997). Vaccinia virus immune evasion. *Immunol Rev* **159**: 137–154.
24. Zou, W (2005). Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat Rev Cancer* **5**: 263–274.
25. Hovgaard, D, Mortensen, BT, Schifter, S and Nissen, NI (1992). Clinical pharmacokinetic studies of a human haemopoietic growth factor, GM-CSF. *Eur J Clin Invest* **22**: 45–49.
26. Sze, DY, Freeman, SM, Slonim, SM, Samuels, SL, Andrews, JC, Hicks, M et al. (2003). Dr. Gary J. Becker Young Investigator Award: Intraarterial adenovirus for metastatic gastrointestinal cancer: activity, radiographic response, and survival. *J Vasc Interv Radiol* **14**: 279–290.
27. Wolchok, JD, Hoos, A, O'Day, S, Weber, JS, Hamid, O, Lebbé, C et al. (2009). Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. *Clin Cancer Res* **15**: 7412–7420.
28. Kulesh, DA, Baker, RO, Loveless, BM, Norwood, D, Zwiers, SH, Mucker, E et al. (2004). Smallpox and pan-orthopox virus detection by real-time 3'-minor groove binder TaqMan assays on the roche LightCycler and the Cepheid smart Cyclo platform. *J Clin Microbiol* **42**: 601–609.
29. Therasse, P, Arbrick, SC, Eisenhauer, EA, Wanders, J, Kaplan, RS, Rubinstein, L et al. (2000). New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* **92**: 205–216.

APPENDIX V: EX VIVO INFECTION OF LIVE TISSUE WITH ONCOLYTIC VIRUSES.

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Contribution: I received and infected sarcoma samples provided by Dr. Abdelbary. Dominic Roy and I infected and imaged samples.

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Video Article

Ex Vivo Infection of Live Tissue with Oncolytic Viruses

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Abstract

Oncolytic Viruses (OVs) are novel therapeutics that selectively replicate in and kill tumor cells¹. Several clinical trials evaluating the effectiveness of a variety of oncolytic platforms including HSV, Reovirus, and Vaccinia OVs as treatment for cancer are currently underway²⁻⁵. One key characteristic of oncolytic viruses is that they can be genetically modified to express reporter transgenes which makes it possible to visualize the infection of tissues by microscopy or bio-luminescence imaging^{6,7}. This offers a unique advantage since it is possible to infect tissues from patients *ex vivo* prior to therapy in order to ascertain the likelihood of successful oncolytic virotherapy⁸. To this end, it is critical to appropriately sample tissue to compensate for tissue heterogeneity and assess tissue viability, particularly prior to infection⁹. It is also important to follow viral replication using reporter transgenes if expressed by the oncolytic platform as well as by direct titration of tissues following homogenization in order to discriminate between abortive and productive infection. The object of this protocol is to address these issues and herein describes 1. The sampling and preparation of tumor tissue for cell culture 2. The assessment of tissue viability using the metabolic dye alamar blue 3. *Ex vivo* infection of cultured tissues with vaccinia virus expressing either GFP or firefly luciferase 4. Detection of transgene expression by fluorescence microscopy or using an *In Vivo* Imaging System (IVIS) 5. Quantification of virus by plaque assay. This comprehensive method presents several advantages including ease of tissue processing, compensation for tissue heterogeneity, control of tissue viability, and discrimination between abortive infection and bona fide viral replication.

Protocol

1. Tissue processing

- For best results, this protocol should be performed using freshly isolated tissue deposited in DMEM media containing 10% FBS, 1% Penicillin/Streptomycin solution, and 0.1% Amphotericin B solution immediately following surgery prior to processing. If this is not possible, tissues can be left overnight in this medium at 4 degrees prior to processing.
- Prior to processing the sample, sterilize metal forceps and disposable blades by depositing them in a 70% ethanol solution for at least 5 minutes. Also prepare a 24-well plate with 2 ml of DMEM media containing 10% FBS, 1% Penicillin/Streptomycin solution, and 0.1% Amphotericin B.
- Collect the tissue sample in a laminar flow cell culture hood using sterilized forceps and deposit the tissue in an empty 15 cm petri dish, keeping the lid on the side, sterile side up.
- In the cell culture hood, use a 2 mm biopsy punch to obtain different cores from a variety of regions within the tissue as in Figure 1. Deposit the cores in the lid of the 15 cm petri with the help of forceps, leaving enough space in between each core so that they can be easily cut along the horizontal axis.
- Split each core into 4 even quarters using a sterilized razor blade as in Figure 1.
- Using a pipet tip, put each core quarter from a given core in a different well from A1 to A4 as in figure 1, already containing 1.5 ml of DMEM containing 10% FBS, 1% Penicillin/Streptomycin solution, and 0.1% Amphotericin B solution. Repeat for each core. This should allow representative sampling of the tumor, while minimizing bias in a given well/condition. For better representation, increase the number of cores.

2. Assessment of tissue viability

- Following tissue processing, add 25 μ l Alamar blue to well # A1 and # A2 as shown in figure 1 and incubate for 1 hour at 37 degrees in a humidified incubator with 5% CO₂.
- Following incubation with alamar blue, remove 3 times 100 μ l from each A1 and A2 well and transfer to 6 different wells of a 96-well plate. Read the signal using a fluorescence plate reader (530 excitation, 590 emission) and keep the data for your records.
- After Alamar blue signal has been read, transfer all pieces of tissue using a pipet tip, from well A1 to C1 that contains DMEM, 10% FBS + PS + AmphoB. Be careful not to transfer an excessive amount of media from well A1.
- Infect wells A3 and A4 respectively with 10⁵ PFU of GFP-expressing and luciferase-expressing vaccinia virus diluted in 25 μ l of media. Well A2 can be infected with a virus that expresses any given transgene including none at all.
- 72 hours later, add 25 μ l Alamar blue to wells C1 and D1 and repeat step 2.2

3. Visualization of GFP transgene expression by fluorescence microscopy

- Remove all cell culture media covering the tissue pieces in order to take good fluorescence pictures.
- Using a fluorescence-capable dissection microscope, first take a phase contrast image at an appropriate resolution
- Switch to fluorescence mode and take a picture using the appropriate filter to visualize the transgene of interest, in this case GFP.
- Use a fluorescence filter for another wavelength as far as possible from that of the transgene of interest (such as RFP) to take a picture of background fluorescence

4. Visualization of luciferase expression using an *In Vivo* imaging System (IVIS)

1. Make sure that the IVIS is initialized before you begin.
2. In wells A4 and B4, add 5 μ l of luciferin substrate 10 mg/ml, mix well and incubate for 5 minutes at room temperature.
3. Set the IVIS exposure time to 5 seconds and take a picture of your wells. If the image is saturated, repeat with lower exposure time. If there is no signal, increase the exposure time.
4. Using the IVIS imaging software, you may remove background using the signal from well B4 and select the region of interest to quantify the luminescence signal.

5. Assessing viral titers by plaque assay

1. Prior to quantifying viral titers, tissues must first be homogenized to release viral particles. This can be done several months later in the infected tissue samples are stored at -80.
2. Weigh the sample that needs to be homogenized using an analytical scale. In this case, we will use the sample collected from well A2.
3. Place the tissue in a 5 ml polystyrene round-bottom falcon tube and add 1 ml of PBS. Homogenize the tissue using a tissue homogenizer. If necessary, store the homogenate at -80 for assessment of viral titers at a later time.
4. To titer vaccinia virus, first plate 1 million U2OS cells in a 6-well plate and incubate overnight in a 37 degree, 5% CO₂ humidified incubator such that they have reached approximately 95 % confluency the following day.
5. Use serum free media to do serial dilutions of virus, making sure to change tips between each dilution step. Typically we do 1 in 10 dilutions and use 100 μ l to transfer into 900 μ l. How many dilutions are made depends on the anticipated virus yield.
6. Following making the dilutions, remove the media covering the plated U2OS cells then add 500 μ l of diluted virus stock (for each dilution) to infect the U2OS cells. Incubate the cells for 1 hour minutes at 37 degrees Celsius in a 5% CO₂ humidified incubator.
7. During this time, warm up the 2 X concentrated DMEM containing 20% FBS, and the 3% CMC solution in a 37 degree water bath.
8. After the 1 hour incubation, remove the media covering infected U2OS cells. Mix 1:1 volumes of 3% CMC : 2X DMEM, 20% FBS together and use 2 ml of this mixture to cover each well of the infected U2OS cells.
9. Put cells back in a 37 degree 5% CO₂ humidified incubator for another 48 hours.
10. After 48 hours, add 2 ml of methanol-acetic acid fixative on top of the CMC overlay in each well and incubate at room temperature for 10 minutes in a cell culture hood.
11. Discard the fixed overlay and wash remainder off of the wells using tap water.
12. Stain the fixed U2OS cells with 2ml of a coomassie blue solution per well and incubate for 30 minutes at room temperature at low speed on a plate shaker.
13. Remove the coomassie stain from the wells and wash the plates using tap water. Allow to dry with lid off for about an hour.
14. The Resulting viral plaques can be easily visualized in figure 2C. Plates can be stored indefinitely at this stage.
15. Count plaques at the dilution step where between 10 and 100 plaques are visible.
16. Multiply the number of plaques counted by the dilution used and multiply the resulting number by 2 to give a titer in PFU/ml. For example, if 25 plaques are counted in the well where a million-fold dilution was used, the titer of the initial undiluted sample is 25 multiplied by 1 million multiplied by 2 or 50 million PFU/ml. Further divide this number by the weight initially measure for the sample to report titers in PFU/g

6. Representative Results:

In order to accurately determine whether a surgically obtained normal/tumor tissue sample is or is not infectable with virus, one must first ensure that the tissue sample is at least viable. Fig 1A shows that viability can be assessed using a metabolic dye (Alamar blue) and that both normal and tumor tissue can remain metabolically active over a period of at least 72h. This suggests that tissues cultured ex vivo can support viral replication. A significant advantage of oncolytic viruses is that they can be engineered to express therapeutic or imaging transgenes. Fig 2D-E shows that GFP or Luciferase can be detected from tissues infected ex vivo, further supporting that these tissues are viable and also infectable. Although increased transgene expression is generally associated with viral replication, it does not necessarily equate to a productive viral life cycle that leads to self-amplification and spread, which is thought to be important for therapeutic activity. For this reason, it is necessary to determine whether more virus is produced than what was used to initially infect the tissue. Fig 2B shows that upon viral quantification by plaque assay, more virus is obtained after 72 hours infection than tissue collected immediately after infection. Overall, these data show that surgically excised tumor tissue can remain viable in cell culture for a period of at least 72 hours, during which time viral replication can be supported.

