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Neoadjuvant Onoclytic Virus Therapy in a Murine Model of Cancer Surgery

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By

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Abstract

Surgery is the primary treatment modality for most solid tumours. Despite complete resection, the development of metastatic disease limits it curative potential and provides the rationale for neoadjuvant (preoperative) therapies. Oncolytic Viruses (OVs) are replicating therapeutics that are selected or engineered to grow in malignant cell types and are capable of killing the infected target cell, while leaving normal, adjacent cells unharmed. OVs may be an ideal candidate for generating a potent anti-tumour immune response due to effective recruitment of immune cells into the tumour microenvironment, proper activation of immune cells, and generation of tumour antigen-specific T cells. Preoperative OV therapy may serve as a neoadjuvant immunotherapy capable of preventing the development of metastatic disease by generating an effective immune response. Although VSVΔ51GM-CSF treatment of CT26lacZ tumours is able to generate an anti-tumour immune response capable of preventing the growth of a subsequent CT26lacZ tumour, preoperative VSVΔ51GM-CSF treatment of primary tumours in a CT26lacZ surgery model was unable to generate an immune response capable of rejecting a secondary tumour. This abrogation of protection against the secondary CT26lacZ tumour was observed to be a result of the surgical intervention. Preoperative VSVΔ51GM-CSF and VSVΔ51 treatment IV of primary tumours in a B16-F10 surgery model was unable to generate an immune response capable of reducing the number of secondary surface lung metastases. JX594mGMCSF treatment IT of a primary tumour in a B16-F10 surgery model was able to reduce the number of secondary surface lung metastases, while JX594mGMCSF injected IV was not. This suggests that one of the factors in the induction of an anti-tumour

immune response by neoadjuvant therapy in a surgical model may be the route of administration of OV.

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List of Abbreviations

bFGF: basic fibroblast growth factor
CEV: cell-associated enveloped virus
CTLs: cytotoxic T lymphocytes
DC: dendritic cell
DMEM: Dulbecco's Modified Eagle Medium
EEV: extracellular enveloped virus
EGF: epidermal growth factor
eIF2α: eukaryotic initiation factor 2
FBS: fetal bovine serum
FP: footpad
GFP: green fluorescent protein
GM-CSF: granulocyte-macrophage colony-stimulating factor
HIV: human immunodeficiency virus
HSV: herpes simplex virus
IDO: indoleamine-pyrrole 2,3-dioxygenase
IFN: interferon
IFN-γ: interferon-γ
IGF I: insulin-like growth factor I
IGF II: insulin-like growth factor II
IH: intrahepatic
IL-6: interleukin 6
IL-10: interleukin 10
IL-12: interleukin 12
IMV: intracellular mature virus
IP: intraperitoneal
ISG: interferon stimulating gene
IT: intratumoural
IV: intravenous
LMP2: low-molecular-mass protein 2
LMP7: low-molecular-mass protein 7
LPS: lipopolysaccharide
M-CSF: macrophage colony-stimulating factor
MHC: major histocompatibility complex
MOI: multiplicity of infection
NE: norepinephrine
NK cell: natural killer cell
NKT cell: natural killer T cell
NOD: nucleotide oligodimerization domain
OV: oncolytic virus
PAMP: pathogen-associated molecular pattern

pAPC: professional antigen presenting cell
PBS: phosphate-buffered saline
PCR: polymerase chain reaction
PDGF: platelet-derived growth factor
PGF: placental growth factor
PRR: pattern recognition receptor
RIG-1: retinoic acid inducible gene 1
RLRs: retinoic acid inducible gene I-like receptors
SQ: subcutaneous
Sx: surgery
TAP protein: transporter associated with antigen processing protein
TCR: T cell receptor
TDLNs: tumour-draining lymph nodes
TGF-α: transforming growth factor-α
TGF-β: transforming growth factor-β
TILs: tumour-infiltrating lymphocytes
TLR: toll-like receptor
TRAIL: TNF-related apoptosis inducing ligand
Treg: regulatory T cell
Tx: treatment
VEGFA: vascular endothelial growth factor A
VSV: vesicular stomatitis virus
VV: vaccinia virus

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Chapter 1: Introduction

1.1 Cancer

1.1.1 Overview

Cancer is defined as the abnormal growth of cells, which have gained the ability to proliferate uncontrollably and to metastasize. This definition refers to the disease in general, however there are over 100 distinct types of cancer(1). Cancer is characterized by the tissue and cell of origin. For example, for a lung carcinoma, lung refers to the tissue and carcinoma refers to the cell of origin, which in this case is derived from the epithelial cells. There are also subtypes of cancer that can be found within specific tissues, such as small cell lung carcinoma and non-small cell lung carcinoma. Malignant cells are derived from normal tissues after a series of genetic alterations. The development of tumours is a complex, multi-step process, where a normal tissue evolves progressively into one that is highly malignant(1). There are six accepted hallmarks of cancer which distinguishes them from normal, healthy cells. These essential alterations include self-sufficiency in growth signals, insensitivity to anti-growth signals, uncontrollable proliferation, angiogenesis, the evasion of apoptosis, and the ability to invade tissues and metastasize(1). The seventh hallmark of cancer has been considered by some immunologists to be the ability of cancer cells to evade the immune system. Immune evasion strategies by cancer cells will be discussed at length shortly.

1.1.2 Metastasis

Although primary tumours are insidious and can induce numerous effects on normal tissue function, the majority of deaths from cancer are not from primary tumour growth, but from the growth of distant metastases. Metastasis is an intricate, multistage process, where cancer cells face numerous challenges during their spread from the tumour of origin to a new distant organ. Despite

these challenges and the inefficiencies during the process of metastasis, cancer cells can eventually succeed in generating a secondary tumour mass. The proportion of patients that will develop metastasis is specific to the type of cancer and the stage of the disease at the time of diagnosis.

Before a cancer cell can metastasize, it must first gain the ability to invade into adjacent tissue and neighbouring blood or lymphatic vessels. The process of invasion into vessels is termed intravasation (2, 3). Cancer cells face a hostile environment in the blood – lacking a solid substrate to attach to, facing hydrodynamic shear forces in the circulation, and by potential cell killing by immune cells (4, 5). Studies have shown that many tumour cells grow best when attached to a plate or a solid substrate. Anoikis, a form of apoptosis that is triggered by the detachment of a cell from a solid substrate, is hypothesized to occur when tumour cells are free in the bloodstream (6). There is also evidence that cancer cells in the circulation are more likely to survive if they can attract blood platelets to shield them from the immune system as they travel (7). Specifically, cancer cells are protected from natural killer (NK) cell-mediated lysis through the formation of aggregates with platelets (8, 9).

If the cancer cells survive in the bloodstream, they must arrest in the circulatory system and extravasate. Some studies suggest that arrest occurs by binding receptors or by size restriction in capillary beds (10). Extravasation is the process of penetrating the surrounding tissue from the lumen of the vessel. In some cases, proliferation of the cancer cells occurs in the lumen of the vessel and subsequently extravasation occurs as a small tumour mass. Additionally, cancer cells can extravasate into the surrounding tissue first, and then proliferate (11, 12). Following extravasation into a tissue, cancer cells can survive for extended periods of time as single cells or small clusters of cells, which are termed micrometastases or the cancer cells may form a tumour mass in a process termed colonization.

The period of time between infiltration and colonization when small micrometastases can survive without progression is termed metastatic latency or dormancy (4). Dormancy has been observed in the clinic in breast cancer, prostate cancer, and melanoma (13). Experimental models have shown that these dormant tumour cells can remain viable for extended periods of time in a non-dividing, inactive state (14-16). Another theory, termed angiogenic dormancy, suggests that there is a balance of proliferation and apoptosis owing to poor vascularization, which prevents progression of the micrometastases (17, 18).

Colonization is challenging for cancer cells as they are not placed in the favourable environment in which they thrived at the primary tumour site. A study that used a melanoma experimental metastasis model showed that although the majority of tumour cells injected intravenously successfully survived in the bloodstream and extravasated into the liver, only 1 in 100 micrometastases progressed to form macroscopic metastases 10 days later (14). The success of colonization has been attributed to the interaction of the cancer cells and the microenvironment of the new organ. It is not known exactly how cancer cells are able to develop the ability to colonize an organ. The proficiency of cancer cells to survive the period of metastatic latency until they are able to form into a tumour mass is also not well understood.

There is debate about when the process of metastasis is initiated. There is evidence that seeding of distant micrometastases can occur years before discovery of the primary tumour (16, 19-23). However, patients that have no evidence of tumour dissemination at the time of diagnosis of the primary tumour can still develop metastatic disease. Roughly one-third of breast cancer patients that receive surgical resection of their primary mass and who have negative lymph node status at the time of resection, develop secondary tumours. These patients with node negative

status and small primary tumours still have a 15-25% chance of developing distant metastases(24, 25).

Several studies have examined the interactions between the primary tumour and the metastatic site. In some cases, it has been shown that the primary tumour secretes products to create favourable environments distantly for the development of micrometastases. These distant sites that house a favourable atmosphere for metastatic growth are termed pre-metastatic niches (26-28). Recent evidence has shown that the primary tumour secretes growth factors, including vascular endothelial growth factor A (VEGFA), placental growth factor (PGF), and transforming growth factor-β (TGF-β), which upregulate inflammatory S100 chemokines and serum amyloid A3(29). The upregulation of these factors results in the recruitment of tumour-associated cells, such as haematopoietic progenitor cells and macrophages. These cells and soluble factors create an environment that is favourable for tumour cell adhesion, invasion, and growth (5).

Conversely, studies have shown that the presence of the primary tumour inhibits metastasis, and the removal of the primary tumour can promote metastatic growth (18, 30, 31). Angiostatin, an anti-angiogenic molecule, has been identified to be secreted from primary tumours (32, 33). This molecule has been shown to induce dormancy in a variety of tumour models(32). This would suggest that the secretion of angiostatin by the primary tumour keeps the distant metastases in a dormant state. Upon resection of the primary tumour and removal of the source of angiostatin, the metastases would then be able to grow.

Since metastasis contributes to the majority of cancer deaths, the importance of understanding how cancer cells develop the ability to metastasize and how they survive during the period of dormancy is crucial. Our understanding of these processes could lead to therapies that specifically target disseminated disease.

1.2 The Immune System

1.2.1 Overview

The mammalian immune system is highly effective in detecting and eliminating foreign infectious agents such as viruses, bacteria, and fungi. Antigens are specific molecular entities which make up these infectious agents. Infectious particles or infected cells displaying these antigens are destroyed by the immune system. There are two arms of the immune system – the innate immune response and the adaptive immune response. The former is the first line of defence against infectious particles and does not require prior sensitization towards a foreign particle. The latter response is initiated by cells of the innate immune system and the adaptive immune cells, which recognize specific antigens, are educated through the initial encounter with an antigen to recognize the same antigen in the future (3). Some of the cells of the innate immune system that will be discussed are dendritic cells (DCs), NK cells, and macrophages. The adaptive immune cells include T cells and B cells.

The adaptive immune response is initiated when specialized phagocytic cells, mainly macrophages and DCs, engulf infectious particles or abnormal cells. These cells, which are termed professional antigen presenting cells (pAPCs) have the capability to process antigen and present it in the form of peptides to T cells. T cells only recognize peptides that are displayed on cell surfaces complexed with the major histocompatibility complex (MHC) molecule. Peptides presented by the MHC Class I molecule activate $CD8^+$ T cells, which are also called cytotoxic T lymphocytes (CTLs) when activated to be cytolytic, while peptides presented by the MHC Class II molecule activate $CD4^+$ T cells, which are also called helper T cells when activated. T cells recognize the MHC-antigen complexes through their T cell receptor (TCR), which are specific for an antigenic peptide (34). This MHC-restricted T cell recognition causes activation of T lymphocytes after MHC-antigen complex

binding (35)with the addition of co-stimulatory molecules, CD80 or CD86, on the pAPC binding to CD28 on the T cell (36).

After ingestion of foreign fragments via phagocytosis or endocytosis, these pAPCs travel to the lymph node, where they inform the immune system of the antigen that they have taken up. Antigen is digested in cytoplasmic vesicles by endocytic proteases (36) into peptides that are 18-22 amino acid residues long. The peptides are then loaded onto MHC class II molecules as they are being shuttled to the cell surface. The MHC class II–antigen complex is presented to $CD4^+$ T cells which when activated, stimulate B cells that can manufacture antibodies. Neutralization by antibody recognition and binding of infectious particles can then occur by complement-mediated killing. The adaptive immune response that involves the production of antibodies is called the humoral immune response.

Another adaptive immune response, which is the cellular immune response, leads to the formation of cytotoxic cells, where antigens are processed and presented on MHC Class I molecules. There are two pathways by which this activation can occur - the direct presentation pathway and the cross presentation pathway. Direct presentation is carried out by all nucleated cells, including pAPCs. In this pathway, endogenous peptides are presented on MHC class I molecules via the cytosolic pathway.

In direct presentation, cytosolic protein is processed by the ubiquitin-proteasome system. Protein, which is tagged to be degraded, is led into the core of the proteasome and degraded into short peptides, ranging in size from about 8-11 amino acids (37). MHC Class I molecules are assembled in the lumen of the endoplasmic reticulum by a variety of chaperone proteins. After assembly, the MHC Class I molecule forms a complex with the transporter associated with antigen processing (TAP) protein, which delivers the peptide. The complex can then be transported to the

cell surface via the Golgi Apparatus (36). Peptides of proteins that are synthesized normally by cells as well as those made by foreign infectious agents within the cell are presented on the cell surface.

In the cross presentation pathway, the peptide antigens derived from exogenous materials are presented on the MHC Class I receptor. The exogenous antigen enters the pAPC via phagocytosis, endocytosis, or macropinocytosis and is taken up into a phagosome (38). As in the direct presentation pathway, the antigen then undergoes proteasome degradation. Assembly of the peptide-MHC class I complex in the endoplasmic reticulum, followed by transport to the cell surface via the Golgi Apparatus is no different from the direct presentation pathway.

Activation of CTLs requires two signals – 1. Recognition of a foreign peptide-MHC class I complex with the TCR and 2. binding of the co-stimulation molecules. In order for pAPCs to upregulate their co-stimulatory molecules they must undergo maturation through inflammatory stimulus or toll-like receptor (TLR) ligation which will be discussed in more detail shortly. Activating cytokines from helper T cells can assist this second signal. This activation results in T cell mediated killing by perforin or by the Fas death receptor (3).

The proper function of the immune system is of crucial importance in protecting the host from various pathogens, such as bacteria, viruses, and fungi. Some of these pathogens have evolved mechanisms to evade the immune system or actively suppress it. The mechanisms in which cancer avoids the immune system will now be discussed.

1.2.2 Immune Tolerance in Cancer

1.2.2.1 Immunosurveillance

In addition to the six hallmarks of cancer, immunologists have suggested that avoidance of immunosurveillance might be the seventh hallmark of cancer. Cancer immunosurveillance refers to

the concept that numerous innate and adaptive immune effector cells and molecules participate in the recognition and destruction of cancer cells. The concept of immunosurveillance was validated by studies that showed that animals which lack various components of the immune system are more susceptible to the development of spontaneous or chemically induced tumours (39-42). Immune effector cells such as B cells, T cells, NK cells, and natural killer T (NKT) cells have been shown to be key players in the process of immune surveillance (42-44). It has also been observed that patients with immunodeficiencies (45) and transplant patients, who are immunosuppressed (46), are predisposed to developing certain types of cancer (39).

It is widely accepted that tumour-infiltrating lymphocytes (TILs) have an ability to attack and eradicate tumour cells in cancer patients. Evidence for this is based on studies that have examined $CD8^+$ T cell infiltration and correlated this with prognosis. The presence of TILs was able to predict a better survival in a variety of cancers (42). Importantly, it has been shown that these intratumourally located $CD8^+$ T cells are important for tumour cell eradication, unlike $CD8^+$ T cells located around the tumour site (47). Despite an intact immune system, malignancies continue to develop, grow, and spread.

Cancer cells escape immunosurveillance by immunoselection, which is the selection of poorly immunogenic tumour-cell variants, and by immunosubversion, which is the active suppression of the immune response. Tumours have a variety of mechanisms to escape immunosurveillance. Immunoediting, which has three temporally distinct stages termed elimination, equilibrium, and escape, refers to the process responsible for both eliminating tumours and sculpting the immunogenic phenotypes of tumours that form in immunocompetent hosts.

1.2.2.2 Immunoediting

1.2.2.2.1 Elimination

Elimination includes innate and adaptive immune responses to tumour cells, and is the hallmark of the concept of cancer immunosurveillance. Innate effector cells, such as NK cells, NKT cells, and γδ-T cells, are activated by inflammatory cytokines. These inflammatory cytokines are released by the growing tumour cells, macrophages, and stromal cells surrounding the tumour mass during stromal remodeling. Killing of tumour cells by NK cells leads to the release of tumour antigens, which can initiate adaptive immune responses. Immature DCs phagocytose lysed tumour cell fragments. Cross talk occurs between NK cells and DCs, where NK cells can promote the maturation of DCs and subsequently their migration to tumour-draining lymph nodes (TDLNs). Antigen processing and presentation occurs in the pAPCs and naive T cells can be primed and expanded. Tumour antigen specific T cells are recruited to the primary tumour site and can directly attack and kill tumours cells by producing interferon-γ (IFN-γ) (42), which exerts a limited cytotoxicity through antiproliferative and anti-angiogenic effects ,or through their other killing mechanisms previously discussed.

One of the controversial topics in the oncology field has been how the immune system can be activated by a developing tumour. There are two theories for immune recognition for DC activation: Matzinger's 'danger' theory of immune activation and Janeway's theory that activation occurs via pattern recognition receptors (PRR) binding to pathogen-associated molecular patterns (PAMPs). In the former theory, Matzinger suggested that the immune system reacts to cellular or tissue distress as opposed to non-self. In this model, activation of pAPCs occurs by endogenous 'danger' signals from stressed or dying cells. In the latter theory, pathogen sensors such as TLRs, retinoic acid inducible gene 1 (RIG-1)-like receptors (RLRs), and nucleotide oligodimerization domain

(NOD)-like receptors recognize PAMPs. These PRRs are found on pAPCs, and when ligated, cause activation of the pAPC. It has been thought that cellular transformation does not provide sufficient proinflammatory signals to activate the immune system in response to a developing tumour. The proinflammatory signals required to recruit and activate innate immune cells are thought to be made by minor disruptions within the surrounding tissue by invasive growth (48-50).