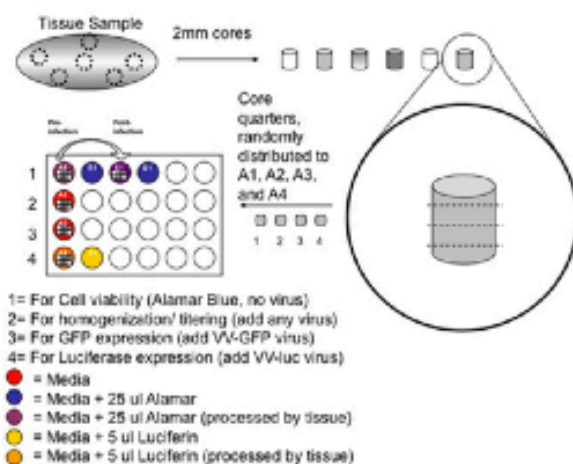


Figure 1. Overview of the tissue sampling / sectioning protocol. Tissue sample is processed by removing several 2 mm cores which are subsequently divided into four quarters which are randomly distributed into wells A1-A4. The color codes indicate the reagents used in each well. The grey-shaded squares within the wells represent each quarter from the 8 individual cores depicted. The tissue in well A1 is transferred to a new well (C1) with media after the initial alamar blue reading. A second reading is done at the end of the 72 hour incubation with viruses. Wells A4 and B4 are supplemented with Luciferin after the 72 hour incubation post-infection

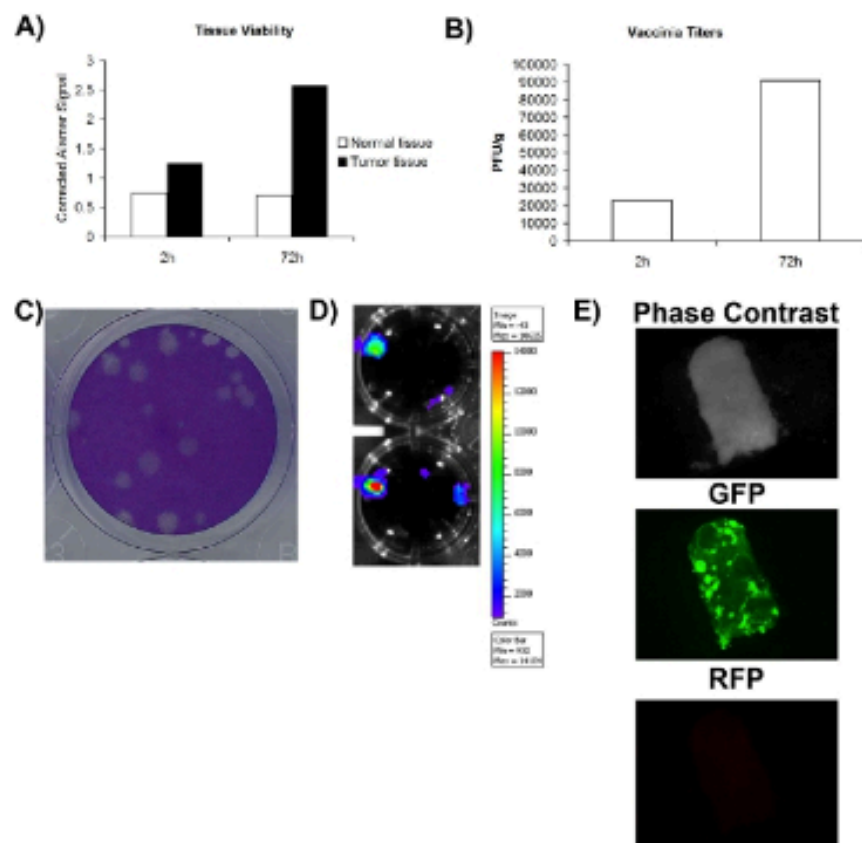


Figure 2. Viability and infectability of patient tissue samples. **A)** Tissue viability as measured by alamar blue signal at 2 hours and 72 hours post collection. Y-axis represents the blank corrected alamar blue signal. **B)** Titer of vaccinia virus collected per gram of tissue samples 2 and 72 hours post-infection. **C)** Example of vaccinia virus plaques on U2OS cells following coomassie staining. **D)** Luminescence signal obtained from patient VV-luciferase-infected tissue imaged using IVIS. **E)** Fluorescence microscopy pictures of patient tissue sample infected with VV-GFP.

Discussion

One of the critical steps in this protocol is obtaining fresh tissue sample. If the sample is put into cell culture following a long wait at the operating room in an inappropriate media (eg. PBS), this may compromise tissue viability and prevent infectability. Of note, normal tissue is inherently more prone to these effects than tumor tissues. Another critical point is how many cores are used to sample the tissue and the consistency of their size. Inconsistencies in size will lead to variability across patient samples since factors such as tissue hypoxia and infectable surface will fluctuate depending on the size of the cores and core quarters. While this can be partially resolved by using tissue slicers, one advantage of the method presented here is that it is relatively easy, less prone to contamination, and widely applicable to a variety of tissue types, including soft or viscous tissues which are not easily amenable to tissue slicing. Notably, the number of cores can be increased in order to get better tissue representation. Also, the cores can be cut into more pieces (eg. 5-8) to accommodate other potential assays to be done in parallel such as DNA, RNA, or protein extraction. However, the number of pieces in which the cores can be cut to reproducible sizes will depend on the actual size of the cores, which can be modified by using different sized corers. Optionally, one way to help obtain reproducibly sized tissue pieces is to even out the length of the cores using a ruler prior to further subdividing them into smaller pieces. The protocol can naturally be modified to accommodate other viruses and we have found that tissues can be viable and support replication for up to 6 days. The protocol can further be extended to measurements of other virally-expressed transgenes including cytokines. Following infection, tissues can also be embedded in paraffin for further sectioning and staining by immunohistochemical methods, which allows for further refinement of the tissue histology and how it relates to viral infection¹²⁻¹².

Disclosures

No conflicts of interest declared.

Acknowledgements

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References

1. Parato, K.A., Senger, D., Forsyth, P.A., Bell, J.C. Recent progress in the battle between oncolytic viruses and cancer. *Nat Rev Cancer*. 5 (12) : 965-76 (2005).
2. Renouf, L.C., Thway, K., Sibbain, A., McNeish, I.A., Newbold, K.L., Goldswieg, H., Coffin, R. and Nutting, C.M. *Phase III study of oncolytic HSV GM-CSF in combination with radiotherapy and cisplatin in untreated stage III/IV squamous cell cancer of the head and neck*. *Clin Cancer Res*. 16 (15) : 4005-15 (2010).
3. Lal, R., Harris, D., Postel-Vinay, S. and de Bono, J. *Reovirus: Rationale and clinical trial update*. *Curr Opin Mol Ther*. 11 (5) : 532-9 (2009).
4. Park, B.H., Hwang, T., Liu, T.C., Sze, D.Y., Kim, J.S., Kwon, H.C., Oh, S.Y., Han, S.Y., Yoon, J.H., Hong, S.H., Moon, A., Speth, K., Park, C., Ahn, Y.J., Daneshmand, M., Rhee, B.G., Pinedo, H.M., Bell, J.C. and Kim, D.H. *Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: a phase I trial*. *Lancet Oncol*. 9 (6) : 533-42 (2008).
5. Breitbach, C.J., Reid, T., Burke, J., Bell, J.C., Kim, D.H. Navigating the clinical development landscape for oncolytic viruses and other cancer therapeutics: no shortcuts on the road to approval. *Cytokine Growth Factor Rev*. 21 (2-3) : 85-9 (2010).
6. Le Boeuf, F., et al. Synergistic interaction between oncolytic viruses augments tumor killing. *Mol Ther*. 18 (5) : 888-95 (2010).
7. De Silva, N., Atkins, H., Kim, D.H., Bell, J.C., Breitbach, C.J. Double trouble for tumours: exploiting the tumour microenvironment to enhance anticancer effect of oncolytic viruses. *Cytokine Growth Factor Rev*. 21 (2-3) : 135-41 (2010).
8. Diallo, J.S., et al. A high-throughput pharmacoviral approach identifies novel oncolytic virus sensitizers. *Mol Ther*. 18 (6) : 1123-9 (2010).
9. Cooke, S.L., et al. Intra-tumour genetic heterogeneity and poor chemoradiotherapy response in cervical cancer. *Br J Cancer*. Epub Nov 9 (2010).
10. Pennington, K., Chu, Q.D., Curiel, D.T., Li, B.D.L., Mathis, J.M. The Utility of a Tissue Slice Model System to Determine Breast Cancer Infectivity by Oncolytic Adenoviruses. *J Surg Res*. 1-6 (2010).
11. Hochberg M., et al. Tropism of herpes simplex virus type 1 to non-melanoma skin cancers. *Br J Dermatol*. Epub Oct 26 (2010).
12. van der Kuip, H., et al. Short term culture of breast cancer tissues to study the activity of the anticancer drug taxol in an intact tumor environment. *BMC Cancer*. 6 : 86 (2006).

APPENDIX VI: ENHANCEMENT OF VACCINIA VIRUS BASED ONCOLYSIS WITH HISTONE DEACETYLASE INHIBITORS.

MacTavish H, Diallo JS, Huang B, Stanford M, Le Boeuf F, De Silva N, Cox J, Simmons JG, Guimond T, Falls T, McCart JA, Atkins H, Breitbach C, Kirn D, Thorne S, Bell JC.

Contribution: I performed ex vivo infections of normal tissues that I extracted from mice under various treatment conditions as indicated in the manuscript.

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Enhancement of Vaccinia Virus Based Oncolysis with Histone Deacetylase Inhibitors

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Abstract

Histone deacetylase inhibitors (HDI) dampen cellular innate immune response by decreasing interferon production and have been shown to increase the growth of vesicular stomatitis virus and HSV. As attenuated tumour-selective oncolytic vaccinia viruses (VV) are already undergoing clinical evaluation, the goal of this study is to determine whether HDI can also enhance the potency of these poxviruses in infection-resistant cancer cell lines. Multiple HDIs were tested and Trichostatin A (TSA) was found to potentially enhance the spread and replication of a tumour selective vaccinia virus in several infection-resistant cancer cell lines. TSA significantly decreased the number of lung metastases in a syngeneic B16F10LacZ lung metastasis model yet did not increase the replication of vaccinia in normal tissues. The combination of TSA and VV increased survival of mice harbouring human HCT116 colon tumour xenografts as compared to mice treated with either agent alone. We conclude that TSA can selectively and effectively enhance the replication and spread of oncolytic vaccinia virus in cancer cells.

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Introduction

As biological tumour killing machines, oncolytic viruses (OVs) often display an array of anti-cancer activities including direct tumour lysis, immune cell recruitment and anti-vascular activity [1,2]. In order to safely implement OVs in the clinic it is critical to restrict their replication and activity to tumours. To date, this has been achieved in part by the engineering or selection of virus variants that have mutations or deletions in viral virulence genes. The proteins encoded by virulence genes often attack or antagonize normal cellular anti-viral programs facilitating the invasion and ultimate destruction of the infected cell. Since OVs have impaired virulence genes they are unable to productively infect normal cells, however, since tumour cells frequently have acquired defects in anti-viral signaling pathways, they remain uniquely sensitive to OV infection and killing. One signaling pathway that is defective in a large proportion of cancer cells (~70–75%) is the interferon (IFN) pathway, which mediates the first line of cellular anti-viral response [3,4,5,6,7]. However we and others have shown that the extent of interferon non-responsiveness is variable in tumour cell lines and patient tumour explants and this may lead to less than optimal therapeutic benefit from some OVs [2,8,9].

Vaccinia virus (VV) has many of the biological properties that an ideal oncolytic or cancer killing virus should have. It has an extensive safety history in humans, a large cloning capacity for insertion of therapeutic transgene payloads, is active as a systemic agent, lacks any known genotoxic activity and expresses a sophisticated array of immune modulating genes that can be exploited for therapeutic benefit [1]. A Phase I trial of an oncolytic vaccinia virus JX-594 demonstrated acceptable safety and promising anti-cancer activity in patients with advanced liver tumours [10].

Vaccinia encodes close to two hundred genes, some of which are now known to be redundant for growth in tumour cells [1,11,12]. For example VV mutants with deletions in the thymidine kinase gene (TK) and/or the vaccinia growth factor gene (VGF) are well advanced in pre-clinical and clinical studies [1,10,13,14]. These mutants grow selectively in cancer cells in which high levels of cellular TK and constitutively activated EGFR/Ras pathway signaling complements the loss of the viral gene products [12]. Another vaccinia gene that can be manipulated to enhance virus selectivity for cancer cells is B18R which encodes a soluble mimetic of the type-1 interferon receptor. When produced and secreted from VV infected cells the B18

protein locally blunts the cellular interferon response by sequestering interferon produced by the infected cell [15,16,17]. Previously, we have shown that a VV strain with an engineered deletion of the B18R gene is more rapidly cleared from normal tissues than the parental strain while remaining active within tumours [18]. A natural truncation of the B18R gene of the clinical vaccinia candidate JX-594, has been shown by others to have reduced ability to antagonize interferon activity [16] and this likely contributes to its acceptable safety profile in humans [10].

As mentioned above, while defects in innate anti-viral responses are common in malignant cells the extent of the defect is variable and can affect the growth of OV in tumours. In an earlier study we showed that a Histone Deacetylase Inhibitor (HDI) can specifically enhance the growth of an interferon sensitive version of vesicular stomatitis virus (VSVΔ51) in tumour cells. HDIs block the activity of histone deacetylases (HDACs), leading to increased acetylation of histones and other proteins [4,19,20,21] and importantly inhibit the ability of tumour cells to mount a productive anti-viral response [22,23,24]. In the current study we set out to examine the ability of a panel of HDIs to augment oncolytic activity of vaccinia virus. We present evidence that the growth of vaccinia virus is most potently and selectively enhanced in tumour cells both *in vitro* and *in vivo* by the HDI trichostatin A.

Results

Trichostatin A is a potent enhancer of vaccinia virus spread

A number of HDIs are in clinical development and we assessed a panel of candidates for their ability to enhance vaccinia virus replication and spread in tumour cells. 4T1 murine breast cancer cells were pre-treated with individual HDIs over a range of concentrations then challenged with a Green Fluorescent Protein (GFP)-expressing vaccinia virus (herein referred to as VVdd) [12], at a multiplicity of infection (MOI) of 0.1 plaque forming units (pfu) per cell. The percentage of GFP positive cells visible (indicating active virus replication) following 120 h incubation was subsequently assessed from fluorescence microscopy images quantified using image analysis software. Each condition was related to percent GFP-positive area in the vehicle control. Table 1 shows that several HDIs enhance the spread of VVdd albeit to varying extents. Overall, we found that Valproic Acid, SBHA, M344 and Trichostatin A (TSA) elicited the greatest response however clearly TSA was the most potent enhancer of VVdd replication (Table 1). We confirmed by western blot analysis that all the HDIs were able to affect histone acetylation in treated cells and the extent of histone 4 acetylation generally corresponded with the ability of the HDI to enhance VVdd growth. Since TSA seemed to be the most potent HDI in these studies we chose to test it in a variety of *in vitro* and *in vivo* models.

TSA enhances the spread of VV strains specifically in cancer cells

Murine 4T1 breast cancer cells and B16 melanoma cells were plated *in vitro* and confluent cells were pre-treated with low-dose TSA (37.5 nM) before infection with VVdd-GFP at MOIs of 0.01, 0.1 and 1 pfu/cell. Figure 1a shows that TSA enhanced VVdd-associated GFP expression in both murine cancer cell lines. TSA treatment resulted in visibly more and larger plaques (Figures 1a and b) and increased viral titers of up to 100-fold (Figure 1c) in tumour cells. Performing single-step and multi-step growth curves (starting MOI = 0.1 and 3 respectively) further revealed that TSA-enhanced VVdd growth was more substantial when the virus was

Table 1. TSA is a potent enhancer of vaccinia virus spread.

HDI	Maximum effect	Effective Dose (μM)	Level of Acetylated H4
SAHA	++	0.8	33.7
MS-275	+	1.6	26.6
Oxamflatin	++	2.5	28.0
Apicidin	+	0.25	26.7
SBHA	+++	25	50.8
Scriptaid	++	0.5	16.0
CHAH	+	0.6	4.7
Valproic Acid	+++	1250	1.5
M344	+++	0.6	66.2
TSA	+++	0.08	52.7

4T1 cells were plated in 96-well plates then pre-treated with a concentration gradient of the indicated drugs. DMSO was used as a control. Following pre-treatment with drugs, cells were challenged with VVdd-GFP at an MOI of 0.1. After 120 h incubation period, fluorescence pictures were taken of each well, spanning the entire well-surface. Green fluorescence, indicating vaccinia spread, was measured using an image analysis software (Image J, NIH) and reported as a fold change in GFP-positive comparison to control (average of triplicate). + indicates increase in spread <2-fold, ++ indicates between 2 and 3-fold increase, +++ indicates >3-fold increase. TSA was the most active compound at the lowest dose tested. 10 cm plates of confluent 4T1 cells were treated with indicated HDI and samples were probed for hyper-acetylated histone H4 by Western Blot and reported as level of acetylated H4 relative to the untreated control.

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provided at a low MOI and allowed to spread (Figure 1d). In earlier studies we and others, demonstrated that VVdd presents attenuated growth in normal cells when compared to the wild type Western Reserve strain [12]. In figure 1e it is evident that the attenuated growth of VVdd in normal MRC-5 fibroblasts is not affected by treatment with TSA. Combination indices calculated as described by Chou and Talalay [25] revealed that TSA and VVdd combination therapy results in synergistic killing in 4T1 cells (Figure 1f).

TSA enhances VVdd efficacy in a syngeneic lung metastasis model

We next tested the ability of TSA to enhance VVdd activity in a mouse tumour model. To this end, C57/B6 mice were injected intravenously with 3×10^5 B16F10LacZ melanoma cells. Following tumour seeding in the lungs, mice were treated with either TSA (4 daily intraperitoneal doses of 0.05 mg) and/or VVdd (2 intravenous injections of 1×10^7 pfu/mouse). This treatment schedule can be visualized in figure 2a. At the doses used, mice did not display any discernable side effects from treatment with TSA, VVdd, or the combination of both. Two weeks following implantation, lungs were collected and tumours identified (figure 2c) by using established X-gal staining procedures [26,27]. As single agents at the doses used, both TSA and VVdd reduced the number of lung tumours in treated animals compared to vehicle treated controls. When used in combination however a further therapeutic benefit was obvious with significantly fewer lung metastases observed than with either agent alone (Figure 2b, $p < 0.05$). To determine if the effect of TSA was limited to VVdd growth in tumour tissues, we carried out bio-distribution studies in a sampling of normal tissues in infected animals. At the indicated times (figure 2d) infected animals were sacrificed and virus titers in selected organs determined by plaque assay. We found that

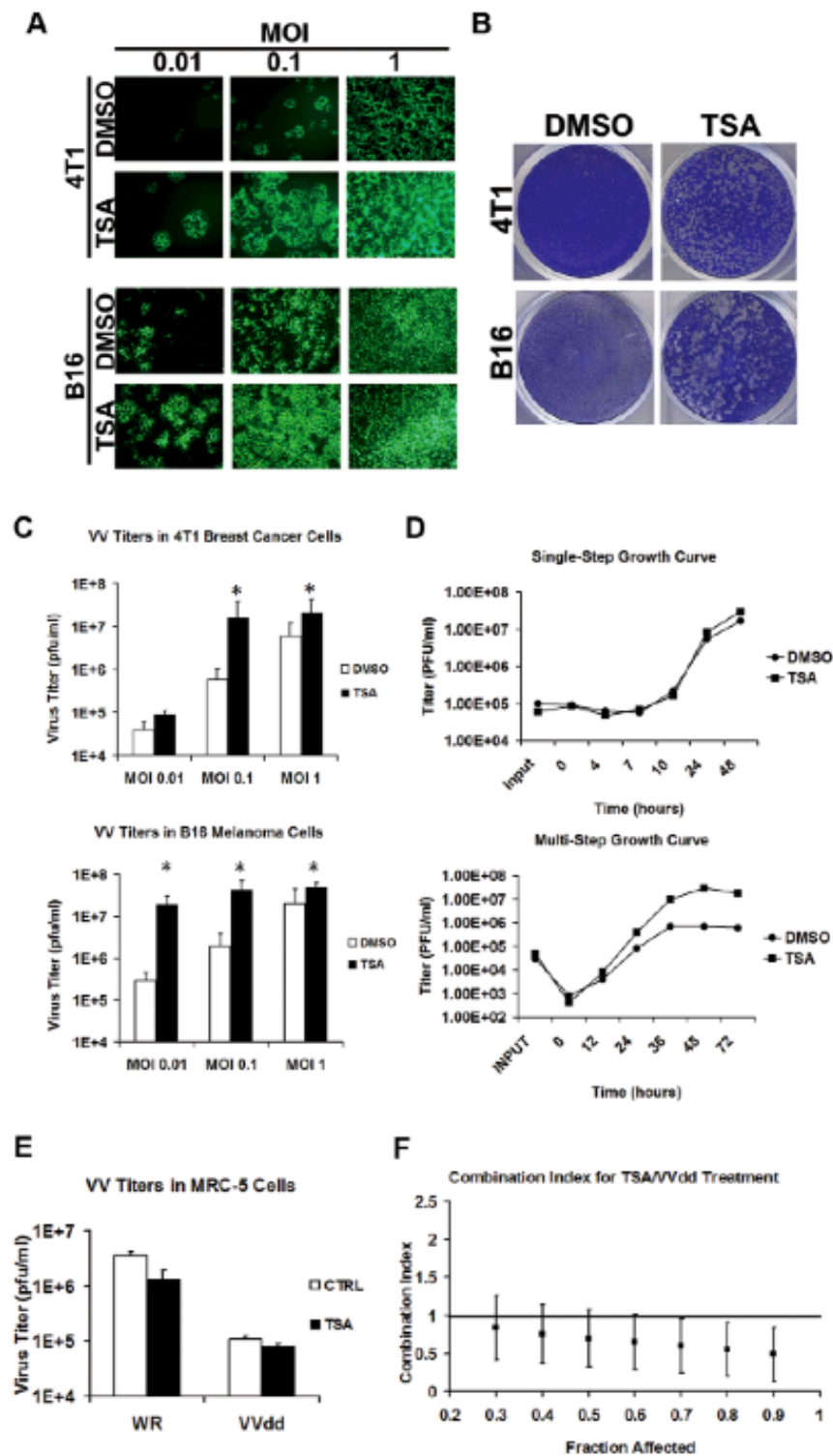


Figure 1. TSA enhances the spread and replication of VVdd *in vitro*. (A) 4T1 breast cancer and B16 melanoma cells were pretreated with TSA (0.0375 μ M) for 3 hr and infected with VVdd at an MOI of 0.01, 0.1 or 1 pfu/cell. Fluorescence microscopy pictures were taken at 72 hours post infection. (B) 4T1 and B16 cells were infected with VVdd at an MOI of 0.1 and 0.01 respectively and plaques were stained for visualization with Coomassie Blue 72 hours post-infection. (C) 4T1 and B16 cells were infected with VVdd-GFP (MOI of 0.01, 0.1, and 1) and samples were collected after 72 hours. Viral titers were measured by standard plaque assay on U2OS cells. The experiment was done in triplicate. Star indicates P value <0.05. (D) B16 cells were pre-treated with TSA 0.0375 μ M for 3 hours then challenged with VVdd at an MOI of 3 (Single step growth curve, top panel) or an MOI of 0.1 (Multi-step growth curve, bottom panel). Samples were collected at the indicated times and viral titers assessed by plaque assay on U2OS cells. (E) MRC-5 normal human fibroblasts were plated into 6-well plates and treated with PBS or a non-toxic concentration (1 μ M) of TSA for 6 h. At the end of the pre-treatment, cells were infected at an MOI of 1.0 by vaccinia strain VVdd or wild type WR. Cells and media were collected after 72 h and viral pfu/cell titrated by plaque assay on BSC-1 cells. (F) B16 cells were pre-treated for 4T1 cells were treated with serial dilutions of a fixed ratio combination mixture of VVdd and TSA (1562 PFU: 1 μ M VVdd:TSA). Cytotoxicity was assessed using alamar blue reagent after 96 h. Combination indices (CI) were calculated according to the method of Chou and Talalay using Calcsyn. Plots represent the algebraic estimate of the CI in function of the fraction of cells affected (Fa). Error bars indicate the estimated standard error. doi:10.1371/journal.pone.0014462.g001

treatment with TSA did not generally lead to increased VVdd replication in normal tissues, although clearance of VVdd appeared to be slightly quicker in lymph nodes and heart while prolonged in ovaries. In light of this, we further tested the effect of TSA on VVdd growth in mouse lymph node, heart and ovarian tissue explants as well as three normal primary ovarian cell lines. We found that TSA treatment did not significantly change virus yields from tissue explants of any origin, nor did it increase titers obtained from normal ovarian cells (Supplemental figure S1a-c). These findings coupled with the increased anti-tumour activity and minimal toxicity suggests the TSA/VVdd combination therapy may be clinically beneficial.