1.2.2.2.2 **Equilibrium**

In the equilibrium phase, the host immune system and the tumour variants that were not eliminated in the first stage of immunoediting enter into a dynamic equilibrium. In this phase, T cells exert selection pressure on the tumour cells – eliminating certain immunogenic variants, but ignoring less immunogenic variants produced by random gene mutations (42, 43). Experimental mouse studies that had different deficiencies of effector molecules indicated various degrees of immune selection pressure (51, 52). Chemically induced sarcomas in both nude and SCID mice were more immunogenic in comparison to tumours formed in immunocompetent mice, suggesting that tumours formed in the absence of an intact immune system are more immunogenic than tumours developed in immunocompetent hosts (52-54). Of the stages in immunoediting, it has been thought that the equilibrium phase may be the longest - occurring over a period of many years (42, 43).

1.2.2.2.3 **Escape**

In the escape phase, the tumour cell variants that escaped immune recognition begin to expand in an uncontrolled manner. Here, the surviving tumour variants have developed several escape mechanisms from immune surveillance. Commonly, tumour cells downregulate or lose expression of MHC class I molecules in order to elude T cell mediated killing (55). Tumour cells can also develop defects in molecules necessary for antigen processing and presentation such as TAP protein, β2-microglobulin protein, low-molecular-mass protein 2 (LMP2), LMP7, and tapasin (56).

These strategies are employed by tumour cells in order to avoid recognition and activation by the immune system. In addition, tumour cells also employ mechanisms to avoid CTL killing. Blockade of the granzyme-B-perforin pathway by overexpression of the serine-protease inhibitor P19 by tumour cells renders CTLs unable to lyse the cancer cells (57). Additionally, resistance to CTL-induced killing of tumour cells can include downregulation or mutation of death receptors, mutation of the gene that encodes caspase-8, and decoy receptors for TNF-related apoptosis inducing ligand (TRAIL) (58). Caspase-8 is involved in executing apoptosis when induced by Fas or other apoptotic stimuli. TRAIL is a protein that, when ligated, induces apoptosis. These are some of the many ways that tumour cells are able to escape cell killing by selecting for tumour cell variants with desirable immune evasion properties.

Tumour cell products also actively suppress the immune response. In an experimental mouse model, tumour-specific $CD8^+$ T cells were shown to be active at the initial stages of tumour development, but gradually showed loss of cytolytic function as the tumour progressed (59). Likewise, $CD4^+$ T cells have also been shown to progressively lose their anti-tumour activity (60). Numerous factors are secreted by tumour beds that act to inhibit the maturation or function of various immune cells (61). Some of these factors include VEGF, interleukin-6 (IL-6), interleukin-10 (IL-10), TGF-β, macrophage colony-stimulating factor (M-CSF), arginase-1, and indoleamine-pyrrole 2,3-dioxygenase (IDO). Although, VEGF was described previously is a pro-angiogenic factor, this factor also plays a role in active suppression of the immune system by inhibiting T cell activation and preventing the maturation and proper function of DCs(39). TGF-β has numerous roles in suppressing the immune system such as inhibiting antigen presentation, T cell proliferation, inhibiting NK cell cytotoxicity, and activating regulatory T (Treg) cells (36, 39). Treg cells are found in great numbers in the tumour microenvironment and act to suppress activation of CTLs in two ways – by cell contact dependent and by contact independent, cytokine-mediated mechanisms (62, 63).

T cell function can be inhibited by tumour cell overproduction of nitric oxide and increased arginase-1 activity (64). IDO, which has been shown to block proliferation of $CD8^+$ T cells, is commonly constitutively expressed by a variety of human tumours (65). IDO has also been shown to promote the apoptosis of $CD4^+$ T cells (66). The immunosuppressive cytokine, IL-10, can reduce DC development and activity (36). As previously emphasized, the development of a robust adaptive immune response towards the tumour relies on the proper antigen processing by DCs, maturation of DCs, and expansion and activation of T cells. Cancer cells are able to impede the generation of an adaptive immune response at many stages.

Another property of tumour cells is the ability to induce T cell tolerance. At this stage in tumour development, it has been thought that tumour cells that are taken up by DCs fail to provide the required PAMP to activate pAPCs by PRRs or fail to provide 'danger' signals by stressed or dying cells since the tumour is 'healthy' and growing. As discussed previously, DCs play a critical role in initiating adaptive immune responses by antigen processing and presentation to T cells. If tumour cells do not provide the required signal for DC maturation, DCs are then unable to express sufficient levels of co-stimulatory molecules required for T cell activation or to produce the cytokines required to support the survival and effector functions of tumour-specific T cells (67-70). DC presentation to T cells in the absence of co-stimulatory signals and cytokine secretion leads to T cell tolerance (36, 71, 72). These tolerized T cells are unable to mount a response against the target. It has also been shown that injection of immature antigen-pulsed DCs can induce T cell tolerance specific to that antigen (73).

Altogether, these studies show some of the numerous ways that tumour cells work to evade the immune system. Less immunogenic tumour cell variants are selected for their ability to evade immune cell killing. In addition, active suppression of the immune system occurs by tumour cell

secretions. Finally, cancer cells are able to induce T cell tolerance by the improper maturation of DCs, which leads to improper activation of T cells. Many cancer immunotherapies aim to break this tolerance and to generate a functional adaptive immune response specific for the tumour cells.

1.3 Cancer Therapeutics

1.3.1 Conventional Cancer Treatment

Although cancer treatment has dramatically changed over the last century, some of the initial treatment strategies have remained key players in the fight against cancer. Currently, surgical resection is the most effective treatment of a solid primary tumour, however this option is limited to localized disease. Additionally to treat localized disease, radiation therapy can be given as a single modality or in combination. Chemotherapy can be used to treat disseminated disease as it is administered systemically. Chemotherapy can also be administered as a single modality or in combination with surgery or radiation therapy (74).

1.3.1.1 Surgery

Surgery is the primary treatment modality for most solid tumours that are localized or have spread to regional lymphatics. As the goal of cancer surgery is complete eradication of the local tumour, the most efficacious method for preventing local recurrence is to resect a wide surgical margin of normal uninvolved tissue. Most solid tumours also have a tendency to disseminate to regional lymph nodes via local lymphatics so regional lymph nodes may need to be surgically excised as well. Despite complete resection, the development of metastatic disease limits it curative potential. The majority of cancer mortality is associated with disseminated disease rather than the primary tumour (3).

1.3.1.1.1 The Effect of Perioperative Factors on Cancer Recurrence

Surgical wounding, which can occur to various degrees depending on the type and invasiveness of the surgery, may provide favourable conditions for tumour recurrence or tumour growth. There are numerous stages in wound healing that involve the activation of epithelial, endothelial, and inflammatory cells as well as the production of a variety of growth factors. Similarly, in tumour growth, activation of these cells and production of various growth factors occurs as well (75).

Growth factors are small peptides that bind to their respective receptors and produce an intracellular response that results in either proliferation or inhibition of the cell. Growth factor stimulation may be autocrine, paracrine, or endocrine. Growth factors that are involved in wound healing which can also play a role in the development of tumours include epidermal growth factor (EGF), TGF-α and β, basic fibroblast growth factor (bFGF), insulin-like growth factor I and II (IGF I and II), and platelet-derived growth factor (PDGF).

During wound healing, EGF is released from platelets to initiate mitosis and migration of various types of cells. In tumourigenesis, EGF has been shown to mediate increased tumourigenicity and metastatic potential in numerous experimental studies upon interaction with normal fibroblasts and tumour cells (76-78). Injury healing is accelerated by the secretion of TGF-α by macrophages, keratinocytes, and platelets (79). Many cancer cells secrete TGF-α, such as breast carcinoma cells as they proliferate (80). Additionally, TGF-α causes neovascularization which aids in the growth of a tumour mass(81). TGF-β is a known player in wound healing and tumourigenesis (82). It is released in wound healing to induce the synthesis of collagen and stimulate extracellular matrix synthesis. In tumourigenesis, it has been found to be stimulatory to tumour cell growth and has the capabilities to alter the metastatic potential of the cells (83, 84). PDGF and bFGF both induce angiogenesis in

wound healing and in tumour growth (85, 86). Finally IGF I and IGF II, which are released during wound healing have been also shown to be overexpressed in a number of cancers (80, 87-89).

These growth factors which are involved in wound healing have also been implicated in tumourigenesis. During surgery, these growth factors are released locally and systemically. The release of these growth factors could affect the longer-term outcome after cancer surgery.

There have been numerous studies that show alterations in immune system function after surgery. After surgical procedures involving general anaesthesia and tissue injury, activation of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system is thought to suppress the immune system. The suppression of NK cell activity after surgery has been shown to promote tumour development in a number of rat models (90). In humans, surgical procedures for localized tumour resection have led to the suppression of NK cell activity during the post-operative period. Specifically, defects in NK cell lysis and diminished responses to recombinant IFN-γ have been observed (91-93). In addition to changes in NK cell activity, this clinical study also observed that the ability for T cells to proliferate was decreased after surgery (91). Catecholamines, which are released after tissue injury, have also been found to suppress NK cell activity (94). As NK cells have been shown to play a key role in controlling malignancies, defects in this vital cell population could lead to the favourable growth of tumours (95).

After tissue injury, norepinephrine (NE), which is a catecholamine, is released into the peripheral circulation (96). Macrophage function has been shown to be inhibited by NE by adrenergic receptors (97). Macrophages are phagocytic cells and pAPCs which are capable of cross-presentation. Suppression of macrophage function could inhibit initiation of the adaptive immune response.

There have been many studies suggesting alteration in a number of immune cells following surgery. Importantly, the number of lymphocytes has been shown to decrease as well as their capability to proliferate and produce cytokines (98). There has also been observance of reduced expression of HLA-DR, which is the MHC class II molecule in humans, by lymphocytes and monocytes, possibly indicating that antigen presentation may be reduced (99).

Although the initial postoperative cytokine response is pro-inflammatory at the site of injury, induction of a systemic anti-inflammatory response ensues, which in turn causes cellular immune suppression. This anti-inflammatory response is thought to occur to restrict inflammation to the site of injury, prevent inflammatory damage to other tissues and organs, and to limit unwanted systemic immune reactions (100). This systemic immune suppression after surgery could aid the growth of disseminated disease by thwarting an active immune response.

It has been shown that handling and disrupting the tumour during surgical resection releases tumour cells into the circulation. An increase in the presence of tumour cells in the blood of patients was detected by polymerase chain reaction (PCR) (101). As previously discussed, metastasis is a multistep and inefficient process, but if enough tumour cells are released into the bloodstream, some will eventually succeed in extravasating and forming a secondary mass.

Together, these studies suggest that suppression of immune functions or facilitation of tumour growth may increase the susceptibility to tumour metastasis during or shortly after surgery. As surgery is the primary treatment modality for most solid tumours, the long-term outcome after cancer surgery could be affected.

1.3.1.2 Radiation Therapy

Radiation therapy uses high-energy radiation to create highly reactive radicals that produce DNA damage. Radiochemical reactions with water produce products such as hydrogen free radicals, hydoroxyl free radicals, hydroxyl ions, hydrogen, and hydrogen peroxide. Most commonly, the DNA injury is lethal due to the interruption of the cell's capacity to reproduce indefinitely (reproductive death). Less commonly, interphase death occurs by structural degeneration. The rate at which cells die reflects the rate of their division, where rapidly dividing normal tissues show an early response while slowly proliferating tissues may not manifest injury for months or years. The rate of cell killing of cancer cells is higher than normal cells as they divide more rapidly and death of the cell occurs upon cell division (102). One of the major limitations of radiation therapy is decreased radiosensitivity due to hypoxia in the tumour. Oxygen is necessary for effective radiation therapy acting as a potent radiosensitizer involved in mediating DNA damage (103).

1.3.1.3 Chemotherapy

Chemotherapy is administered systemically and can be used to control disseminated disease. Chemotherapeutic drugs affect cell division or DNA synthesis and function. The selectivity of chemotherapy selectivity is based on quantitative differences between malignant and normal cells (102). The rate of DNA synthesis is much higher in malignant cells compared to normal cells, allowing selective targeting by chemotherapeutics. Unfortunately, there are some normal tissues with high proliferation rates such as bone marrow, gastrointestinal epithelium, and hair follicles that are targeted by this therapy as well (102). There is a vast number of chemotherapies, which vary in the molecule or pathway targeted, but the majority of chemotherapies act on cell division or DNA synthesis and function.

1.3.2 Cancer Immunotherapies

For decades, research has been aimed at breaking immune tolerance of cancer and eliciting an anti-tumour immune response by instruction of the patients' own immune system through various modes of vaccination or inflammatory stimulus.

1.3.2.1 *Tumour cell Vaccination with Viral Adjuvants*

Tumour lysates combined with viruses as an adjuvant have been examined as an immunotherapy. Anti-tumour effects have been observed in animal models using vaccinia virus and Newcastle disease virus as adjuvants in a melanoma cell lysate (104, 105). Whole cell viral adjuvant vaccinations have also been studied in our lab. Vesicular stomatitis virus is able to act as an adjuvant in an autologous whole cell vaccine given prophylactically to protect against a subsequent live tumour cell inoculation in immunocompetent mice (CL unpublished data).

1.3.2.2 Viral Vectors encoding Tumour-associated Antigens

Viral vectors encoding tumour-associated antigens have been used recently in the clinic. Although there were indications of stabilized disease, there were very few examples of tumour regressions. Repeated administration of a single viral vector or homologous boosting caused the immune response towards the viral vector to be boosted as well. The response generated towards the viral antigens tends to be more robust in this scenario than the response against the tumour antigen (106). Clinical trials using heterologous viruses for the prime-boost aim to boost the immune response towards the tumour antigen and not the viral antigen. This strategy has shown some enhancement of the immune response generated towards the tumour antigen, but optimization of virus pairing is still required (107).

Xenoimmunization has been used as a strategy for breaking tolerance. This technique elicits an immune response to a self-antigen by vaccination with a homologous antigen from a different species (108, 109). The careful selection of xenoantigens is critical for a number of reasons. The antigen selected must remain sufficiently foreign to maximize the immune response generated. However, in order to ensure a cross-reactive response, the antigen must also remain similar to the target antigen.

1.3.2.3 Dendritic Cell Vaccines

It has been observed in patients with melanoma, ovarian, breast, renal, prostate, lung and head and neck cancer (68, 110-112) that their endogenous DCs are dysfunctional. This has prompted research on the use of *ex vivo*-generated DCs as carriers of cancer vaccines (113). First generation DC vaccines utilised immature or partially mature DCs that were loaded with tumour antigen for presentation to T cells. It was found that these DCs were suboptimal at homing to the lymph node and activating T cells. Second generation DC vaccines utilised mature antigen loaded DCs, which were matured in the presence of an activating cytokine cocktail (114). Compared to the first generation DC vaccines, the second generation DC vaccines were superior at homing to the lymph node, however, they were found to exhibit a reduced ability to produce interleukin 12 (IL-12)p70, which activates the Th1 pro-inflammatory response in T cells and NK cells (115-117).

While many of these strategies elicit anti-tumour immunity, a limitation of these therapies is the recruitment of activated, effector T cells to the tumour where their effector functions would be fulfilled. Activated, effector T cells which do reach the tumour are faced with the immunosuppressive tumour microenvironment leading to the blunting of the anti-tumour immune response (118, 119).

1.3.2.4 Local Adjuvants

Other studies have focused on providing an inflammatory stimulus directly to the local tumour microenvironment in the hopes of recruiting immune cells or allowing them to fulfill their effector function in an inflammatory environment. Intratumoural injections of lipopolysaccharide (LPS), which ligates TLR4, into subcutaneous glioma tumours caused regressions of both injected and uninjected tumours. In addition to observing regression, histology of the tumours showed lymphocytic infiltration. Together, these findings suggested that the local inflammatory stimulus generated immune-mediated anti-tumour activity (120, 121). Another example of successful anti-tumour immune response generation by intratumoural injection to create local inflammation is a semliki forest virus vector encoding IL-12. This local therapy caused complete CT26 colon carcinoma tumour regression and the inhibition of metastasis in the spontaneously metastasizing 4T1 mammary carcinoma (122). In addition to initiating local inflammation, the importance of recruiting and activating DCs has also been shown. Intratumoural treatment with CpG DNA, a TLR ligand, was successful in the regression of B16 melanoma tumours, but recruitment of DCs with the chemokine CCL20 was also required (73). Taken together these studies show the importance of local inflammation and dendritic cell activation. A limitation to these approaches is the accessibility of the tumour to intratumoural injection.

Some immunotherapies are often successful at generating antigen specific T cells while others are often successful in causing inflammation for the recruitment of immune cells to the site of the tumour. With the recent exception of sipuleucel-T (123), which is an active cellular immunotherapy, most immunotherapies have seen little success in the clinic (124). Sipuleucel-T is a cancer vaccine that comprises of peripheral-blood monomuclear cells, including pAPCs. These cells have been activated *ex vivo* with a recombinant fusion protein, which consists of prostate antigen, prostatic

acid phosphatase, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Even with the promising results of this new immunotherapy, innovative strategies and novel immunotherapies need to be actively sought out.

1.4 Oncolytic Viruses

1.4.1 Overview

Oncolytic viruses (OVs) are replicating therapeutics that are selected or engineered to grow in malignant cell types and capable of killing the infected target cell. OVs may be tumour selective in wild-type or attenuated forms. They may also be engineered to provide tumour selectivity. Tumour selectivity of OVs is based on tumour antigen expression, tumour-specific mutations in anti-viral programs, or the targeting of signalling pathways upregulated in malignant cells. The cellular translational and transcriptional machinery is taken over by the virus and ultimately leads to the induction of cell necrosis or apoptosis (125). Cancer-cell killing can also occur through the induction of tumour-specific immunity and antivascular effects (126).

OV therapy offers several advantages over conventional cancer treatment. OVs have the ability to self-replicate and amplify in the tumour, which is unique from the pharmacokinetic properties of conventional therapeutics. Viruses can also be designed to express various transgenes, such as suicide genes or immune-stimulatory genes.

1.4.2 Vesicular Stomatitis Virus

Vesicular Stomatitis Virus (VSV) is a small negative-stranded RNA virus that is a member of the rhabdoviridae family. VSV replicates entirely in the cytoplasm of infected cells. The virus does not undergo genetic recombination, has no known transforming potential, and does not integrate any part of its genome into the host (127). VSV comprises of only five proteins; the nucleocapsid

(N), polymerase proteins, (L) and (P), surface glycoprotein (G) and the matrix protein (M) (127). VSV-N in combination with VSV-L, VSV-P and specific host proteins is responsible for viral transcription and replication. Viral binding to target cells occurs through VSV-G. The matrix protein has numerous functions, including regulation of transcription (128, 129), virus assembly, virus budding (130, 131), cellular apoptosis (127, 132), and avoidance of cellular anti-viral programs.

VSV enters the host cell by VSV-G binding to phosphatidylserine, which is found on nearly all cell-surface membranes (133). The virus enters the cell via endocytosis and subsequently the endosomes are acidified. VSV-G also fuses the viral and cellular membranes upon the drop in endosomal pH, enabling the viral genome and replicase to enter to the cytoplasm (127). The nucleoprotein in combination with the phosphoprotein (P) and large polymerase protein (L) transcribe the viral genes. The viral proteins and genomic RNA are assembled into complete virus particles. Following the budding of VSV through the plasma membrane, the host cell undergoes cell death (134).