TSA enhances an attenuated B18R-deleted vaccinia strain

Although vaccinia encodes a number of interferon signaling antagonists [15,16,17,28,29,30], it nonetheless can have its growth attenuated by pre-treatment of normal cells with interferon (IFN) prior to the time when virus can initiate the production of its armament of innate immune suppressing proteins. As shown in Figure 3a, pre-treatment of normal human fibroblast GM38 and MRC-5 cells with IFN led to strong inhibition of virus replication and spread. Importantly, the protective effect of IFN was not overcome by TSA in these same cells (Figures 3a, c). In contrast, TSA enhanced VVdd spread as evidenced by vaccinia-associated GFP expression in several cancer cell lines even in the presence of

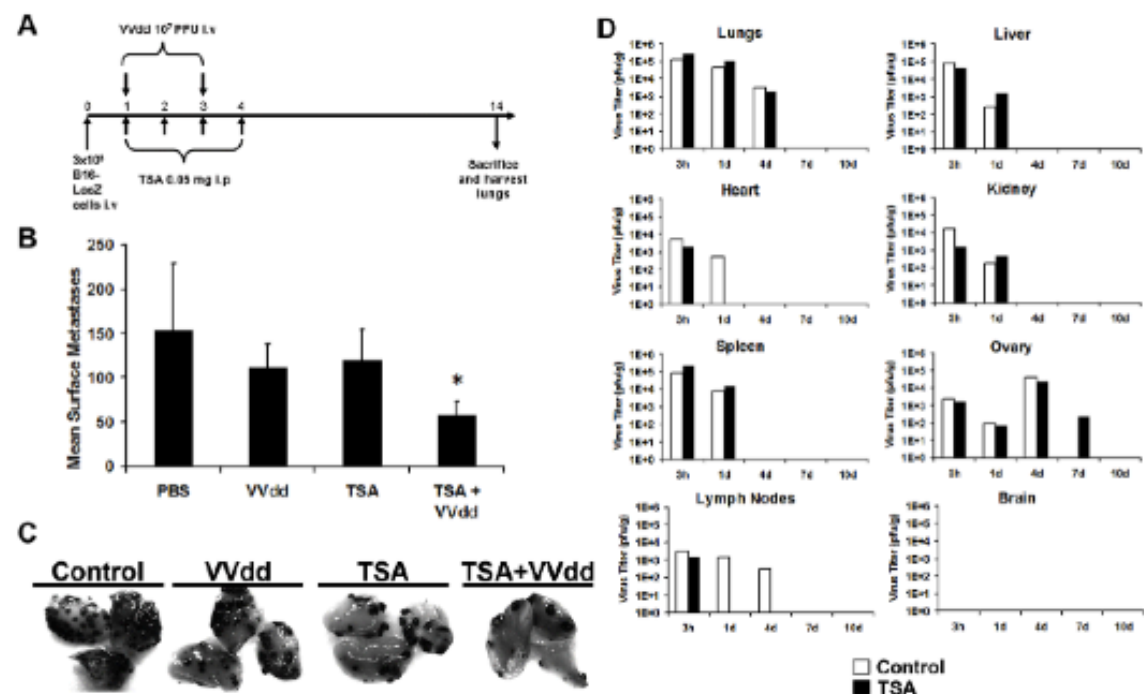


Figure 2. TSA is an effective enhancer of vaccinia *in vivo*. (A) C57BL/6 mice were injected intravenously with B16F10LacZ melanoma cells (3×10^5 cells/mouse) and TSA (4 daily intraperitoneal doses of 0.05 mg/mouse) alone or in combination with VVdd (1×10^7 pfu/mouse). (B) The mice were sacrificed after 14 days and the lung metastases were counted after staining with X-gal. Asterisk * indicates a P value <0.05 and is significantly different than PBS group and each of the single treatment groups. (C) Lung lobes from control or VVdd and TSA treated mice. B16F10LacZ cells stained with X-Gal. (D) Balb/C mice pre-treated or not with TSA (0.05 mg/mouse) on days 0 through 3. After 3 hr pre-treatment on day 0, mice were given an intra-venous dose of VVdd-luciferase of 1×10^8 pfu/mouse. One mouse per group was sacrificed at each time point and organs were titrated for virus content by standard plaque assay on U2OS cells. doi:10.1371/journal.pone.0014462.g002

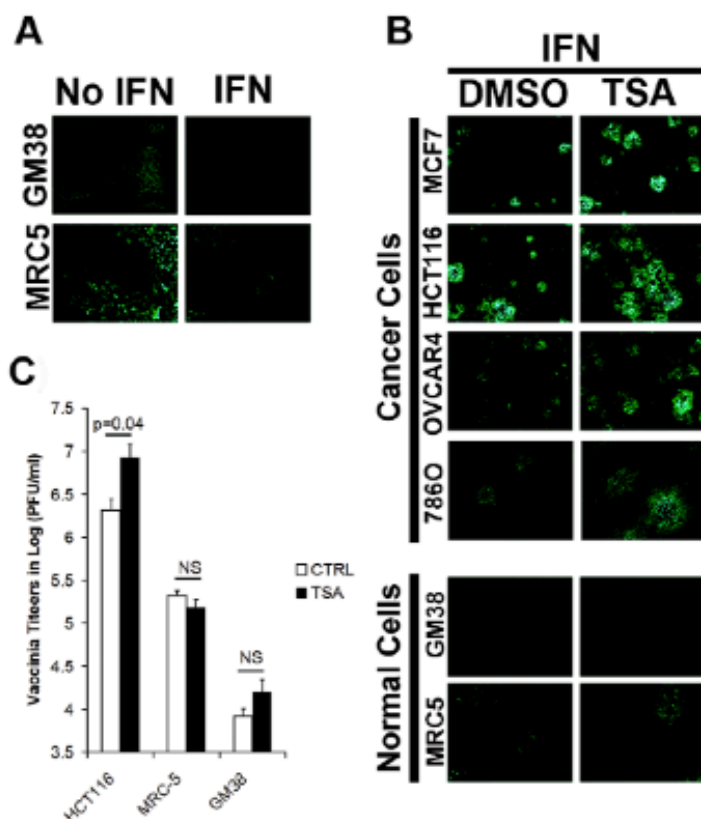


Figure 3. TSA enhances vaccinia in presence of IFN in cancer cells. (A) Normal cells GM38 and MRC-5 were treated or not with 200 IU/ml of IFN for 16 hrs. Cells were then infected with VVd at MOI 0.001 and fluorescent pictures were taken after 72 h. (B) Cells were pre-treated for 3 h with TSA (0.04 μ M) and then 200 IU/ml of IFN for 16 h. Cells were subsequently infected with VVd at MOI 0.001 and fluorescent pictures were taken after 72 h. (C) Cells were treated as in (B) but samples were collected after 72 h for titrating on U2OS cells. Error bars indicate the standard error. NS stands for non-significant ($n=3$, ANOVA). doi:10.1371/journal.pone.0014462.g003

IFN (Figure 3b). In the HCT116 cell line, we further confirmed that this leads to significant increases in viral titers as expected (Figure 3c). These results demonstrate that TSA can enhance vaccinia spread even in the presence of IFN in cancer cells but that TSA cannot overcome an IFN-induced anti-viral state in normal cells.

Since the vaccinia virus B18 protein (encoded by the B18R gene) plays a major role in scavenging IFN secreted from infected cells [15,16,17], we predicted that TSA may be particularly effective in enhancing the growth of vaccinia strains in which the B18R gene has been deleted [18]. We have previously created an oncolytic version of vaccinia virus by deletion of the B18R gene and while as predicted, this virus replicates poorly in normal cells it still replicates in and kills a wide spectrum of cancer cells [18]. However in tumour cells that retain some interferon responsiveness the B18R deleted virus is less effective than a B18R-replete virus. To investigate the growth of a B18R deleted vaccinia in combination with TSA, the fold induction in plaque forming units produced was compared between TK-deleted and TK/B18R-deleted WR strains in several cell lines. Whereas TSA did not lead to any increase in viral production in normal MRC-5

cells for either strain, it increased viral titers for both strains in human UCI-101 ovarian cancer cells, HeLa cervical cancer cells, and HCT116 colon cancer cells (Figure 4a). In these three cancer cell lines the growth of B18R deleted virus is significantly less than WR parental strain however this deficit can be overcome by incubation of infected cells with TSA. Thus the relative fold induction of B18R deleted virus by TSA is significantly higher than in WR infected cells (figure 4a). This TSA-induced increase in B18R-deleted virus replication led to synergistic cytotoxicity in HCT116 cells as determined by isobologram analysis (Figure 4b). These results support the idea that the spectrum of cancer cells a B18R deleted virus can effectively destroy can be enhanced with TSA without compromising the superior safety of this oncolytic virus, as evidenced from viral bio-distribution studies done for this virus in control conditions or in presence of TSA (supplemental Figure S2a). We further tested the possibility that the combination of B18R-deleted vaccinia virus and TSA could be effective in a human xenograft tumour model. Immunocompromised mice with palpable HCT-116 colon cancer tumours were treated with TSA (or vehicle) and a luciferase-expressing B18R/TK-deleted virus and IVIS imaging was used to examine

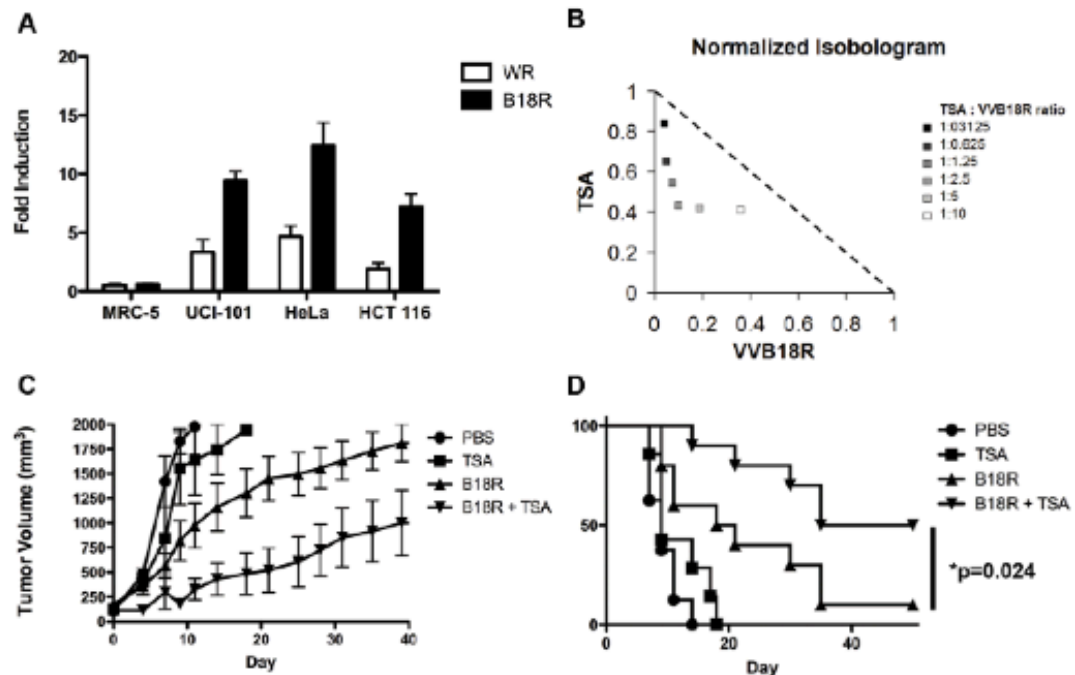


Figure 4. TSA enhances B18R-deleted vaccinia *in vitro* and increases survival in a xenograft model. (A) Human cell lines (MRC-5; UCI-101, HeLa or HCT116) treated with PBS or TSA (1 μ M) for 6 h. At the end of the pre-treatment, cells were infected at an MOI of 1.0 by vaccinia strains Western Reserve or Western Reserve with a deletion in the B18R gene. Cells and media were collected after 72 h and viral pfu/cell titered by plaque assay on BSC-1 cells. Fold induction of titer upon TSA treatment relative to control is reported. (B) HCT116 cells were plated in 96-well plates and pre-treated with 0.1 μ M TSA for 3 hours then infected with B18R-deleted vaccinia virus at varying MOIs, resulting in the TSA: VVB18R ratios indicated in the figure legend. Cytotoxicity data was obtained using alamar blue 72 hours later and data was analyzed using CalcuSyn according to the Chou and Talalay method and the resulting isobologram was plotted. The fraction of the EC₅₀ doses for each VVB18R and TSA required in order to result in 50% cell killing for the combination are drawn on the x-axis and y-axis, respectively. Notably, if the combination data point falls on the diagonal (dashed line), an additive effect is indicated; if it falls on the lower left, synergism is indicated; and if falls on the upper right, antagonism is indicated. (C–D) Athymic nu/nu- mice were implanted subcutaneously with HCT-116 cells (5×10^5 cells/mouse). Once palpable tumours had formed (50–100 mm³), mice were treated with either (i) intraperitoneal PBS; (ii) intraperitoneal TSA (6 μ g/mouse) on days –1, 0 and 2; (iii) intravenous injection of WR B18R–TK–Luc+ (1×10^8 pfu/mouse) on day 0; or (iv) both TSA and WR B18R–TK–Luc+ (n = 10 mice/group). Subsequent tumour burden was followed by caliper measurement (C) and mice were sacrificed when their tumours reached 1400 mm³. Percent survival of mice is graphed (D, p = 0.024). doi:10.1371/journal.pone.0014462.g004

the growth of the virus in tumour bearing mice. Treatment with TSA resulted in increased virus-associated luciferase activity within HCT-116 tumours when compared to treatment with vaccinia virus alone (Supplemental Figure S2b). The low virus signal in the lungs after 48 h is consistent with the biodistribution data; however this signal is gone by 4 days. Importantly the signal in the lungs is not enhanced by TSA treatment whereas the signal is greatly enhanced in the tumour. Consistent with this observation and the results obtained in the lung metastasis model (Figure 2a), mice treated with the combination of TSA and TK-/B18R-deleted WR had delayed tumor progression (Figure 4c) and demonstrated increased survival versus mice treated with either agent alone (Figure 4d, p = 0.024).

Discussion

Trichostatin A (TSA) was one of the first HDAC inhibitors to be discovered and although its anti-cancer properties are well documented, its sub-optimal *in vivo* stability has made it less attractive for use as a chronically administered anti-cancer drug

[31,32]. A considerable effort in the HDI field has led to the development of more stable TSA derivatives such as Vorinostat® (SAHA), which was recently approved for limited applications like treatment of CTCL [33,34,35,36]. In this study we found that SAHA was significantly less effective at augmenting vaccinia virus spread *in vivo* than TSA. In this context, we also found that TSA interacted synergistically with vaccinia virus, leading to better cell killing. Theoretically, this synergistic interaction predicts that the effective dose used for each therapeutic *in vivo* could be reduced while retaining efficient anti-tumour activity. However, since OV's need to overcome numerous physiological barriers in order to reach tumors, it is likely that TSA/Vaccinia combination therapy would be best used as a means to increase efficacy as opposed to dose reduction. Nonetheless, this suggests that the relatively short half-life of TSA *in vivo* may not be a concern for the therapeutic application described here in light of the relative potency of its vaccinia-enhancing effect.

Oncolytic virus therapy is an acute treatment with curative intent. Indeed the activity of OV's involves not only replication in, and destruction of tumour cells but also the recruitment of host

immune cells to the tumour bed leading to the initiation of anti-tumour immunity. It is known that HDIs can impact the patient's immune cells [37,38,39] and thus a fast acting, virus enhancing, compound that is rapidly cleared once an infection is established may be preferred. Furthermore the short half-life of TSA allows for better control over the OV dose should treatment need to be stopped abruptly. Given that our data shows anti-tumour activity of several different VV strains can be enhanced by TSA *in vivo* the clinical application of TSA may need to be re-visited. This is of considerable interest since VV strains such as JX-594 (Wyeth TK-deleted, GM-CSF expressing) and JX-929 (Western Reserve TK/VGF-deleted, GM-CSF expressing) are currently undergoing Phase I/II clinical trials.

The effect of TSA on the IFN response is well documented [40,41] and the enhancing effect of HDIs such as TSA on IFN-sensitive strains including VSV and HSV has been previously reported [22]. It is therefore not surprising that TSA can increase the activity of B18R-deleted VV strains. However our finding that vaccinia with an intact B18R gene is still enhanced by TSA suggests that either the anti-interferon activities of B18R are insufficient to completely impair the cellular interferon response or alternatively the effects of TSA go beyond interferon induced anti-viral responses. While we cannot distinguish between these possibilities at the present time, the latter explanation seems likely to us since we and others have found using microarray analysis of HDI treated cells, that hundreds of cellular transcripts induced by viral infection are affected by blocking histone deacetylase activity [23,42].

Using small molecule OV-enhancers is attractive from a clinical standpoint. In principle, this strategy allows for quite significant genetic attenuation of OVs to restrict growth in normal tissues with conditional rescue of the virus replication in tumour cells following treatment with an enhancing compound like an HDI or other classes of molecules that can complement viral defects [42]. In this and our previous studies, one of the key features of small molecule complementation of attenuated viruses is that the effect of virus enhancers is restricted to tumour cells with minimal impact on the anti-viral programs of normal tissues. This clearly is the case for TSA in the studies presented here where we demonstrate both *in vitro* and in animal models that TSA does not enhance virus growth in normal tissues. The reason for this selectivity is unclear at this time however tumour cells are known to have elevated levels of histone deacetylases suggesting that perhaps malignancies have evolved more dependency upon this type of epigenetic modification to control gene expression [43]. Alternatively since tumour cells have often inactivated at least some components of their anti-viral programs, it may be that the addition of HDIs simply "break the camel's back" in tumour cells but are ineffective in normal tissues that have fully intact multi-layered protection systems. Further studies are currently under way to address some of these issues.

We conclude that TSA is a potent enhancer of VV *in vitro* and *in vivo*. We propose that HDIs such as TSA could be used to enhance the effectiveness of OVs *in vivo* and that further clinical evaluation of this possibility is warranted.

Materials and Methods

Cells and Viruses

4T1 murine breast cancer cells, HCT116, MCF7, OVCAR4, 786O and MRC5 cells were obtained from ATCC. Maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% 3:1 calf serum: fetal bovine serum and grown at 37°C with 5% CO₂. B16F10-LacZ murine melanoma cells were obtained

from ATCC and maintained in α MEM supplemented with 10% 3:1 calf serum: fetal bovine serum. GM38 cells were kindly provided by Dr. Bruce McKay (Ottawa Hospital Research Institute, Ottawa, ON) and maintained in DMEM supplemented with 15% 3:1 calf serum: fetal bovine serum. Human lines MRC-5, UCI-101 and HeLa were provided by Stephen Thorne. NOV2963D, NOV3128D and NOV3198G normal ovarian cell lines were kindly provided by Dr. Anne-Marie Mes-Masson (Institut du Cancer de Montreal, Montreal, QC, Canada) and were grown in OSE (Wisent, QC, CA) media supplemented with 10 ng/ml endothelial growth factor, 34 μ g/ml bovine pituitary extract, 5 μ g/ml insulin and 0.5 μ g/ml hydrocortisone. VVdd was derived from the wild type strain Western Reserve with a double deletion of the genes thymidine kinase and vaccinia growth factor [12]. Green fluorescent protein was inserted at the TK locus. Virus was propagated in U2OS cells. Lastly, the wild type Western Reserve (WR) and WR B18R-TK-LacZ were also used in *in vitro* and *in vivo* experiments.

Fluorescence Microscopy and Fluorescence Quantification

A fluorescent microscope (Zeiss Axiovert S 100) was used to photograph the cells. The GFP expressing virus can be visualized with a fluorescence microscope. Infected cells with actively replicating virus appear green under the fluorescent microscope. Images were quantified for green fluorescence using the image analysis software Image J (NIH).

In Vitro Assays and Cell Staining

The HDI screening was done in 96-well plates with 20,000 cells per well. Cells were plated and 24 hours later were pre-treated for 3 hours with indicated HDI. The drugs: SAHA (Exclusive Chemistry, Obninsk, Russia), MS-275 (Selleck Chemicals, Houston, TX, USA), Oxamflatin (Alexis Biochemicals, Plymouth Meeting, PA, USA), Apicidin (Alexis Biochemicals, Plymouth Meeting, PA, USA), SBHA (Enzo Life Sciences International Inc., Plymouth Meeting, PA, USA), Scriptaid (Sigma), Valproic acid (Sigma), CHAHA (Sigma), M344 (Sigma) and Trichostatin A (Sigma) were added to wells at indicated concentrations and cells were infected with VV. Images spanning well surface were quantified as described above. For further TSA testing *in vitro* cells were plated in 12 well plates with 2.5×10^5 cells per well. Once confluence was reached, the cells were pre-treated with TSA (0.0375 μ M) and DMSO as the drug vehicle control. After 3 hours of pre-treatment, the virus was added at the indicated multiplicity of infection. Fluorescence images were taken after 24, 48 and 72 hours. Cells were collected after 72 hours and frozen at -80°C for titrating on U2OS cells. Other wells were stained for plaques by first rinsing each well with PBS, then fixing the cells for 10 minutes using 3:1 ratio of methanol to acetic acid. After the cells were fixed, they were stained with Coomassie Blue to visualize viral plaques. Cell lines GM38, MRC5, HCT116, MCF7, 786O and OVCAR4 were pre-treated for 3 hrs with TSA and then treated with 200 IU/ml of IFN (Intron A from Schering, Kenilworth, NJ) overnight (16 hrs) and then infected with vaccinia at various MOIs. For combination index 20,000 4T1 cells were plated in 96-well plates. Cells were treated with serial dilutions of a fixed ratio combination mixture of VVdd and TSA (1562 PFU: 1 μ M VVdd-TSA). Alternately in Figure 4a, HCT116 cells were plated similarly but treated with drug-virus at the indicated ratio, where the TSA concentration was 0.1 μ M. Cytotoxicity was assessed using alamar blue reagent after 96 h. Combination indices (CI) were calculated according to the method of Chou and Talalay using CalcuSyn [25]. In Figure 1f, Plots represent the algebraic

estimate of the CI in function of the fraction of cells affected (Fa). Error bars indicate the estimated standard error.

Titration of virus samples

Each sample (cells and supernatants) was collected from the well and cells were lysed by freezing and thawing (-80°C to 37°C) three times. Samples were diluted serially by a factor of 10 and 500 μL of each dilution were put on confluent U2OS or BSC-1 cells in a 12 well plate (5×10^5 cells per well plated 24 hours prior). The samples are placed in an incubator for 2 hours at 37°C to allow the virus to enter the cells. After the 2 hour incubation, the virus sample was removed from the U2OS cells and an 1 mL of an overlay solution was put on the cells (1:1 ratio of 3% CMC: 2XDMEM +20% FBS). The plates were left to incubate at 37°C for 72 hours. After 72 hours, the overlay was removed and each well was stained with Coomassie Blue to visualize and count the plaques (see above for staining).

Western Blot

4T1 cells were plated in 10 cm dishes and treated with HDI at the indicated concentrations. The following day cells were lysed with Radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor (Sigma, P2714). Equal amounts of proteins collected from samples were electrophoresed on a 15% SDS-polyacrylamide gel. Gels were blotted on nitrocellulose membranes and detected by Western blot analysis probing with the antibody Anti-hyperacetylated Histone H4 (Penta, 06-946) diluted 1:5,000. Actin was used as the loading control and was detected by mouse actin antibody (Sigma) diluted 1:10,000.