The matrix protein helps VSV to avoid cellular antiviral programs in two ways: interruption of cellular transcription (135) and by blocking mRNA transport from the nucleus (136). Both of these actions serve to permit free virus replication by blocking the expression of antiviral gene products.

VSV is well known for its sensitivity to treatment with interferon (IFN) (137). IFNs are cytokines that, when bound to their IFN receptor, confer an antiviral state on host cells. This IFN-induced antiviral response can be mediated through a variety of proteins. The critical interferon stimulated gene (ISG) that inhibits VSV infection is the double-stranded RNA-dependent protein kinase PKR (138). Following interaction with dsRNA species, PKR is able to phosphorylate its substrate, the eukaryotic initiation factor 2 (eIF2α). Upon phosphorylation of eIF2α, viral protein

translation is inhibited (133). By inhibiting viral protein translation, PKR allows the host time to mount an innate immune response (IFNs) and an adaptive immune response (neutralizing antibodies (139).

VSV-WT is able to prevent translation of IFN transcripts by the ability of the matrix protein to block mRNA transport from the nucleus. The key limitation to VSV-WT as a cancer therapeutic was the possibility of uncontrolled virus growth in normal tissues. Three VSV variants, AV1 (M51R), AV2 (V221F and S226R), and AV3 (MΔ51) were created with changes in the M protein. These variants were not able to block mRNA transport from the nucleus, enabling the IFN response to proceed (136). The majority of tumours are non-responsive to IFN treatment or develop resistance (140). 80% of the NCI60 panel were not able to produce or respond to IFN allowing the variants to spread and replicate in these malignant cells. Due to the variants' inability to shutdown the IFN response, normal cells with a functioning anti-viral program were able to prevent replication.

1.4.3 Vaccinia Virus

Vaccinia Virus (VV) is a large double-stranded DNA virus that is a member of the poxvirus family. Vaccinia is comprised of approximately 200 genes and 200 kilobase pairs of DNA (141). There are several features that make VV an attractive candidate as an oncolytic. Like VSV, VV does not integrate any part of its genome into the host as the virus replicates in mini-nuclear structures or virus factories in the cytoplasm (142). Due to its size, VVs are able to accommodate multiple large transgenes to add therapeutic benefit (143). Vaccinia virus was used in the important medical feat – the eradication of smallpox (144). The extensive vaccination campaign has provided us with thorough safety information for humans (145, 146). Additionally, in the event of a rare adverse event, there are a number of antiviral agents, such as vaccinia immune globulin, cidofovir, and ST-246, available to treat poxvirus infections (147-149).

VV forms three infectious particles: intracellular mature virus (IMV), extracellular enveloped virus (EEV), and cell-associated enveloped virus (CEV) (150). IMV is surrounded by a single membrane and waits within the host cell until cell lysis. EEV is an IMV surrounded by a second membrane containing several viral antigens and this virion exits the cell prior to cell death. CEV, like EEV, is surrounded by a second membrane, but only differs from EEV as it is retained on the cell surface and it is pushed into the next cell. IMV and EEV are structurally, antigenically, and functionally different as they vary in the viral proteins found within either membrane. EEV and CEV virions are vital for virus dissemination. EEV virions have been shown to mediate longer-range dissemination *in vitro* and have been speculated to do so *in vivo* as well (151). CEVs induce the formation of actin tails from the cell surface that are responsible for propelling virions towards uninfected cells (151).

VV enters the host cell by membrane fusion pathways (152, 153) allowing the virus to enter a wide variety of cells, since it does not require a defined cell surface receptor. After successful entry of the naked viral core into the cell, the core interacts with microtubules to be shuttled deeper into the cytoplasm (154). Cores are partially uncoated in the perinuclear region of the cell. At this location, virus-associated DNA-dependent RNA polymerase transcribes the virus genome into early mRNAs. Early genes encode proteins which function to replicate virus DNA and create favourable conditions for the virus. Intermediate genes encode proteins which function to initiate the transcription of late genes. Finally, late genes encode proteins which function to produce new virus particles (155). Viral progeny are formed in mini-nuclear structures in the cytoplasm called viral factories. At this location, immature virions are assembled and processed to form IMV. The majority of virions are IMV and are released by the cell upon cell lysis. The remaining virions are transported by microtubules (156, 157) to a nearby site where the virions become enveloped by a

double cellular membrane (150). These enveloped virions are then transported to the cell surface by microtubules where a CEV can be exposed or an EEV can be released.

VV strains have been shown to inherently target tumours (158, 159). This inherency towards cancer cells may be due to the hallmarks of cancer described previously creating optimal cellular conditions for poxvirus replication. There are several strains of vaccinia such as Wyeth, Lister, Copenhagen, and Western Reserve, which were used in vaccination during the smallpox eradication. JX594, our clinical candidate, is of the Wyeth strain. In order to enhance the specificity of JX594 to cancer cells, a thymidine kinase gene was deleted forcing the virus to be dependent on cellular thymidine kinase expression (160, 161). In normal proliferating cells, cellular thymidine kinase is transiently expressed during the S phase of the cell cycle. In the majority of cancer cells, cellular thymidine kinase is constituitively expressed at high levels irrespective of cell cycle phase (162). JX594 also expresses the transgene, granulocyte-monocyte colony-stimulating factor (GM-CSF). GM-CSF stimulates the production, proliferation, maturation, and activation of granulocytes, macrophages, and DCs, which can lead to the subsequent induction of tumour-specific CTLs. Intravenous JX594 has been shown to be well tolerated and highly efficacious against two immunocompetent liver tumours models in rabbits and rats (163).

1.4.4 Clinical Trials

Remissions of malignancies were reported after vaccinations or natural viral infections over the past century (126). Case reports on these remissions led to treatment of patients with non-engineered viruses or first-generation OVs. Attenuation of these viruses was achieved by serial passage *in vitro*. Some of these first-generation OVs include Reovirus, Mumps, Adenovirus, and Vaccinia Virus. No genetic modifications were made to the virus backbone and no transgenes were added to these viruses. Various routes of administration were tested, although intravenous and

intratumoural were the main administration routes. Anti-tumoural activity ranged from 3-80%. Adverse effects in these clinical trials ranged from none reported to mild effects such as flu-like symptoms, rashes, or fevers (126).

The second generation OVs were engineered viruses that were targeted and replication selective, but remained un-armed, without the addition of transgenes. Dl1520, which is an E1B-55K gene-deleted adenovirus, was the first engineered replication-selective virus to be used in humans. This virus produced by Onyx Pharmaceuticals was designed to be specific for cells with inactive p53. Adenoviruses with genetic modifications were designed with specificity for prostate cancer through prostate specific promoter/enhancer elements (164, 165). Finally, a herpes virus with deletions in the gamma-34.5 gene, whose products are responsible for inhibiting the IFN/dsRNA-dependent protein kinase R reponse mechanism, were examined. The second generation OVs were also well-tolerated and showed varied amounts of tumour responses.

Finally, the third generation oncolytic viruses are engineered viruses that are both targeted and armed. JX594 is tumour selective based on its thymidine kinase deletion and is armed with the transgene encoding GM-CSF in order to stimulate the immune system. Third generation adenoviruses are specific based on p53 pathways defects and late RNA transport defects. They also come armed with a CD/TK fusion protein that acts like a local chemotherapeutic. Anti-tumoural effects have been seen after intratumoural injection with all viruses clinically evaluated to date, while variable effects were seen after intravenous injection of some viruses. Importantly, all clinical trials have established the safety of OVs(126).

1.4.5 Anti-tumour Immunity generated by Oncolytic Virotherapy

Although most interest in OVs has focussed on their direct oncolytic properties, there is an increasing number of reports showing that anti-tumour immunity is induced following treatment

with OVs. It has been shown that intravenous reovirus therapy is able to generate anti-tumour immunity and reduce metastatic disease burden in an immunocompetent murine melanoma model through the phenotypic and functional activation of DCs and subsequent activation of CTLs (166). $CD8^+$ T cells have been shown to be essential for the efficacy of VSV injected IT. VSV virotherapy IT has also shown priming of a T cell response toward the dominant viral epitope and tumour epitope (167). Oncolytic herpes simplex virus has been shown to initiate anti-tumour immunity in several tumour models (168-171). Oncolytic adenovirus efficacy was dependent on the generation of anti-tumour immunity, however only after depletion of Tregs (172). From these studies and additional studies, it is clear that an association between oncolysis and subsequent anti-tumour immunity exists.

Previously we have found that 6 treatments of VSVΔ51 can completely regress a primary CT26lacZ, colon carcinoma, tumour in Balb/c mice (136). Subsequently, these mice can reject a secondary CT26lacZ tumour challenge (KP unpublished data). Mice that have been cured of their CT26lacZ tumours have been shown to transfer this protective immunity to naïve mice by an adoptive splenocyte transfer (KP unpublished data). Mice that were cured of their CT26lacZ primary tumours by VSVΔ51 treatment were unable to reject a syngeneic 4T1 mammary carcinoma tumour inoculation (KP unpublished data) suggesting that the resulting immune response is tumour specific. It has also been shown that 6 treatments of VSVΔ51 cannot completely regress a primary CT26lacZ tumour in CD1d nude mice, suggesting that an intact immune system is necessary for the primary tumour regression (CL unpublished data). Altogether, these results suggest that regression of a CT26lacZ primary tumour with VSVΔ51 leads to a specific anti-tumour immune response that is capable of rejecting a secondary CT26lacZ tumour.

1.4.6 Oncolytic Viruses as an Immunotherapy

The immune system typically poses as an obstacle to oncolytic virotherapy as the immune system works efficiently to clear the virus. However, with the mounting evidence of anti-tumour immunity generated through oncolytic virotherapy, the importance of harnessing this property of the immune system is at the forefront of OV research. OVs could be a multipronged therapeutic for targeting cancer as they specifically target tumour cells for destruction and could be ideal immunotherapeutics for cancer.

OVs would make for a more attractive immunotherapy than a cancer vaccine against a tumour antigen as there are so many types of cancer and subtypes within tissues. Identifying a tumour antigen for each type and expressing them in vaccine vectors is unlikely to be a feasible strategy in the clinic. Although cancer cells have various levels of susceptibility to OVs, viral infection and lysis will create cell-associated antigen for pAPCs to take up and present.

There are several criteria that have been proposed that are required for the successful immunologic destruction of established tumours. Firstly, the therapy must be able to generate sufficient numbers of immune cells with specificity for tumour antigens *in vivo*. Immune cells must be recruited to the tumour site to execute their effector function. Finally, effector immune cells must be activated at the tumour site in order to fulfill their effector functions without the immunosuppressive environment of the tumour counteracting direct lysis or cytokine secretion (173).

Although it is not exactly clear how OVs generate anti-tumour immunity, it is hypothesized that tumour cell lysis in an inflammatory setting and proper DC activation by TLR ligation prime effective T cell responses. Oncolytic virus-induced tumour cell death is expected to release tumour antigens into the tumour microenvironment. Cell associated tumour antigens would then be

available for uptake by DCs. Viral infection of tumours has been shown to enhance the phagocytosis of tumour-derived material (174, 175).

By altering the cytokine profile, OVs may alter the immunology of the tumour microenvironment. As previously discussed, the tumour microenvironment is extremely immunosuppressive. VSV infection of the tumour creates acute inflammation causing the secretion of pro-inflammatory cytokines and chemokines (KP unpublished data, (176)). It has been shown that reovirus infection of human melanoma cell lines reduced the secretion of the anti-inflammatory cytokine, IL-10, while inducing the production of pro-inflammatory cytokines, IL-6, TNF-α, RANTES, and type I interferons (177). This inflammation in the tumour microenvironment could promote immune cell trafficking and proper immune cell activation and function.

OVs could provide the necessary TLR ligation or danger signal needed to activate APCs and avoid T cell anergy upon APC/T cell interaction. As pathogens, viruses elicit TLR signalling through a variety of TLRs found on APCs (178-181). Viral particles and virally lysed tumour fragments could be presented to T cells with the required co-stimulation (72). It has been shown that virally infected cells are superior at delivering nonviral antigen for cross presentation and cross-priming adaptive immune response (182).

In addition to initiating the adaptive immune response by maturing DCs, OVs can influence the nature of the innate tumour response. In the cross-talk between DCs and NK cells, reciprocal activation can occur, where activated DCs aid in the activation of NK cells. Reovirus-infected DCs have been shown to enhance cytotoxicity of NK cells towards tumour cells (183).

In an immune competent host, it has been proposed that therapeutic efficacy of viral therapy will depend on 1) the amount of viral replication inside the tumour, which subsequently leads to tumour cell destruction. 2) The anti-viral immune mechanisms which function to eliminate

the virus - minimizing viral spread in the tumour and the host. 3) The anti-tumour immune response, which can act locally at the tumour to clear tumour cells and can also act at distant sites of tumour growth (167).

Rationale

Surgery is an effective treatment modality for most solid primary tumours. Despite complete resection, the development of metastatic disease limits its curative potential and provides the rationale for neoadjuvant (preoperative) therapies. Oncolytic viruses may be an ideal candidate for generating a potent anti-tumour immune response due to effective recruitment of immune cells into the tumour microenvironment, proper activation of immune cells, and generation of tumour antigen-specific T cells. Preoperative oncolytic virus therapy may serve as a neoadjuvant immunotherapy capable of preventing the development of metastatic disease by generating an effective immune response. Disseminated cells may be good candidates for immune cell killing as they do not have the complete immunosuppressive tumour microenvironment in place yet.

The use of viruses expressing the transgene, GM-CSF, was selected in order to aid in stimulating the immune response. A study using a murine hepatoma cell line, Hepa 1-6, in a surgical model of cancer found that preoperative treatment with oncolytic herpes simplex virus (HSV) expressing GM-CSF was superior in protection in comparison to HSV without GM-CSF(184). A VSVΔ51GM-CSF-infected autologous cell vaccine given prophylactically to mice has been shown to increase protection from subsequent tumour challenge in mice in comparison to a VSVΔ51-infected autologous cell vaccine (CL unpublished data). For these reasons, viruses expressing GM-CSF were used in order to aid in stimulating the immune response in our OV/surgery models.

Hypothesis

The treatment of tumours with oncolytic viruses prior to surgical resection will generate an *in situ* anti-tumour immune response which may protect immune competent hosts from recurrence or metastasis.

Objectives

1. To establish a mouse model of surgically resectable cancer and subsequent development of metastatic disease

2. To determine if an anti-tumour response can be generated by preoperative viral therapy in this surgical model

Chapter 2: Materials and Methods

2.1 **Viruses**

VSVΔ51GM-CSF and VSVΔ51 (Indiana serotype) were propagated in Vero cells (American Type Culture Collection). Confluent vero cells were infected at a multiplicity of infection (MOI) of 0.01 for 24h. The supernatant was collected and the virus was pelleted. The virus was purified on a sucrose gradient using an ultracentrifuge, and finally resuspended in phosphate-buffered saline (PBS). The virus was titered on confluent vero cells by making serial dilutions in serum free media. After incubation at 37°C with 5% CO_2 for 1 hour, the virus was overlayed with a 1:1 ratio of 1% agarose to 2x Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) (Cansera). After 24 hours of incubation, the overlay was removed and the cells were stained with Coomasie Brilliant Blue in order to visualize the plaques. Plaques were counted by visual inspection.

JX594GM-CSF (Wyeth strain) was propagated in Hela cells (American Type Culture Collection). Confluent hela cells were infected at a MOI of 0.01 for 72h. The supernatant and cells were collected and the virus was pelleted. The virus was purified on a sucrose cushion using an ultracentrifuge, and finally resuspended in PBS. The virus was titered on U2OS cells (American Type Culture Collection) by making serial dilutions in serum free media. After incubation at 37°C with 5% CO_2 for 2 hours, the virus was overlayed with a 1:1 ratio of 3% CMC to 2x DMEM supplemented with 20% FBS. After 48 hours of incubation, the overlay was removed and the cells were stained with Coomasie Billiant Blue in order to visualize the plaques. Plaques were counted by visual inspection.

2.2 Cell lines

CT26WT (murine colon adenocarcinoma), CT26lacZ (murine colon adenocarcinoma), expressing the reporter gene lacZ, and B16-F10 (murine melanoma) were purchased from American Type Culture Collection, cultured in HyQ DMEM (High glucose) (Hyclone) supplemented with 10% FBS, and grown at 37°C with 5% CO_2.

2.3 *In vitro* Infections

Cells were plated in 6-well plates 24 hours prior to infection. Cells were infected with VSVΔ51 expressing green fluorescent protein (GFP) or JX594hGM-CSFGFP at various MOIs and incubated for 12, 24, or 48 hours at 37°C with 5% CO_2.

2.4 Fluorescence Microscopy

Images of cells in both phase contrast and fluorescence were taken using a fluorescent microscope (Zeiss Axiovert S 100). Images were taken at 12 hours, 24 hours, 48 hours, and 72 hours.

2.5 XGal Substrate Staining of Lung Tissues

Lungs were harvested from mice and rinsed in sodium phosphate buffer. The lungs were subsequently fixed for 20 minutes with fixative solution, and stained overnight with XGal substrate (BioShop) at 37°C. Lungs were incubated with wash buffer at 4°C for 24 hours. Finally, the lungs were placed in 10% buffered formalin for preservation.

2.6 Animals

Female 6-8 week old Balb/c, C57/Bl6, and CD1d athymic nude mice were obtained from Charles River Laboratories (Wilmington, MA). Mice were housed in groups of 5-6 mice in a level 2 biocontainment room at the Animal Care facility at the University of Ottawa.

2.7 *Surgical Implant of Primary Tumours*

Syngeneic CT26lacZ primary tumours were established by injection of $1x10^6$ CT26lacZ cells in 100µl of PBS SQ bilaterally. After 2 weeks of tumour growth, donor mice were euthanized by CO_2 overdose. Tumours were extracted and stored in PBS. CT26lacZ tumours were cut into 1x1x1mm pieces using a surgical scalpel. Recipient mice received buprenorphine pre- and post-surgery. Recipient mice were placed under isoflurane anaesthetic and their right hind flank was prepared by disinfection with 70% ethanol. A 1-2cm incision was made in their skin in the right hind flank, and adhesions were broken by blunt dissection. The 1x1x1mm CT26lacZ tumour was introduced into the recipient mouse with a spinal catheter introducer (BD Biosciences). The incision was closed with surgical wound clips (BD Biosciences) and the mice received 1ml of PBS subcutaneously.

2.8 Surgical Resection of Primary Tumours

Mice received buprenorphine pre and post-surgery. Mice were placed under isoflurane anaesthetic and their right hind flank was prepared by disinfection with 70% ethanol. An incision was made in the skin at the base of the visible and palpable tumour followed by blunt dissection of the adhesions attaching the tumour to the muscular wall of the mouse. Tumours were completely resected with the skin surrounding the tumour to ensure that no tumour cells were left behind. The incision was closed with surgical wound clips (BD) or polysorb taper needle sutures (Syneture) and the mice received 1ml of PBS subcutaneously.