Lung metastasis model

B16F10 LacZ cells were injected intravenously into female C57BL/6 mice from Charles River Laboratories (Wilmington, MA). Each mouse was injected with 3×10^5 cells into the tail vein on day 0. On days 1–4 mice were given intra-peritoneal injections of TSA of 0.05 mg/mouse. On days 1 and 3 mice were given 1×10^7 pfu/mouse of VVdd intravenously via the tail vein. On day 14 mice were sacrificed and lungs were dissected. Lung tumours were stained with the substrate X-gal and each metastasis was counted [26,27].

Colon Cancer Survival Model

Athymic nu/nu- mice were implanted subcutaneously with HCT-116 cells (5×10^6 cells/mouse). Once palpable tumours had formed (50–100 mm³), mice were treated with either (i) intraperitoneal PBS; (ii) intraperitoneal TSA (6 μg /mouse) on days –1, 0 and 2; (iii) intravenous injection of WR B18R- TK- Lac+ (1×10^8 pfu/mouse) on day 0; or (iv) both TSA and WR B18R- TK- Lac+ ($n = 10$ mice/group). Subsequent tumour burden was followed by caliper measurement and mice sacrificed when their tumours reached 1400 mm³. In addition, mice receiving viral treatment were imaged by bioluminescence imaging at regular times after treatment to assess viral gene expression. Mice were injected with D-luciferin (Molecular Imaging Products, Ann Arbor, MI) for firefly luciferase imaging. Mice were anesthetized under 3% isoflurane (Baxter, Deerfield, IL) and imaged with (IVIS200, Xenogen, part of Caliper Life Sciences). Data acquisition and analysis were performed using Living Image v2.5 software.

Biodistribution

Balb/C mice were pre-treated with 0.05 mg of TSA per mouse or a control by intra-peritoneal injections on day 0. Mice were given the same dose of TSA or control each day for days 0 through 3. Mice were given 1×10^8 pfu/mouse of VVdd-luciferase or

luciferase-expressing B18R/tk-deleted vaccinia virus by intravenous injection into the tail vein on day 1 after a 3 hour pre-treatment with TSA. One mouse from each condition (treated or not with TSA) was sacrificed at various time points. Mice were sacrificed 3 hours after virus injection and on day 1, 4, 7 and 10. The following organs were collected for titering: lymph nodes (brachial and inguinal), ovaries, spleen, kidney, liver, lungs, heart and brain. Organs were homogenized in PBS and titered by standard plaque assay on U2OS cells (see above).

Supporting Information

Figure S1 TSA does not increase in vitro infection of normal mouse tissues or human normal ovarian primary cell lines. (A) Mouse Lymph nodes (LN), Heart, and Ovaries were obtained by dissection and immediately put in cell culture. Tissues were subsequently pretreated with 0.1 μM TSA for 24 hours and infected with 1×10^7 PFU VVdd-GFP. 72 hours later, tissues were collected, weighed, and homogenized in PBS using a tissue homogenizer. Homogenates were subsequently titered on U2OS cells and VVdd PFU/g of tissue was graphed. (B) Normal human ovarian primary cells (NOV2963D, NOV3128D, and NOV3198G) were plated in 96-well plates (25 000 cells/well) overnight and pre-treated with TSA 0.04 μM for 3 hours. Subsequently, cells were infected with VVdd-GFP at an MOI of 0.001. 72 hours later, pictures were taken by fluorescence microscopy. Cells and supernatant were subsequently harvested and titered on U2OS cells. Titers are presented in (C). Error bars represent the standard error. NS stands for non significant (ANOVA, $n = 3$).

Found at: doi:10.1371/journal.pone.0014462.s001 (1.68 MB TIF)

Figure S2 TSA increases virus-associated luciferase activity in subcutaneous tumour. (A) Balb/C mice pre-treated or not with TSA (0.05 mg/mouse) on days 0 through 3. After 3 hr pre-treatment on day 0, mice were given an intra-venous dose of B18R-deleted at 1×10^8 pfu/mouse. One mouse per group was sacrificed at each time point and organs were titered for virus content by standard plaque assay on U2OS cells. (B) Athymic nu/nu- mice were implanted subcutaneously with HCT-116 cells (5×10^6 cells/mouse). Once palpable tumours had formed (50–100 mm³), mice were treated with either (i) intraperitoneal PBS; (ii) intraperitoneal TSA (6 μg /mouse) on days –1, 0 and 2; (iii) intravenous injection of WR B18R- TK- Lac+ (1×10^8 pfu/mouse) on day 0; or (iv) both TSA and WR B18R- TK- Lac+ ($n = 10$ mice/group). Viral replication at tumour sites was imaged using in vivo imaging system 48 hours after virus injection for luciferase.

Found at: doi:10.1371/journal.pone.0014462.s002 (1.46 MB TIF)

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Author Contributions

Conceived and designed the experiments: HM JSD BH HA SHT JCB. Performed the experiments: HM JSD BH FLB NDS JC JGS TG TF. Analyzed the data: HM JSD BH. Contributed reagents/materials/analysis tools: FLB JAM DK SHT JCB. Wrote the paper: HM JSD CB JCB.

References

- Kim DH, Thorne SH (2009) Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer. *Nat. Rev. Cancer* 9: 64–71.
- Parato KA, Senger D, Forsyth PA, Bell JC (2005) Recent progress in the battle between oncolytic viruses and tumours. *Nat. Rev. Cancer* 5: 965–976.
- Xu B, Grandt D, Sangfelt O, Einhorn S (1994) Primary leukemia cells resistant to alpha-interferon in vitro are defective in the activation of the DNA-binding factor interferon-stimulated gene factor 3. *Blood* 84: 1942–1949.
- Yoshida M, Kijima M, Akita M, Beppu T (1990) Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. *J Biol. Chem.* 265: 17174–17179.
- Wong LH, Krausz KG, Hatzinikolaou I, Estcourt MJ, Hersey P, et al. (1997) Interferon-resistant human melanoma cells are deficient in ISGF3 components, STAT1, STAT2, and p48-ISGF3gamma. *J Biol. Chem.* 272: 28779–28785.
- Stojdl DF, Lichy BD, tenOver BR, Paterson JM, Power AT, et al. (2003) VSV strains with defects in their ability to shut down innate immunity are potent systemic anti-cancer agents. *Cancer Cell* 4: 263–275.
- Dunn GP, Koebel CM, Schreiber RD (2006) Interferons, immunity and cancer immunosuppression. *Nat. Rev. Immunol.* 6: 836–848.
- Naik S, Russell SJ (2009) Engineering oncolytic viruses to exploit tumor specific defects in innate immune signaling pathways. *Expert Opin. Biol. Ther.* 9: 1163–1176.
- Stojdl DF, Lichty B, Knowles S, Mariani R, Atkins H, et al. (2000) Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nat. Med.* 6: 821–825.
- Park BH, Hwang T, Liu TC, Sze DY, Kim JS, et al. (2008) Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: a phase I trial. *Lancet Oncol* 9: 533–542.
- Shen Y, Nemanishi J (2005) Fighting cancer with vaccinia virus: teaching new tricks to an old dog. *Mol. Ther.* 11: 180–195.
- McCart JA, Ward JM, Lee J, Hu Y, Alexander HR, et al. (2001) Systemic cancer therapy with a tumor-selective vaccinia virus mutant lacking thymidine kinase and vaccinia growth factor genes. *Cancer Res* 61: 8751–8757.
- Thorne SH, Hwang TH, O'Gorman WE, Bartlett DL, Sei S, et al. (2007) Rational strain selection and engineering creates a broad-spectrum, systemically effective oncolytic poxvirus, JX-963. *J Clin. Invest* 117: 3350–3358.
- Liu TC, Hwang T, Park BH, Bell J, Kim DH (2008) The targeted oncolytic poxvirus JX-594 demonstrates antitumoral, antivascular, and anti-HBV activities in patients with hepatocellular carcinoma. *Mol. Ther.* 16: 1637–1642.
- Symons JA, Alami A, Smith GL (1995) Vaccinia virus encodes a soluble type I interferon receptor of novel structure and broad species specificity. *Cell* 81: 551–560.
- Alami A, Symons JA, Smith GL (2000) The vaccinia virus soluble alpha/beta interferon (IFN) receptor binds to the cell surface and protects cells from the antiviral effects of IFN. *J Virol* 74: 11230–11239.
- Colaninici OR, Domanski P, Schweizer SM, Larner A, Buller RM (1995) Vaccinia virus B18R gene encodes a type I interferon-binding protein that blocks interferon alpha transmembrane signaling. *J Biol. Chem.* 270: 15974–15978.
- Kim DH, Wang Y, Le Boeuf F, Bell J, Thorne SH (2007) Targeting of interferon-beta to produce a specific, multi-mechanistic oncolytic vaccinia virus. *PLoS Med* 4: e353.
- Bolden JE, Peart MJ, Johnstone RW (2006) Anticancer activities of histone deacetylase inhibitors. *Nat. Rev. Drug Discov* 5: 769–784.
- Marka P, Rifkin RA, Richon VM, Breslow R, Miller T, et al. (2001) Histone deacetylases and cancer: causes and therapies. *Nat. Rev. Cancer* 1: 194–202.
- Minacci S, Pellici PG (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. *Nat. Rev. Cancer* 6: 38–51.
- Nguyen TL, Abdelhady H, Arguello M, Breithach C, Leveillé S, et al. (2008) Chemical targeting of the innate antiviral response by histone deacetylase inhibitors renders refractory cancers sensitive to viral oncolysis. *Proc. Natl. Acad. Sci U S A* 105: 14981–14986.
- Chang HM, Paulson M, Holko M, Rice CM, Williams BR, et al. (2004) Induction of interferon-stimulated gene expression and antiviral responses require protein deacetylase activity. *Proc. Natl. Acad. Sci U S A* 101: 9578–9583.
- Otsuki A, Patel A, Kassi K, Suzuki M, Kurosumi K, et al. (2008) Histone deacetylase inhibitors augment antitumor efficacy of herpes-based oncolytic viruses. *Mol. Ther.* 16: 1546–1555.
- Chou TC, Talalay P (1977) A simple generalized equation for the analysis of multiple inhibitions of Michaelis-Menten kinetic systems. *J Biol. Chem.* 252: 6438–6442.
- Kirstein JM, Graham KC, Mackenzie LT, Johnston DE, Martin LJ, et al. (2009) Effect of anti-fibrinolytic therapy on experimental melanoma metastasis. *Clin. Exp. Metastasis* 26: 121–131.
- Stanford MM, Shahan M, Barrett JW, Werden SJ, Gilbert PA, et al. (2008) Myxoma virus oncolysis of primary and metastatic B16F10 mouse tumors in vivo. *Mol. Ther.* 16: 52–59.
- Arsenio J, Deschambault Y, Cao J (2008) Antagonizing activity of vaccinia virus E3L against human interferons in Huh7 cells. *Virology* 377: 124–132.
- Meng X, Jiang C, Arsenio J, Dick K, Cao J, et al. (2009) Vaccinia virus K1L and C7L inhibit antiviral activities induced by type I interferons. *J Virol* 83: 10627–10636.
- Zhang Z, Abrahams MR, Hunt LA, Sattles J, Marshall W, et al. (2005) The vaccinia virus N1L protein influences cytokine secretion in vitro after infection. *Ann N Y Acad. Sci.* 1056: 69–86.
- Vanhaecke T, Papeleu P, Elaut G, Rogiers V (2004) Trichostatin A-like hydroxamate histone deacetylase inhibitors as therapeutic agents: toxicological points of view. *Curr. Med. Chem.* 11: 1629–1643.
- Vignashin DM, Ali S, Pace PE, Mirsaeidi N, Ito K, et al. (2001) Trichostatin A is a histone deacetylase inhibitor with potent antitumor activity against breast cancer in vivo. *Clin. Cancer Res* 7: 971–976.
- Olsen EA, Kim YH, Kuzel TM, Pacheco TR, Foss FM, et al. (2007) Phase IIb multicenter trial of vorinostat in patients with persistent, progressive, or treatment refractory cutaneous T-cell lymphoma. *J Clin. Oncol* 25: 3109–3115.
- Batty N, Malouf GG, Issa JP (2009) Histone deacetylase inhibitors as anti-neoplastic agents. *Cancer Lett* 280: 192–200.
- Nasimzou I, Horvath CM (2006) Positive and negative regulation of the innate antiviral response and beta interferon gene expression by deacetylation. *Mol. Cell. Biol.* 26: 3106–3113.
- Sun WH, Pabon C, Alsayed Y, Huang PP, Jandeska S, et al. (1998) Interferon-alpha resistance in a cutaneous T-cell lymphoma cell line is associated with lack of STAT1 expression. *Blood* 91: 570–576.
- Adcock IM (2007) HDAC inhibitors as anti-inflammatory agents. *Br. J. Pharmacol.* 150: 829–831.
- Moreira JM, Scheipers P, Sorensen P (2003) The histone deacetylase inhibitor Trichostatin A modulates CD4+ T cell responses. *BMC Cancer* 3: 30.
- Wang L, de Zorzi EF, Greene MI, Hancock WW (2009) Immunomodulatory effects of deacetylase inhibitors: therapeutic targeting of FOXP3+ regulatory T cells. *Nat. Rev. Drug Discov* 8: 969–981.
- Genin P, Morin P, Civas A (2003) Impairment of interferon-induced IRF-7 gene expression due to inhibition of ISGF3 formation by trichostatin A. *J Virol* 77: 7113–7119.
- Vasakova J, Novakova Z, Rosamirou L, Kahle M, Hozak P, et al. (2007) Histone deacetylase inhibitors suppress IFNalpha-induced up-regulation of promyelocytic leukemia protein. *Blood* 109: 1373–1380.
- Diallo JS, Le Boeuf F, Lai F, Cox J, et al. (2010) A High-throughput Pharmacoviral Approach Identifies Novel Oncolytic Virus Sensitizers. *Mol. Ther.* Jun 18(6): 1123–9.
- Mehner JM, Kelly WK (2007) Histone deacetylase inhibitors: biology and mechanism of action. *Cancer J* 13: 23–29.