2.9 CT26WT Tumour model

Syngeneic CT26WT primary tumours were established by injection of $3x10^5$ CT26WT cells in 100µl of PBS SQ in the right hind flank of Balb/c mice. When tumours reached a palpable size, mice were treated with 100µl PBS or $5x10^8$pfu VSVΔ51GM-CSF intratumourally or intravenously by tail vein injection. Right hind flank tumours were removed by surgical resection as described above. A challenge of $1x10^6$ CT26WT cells in 100µl of PBS was injected SQ in the left hind flank. Tumours were measured using digital callipers and mice were euthanized using CO_2 overdose when tumour volume surpassed 750mm^3.

2.10 CT26lacZ Tumour model

Syngeneic CT26lacZ primary tumours were established by injection of $3x10^5$ CT26lacZ cells in in 100µl of PBS SQ in the right hind flank or by surgical implant of a 1x1x1mm CT26lacZ tumour as described above. When tumours reached a palpable size, mice were treated with 100µl PBS or $5x10^8$pfu VSVΔ51GM-CSF intravenously by tail vein injection. Right hind flank tumours were removed by surgical resection as described above in Balb/c mice. A challenge of $1x10^6$ CT26lacZ cells in 100µl PBS was injected IV by tail vein or SQ in the left hind flank. Mice given an IV challenge were euthanized using euthanyl injection after 9 days. Mice that received a SQ challenge had their tumours measured using digital callipers and mice were euthanized using CO_2 overdose when tumour volume surpassed 750mm^3.

2.11 B16-F10 Tumour model

Syngeneic B16-F10 primary tumours were established by injection of $3x10^5$ B16-F10 cells in 100µl of PBS subcutaneously in the right hind flank of C57/Bl6 mice. When tumours reached a palpable size, mice were treated with 100µl PBS or $5x10^8$ VSVΔ51 or VSVΔ51GM-CSF intravenously

or $1x10^7$ JX594mGM-CSF intratumourally or intravenously. Right hind flank tumours were removed by surgical resection as described above. A challenge of $3x10^5$ B16-F10 cells in 100µl of PBS was injected intravenously. Mice were euthanized 2 weeks later using euthanyl injection. Lungs were extracted and fixed using 10% buffered formalin for preservation. Surface lung metastases were counted using a dissecting microscope.

2.12 IFN-γ production of $CD3^+CD8^+$ cells to measure antigen-specific T cell responses

Balb/c mice were euthanized and their spleens extracted. Spleens were homogenized through a 100µm strainer (Fisher Scientific). Splenocytes were isolated and cleared of red blood cells and dying cells using a lympholyte (Cedarlane) gradient. Splenocytes were plated in round bottom 96-well plates (Costar) at 1 million cells/well in RPMI supplemented with 10% FBS. The $CD8^+$ T cells immunodominant epitope for VSV N protein (Biosynthesis), herein referred to as VSV-N2 peptide, was incubated at a concentration of 1mg/ml with anti-CD28. The peptide mix was incubated with the isolated splenocytes for 1.5 hours prior to the addition of GolgiPlug (BD Biosciences). The mixture was incubated together for an additional 3.5 hours at which point, the cells were resuspended in PBS supplemented with 5% FBS overnight.

Intracellular cytokine staining was executed using a Cytofix/Cytoperm plus Fixation/Permeabilization kit (BD Biosciences). Splenocytes were stained with FITC-conjugated rat anti-mouse IFN-γ (1:200 dilution), PE-conjugated rat anti-mouse CD3 (1:50 dilution), PerCP-Cy-conjugated Rat anti-mouse CD8 (1:50 dilution) (BD Pharmingen). Forward and side scatter in addition to the three colours of fluorescence were measured for each sample on an Epics XL flow cytometer (Beckman Coulter). 20,000 events were acquired for each sample and the data was analyzed using Quanta Analysis Software(Beckman Coulter).

2.13 Statistical Analyses

Statistical Analyses were performed using Graphpad Prism 3.0 software. For Kaplan Meier Survival Curves, statistics were done using a logrank test. All other statistics were done using an unpaired, non-parametric, two-tailed t test. Error bars represent SD. In the B16-F10 surgery model, one mouse from the PBS treated group was excluded from the statistical analysis as it did not develop lung metastases.

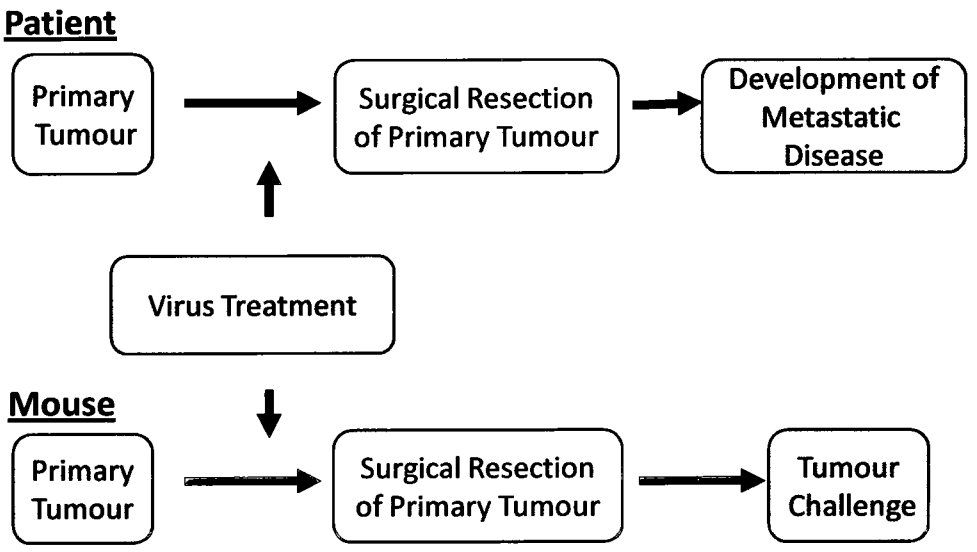
3.1 CT26WT Surgical model

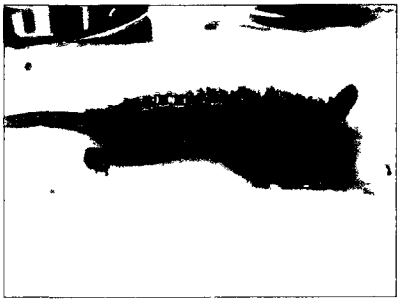
3.1.1 A CT26WT primary tumour does not protect against a subsequent CT26WT subcutaneous tumour inoculation

In establishing a mouse surgical model we examined the CT26 colon adenocarcinoma cell line that is commonly used in our lab. Two CT26 tumour models are routinely used and differ by the presence of a transgene – CT26WT and CT26lacZ. Although our previous data, indicating VSVΔ51 could induce an anti-tumour immune response was demonstrated with the CT26lacZ cell line in Balb/c mice, we first considered the parental CT26WT cell line in an attempt to get around the use of a transgene. Previous data from our lab indicates that CT26lacZ cells are more immunogenic than the parental CT26WT cells, possibly due to the lacZ transgene (CL unpublished data). Irradiated CT26lacZ cells given prophylactically to mice protect all mice against a subsequent live CT26lacZ tumour inoculation. Irradiated CT26WT cells given prophylactically protect approximately only 50% of mice from a subsequent live CT26WT tumour inoculation.

In Figure 1, we delineate this murine surgical cancer model. In order to assess whether the CT26WT colon carcinoma cell line, syngeneic in Balb/c mice, could be utilised in a surgical model of cancer, we examined whether a CT26WT primary tumour protects the mice from subsequent CT26WT tumours inoculated subcutaneously. Primary CT26WT tumours were established in a group of Balb/c mice subcutaneously in the right hind flank. On day 13, these mice underwent surgery to remove the primary tumour. On day 14, all mice, including a naïve control group of mice, were inoculated with a CT26WT tumour on the left hind flank. This experimental design is shown in Figure 2a. It was determined that some of the experimental mice had an extension in survival,

Figure 1. **Experimental Mouse Model of Surgically Resectable Cancer and Subsequent Development of Metastatic Disease**. Mice establish a primary tumour at a resectable location. Following neoadjuvant oncolytic virus treatment, the tumour is completely surgically resected. Subsequently the mice receive a live tumour challenge.


Patient
Primary Tumour
Surgical Resection of Primary Tumour
Development of Metastatic Disease
Virus Treatment
Mouse
Primary Tumour
Surgical Resection of Primary Tumour
Tumour Challenge



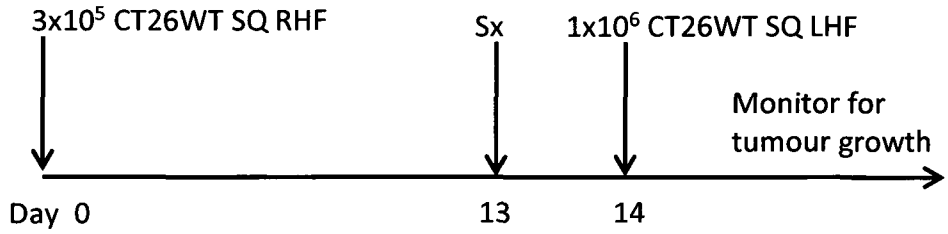
which did not reach statistical significance (p=0.1374), in comparison to the naïve mice, which only received a CT26WT challenge tumour on day 14. This data suggests that a CT26WT primary tumour does not protect against a subsequent CT26WT tumour inoculation injected subcutaneously, prompting us to utilise the CT26WT cells in a surgical model of cancer.

3.1.2 Preoperative treatment of CT26WT primary tumours with VSVΔ51GM-CSF does not protect against a subsequent CT26WT flank tumour

In order to determine if VSVΔ51GM-CSF treatment could generate an anti-tumour immune response capable of preventing the growth of a secondary tumour, we preoperatively treated primary tumours with VSVΔ51GM-CSF in the CT26WT surgical model. Primary CT26WT tumours were established in Balb/c mice subcutaneously in the right hind flank (Figure 3a). On day 11, the CT26WT tumours were injected with VSVΔ51GM-CSF or PBS intratumourally. Mice were injected with VSVΔ51GM-CSF or PBS intravenously on day 16. On day 18, mice underwent surgery to resect the primary tumour. Primary tumour volumes were measured and the average tumour volume is graphed in Figure 3b. The primary tumour volume did not differ in mice that received treatment with VSVΔ51GM-CSF or PBS. All mice, including a naïve control group of mice, received a subsequent CT26WT tumour inoculation in the left hind flank. It was determined that there was no extension in survival in mice that received VSVΔ51GM-CSF treatment of the primary tumour from the mice that received preoperative treatment with PBS (Figure 3c). This result suggests that VSVΔ51GM-CSF treatment did not significantly impact the progression of the primary CT26WT tumour cells. This result also suggests that VSVΔ51GM-CSF treatment of the primary CT26WT tumour was unable to generate an anti-tumour immune response capable of preventing the growth of a secondary CT26WT tumour.

Figure 2. **A *CT26WT primary tumour does not protect against a subsequent CT26WT subcutaneous* tumour challenge**. Balb/c mice were injected with $3x10^5$ CT26WT cells SQ in the right hind flank on day 0. On day 13, mice received surgery to remove their tumour. On day 14, control mice and experimental mice were injected with $1x10^6$ CT26WT cells SQ in their left hind flank. Tumour growth was monitored and mice were euthanized when tumour size exceeded $750mm^3$. (A) Experimental setup (B) The percent survival is plotted in a Kaplan Meier Survival Plot.

A


3x10^5 CT26WT SQ RHF
Sx
1x10^6 CT26WT SQ LHF
Monitor for tumour growth
Day 0
13
14

B

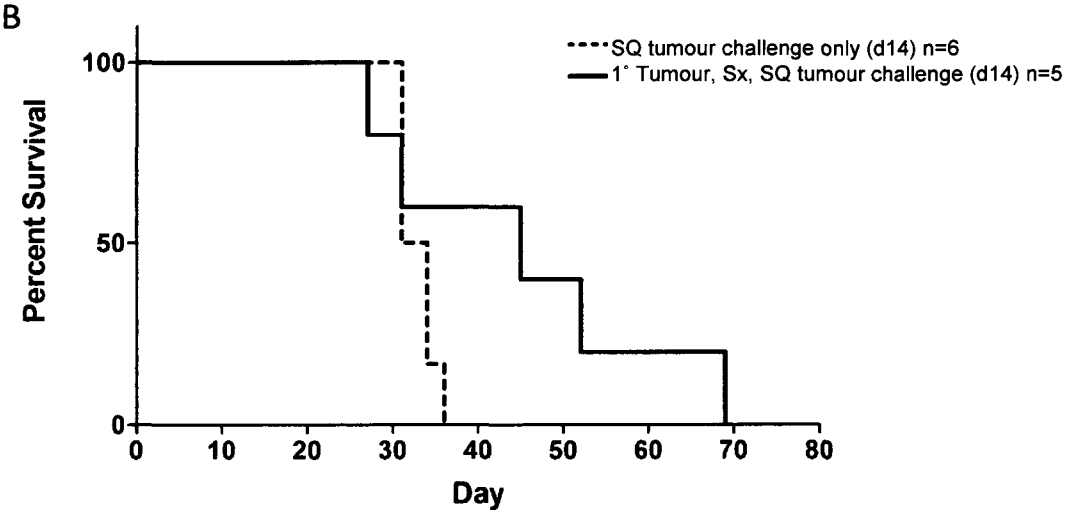
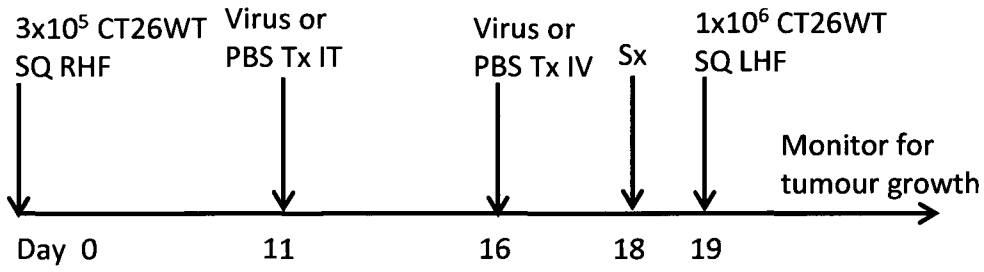
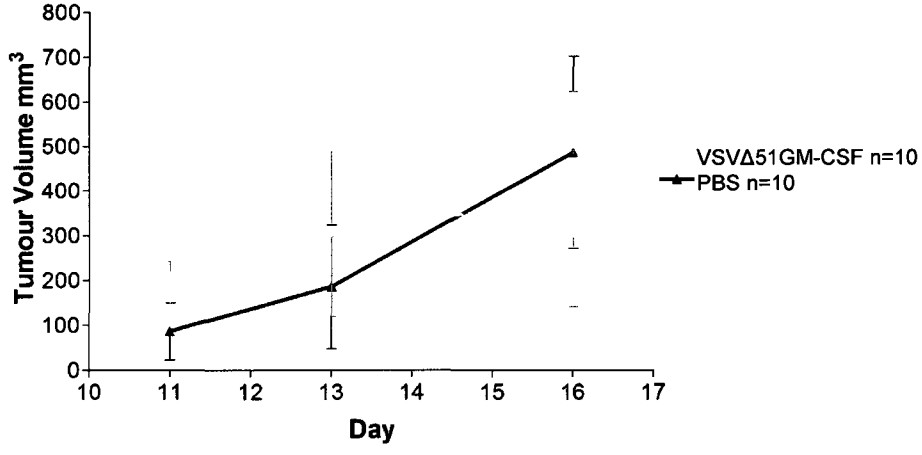

SQ tumour challenge only (d14) n=6
1° Tumour, Sx, SQ tumour challenge (d14) n=5
Percent Survival
100
50
0
0
10
20
30
40
50
60
70
80
Day

Figure 3. **VSVΔ51GM-CSF treatment prior to surgical resection of a CT26WT tumour does not protect against a subsequent CT26WT tumour challenge**. On day 0, Balb/c mice were injected with $3x10^5$ CT26WT SQ in the right hind flank. Mice were injected with 100µl PBS IV or $5x10^8$pfu VSVΔ51GM-CSF IT on day 11. Mice were injected with 100µl PBS IV or $5x10^8$pfu VSVΔ51GM-CSF IV on day 16. Primary tumour volume was measured using digital callipers. On day 18, mice received surgery to remove their tumour. On day 19, all mice were injected with $1x10^6$ CT26WT cells SQ in their left hind flank. Tumour growth was monitored and mice were euthanized when tumour size exceeded 750mm^3. (A) Experimental setup (B) Average primary tumour volume is plotted using tumour volume $x^2y/2$; where x and y are the length and width of the tumour and y>x. (C) The percent survival is plotted in a Kaplan Meier Survival Plot.

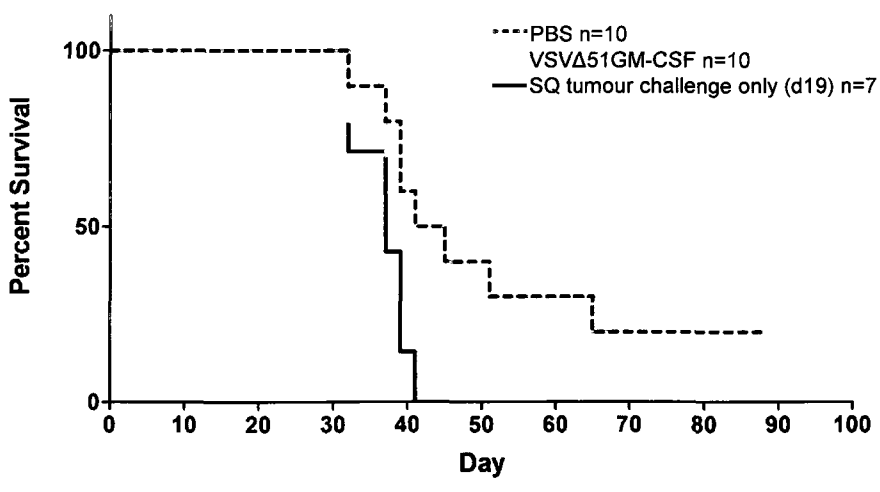
A


3x10^5 CT26WT
SQ RHF
Virus or
PBS Tx IT
Virus or
PBS Tx IV
Sx
1x10^6 CT26WT
SQ LHF
Monitor for
tumour growth
Day 0
11
16
18
19

B


Tumour Volume mm3
800
700
600
500
400
300
200
100
0
10
11
12
13
14
15
16
17
Day
VSVΔ51GM-CSF n=10
PBS n=10

C


Percent Survival
100
50
0
0
10
20
30
40
50
60
70
80
90
100
Day
PBS n=10
VSVΔ51GM-CSF n=10
SQ tumour challenge only (d19) n=7

3.1.3 CT26WT tumour cells are resistant to VSVΔ51gfp infection compared to CT26lacZ tumour cells.

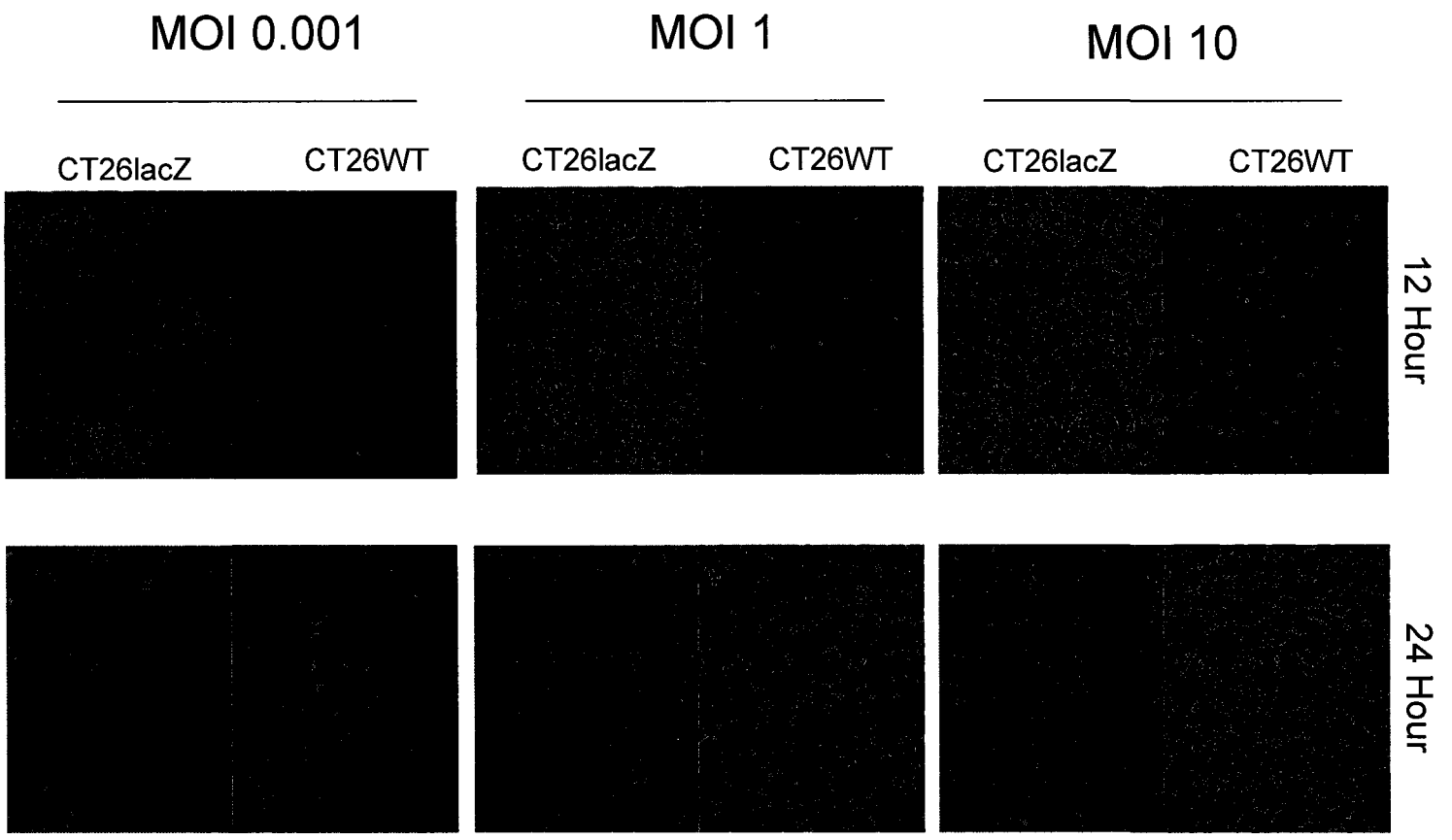
It has been observed that subclones of parental cell lines can vary in susceptibility to infection by viruses (185). The CT26lacZ cells are a subclone of the parental CT26WT cell line. Previous data in our lab has shown that CT26lacZ are very susceptible to VSVΔ51 infection. In addition, it has been shown that VSVΔ51 treatment of CT26lacZ tumours results in a marked reduction in tumour volume (136). Given the lack of *in vivo* response of the CT26WT tumour to VSVΔ51GM-CSF, we examined the susceptibility of CT26WT cells to VSVΔ51 infection. CT26lacZ and CT26WT cells were plated and infected with VSVΔ51gfp at an MOI of 0.001, 1, or 10. Fluorescence microscopy images were taken after 12 and 24 hours of incubation (Figure 4). GFP expression is indicative of viral replication and the relative amount of fluorescence was qualitatively examined. It was determined that CT26WT tumour cells were less susceptible to VSVΔ51gfp infection in comparison to CT26lacZ cells. This observation shows that the heterogeneous parental cell line CT26WT differs in its susceptibility to infection by VSVΔ51 in comparison to the CT26lacZ subclone.

3.2 CT26lacZ Surgical model

3.2.1 A CT26lacZ primary tumour protects against a subsequent CT26lacZ tumour inoculation given intravenously

It has been suggested that tumour infection and cell lysis by oncolytic viruses could be beneficial to the generation of an adaptive immune response due to the phagocytosis, processing, and presentation of virally lysed antigen by pAPCs to activate T cells(70). We wanted to assess whether the CT26lacZ cell line, which is more susceptible to VSV-mediated oncolysis, could be utilised in a surgical model of cancer with the secondary tumour inoculation given intravenously.

Figure 4. **CT26WT are resistant to VSVΔ51 infection in comparison to CT26lacZ**. CT26WT and CT26lacZ cells were plated in 6-well plates. Cells were infected with VSVΔ51GFP at an MOI of 10, 1, or 0.001. After 12 hours and 24 hours of incubation, fluorescence pictures were taken of each well.

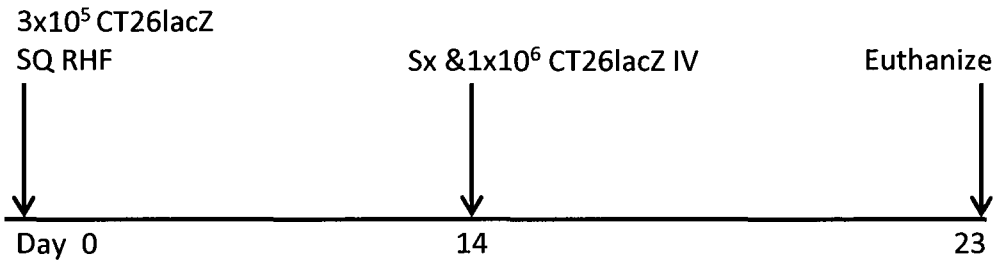

MOI 0.001
MOI 1
MOI 10
CT26lacZ
CT26WT
CT26lacZ
CT26WT
CT26lacZ
CT26WT
12 Hour
24 Hour

To this end we assessed whether a CT26lacZ primary tumour protects the mice from subsequent CT26lacZ tumours injected intravenously. Primary CT26lacZ tumours were established in a group of Balb/c mice subcutaneously in the right hind flank. On day 14, these mice underwent surgery to remove the primary tumour and subsequently all mice, including a naïve control group of mice, were injected with CT26lacZ tumour cells intravenously. All mice were euthanized on day 23, at which point the lungs were removed and stained with Xgal substrate. This experimental design is shown in Figure 5a. We observed that mice, which had received the primary CT26lacZ tumour inoculation, were protected against the development of surface lung metastases (Figure 5b). Control mice, which only received the intravenous inoculation of CT26lacZ tumours, all developed lung metastases. This data suggests that a CT26lacZ primary tumour protects against the subsequent development of CT26lacZ surface lung metastases, preventing us from utilising the CT26lacZ cells, at least intravenously, as the route of inoculation for the secondary tumour.

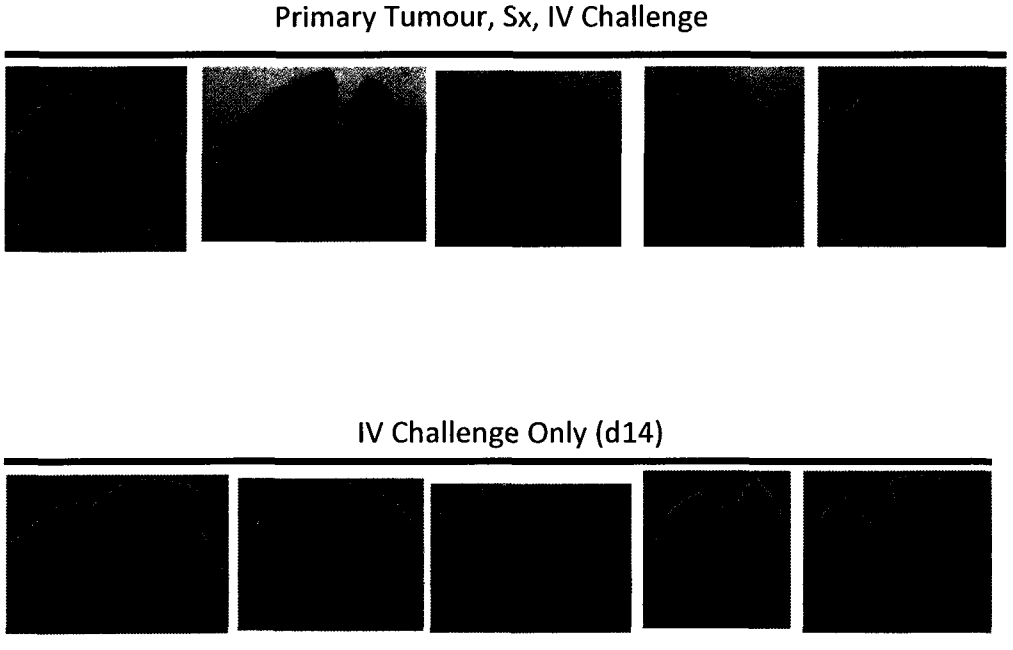
3.2.2 A CT26lacZ primary tumour implant does not protect against a subsequent CT26lacZ subcutaneous tumour inoculation

A sarcoma model showed that tumour cells given as a single-cell suspension were capable of inducing a protective cytotoxic T cell response due to recognition by the immune system. In this study, the transplantation of a small tumour implant did not induce a protective CTL response (186). In order to assess whether the CT26lacZ cell line could be utilised in a surgical model of cancer, where the primary tumour is transplanted as a tumour implant and the secondary CT26lacZ tumour is injected subcutaneously, we assessed whether a CT26lacZ primary tumour implant protects the mice from subsequent CT26lacZ tumours injected subcutaneously. The experimental design is shown in Figure 6a. CT26lacZ tumours were established in donor Balb/c mice on day -14.

A


3x10^5 CT26lacZ
SQ RHF
Sx &1x10^6 CT26lacZ IV
Euthanize
Day 0
14
23

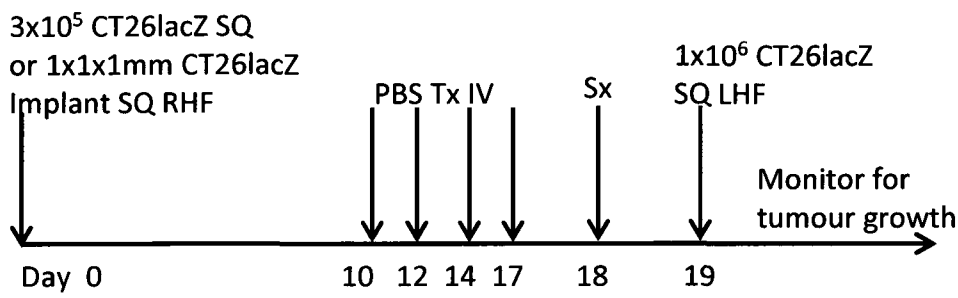
B


Primary Tumour, Sx, IV Challenge
IV Challenge Only (d14)

On day 0, the donor mice were euthanized, and recipient Balb/c mice received a 1x1x1mm CT26lacZ tumour implanted or CT26lacZ cells in a single-cell suspension injected in the right hind flank subcutaneously. Mice were treated with PBS intravenously on day 10, 12, 14, and 17. On day 18, the primary tumour was surgically resected from the right hind flank. On day 19, all mice, including a naïve control group of mice, received a CT26lacZ tumour inoculated subcutaneously on the left hind flank. The percent of secondary tumour outgrowth is graphed in Figure 6b. 60% of mice that received a single-cell suspension of CT26lacZ cells as the primary tumour inoculation were able to develop a secondary CT26lacZ tumour. Mice that received a CT26lacZ tumour implant as the primary tumour were able to develop a secondary CT26lacZ tumour at a success rate of 100%. Finally, all mice, which received the subcutaneous tumour inoculation only on day 19, grew the CT26lacZ tumour. This data suggests that secondary CT26lacZ tumours inoculated subcutaneously can grow consistently after a CT26lacZ primary tumour implant is transplanted. The growth of the CT26lacZ primary tumour was compared between mice that received a CT26lacZ primary tumour by an inoculation with a single-cell suspension of cells or by transplantation of a tumour implant. The experimental design is shown in Figure 7a. It was found that mice that were treated with PBS and received a CT26lacZ single-cell suspension injection as the primary tumour inoculation had a similar average primary tumour volume as mice that were treated with PBS and received the CT26lacZ implant primary tumour (Figure 7b). Mice that were treated with VSVΔ51GM-CSF and received a single-cell suspension injection as the primary tumour inoculation had a similar average primary tumour volume as mice that received a CT26lacZ implant primary tumour and were treated with VSVΔ51GM-CSF. Regardless of the method of CT26lacZ primary tumour establishment, there was a trend towards a smaller tumour volume in the VSVΔ51GM-CSF treated mice in comparison to the mice treated with PBS.

Figure 6. **A CT26LacZ Challenge Tumour SQ grows consistently after a CT26LacZ primary tumour implant SQ**. Balb/c mice were injected with $3x10^5$ CT26lacZ cells SQ in the right hind flank or received a 1x1x1mm CT26lacZ tumour, which were grown in donor mice, surgically implanted SQ in the right hind flank on day 0. Mice were injected with 100µl PBS IV on day 10, 12, 14, and 17. On day 18, mice received surgery to remove their tumour. On day 19, all mice were injected with $1x10^6$ CT26lacZ cells SQ in their left hind flank. Tumour growth was monitored and mice were euthanized when tumour size exceeded $750mm^3$. (A) Experimental setup (B) Percent CT26lacZ Challenge Tumour Outgrowth is graphed for mice that received the challenge tumour on day 19.

A


3x10^5 CT26lacZ SQ
or 1x1x1mm CT26lacZ
Implant SQ RHF
PBS Tx IV
Sx
1x10^6 CT26lacZ
SQ LHF
Monitor for
tumour growth
Day 0
10
12
14
17
18
19

B

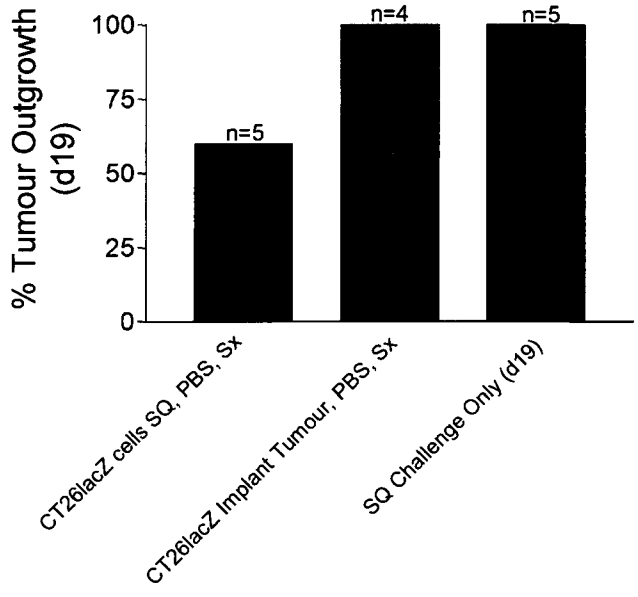
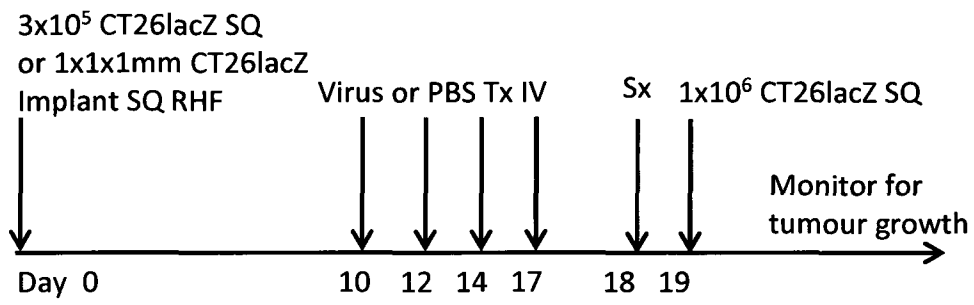
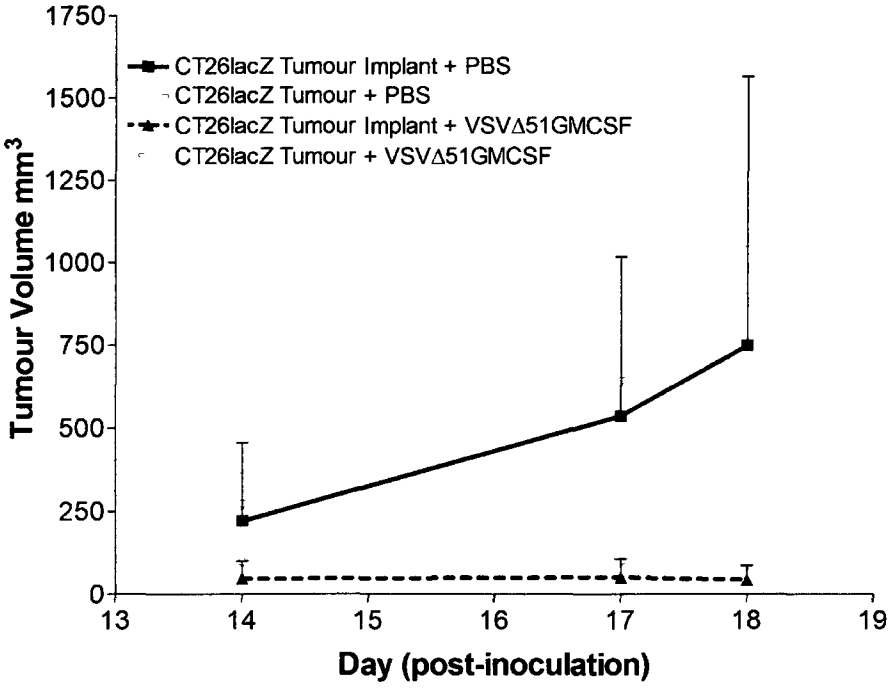

% Tumour Outgrowth
(d19)
100
75
50
25
0
n=5
n=4
n=5
CT26lacZ cells SQ, PBS, Sx
CT26lacZ Implant Tumour, PBS, Sx
SQ Challenge Only (d19)

Figure 7. **There is no difference in the rate of tumour growth between mice that received a single-cell suspension of CT26lacZ cells inoculated SQ and mice that received a CT26lacZ implant tumour SQ**. Balb/c mice were injected with $3x10^5$ CT26lacZ cells SQ in the right hind flank or received a 1x1x1mm CT26lacZ tumour, which were grown in donor mice, surgically implanted SQ in the right hind flank on day 0. Mice were injected with 100µl PBS IV or $5x10^8$pfu VSVΔ51GM-CSF IV on day 10, 12, 14, and 17. (A) Experimental setup (B) Average primary tumour volume is plotted using tumour volume $x^2y/2$; where x and y are the length and width of the tumour and y>x. Tumour volume was measured using digital callipers.