APPENDIX VII: Curriculum Vitae

NAOMI DE SILVA

UNIVERSITY EDUCATIONAL BACKGROUND

2008 – Present

Ph.D. Candidate - University of Ottawa

Ph.D. Candidate in Biochemistry with Specialization in Human and Molecular Genetics.
Thesis “Targeting tumour vasculature with oncolytic viruses.”

2002 - 2008

University of Ottawa

B.Sc. Honours with Specialization in Biochemistry

RESEARCH HONOURS, SCHOLARSHIPS, AND AWARDS

- | | |
|-----------|---|
| 2010-2013 | Vanier Canada Graduate Scholarship. (\$150 000). Awarded through CIHR for demonstrating leadership skills and a high standard of scholarly achievement in the social sciences and humanities, natural sciences and engineering, and health-related fields. |
| 2012 | Ottawa Hospital Research Institute Research Day: Poster Presentation Award – 3rd Place (\$100). Awarded by OHRI for 3 rd place at the doctoral level. |
| 2011 | Ontario Institute of Cancer Research Trainee Award (\$550). Awarded to fund attendance and a poster presentation at the Canadian Cancer Research Conference. |
| 2011 | Canadian Cancer Society – Conference Travel Grant (refused). Awarded to fund attendance and a poster presentation at the Canadian Cancer Research Conference. |
| 2011 | Canadian Institutes of Health Research – Foreign Study Supplement (\$6000).
Awarded to fund research conducted at the University of California, San Francisco. |
| 2010-2011 | Ontario Graduate Scholarship (\$15 000 - refused). Awarded by the Ontario Ministry of Training Colleges and Universities to encourage research excellence. |
| 2009-2010 | Ottawa Hospital Research Institute Research Day: Poster Presentation Award – 1st Place (\$125). Awarded by OHRI for 1 st place at the Masters level. |

- 2009-2010 Ontario Graduate Scholarship (\$15 000).** Awarded by the Ontario Ministry of Training Colleges and Universities to encourage research excellence.
- 2009-2010 University of Ottawa Excellence Scholarship (\$6228).** Awarded by the University of Ottawa for academic excellence.
- 2009-2010 Faculty of Medicine Poster Presentation Award – 1st Place (\$125).** Awarded by the Department of Biochemistry, Microbiology, and Immunology for 1st place at the Masters level.
- 2009-2010 Graduate Students Association Poster Presentation Award.** Awarded for best technological poster in the Department of Biochemistry, Microbiology, and Immunology.
- 2009 Research Travel Grant (\$425).** Awarded by the Faculty of Graduate and Postdoctoral Studies to support a presentation at the 5th International Meeting on Replicating Oncolytic Virus Therapeutics.
- 2009 Research Travel Grant (\$800).** Awarded by the Department of Biochemistry, Microbiology, and Immunology to support a presentation at the 5th International Meeting on Replicating Oncolytic Virus Therapeutics.
- 2007-2008 Merit Scholarship (\$500).** Awarded by the University of Ottawa for Academic Excellence (Dean's List/Honour Roll)
- 2005 Jan-Aug Project Grant Recipient for Tsunami Aid (15 000 USD).** I interrupted my studies to travel to Sri Lanka to work for an NGO providing Tsunami relief. As project manager, I collected demographic data to help World Vision and the UN dispense supplies to camps. I started a program to replace 5000 family photos destroyed by the Tsunami.
- 2004 Children's Hospital of Eastern Ontario Research Institute Award (\$4 000).** Received an award to fund a summer research project supervised by Dr. Martin Holcik and Dr. Michael Geraghty.
- 1996 - 2000 Science Fair Awards (National and Ottawa Region):** Won 1st place for five consecutive years and over 20 special awards. Won a silver medal and a statistics award from 2 national science fairs.

RESEARCH EXPERIENCE

I have worked in research centres within government, academia, and industry. My research interests include cancer, infectious disease, immunology, medical imaging, and vascular diseases. My experience in academic, translational, and clinical trial arenas fuels my keen interest in advancing experimental therapeutics to improve patient survival and quality of life.

- 2008-2013 Graduate Student - Department of Biochemistry, Microbiology, and Immunology.**

- Doctoral research is in the field of oncolytic viruses used for the treatment of cancer. These viruses specifically infect cancer cells. I elucidated the mechanism by which these cancer-fighting viruses can destroy the blood supply to tumours. This finding led to a clinical trial in which the effect was observed in patients.
- Gained expertise in cancer biology, immunology, virology, genetics, vascular biology, pathology, and medical imaging.

2009-2013 Junior Research Associate with a Biotechnology Company for Three Clinical Trials.

- Collaborated with Jennerex Biotherapeutics, Inc. to develop and test oncolytic viruses in patients. Participated in project design and execution through collaborations with scientists, physicians, and students.
- Managed collaboration between the Ottawa Hospital Research Institute and the University of California, San Francisco. Worked at UCSF to exchange knowledge and technical skills.
- Developed standard operating procedures for analysing patient biopsies and developed expertise in histopathological analysis.
- Presented clinical trial data at largest international cancer conference and to investors.

2009-2013 Spokesperson for the Centre for Innovative Cancer Research.

- Frequently asked to communicate with the public regarding issues related to our research.
- Gave interviews and lab tours to patients, investors, CIHR, and national TV stations. Participated in an episode of *Decouverte*, a francophone TV show featuring our research.

2007-2008 Undergraduate Honours Research Project.

- Did research for two summers and my final year of undergrad in Dr. John Bell's lab.
- Gained expertise in medical imaging of cancer. Data were published in the journal *Nature*.
- Collaborated with Dr. Fenster, Robart's Imaging Institute, University of Western Ontario.

2004 May-Aug Undergraduate Summer Student – Apoptosis Research Centre.

- Supervised by Dr. Martin Holcik and Dr. Michael Geraghty.
- Studied Vanishing White Matter Disease, a childhood brain condition.

2003 May-Aug Undergraduate Summer Student – Children's Hospital of Eastern Ontario.

- Supervised by Dr. Martin Holcik. Conducted molecular biological research on IRES elements and the associated regulation of related apoptotic proteins.

2001-Aug Summer Student - National Research Council – Institute of Biological Sciences.

- Microbiology Department: assisted in stroke research, where I monitored neurogenesis in post-ischemic brain cells.

PUBLICATIONS, PRESENTATIONS, AND ABSTRACTS

Publications

9 publications in peer-reviewed journals, including 2 first author (and 2 more in preparation), a co-authorship in Nature, and a paper as a co-supervisor (second last author)

Breitbach CJ, Arulanandam R, **De Silva N**, Thorne SH, Patt R, Daneshmand M, Moon A, Ilkow C, Burke J, Hwang T, Heo J, Cho M, Chen H, Angarita FA, Addison C, McCart A, Bell JC, Kirn DK. Selective targeting and disruption of tumour vasculature in humans with an engineered oncolytic poxvirus. *Cancer Research*. 2013 Feb 15;73(4): 1265-75.

Vaha-Koskela M, Le Boeuf F, Lemay C, **De Silva N**, Diallo JS, Cox J, Becker M, Choi Y, Ananth A, Sellers C, Breton S, Roy D, Falls T, Brun J, Hemminki A, Hinkkanen A, Bell J. Resistance to two heterologous neurotropic oncolytic viruses, semliki forest virus and vaccinia virus, in experimental glioma. *Journal of Virology*. 2013 Feb;87(4):2363-6

Breitbach CJ, Burke J, Jonker D, Stephenson J, Haas AR, Chow LQ, Nieva J, Hwang TH, Moon A, Patt R, Pelusio A, Le Boeuf F, Burns J, Evgin L, **De Silva N**, Cvancic S, Robertson T, Je JE, Lee YS, Parato K, Diallo JS, Fenster A, Daneshmand M, Bell JC, Kirn DH. Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. *Nature*. 2011 Aug 31;477(7362):99-102.

Hwang T, Moon A, Burke J, Ribas A, Stephenson J, Breitbach C, Daneshmand M, **De Silva N**, Parato K, Diallo JS, Lee Y, Liu T, Bell J, Kirn D. A Mechanistic Proof-of-Concept Clinical Trial with JX-594, a Targeted Multi-Mechanistic Oncolytic Poxvirus, in Patients with Metastatic Melanoma. *Molecular Therapy*. 2011 Oct;19(10):1913-22.

Diallo JS, Roy D, Abdelbary H, **De Silva N**, Bell JC. Ex Vivo infection of live tissue with oncolytic viruses. *Journal of Visualized Experiments*. 2011 Jun 25;(52).

Breitbach CJ*, **De Silva N***, Falls T, Aladl U, Evgin L, Paterson, J, Sun Y, Roy DG, Rintoul JL, Daneshmand M, Parato K, Stanford MM, Lichty BD, Fenster A, Kirn D, Atkins H, Bell JC. Targeting tumour vasculature with an oncolytic virus. *Molecular Therapy*. 2011. May 19(5):886-94.

*these authors contributed equally to this manuscript

MacTavish H*, Diallo JS*, Le Boeuf F, **De Silva N**, Stanford-Bleeks M, Cox J, Falls T, McCart A, Atkins H Breitbach C, Kirn D, Thorne S, Bell JC. Enhancement of Vaccinia Virus Based Oncolysis with Histone Deacetylase Inhibitors. *PLoS One*. 2010. Dec 30;5(12):e14462. (*co-first authors)

De Silva N, Atkins H, Kirn DH, Bell JC, Breitbach CJ. Double trouble for tumours: Exploiting the tumour microenvironment to enhance anticancer effect of oncolytic viruses. *Cytokine Growth Factor Rev*. 2010 Mar23.