A


3x10^5 CT26lacZ SQ
or 1x1x1mm CT26lacZ
Implant SQ RHF
Virus or PBS Tx IV
Sx
1x10^6 CT26lacZ SQ
Monitor for
tumour growth
Day 0
10
12
14
17
18
19

B


1750
1500
1250
1000
750
500
250
0
Tumour Volume mm3
CT26lacZ Tumour Implant + PBS
CT26lacZ Tumour + PBS
CT26lacZ Tumour Implant + VSVΔ51GMCSF
CT26lacZ Tumour + VSVΔ51GMCSF
13
14
15
16
17
18
19
Day (post-inoculation)

This trend did not reach statistical significance as there were outliers in the mice that were treated with PBS. Altogether, this data suggests that the CT26lacZ cell line when inoculated as a tumour graft can be utilised to further explore a surgical model of cancer.

3.2.3 Preoperative treatment of CT26lacZ primary implant tumours with VSVΔ51GM-CSF does not protect against a subsequent CT26lacZ flank tumour

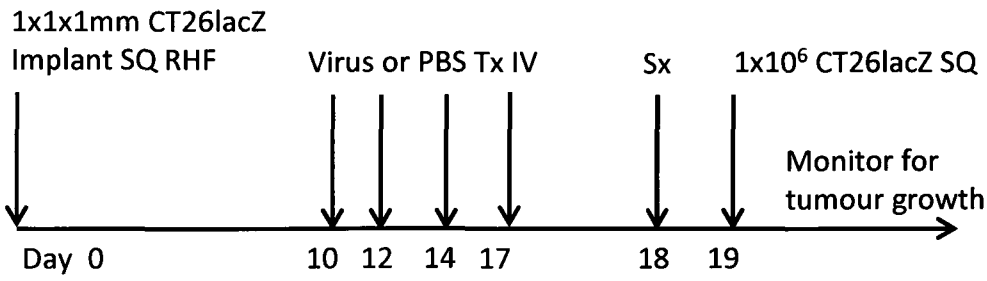
In order to determine whether VSVΔ51GM-CSF treatment could generate an anti-tumour immune response capable of preventing the growth of a secondary tumour, primary tumours were preoperatively treated in the CT26lacZ surgical model. The experimental design is shown in Figure 8a. Primary CT26lacZ tumours were implanted in Balb/c mice subcutaneously in the right hind flank on day 0. On day 10, 12, 14, and 17, the CT26lacZ tumour bearing mice were injected with VSVΔ51GM-CSF or PBS intravenously. On day 18, the primary tumours were removed by surgical resection. On day 19, all mice, including a naïve control group of mice, received a subsequent CT26lacZ tumour inoculation in the left hind flank subcutaneously. No extension in survival was observed in mice that received VSVΔ51GM-CSF treatment of the primary tumour compared to the mice that received preoperative treatment with PBS (Figure 8b). This result suggests that VSVΔ51GM-CSF treatment of the primary CT26lacZ tumour was unable to generate an immune response capable of preventing the growth of a secondary CT26lacZ tumour.

3.2.4 VSVΔ51GM-CSF treatment alone of a CT26lacZ tumour protects against a subsequent CT26lacZ tumour challenge

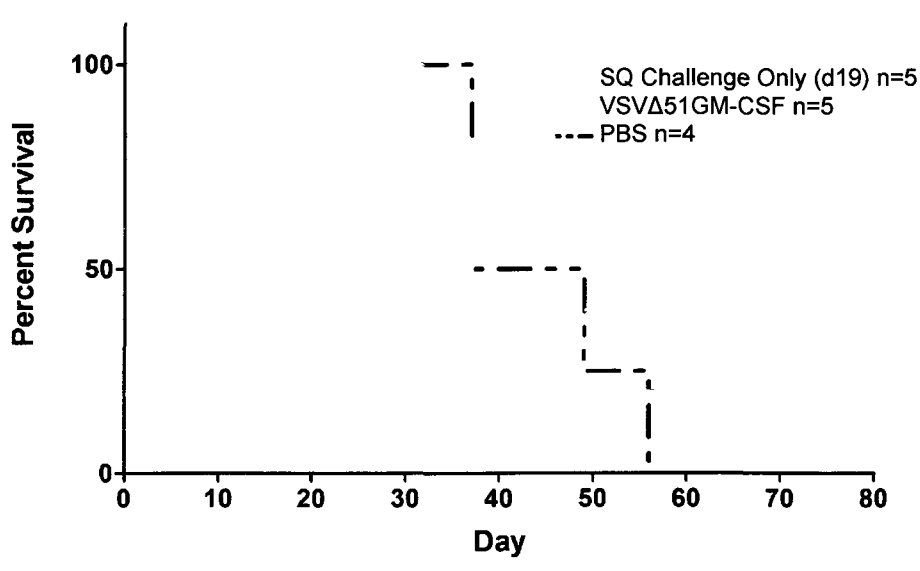
A typical $CD8^+$ T cell response to an infection or vaccination consists of three phases: clonal expansion of specific cells and acquisition of effector functions, contraction of the effector population, and the generation of a population of memory cells. Expansion of effector $CD8^+$ T cells has been shown to peak at approximately day 8 after infection or vaccination(187).

Figure 8. **VSVΔ51GM-CSF treatment prior to surgical resection of a CT26LacZ primary tumour does not protect against a subsequent CT26lacZ tumour challenge**. $1x10^6$ CT26lacZ cells were injected into donor Balb/c mice SQ bilaterally on day -14. On day 0, donor mice were euthanized and their tumours removed. On day 0, recipient Balb/c mice received a 1x1x1mm CT26lacZ tumour surgically implanted SQ in the right hind flank. Mice were injected with 100µl PBS IV or $5x10^8$pfu VSVΔ51GM-CSF IV on day 10, 12, 14, and 17. On day 18, mice received surgery to remove their tumour. On day 19, all mice were injected with $1x10^6$ CT26lacZ cells SQ in their left hind flank. Tumour growth was monitored and mice were euthanized when tumour size exceeded $750mm^3$. (A) Experimental setup (B) The percent survival is plotted in a Kaplan Meier Survival Plot.

A


1x1x1mm CT26lacZ
Implant SQ RHF
Virus or PBS Tx IV
Sx
1x10^6 CT26lacZ SQ
Monitor for
tumour growth
Day 0
10 12 14 17
18 19

B

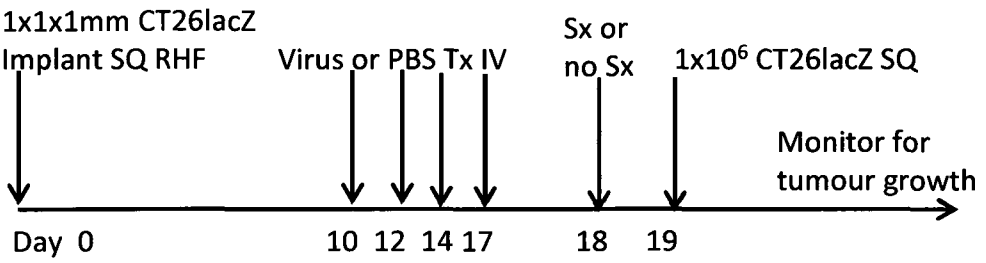

Percent Survival
100
50
0
0 10 20 30 40 50 60 70 80
Day
SQ Challenge Only (d19) n=5
VSVΔ51GM-CSF n=5
PBS n=4

VSVΔ51GM-CSF has been previously shown to completely regress CT26lacZ tumours established subcutaneously (136). Several weeks after tumour regression, mice were able to reject a subsequent CT26lacZ tumour challenge (KP unpublished data). In order to assess the impact of the surgical intervention on the capability of the mouse to reject the secondary tumour inoculation, we undertook an experiment to assess the immune response generated against an established CT26lacZ tumour with or without surgery. This experiment allowed us to validate the presence of a robust anti-tumour immune response on day 19 that would be capable of rejecting the challenge tumour. CD1d nude mice were included to ensure that the rejection of the challenge tumour in mice that received VSVΔ51GM-CSF treatment of CT26lacZ primary tumours with no surgery was not due to direct oncolysis. The experimental design is shown in Figure 9a.

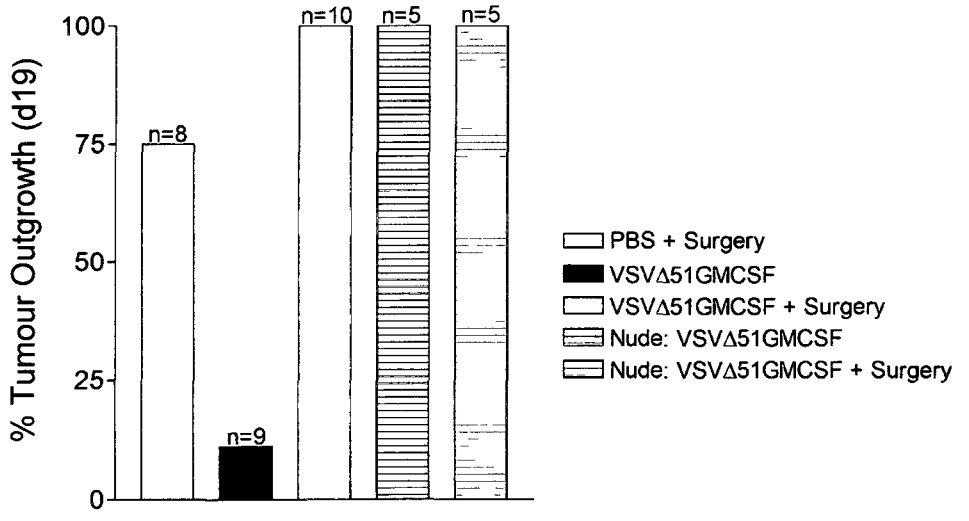
All mice received a primary CT26lacZ tumour implant into the right hind flank on day 0. Mice were treated with PBS intravenously or with VSVΔ51GM-CSF intravenously on day 10, 12, 14, and 17. On day 18, mice received surgery to resect the CT26lacZ primary tumour. On day 19, all mice received a subsequent CT26lacZ tumour inoculation in the left hind flank subcutaneously. Mice that were preoperatively treated with PBS and received surgery to resect the primary tumour, were protected from developing a secondary CT26lacZ tumour at a percentage of 25% (Figure 9b). Mice, which underwent VSVΔ51GM-CSF treatment of their primary tumour and no surgery, were able to reject the development of a CT26lacZ tumour challenge at a percentage of 88.9%. None of the mice that were preoperatively treated with VSVΔ51GM-CSF and received surgery to resect the primary tumour were protected from developing a secondary CT26lacZ tumour. All nude mice that were preoperatively treated with VSVΔ51GM-CSF, regardless of whether they received surgery or not, were not protected from developing a CT26lacZ tumour challenge. Altogether, we have confirmed that mice with established CT26lacZ tumours, when subsequently treated with VSVΔ51GM-CSF, are able to reject a secondary CT26lacZ challenge tumour at day 19.

Figure 9. **VSVΔ51GM-CSF treatment of a CT26lacZ tumour with no surgery protects against a subsequent CT26lacZ tumour challenge. T cells are required for this protection.** $1x10^6$ CT26lacZ cells were injected into donor Balb/c mice SQ bilaterally on day -14. On day 0, donor mice were euthanized and their tumours removed. On day 0, recipient Balb/c and CD1 nude mice received a 1x1x1mm CT26lacZ tumour surgically implanted SQ in the right hind flank. Mice were injected with 100μl PBS IV or $5x10^8$pfu VSVΔ51GM-CSF IV on day 10, 12, 14, and 17. On day 18, mice received surgery to remove their tumour or received no surgery. On day 19, all mice were injected with $1x10^6$ CT26lacZ cells SQ in their left hind flank. Tumour growth was monitored and mice were euthanized when tumour size exceeded $750mm^3$. (A) Experimental setup (B) Percent CT26lacZ Challenge Tumour Outgrowth is graphed for mice that received the challenge tumour on day 19.

A


1x1x1mm CT26lacZ
Implant SQ RHF
Virus or PBS Tx IV
Sx or
no Sx
1x10^6 CT26lacZ SQ
Monitor for
tumour growth
Day 0
10 12 14 17
18
19

B


% Tumour Outgrowth (d19)
100
75
50
25
0
n=8
n=9
n=10
n=5
n=5
PBS + Surgery
VSVΔ51GMCSF
VSVΔ51GMCSF + Surgery
Nude: VSVΔ51GMCSF
Nude: VSVΔ51GMCSF + Surgery

We have also ensured that this effect is not due to direct cell lysis by VSVΔ51GM-CSF as this protective effect is abrogated in nude mice. Finally, this data suggests that the surgical intervention to remove the primary CT26lacZ tumour abrogates the protection against the secondary CT26lacZ tumour challenge.

3.2.5 Surgery ablates protection against the CT26lacZ challenge tumour. Mice that received surgery are protected against the CT26lacZ challenge tumour 1 month post-surgery.

We hypothesized that the ablation of protection of the challenge tumour was due to the surgical intervention and not due to the removal of the primary tumour. We also hypothesized that this abrogation of protection would be transient as has been previously observed (188). In order to test our hypothesis, we assessed the ability of mice treated with VSVΔ51GM-CSF to reject a secondary CT26lacZ tumour after receiving a mock surgery where the primary tumour remains intact.

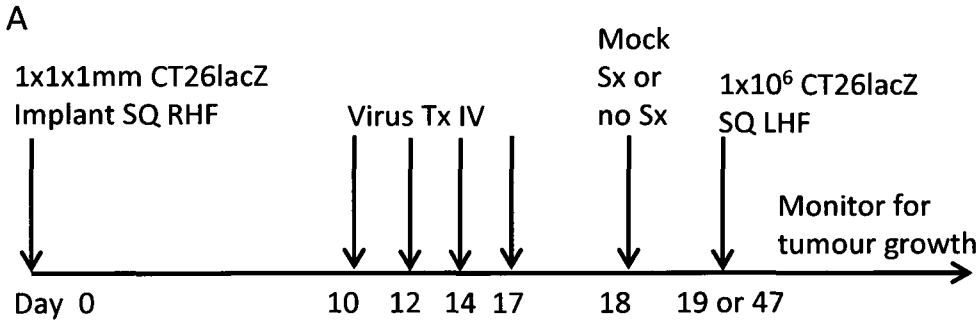
This experimental design is shown in Figure 10a. All Balb/c mice received a CT26lacZ primary tumour implant in the right hind flank, subcutaneously on day 0. Mice were treated with VSVΔ51GM-CSF intravenously on day 10, 12, 14, and 17. On day 18, some mice received a mock surgery which left the primary tumour intact in the flank. On day 19 or 47, mice were inoculated with a CT26lacZ tumour on the left hind flank. It was found that mice that received a mock surgery and a CT26lacZ tumour challenge on day 19, were protected from developing a secondary tumour at a percentage of 25%. All mice that received no surgery and a CT26lacZ tumour challenge on day 19, were protected from developing a secondary tumour. Mice that received a mock surgery and a CT26lacZ tumour challenge on day 47, were protected from developing a secondary tumour at a percentage of 80%. Finally, mice that received no surgery and a CT26lacZ tumour challenge on day 47, were protected from developing a secondary tumour at a percentage of 66%. This data suggests

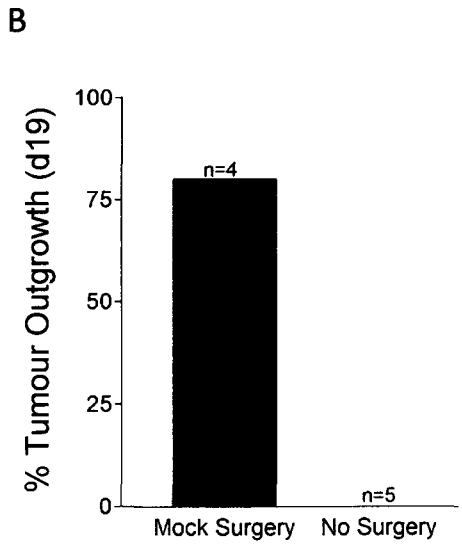
that the development of a CT26lacZ challenge tumour in mice was due to the mock surgery. This data also suggests that this inability to protect against the CT26lacZ challenge tumour is transient.

3.2.6 A CT26lacZ primary tumour without treatment is able to prevent the growth of a CT26lacZ tumour challenge after the transient effects of surgery subside

In order to assess whether VSVΔ51GM-CSF treatment could generate an anti-tumour immune response capable of preventing the growth of a secondary tumour after the transient effects of surgery subside, primary tumours were preoperatively treated in the CT26lacZ surgical model. Following the surgical resection of the primary tumour, the CT26lacZ challenge was injected into mice subcutaneously after 1 month. The experimental design is shown in Figure 11a. Balb/c mice received a CT26lacZ primary tumour implant in the right hind flank subcutaneously on day 0. Mice were treated with VSVΔ51GM-CSF or PBS intravenously on day 10, 12, 14, and 17. On day 18, some mice received surgery to resect the right hind flank primary tumour. On day 48, all mice, including a naïve control group of mice, were inoculated with CT26lacZ cells in the left hind flank. It was found that mice that were preoperatively treated with VSVΔ51GM-CSF IV prior to surgery, were protected from the CT26lacZ challenge tumour outgrowth at a percentage of 85.7%. All mice that received treatment of VSVΔ51GM-CSF IV and received no surgery to remove the primary tumour, were protected from the CT26lacZ challenge tumour outgrowth. Mice that were preoperatively treated with PBS IV prior to surgery, were protected from the CT26lacZ challenge tumour outgrowth at a percentage of 88.9%. None of the mice that only received a subcutaneous CT26lacZ tumour inoculation on day 48, were protected. These results suggest that a CT26lacZ primary tumour without treatment is able to prevent the growth of a CT26lacZ tumour challenge after the transient effects of surgery subside.

Figure 10. **Surgery Effects ablate protection against the CT26lacZ challenge tumour. Mice that receive surgery are protected against the CT26lacZ challenge tumour 1 month post-surgery.** $1x10^6$ CT26lacZ cells were injected into donor Balb/c mice SQ bilaterally on day -14. On day 0, donor mice were euthanized, their tumours removed, and the tumours were cut into 1x1x1mm pieces with a surgical scalpel. On day 0, recipient Balb/c mice received a 1x1x1mm CT26lacZ tumour surgically implanted SQ in the right hind flank. Mice were injected with $5x10^8$pfu VSVΔ51GM-CSF IV on day 10, 12, 14, and 17. On day 18, mice received a mock surgery or no surgery. On day 19 or 47, mice were injected with $1x10^6$ CT26lacZ cells SQ in the left hind flank. Tumour growth was monitored and mice were euthanized when tumour size exceeded $750mm^3$. (A) Experimental Design. (B) Percent CT26lacZ Challenge Tumour Outgrowth is graphed for mice that received the challenge tumour on day 19. (C) Percent CT26lacZ Challenge Tumour Outgrowth is graphed for mice that received the challenge tumour on day 47.


A
1x1x1mm CT26lacZ
Implant SQ RHF
Virus Tx IV
Mock
Sx or
no Sx
1x10⁶ CT26lacZ
SQ LHF
Monitor for
tumour growth
Day 0
10
12
14
17
18
19 or 47


B
% Tumour Outgrowth (d19)
100
75
50
25
0
n=4
n=5
Mock Surgery
No Surgery

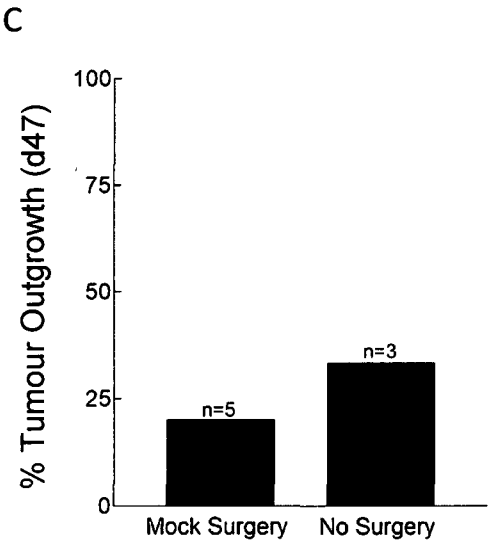
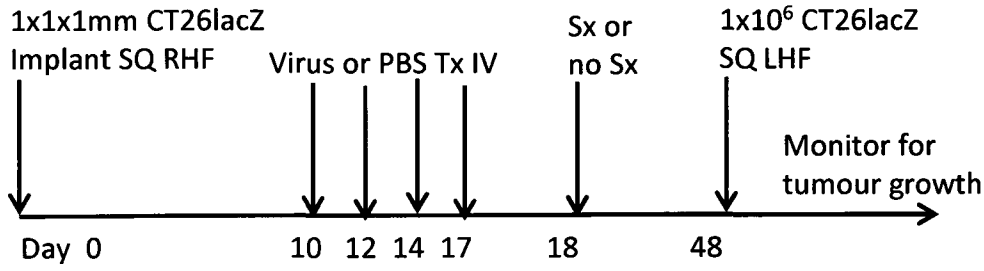
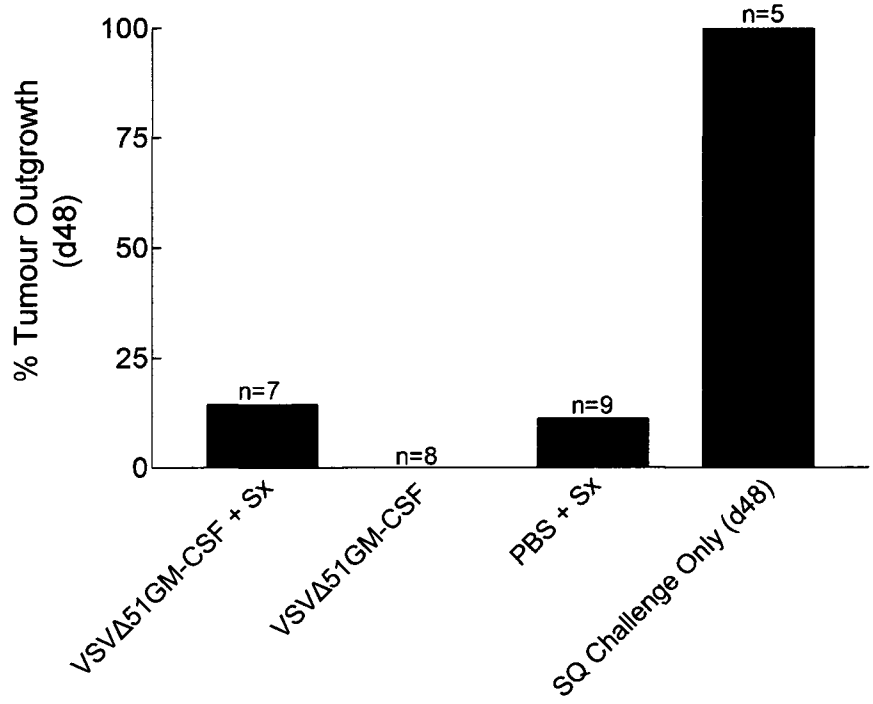

C
% Tumour Outgrowth (d47)
100
75
50
25
0
n=5
n=3
Mock Surgery
No Surgery

Figure 11. A **CT26lacZ primary tumour without treatment is able to prevent the growth of a CT26lacZ tumour challenge after the transient effects of surgery subside**. $1x10^6$ CT26lacZ cells were injected into donor Balb/c mice SQ bilaterally on day -14. On day 0, donor mice were euthanized, their tumours removed, and the tumours were cut into 1x1x1mm pieces with a surgical scalpel. On day 0, recipient Balb/c mice received a 1x1x1mm CT26lacZ tumour surgically implanted SQ in the right hind flank. Mice were injected with 100µl PBS IV or $5x10^8$pfu VSVΔ51GM-CSF IV on day 10, 12, 14, and 17. On day 18, mice received surgery to remove their primary tumour or no surgery. On day 48, all mice were injected with $1x10^6$ CT26lacZ cells SQ in their left hind flank. Tumour growth was monitored and mice were euthanized when tumour size exceeded $750mm^3$. (A) Experimental Design. (B) Percent Outgrowth of the CT26lacZ challenge tumour given on day 48 is graphed.

A


1x1x1mm CT26lacZ
Implant SQ RHF
Virus or PBS Tx IV
Sx or
no Sx
1x10^6 CT26lacZ
SQ LHF
Monitor for
tumour growth
Day 0
10
12
14
17
18
48

B


% Tumour Outgrowth
(d48)
100
75
50
25
0
n=7
n=8
n=9
n=5
VSVΔ51GM-CSF + Sx
VSVΔ51GM-CSF
PBS + Sx
SQ Challenge Only (d48)

We are also unable to evaluate whether VSVΔ51GM-CSF treatment could generate an anti-tumour immune response after the effects of surgery subside, as this neoadjuvant CT26lacZ cancer surgery model is no longer a model we can utilise.

3.3 B16-F10 Surgical Model

3.3.1 A B16-F10 primary tumour does not protect against a subsequent B16-F10 tumour inoculation injected intravenously

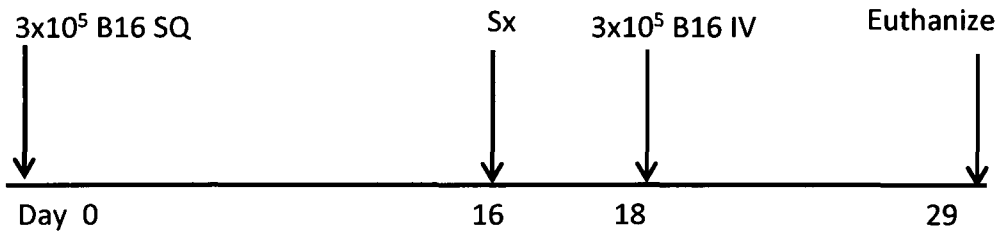
In order to assess whether the B16-F10 melanoma cell line syngeneic in C57/Bl6 mice could be utilised in a surgical model of cancer, we assessed whether a B16-F10 primary tumour protects the mice from subsequent B16-F10 tumours injected intravenously. Primary B16-F10 tumours were established in a group of C57/Bl6 mice subcutaneously in the right hind flank on day 0. On day 16, these mice underwent surgery to remove the primary tumour. On day 18, all mice, including a naïve control group of mice, were inoculated with B16-F10 cells injected intravenously. The mice were euthanized on day 29, and the lungs were removed. This experimental design is shown in Figure 12a. Naïve mice, which only received the B16-F10 intravenous injection, and experimental mice, both grew a similar number of surface lung metastases (Figure 12b). This data suggests that a B16-F10 primary tumour does not protect against a subsequent B16-F10 tumour challenge intravenously, allowing us to employ B16-F10 cells in a surgical model of cancer.

3.3.2 Preoperative treatment of B16-F10 primary tumours with VSVΔ51GM-CSF or VSV Δ51 does not protect against subsequent B16-F10 lung metastases

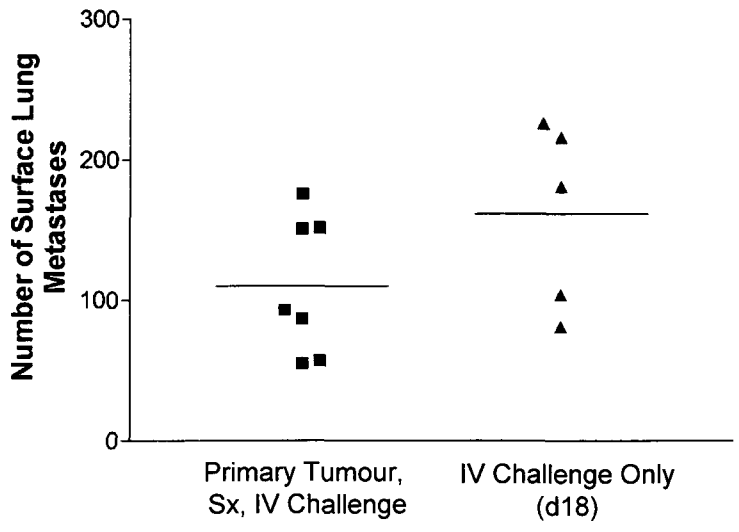
In order to assess whether VSVΔ51GM-CSF or VSVΔ51 treatment could generate an anti-tumour immune response capable of reducing the number of B16-F10 lung metastases, we preoperatively treated primary tumours with VSVΔ51GM-CSF or VSVΔ51 in the B16-F10 surgical model.

Figure 12. **A B16-F10 primary tumour SQ does not protect against subsequent B16-F10 tumour inoculation injected intravenously**. C57/Bl6 mice were injected with $3x10^5$ B16-F10 SQ in the right hind flank. On day 16, B16-F10 tumours were surgically resected from the right hind flank. On day 18, control mice and surgery mice received $3x10^5$ B16-F10 cells IV. On day 29, mice were euthanized, their lungs removed, and examined for lung tumours. (A) Experimental Design. (B) The number of surface lung metastases is graphed.

A


3x10⁵ B16 SQ
Sx
3x10⁵ B16 IV
Euthanize
Day 0
16
18
29

B


Number of Surface Lung Metastases
300
200
100
0
Primary Tumour, Sx, IV Challenge
IV Challenge Only (d18)

Primary B16-F10 tumours were established in C57/Bl6 mice subcutaneously in the right hind flank (Figure 13a). On day 10 and day 17, the B16-F10 tumour bearing mice were treated with PBS or VSVΔ51GM-CSF or VSVΔ51 intravenously. On day 21, mice underwent surgery to resect the primary tumour. All mice, including a naïve control group of mice, received a subsequent B16-F10 tumour inoculation injected intravenously on day 23. On day 37, all mice were euthanized and the lungs were removed. There is a small decrease in the number of surface lung metastases which does not reach statistical significance (P=0.25) in mice that received VSVΔ51GM-CSF treatment of the primary B16-F10 tumour in comparison to mice that received preoperative treatment with PBS. There is also a decrease in the number of surface lung metastases which also does not reach statistical significance (P=0.0571) but may be biologically significant in mice that received VSVΔ51 treatment of the primary B16-F10 tumour in comparison to mice that received preoperative treatment with PBS. This result suggests that VSVΔ51GM-CSF of VSVΔ51 treatment of the primary B16-F10 tumour was unable to generate an anti-tumour immune response capable of reducing the number of surface lung metastases significantly.

In order to assess the magnitude of the $CD8^+$ T cells response towards VSVΔ51GM-CSF and VSVΔ51, we examined the percentage of IFN-γ producing $CD3^+CD8^+$ cells. Balb/c mice were prophylactically treated intravenously with VSVΔ51GM-CSF or VSVΔ51 on day 0,2,4, and 7. On day 8, all mice were euthanized and the spleens were extracted. The splenocytes were isolated and the production of IFN-γ by $CD3^+CD8^+$ T cells was examined in response to stimulation with the VSV-N2 peptide. In this preliminary experiment, it was found that the percent of $CD3^+CD8^+$ cells that were producing IFN-γ was approximately 8% in mice, which were prophylactically treated with VSVΔ51GM-CSF intravenously. The percent of $CD3^+CD8^+$ cells that were producing IFN-γ was approximately 4% in mice, which were prophylactically treated with VSVΔ51 intravenously. It was found that in mice that received no treatment, the percent of $CD3^+CD8^+$ cells that were producing

IFN-γ was approximately 1%. Although this data is not statistically significant due to the number of mice in each group, the study demonstrates a trend that VSVΔ51GM-CSF elicits a higher $CD8^+$ T cell response based on IFN-γ production than VSVΔ51.

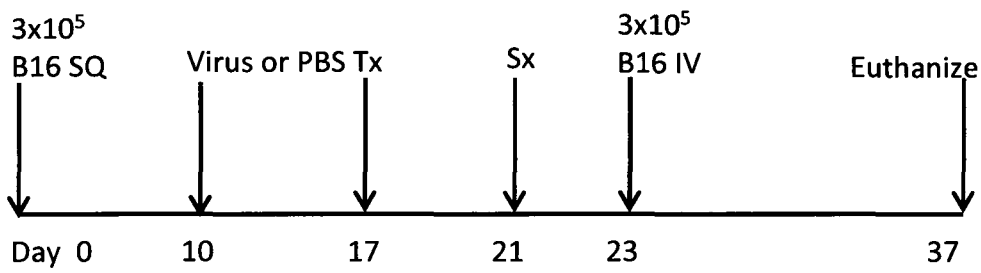
3.3.3 JX594mGM-CSF treatment of a primary B16-F10 tumour injected intratumourally prior to surgical resection reduces the number of B16-F10 lung tumours upon challenge

In order to assess whether another oncolytic virus, specifically JX594mGM-CSF, treatment could generate an anti-tumour immune response capable of reducing the number of B16-F10 lung metastases, we preoperatively treated primary tumours with JX594mGM-CSF intratumourally or intravenously in the B16-F10 surgical model. Primary B16-F10 tumours were established in C57/Bl6 mice subcutaneously in the right hind flank (Figure 14a). On day 10 and day 17, the B16-F10 tumour bearing mice were treated with PBS or JX594mGM-CSF injected intratumourally or intravenously. On day 21, mice underwent surgery to resect the primary tumour. All mice, including a naïve control group of mice, received a subsequent inoculation of B16-F10 tumour cells injected intravenously on day 23. On day 37, all mice were euthanized and the lungs were removed. There was a decrease in the number of surface lung metastases (P=0.0357) in mice that received JX594mGM-CSF treatment of the primary B16-F10 tumour intratumourally in comparison to mice that received preoperative treatment with PBS. There was a small increase in the number of lung metastases which does not reach statistical significance (P=0.1429) in mice that received JX594mGM-CSF treatment of the primary B16-F10 tumour intravenously in comparison to mice that received preoperative treatment with PBS. This result suggests that JX594 treatment injected intratumourally into the primary tumour is able to generate an anti-tumour immune response capable of reducing the number of surface lung metastases.

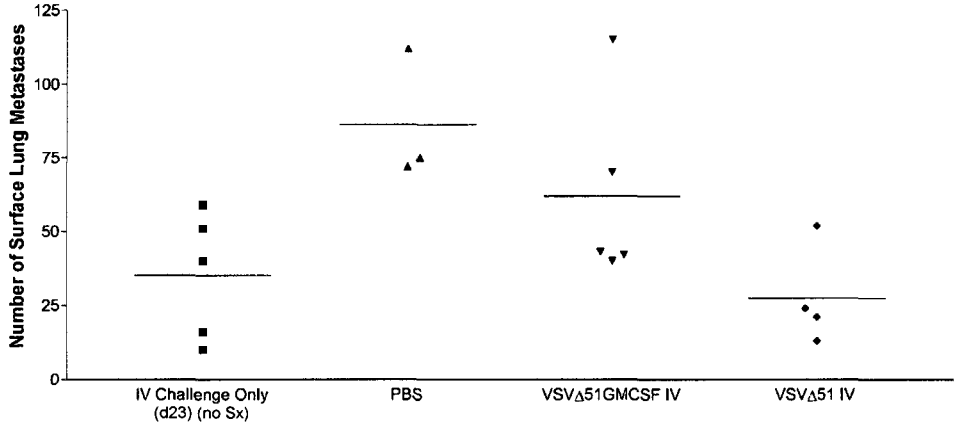
In establishing the model, we investigated the susceptibility of B16-F10 cells to VSVΔ51 and JX594hGM-CSF. B16-F10 were plated and infected with VSVΔ51gfp or JX594hGM-CSFgfp at an MOI of 0.01 and 1. Fluorescence microscopy images were taken after 24 and 48 hours of incubation (Figure 14c). GFP expression in fluorescent images and the corresponding phase contrast images were qualitatively examined. B16-F10 tumour cells were less susceptible to JX594hGM-CSFgfp infection in comparison to VSVΔ51gfp. There appeared to be more cell death based on the phase contrast images in B16-F10 cells infected with VSVΔ51gfp in comparison to JX594hGM-CSFgfp. This observation suggests that B16-F10 cells differ in susceptibility to infection by VSVΔ51gfp in comparison to JX594hGM-CSFgfp.