Hosszu Ungureanu N, Cloutier M, Lewis S, **De Silva N**, Blais J, Bell JC, Holcik M. IRES-mediated translation of Apaf-1, but not XIAP, is regulated during UV-induced cell death. *Journal of Biological Chemistry*. 2006. Jun 281(22):15155-63.

Manuscripts in Preparation

5 manuscripts (2 first-author)

De Silva N, Roy D, Becker M, Storbeck C, Atkins K, Falls T, Daneshmand M, Diallo JS, Ilkow C, Atkins H, Bell JC. Normalizing tumour vasculature improves delivery and efficacy of oncolytic VSV therapy. (in preparation)

De Silva N, Cervi A, Mach H, Susser L, Marguerie M, Roy D, Falls T, Daneshmand M, Atkins H, Bell JC. Macrophage infiltration in response to oncolytic virotherapy potentiates acute bystander cell killing and efficacy. (in preparation)

Rozanne Arulanandam, Cory Batenchuk, Fernando A. Angarita Celis, Kathryn Ottolino-Perry, Sophie Cousineau, Carolina Ilkow, Amelia Mottashed, Emma Burgess, Theresa Falls, Naomi De Silva, Jovian Tsang, Grant Howe, David Conrad, Brian Lichty¹, Christina Addison¹, J. Andrea McCart² and John C. Bell. PRD1-BF1 drives VEGF-mediated innate immune suppression in tumour vasculature - a key determinant of oncolytic virus therapy (in preparation)

Sennino B, Schriver B, Breitbach C, **De Silva N**, Bell J, Kirn D, McDonald D. The oncolytic poxvirus JX-594 rapidly infects tumour endothelial cells, reduces the tumour vasculature, and increases leakiness of surviving tumour blood vessels. (in preparation)

Rozanne Arulanandam, Cory Batenchuk, Chadi Zakaria, Julie Cox, Oliver Varette, Vanessa Garcia, Raunak Karmacharya, Anisha Sinha, Andrew Babawy, Theresa Falls, Andrew Chen, Jeff Hamill, Nicole Forbes, **Naomi De Silva**, David Conrad, Harry Atkins, Kennneth Garson, Carolina Ilkow, Mads Kaern, Barbara Vanderhyden, Nahum Sonenberg, Tommy Alain, Fabrice Le Boeuf, *John Bell, *Jean-Simon Diallo. Interferon Secretion Lag and Enhanced Cytotoxicity Caused by Microtubule Disrupting Agents Improves Efficacy of Oncolytic VSV in Resistant Tumours (in submission)

Conference Presentations/Abstracts

8 poster presentations at international or national conferences, including 4 international conferences and 1st and 3rd place prizes at institution poster days

De Silva N, Breitbach CJ, Falls T, Roy D, Becker M, Daneshmand M, Sennino B, McDonald D, Atkins H, Kirn D, Bell JC. Targeting Tumour Vasculature with Oncolytic Viruses. 7th International Meeting on Oncolytic Viruses as Cancer Therapeutics. June 15-18, 2013. Quebec City, QC.

De Silva N, Breitbach CJ, Fenster A, Kirn D, Bell JC. Targeting Tumour Vasculature with an Oncolytic Virus. Canadian Cancer Research Conference. November 27-30, 2011. Toronto, ON.

Breitbach CJ, Arulanandam R, **De Silva N (presenter)**, Patt R, Parato K, Daneshmand M, Hickman, T, Bell, JC, Kirn DH. JX-594, a targeted multi-mechanistic oncolytic poxvirus, selectively infects tumour

vasculature and causes acute tumour vascular disruption and necrosis in advanced cancer patients. American Association for Cancer Research (AACR) Annual Meeting. April 2-6 2011. Orlando, FL.

De Silva N, Breitbach CJ, Falls T, Sun YY, Evgin L, Roy D, Daneshmand M, Aladl U, Fenster A, Atkins H, Bell JC. Exploiting the Tumour Microenvironment to Enhance the Anticancer Effect of Oncolytic Viruses. 6th International Meeting on Oncolytic Viruses (OVs) as Cancer Therapeutics. March 16-19 2011. Las Vegas, NV.

De Silva N, Breitbach CJ, Falls T, Aladl U, Fenster A, Bell JC. 3D Modelling of Tumour Vascularity During Oncolytic Virus Therapy. Eastern Ontario Cancer Innovation Forum. March 11th 2010. Ottawa, ON.

De Silva N, Breitbach CJ, Falls T, Aladl U, Fenster A, Bell JC. 3D Modelling of Tumour Vascularity During OV Therapy. 5th Canadian Symposium on Gene Therapy and Vaccines. May 2-4 2010. Grenville-sur-la-rouge, QC.

De Silva N, Breitbach CJ, Falls T, Aladl U, Fenster A, Bell JC. 3D Modelling of Tumour Vascularity During Oncolytic Virus Therapy. Ontario Institute of Cancer Research Annual Scientific Meeting. February 28th – March 2nd 2010. Alliston, ON.

De Silva N, Breitbach CJ, Falls T, Aladl U, Fenster A, Bell JC. 3D Modelling of Tumour Vascularity During OV Therapy. 5th Int'l Meeting on Replicating Oncolytic Virus Therapeutics. March 18-22, 2009. Banff, AB.

Co-authored Poster Presentations:

Sennino B, Schriver B, Breitbach C, **De Silva N**, Bell JC, Kirn D, McDonald DM. Widespread endothelial cell infection and tumour cell apoptosis after intravenous injection of oncolytic vaccinia virus JX-594 into RIP-Tag2 mice. American Association of Cancer Research Annual Meeting (2013).

Arulanandam R, **De Silva N**, Breitbach C, Vaha-Koskela M, Batenchuck C, Addison C, Bell JC. Mechanisms of Oncolytic virus targeting of tumour-associated vasculature. 6th International Meeting on Oncolytic Viruses as Cancer Therapeutics. March 16-19 2011. Las Vegas, NV.

Becker M, Rintoul J, **De Silva N**, Falls T, Daneshmand M, Wang J, Bell JC. The effect of tumour vascular modulation on oncolytic virus activity. Mechanisms of Oncolytic virus targeting of tumour-associated vasculature. 6th International Meeting on Oncolytic Viruses as Cancer Therapeutics. March 16-19 2011. Las Vegas, NV.

Breitbach CJ, Falls T, **De Silva N**, Paterson J, Parato K, Atkins H, Bell JC. Tumour targeted inflammation and coagulation following oncolytic virus therapy compromises tumour blood flow and triggers apoptosis of uninfected tumour cells. 4th Canadian Gene Therapy and Vaccines Symposium. May 26-27, 2008. Montreal, QC.

Breitbach CJ, Falls T, **De Silva N**, Paterson J, Parato K, Atkins HL, Bell JC. Tumour targeted inflammation and coagulation following oncolytic virus therapy compromises tumour blood flow and triggers apoptosis of uninfected tumour cells. American Association for Cancer Research (AACR) Annual Meeting. April 12-16, 2008. San Diego, CA.

Hoszu Ungureanu N, Cloutier M, **De Silva N**, Holcik, M. IRES-mediated translational activation of APAF-1, but not XIAP, is regulated during UV-induced cell death. Presenter: M. Holcik. 13th Euroconference on Apoptosis, October 1-4, 2005, Budapest, Hungary.

Hoszu Ungureanu N, Cloutier ML, **De Silva N**, Holcik M. IRES-mediated translational activation of Apaf1 is essential for UV-induced cell death. Keystone Symposium on Apoptosis in Biochemistry and Structural Biology, February 3-8, 2004, Keystone, CO, USA.

Hoszu Ungureanu N, Cloutier M, **De Silva N**, Holcik M. IRES-mediated translational activation of APAF-1 is essential for UV-induced cell death. Cold Spring Harbor Laboratory Symposium on Translational Control, September 7-12, 2004, Cold Spring Harbor, NY, USA.

TEACHING EXPERIENCE

Teaching Environments: I have taught within academia, industry and general public; I have mentored youth and aboriginal groups.

Academic Teaching Experience in a Laboratory Setting (2008 – present)

Over the course of my graduate studies, Dr. John Bell and Dr. Harold Atkins have given me the responsibility of supervising 8 students:

- Medical students - Zi Hymn Mach (2012) and Andrea Cervi (2013)
- Honours student - Naveen Silva (2011-2012)
- Co-op student - Sara Cvancic (2009)
- Undergraduate students - Laura Evgin (2008) and Kelly Sun (2010)
- High school students - Kathleen Atkins (2012) and Leah Susser (2012)

Responsibilities:

Guided research projects through the conceptual design of experiments, teaching of technical skills, and provision of feedback and formal evaluation of presentations, theses, and reports.

Teaching my Research to Research Stakeholders (2010-2012)

- Media relations - My supervisor frequently asked me to represent his lab in interactions with the public.
 - **Decouverte Episode on Oncolytic Viruses: “Un virus contre le cancer” – video interview.** With Dr. Bell, I explained my research to a general audience by participating in the production of the French science show Decouverte. <http://www.radio-canada.ca/emissions/decouverte/2011-2012/Reportage.asp?idDoc=210133> (2012).
 - **CTV National and Local News - video interviews.** Participated in the production of a news segment in where I presented my research with Dr. John Bell. Similar interviews of mine were broadcast on CBC and Global TV. The segments featured our recent publication in *Nature*, where we delivered oncolytic virus to patient tumours by intravenous

administration for the first time. <http://www.ctvnews.ca/canadian-made-virus-shows-promise-as-cancer-treatment-1.690978> (2011).

- **Canadian Institutes of Health Research - video interview.** Participated in the production of a “research spotlight” video for the general public that highlighted Dr. John Bell’s research. <http://www.youtube.com/watch?v=BK7TK14AEdM&feature=related> (2011).
- *Canadian Cancer Society:* Discussed cancer research with the general public, patients, and families during an open house event (2012).
- *Centre for Innovative Cancer Research:* Helped organize and conduct lab tours discussing research and the ‘life of a scientist’ as part of two donor recognition evenings for corporate donors (2010 - 2011).

Teaching Experience in Grade Schools (2007 – 2012)

Science Outreach - Let’s Talk Science

Involved with Let’s Talk Science to improve science literacy through leadership, innovative educational programs, research, and advocacy.

- Helped create interactive “living cell” activity for high school students (2012).
- Young Aboriginal Scientist Program: Mentored Aboriginal high school students through personal discussion and delivered robotics and electrochemistry activities to Akwesasne students in Cornwall, ON (2011).
- Served as a judge in 5 science fairs: Ottawa Regional Science Fair (2009-11), Turnbull Academy (2011), Greenbank Middle School (2009), and Knoxdale Public School (2008).
- Delivered DNA and chemistry lessons at the Public Library in Pembroke, ON (2008).
- Delivered and helped create an interactive presentation on cancer to classes at Castlefrank Elementary (2008).
- Liaison for Knoxdale Public School. Liaised with teachers and LTS volunteers to provide activities for students over the course of the school year (2007-8).

PRACTICUMS

2011 Oct 1-3

Harvard Medical School, Boston, MA, USA

26th Annual Course “Critical Issues in Tumour Microenvironment, Angiogenesis, and Metastasis” offered by the Department of Continuing Medical Education (24 American Medical Association PRA Category 1 Credits).

2011 Jun-Aug

University of California, San Francisco, San Francisco, CA, USA

Completed a foreign study term in the laboratory of Dr. Donald McDonald in the Department of Anatomy. Learned imaging methods and studied tumour vasculature in response to antiangiogenic treatments. Work funded by CIHR Travel Award.

2008 Mar 24-8

Robart's Imaging Institute, University of Western Ontario

Learned how to construct 3D models of tumours in the laboratory of Dr. Aaron Fenster.

2008-2010

Graduate Coursework, University of Ottawa , Ottawa, ON

Developed skills through writing mock grants and participating in grant panels.

Courses:

HMG8103 Advanced topics in molecular biology of human diseases I

BCH8310 Current topics in RNA molecular biology

BCH8105 Advanced topics in molecular biology of human diseases II

MIC8125 Special topics in microbiology and immunology