Figure 13. **Preoperative treatment of B16-F10 primary tumours with VSVΔ51GM-CSF IV or VSVΔ51 IV does not protect against subsequent B16-F10 lung metastases.** C57/Bl6 mice were injected with $3x10^5$ B16-F10 SQ in the right hind flank. On day 10 and 17, mice were treated with $5x10^8$ pfu VSVΔ51GM-CSF IV or VSVΔ51 IV. On day 21, B16-F10 tumours were surgically resected from the right hind flank. On day 23, all mice received $3x10^5$ B16-F10 cells IV. On day 37, mice were euthanized, their lungs removed, and examined for lung tumours. (A) Experimental Design. (B) The number of lung surface metastases is graphed. Balb/c mice were injected with $5x10^8$ pfu VSVΔ51GM-CSF IV or VSVΔ51 IV on day 0, 2, 4, and 7. On day 8, mice were euthanized and their spleens extracted. Splenocytes were isolated and stimulated with the VSV-N2 peptide. (C) The percent of IFN-γ producing $CD3^+CD8^+$ cells is graphed.

A


3x10^5
B16 SQ
Virus or PBS Tx
Sx
3x10^5
B16 IV
Euthanize
Day 0
10
17
21
23
37

B


Number of Surface Lung Metastases
125
100
75
50
25
0
IV Challenge Only
(d23) (no Sx)
PBS
VSVΔ51GMCSF IV
VSVΔ51 IV

C

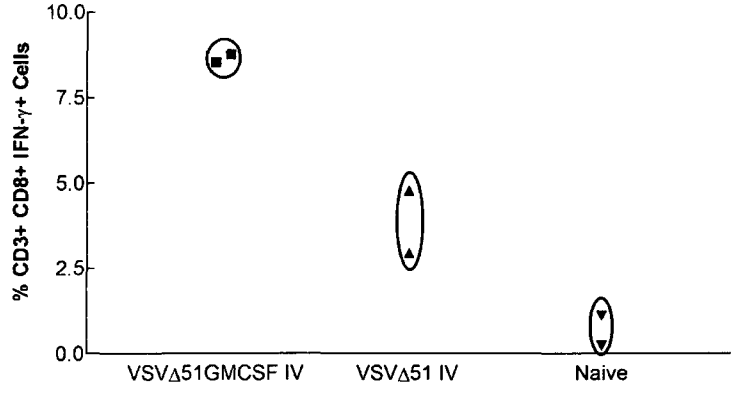
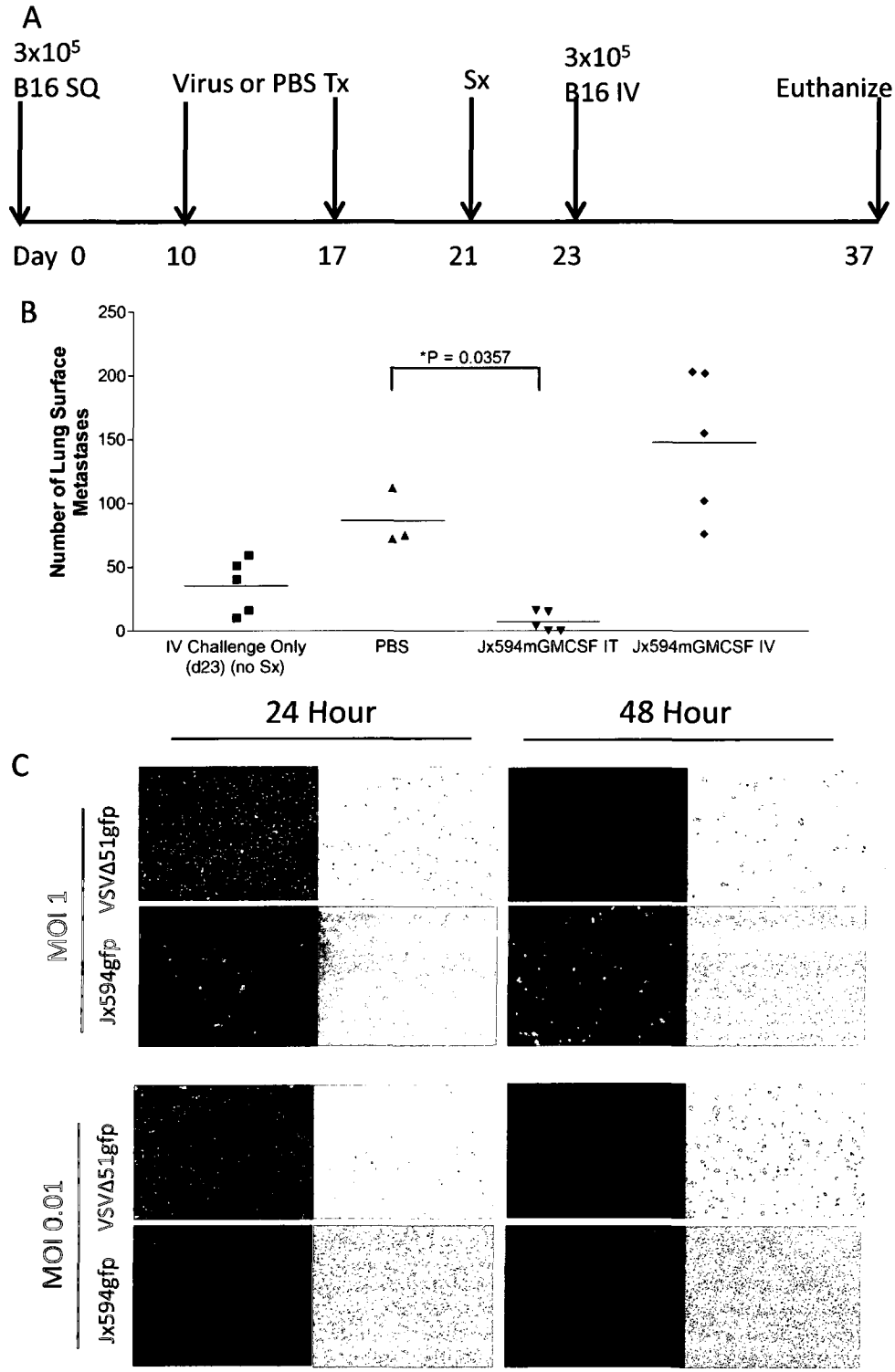

% CD3+ CD8+ IFN-γ+ Cells
10.0
7.5
5.0
2.5
0.0
VSVΔ51GMCSF IV
VSVΔ51 IV
Naive

Figure 14. **JX594mGM-CSF IT treatment prior to surgical resection of a B16-F10 primary tumour reduces the number of B16-F10 lung tumours upon challenge. B16-F10 cells are more susceptible to VSVΔ51 infection than JX594hGM-CSF infection**. C57/Bl6 were injected with $3x10^5$ B16-F10 SQ in the right hind flank. On day 10 and 17, mice were treated with $1x10^7$ JX594mGM-CSF IT or $1x10^7$ JX594mGM-CSF IV or PBS IV/IT. On day 21, B16-F10 tumours were surgically resected from the right hind flank. On day 23, all mice received $3x10^5$ B16-F10 cells IV. On day 37, mice were euthanized, their lungs removed, and examined for lung tumours. (A) Experimental Design. (B) The number of lung surface metastases is graphed. B16-F10 cells were plated in 6-well plates. Cells were infected with VSVΔ51gfp or JX594hGM-CSFgfp at an MOI of 1 or 0.001. After 24 hours and 48 hours of incubation, fluorescence and phase contrast pictures were taken of each well. (C) The fluorescent and phase contrast images are shown.


A
3x10^5
B16 SQ
Virus or PBS Tx
Sx
3x10^5
B16 IV
Euthanize
Day 0
10
17
21
23
37
B
Number of Lung Surface Metastases
250
200
150
100
50
0
*P = 0.0357
IV Challenge Only (d23) (no Sx)
PBS
Jx594mGMCSF IT
Jx594mGMCSF IV
C
24 Hour
48 Hour
MOI 1
VSVΔ51gfp
Jx594gfp
MOI 0.01
VSVΔ51gfp
Jx594gfp

Chapter 4: Discussion

4.1 The establishment of a mouse model of surgically resectable cancer and subsequent development of metastatic disease

We developed a murine model of surgically resectable cancer and subsequent metastatic disease, such that, it would mimic what is observed clinically in recurrent cancer (Figure 1). Typically, in the clinic, a patient presents with an established primary tumour and receives surgery to remove the solid mass. The patient is at risk for the development of metastatic disease. In our mouse model, the mice are injected with tumour cells in order to establish a primary tumour. Subsequently, the mice undergo surgery to resect the primary tumour and are injected with a bolus of tumour cells to establish a secondary "metastatic" tumour. As previously discussed, it is unclear when the process of metastasis is initiated. In some cases, patients have presented with the presence of disseminated disease, however some patients have no evidence of metastatic disease at the time of diagnosis (16, 25). Our model reflects the latter scenario. There are several differences between the murine model that we have developed and the clinical model. The main difference is timing of the secondary tumour or metastatic disease, as we injected the tumour cells after removal of the primary tumour and in patients, the time of initiation of metastatic disease is unknown. Another difference between the clinical model and the murine model is the bolus of cells injected into the mice after the primary tumour is removed. Patients do not receive a bolus of tumour cells, and the number of micrometastases in patients is expected to be lower than the number of tumour cells that we are injecting into mice.

There have been several other surgical models of cancer in the literature that would also be suitable for examining the anti-tumour immune response developed after neoadjuvant therapy. A B16-F10 surgical model was previously published, where mice received a primary B16-F10 tumour

injected intradermally(189). The B16-F10 primary tumour was resected surgically, and the regrowth of a secondary B16-F10 in the same area was seen. In our B16-F10 surgical model, we did not observe regrowth of a secondary tumour in the same area at the same rate or consistency as was previously observed. This difference may be due to the location of the primary tumour, as in our model the B16-F10 was established subcutaneously (Figure 12a). Differences in the removal of a wide surgical margin of normal uninvolved tissue may also contribute to the amount of regrowth that is observed in their study.

The neoadjuvant OV/Surgery strategy was chosen for a number of reasons. Firstly, in a B16-F10 surgical model, neoadjuvant vaccination with a replication-defective adenovirus expressing the human dopachrome tautomerase tumour antigen was found to provide greater protection against tumour relapse compared to adjuvant vaccination(189). Also, virus may not be a candidate for adjuvant treatment (at the time of surgery) or following surgery depending on the extent of metastatic disease at the time of surgery. In some cases, there may not be metastatic disease present to replicate and lyse at the time of primary tumour resection. If there is metastatic disease at the time of surgery, there might not be enough antigen released by viral lysis to stimulate an immune response. Therefore, our CT26WT model, derived from a similar model taken from the literature (184), was the basis for our experimental design. Although there are several differences in the murine model that we have developed from what is observed clinically, the model serves us appropriately for examining a neoadjuvant strategy with OVs to generate an anti-tumour immune response.

The poorly immunogenic 4T1 mammary carcinoma has also been used in a surgical model of cancer, where mice received a primary 4T1 tumour injected subcutaneously (190). 4T1 tumours can

metastasize to the lung as early as day 10 after inoculation. This may be a good model of spontaneous metastasis to examine in the future.

4.2 An anti-tumour immune response capable of preventing a secondary CT26WT tumour was not generated with preoperative VSVΔ51GM-CSF treatment of a CT26WT tumour

We established a CT26WT mouse model of surgically resectable cancer and subsequent development of metastatic disease in order to address the impact of neoadjuvant VSVΔ51GM-CSF viral therapy on anti-tumour immunity. We demonstrated that a CT26WT primary tumour does not protect against a subsequent CT26WT tumour challenge (Figure 2b). Some mice that previously grew a primary tumour had an extension in survival, most likely because the immune system was able to generate a specific adaptive immune response. However, this specific immune response generated was however not robust enough to prevent the development of a secondary tumour. Preoperative VSVΔ51GM-CSF treatment of the primary tumour showed no impact on the growth rate of the primary tumour (Figure 3a), which could be due to the resistance in infectivity of the CT26WT cells with VSVΔ51 as observed *in vitro* (Figure 4) and also *in vivo* (191).

Preoperative VSVΔ51GM-CSF treatment of the primary tumours was also unable to extend the survival of the mice (Figure 3b) as they succumbed to the secondary CT26WT tumour. The lack of efficacy in this model was thought to be due to the resistance in infection of the CT26WT cells by VSVΔ51. A study using a neoadjuvant therapy strategy in a CT26WT surgical model showed protection against developing a secondary CT26WT tumour after intratumoural treatment with herpes simplex virus (HSV) (184). This study also showed that, *in vitro*, at an MOI of 1, HSV was able to infect and kill 100% of CT26WT cells by day 5 and day 7 respectively. In addition, *in vivo*, CT26WT cells were able to support viral replication of HSV. The difference in infection of CT26WT cells by

VSVΔ51 and HSV could contribute to the lack of efficacy in rejecting a secondary CT26WT tumour observed in our model after preoperative VSVΔ51 treatment of the primary CT26WT tumour.

Whether robust oncolysis is required for the generation of a robust anti-tumour immune response nonetheless remains an open question. Although few studies have been carried out to address this directly, it has been proposed that robust viral replication and lysis is important in initiating an adaptive immune response(70). Viral infection of cells leads to cell lysis and the release of cell-associated tumour antigens to be phagocytosed by pAPCs. These pAPCs can then process and present the antigen to CTLs through the cross-presentation pathway. Recently, it has been shown that the anti-tumour activity of reovirus is independent of direct viral oncolysis and replication(185). The study used B16OVA cells, which are resistant to direct oncolysis of reovirus and do not support replication *in vitro* or *in vivo*. Reovirus loaded T cells were able to purge B16OVA lymph node metastases in immunocompetent animals, but the therapeutic was unable to do so in immunodeficient mice. However, much like CT26lacZ, B16OVA are highly immunogenic due to the expression of a foreign transgene.

4.3 An anti-tumour immune response capable of preventing a secondary CT26lacZ tumour was not generated with preoperative VSVΔ51GM-CSF treatment of a CT26lacZ tumour

We initially assessed the feasibility of using a CT26lacZ mouse model of surgically resectable cancer and subsequent development of metastatic disease in order to address the impact of neoadjuvant VSVΔ51GM-CSF viral therapy on anti-tumour immunity. We first assessed whether a CT26lacZ primary tumour could protect the mice from subsequent CT26lacZ tumours injected intravenously. It was found that the majority of mice were protected from developing CT26lacZ tumours in the lung after previously growing a CT26lacZ primary tumour subcutaneously (Figure 5b), which suggests that a robust specific anti-tumour immune response was generated capable of

preventing the growth of lung surface metastases. In contrast, mice that received CT26lacZ tumour cells in order to establish a primary tumour, were able to grow a subsequent subcutaneous challenge tumour at a success rate of 60% (Figure 6b).

This difference may be attributed to the site of the secondary tumour and the ability of the immune response to access the site. Literature on the topic of the types of immune cells and their ability to access the subcutaneous area compared to the lung is sparse. We can speculate that tumour cells may be more likely to be cleared by the immune system with exposure in the circulation and in the lungs. The lung is normally a crucial entry point for a variety of infectious pathogens. The bloodstream is also an important route of dissemination of invading pathogens. The bloodstream has circulating immune cells and the lung tissue is rich in resident immune cells to combat these pathogens(192). The subcutaneous compartment may not be as well vascularized as the lung, and may not have a population of resident immune cells as it is not a typical entry point for pathogens. There are also abundant reports that the site of the primary tumour (193)and route of administration of an immunotherapy (data not shown, (194, 195)) impacts the immune response produced. We have shown that successful vaccination with a whole cell vaccine with VSVΔ51 as an adjuvant is dependent on the route of administration. The whole cell vaccine with a VSVΔ51 adjuvant given prophylactically is able to protect the mice from subsequent tumour challenge when injected intraperitoneally or intrahepatically. This vaccination is unable to protect the mice from subsequent tumour challenge when injected subcutaneously, suggesting differences in eliciting an immune response to be site-specific (data not shown).

In order to establish a CT26lacZ surgical model with consistent secondary tumour growth without OV treatment, we aimed to reduce the immunogenicity of the primary tumour. A sarcoma model showed that tumour cells given as a single-cell suspension were capable of inducing a

protective CTL response, while the transplantation of a small tumour implant was not able to induce this specific immune response(186). In order to reduce the immune recognition of the primary tumour, we transplanted a solid 1x1x1mm CT26lacZ tumour from donor mice to recipient mice. A primary CT26lacZ tumour implant followed by surgical resection did not protect any mice from the development of a secondary CT26lacZ tumour (Figure 6b). Interestingly, the sarcoma study found that in mice that were inoculated with a single-cell suspension of sarcoma cells, there was presence of tumour cells in the secondary lymphoid organs. Conversely, mice that received a tumour implant were found to lack tumour cells in the lymph nodes. The reduction in immunogenicity in our model may be due to the lack of tumour cell trafficking to the draining lymph nodes at implantation. Another possibility is the tumour implant may already have established an immunosuppressive microenvironment that is capable of actively suppressing the immune cells. Tumour cells that are injected as a single-cell suspension may not be capable of producing immunosuppressive factors immediately after inoculation.

The growth of the CT26lacZ primary tumour, regardless of the method of inoculation – single-cell suspension or tumour implant, grew at a similar rate. This indicates that the mice received a similar tumour burden (Figure 7b). In keeping with the robust susceptibility of CT26lacZ cells *in vitro* (Figure 4), these CT26lacZ primary tumours seem more responsive *in vivo* to infection with VSVΔ51GM-CSF (Figure 7b) than the CT26WT primary tumours (Figure 3b).

In order to determine whether VSVΔ51GM-CSF treatment is capable of initiating an anti-tumour immune response capable of preventing the growth of a secondary CT26lacZ tumour, primary CT26lacZ tumours were preoperatively treated with VSVΔ51GM-CSF. No difference in secondary tumour growth was seen in mice preoperatively treated with VSVΔ51GM-CSF compared to mice preoperatively treated with PBS (Figure 8b). Due to the evidence that VSVΔ51GM-CSF

impacted the progression of the CT26lacZ primary tumour (Figure 7b), it does not seem likely that this lack of efficacy was due to insufficient viral replication in the primary tumour. We have previously found that 6 treatments of VSVΔ51 given intravenously to CT26lacZ tumour bearing mice is able to cause complete regression of the CT26lacZ tumour in a viral lysis-dependent and immune-mediated manner (CL unpublished data). We have also found that mice that are cured of the CT26lacZ tumours after VSVΔ51 treatment are capable of rejecting a subsequent CT26lacZ tumour challenge (KP unpublished data). In the CT26lacZ surgery model, we speculated that the lack of extension in survival of VSVΔ51GM-CSF treated mice was not due to the timing of the tumour challenge, but perhaps due to the surgical intervention. We have not shown that an earlier CT26lacZ challenge is rejected after VSVΔ51 treatment of CT26lacZ primary tumours. Since the effector CTL response should peak approximately 1 week after viral treatment, the CT26lacZ challenge should be rejected at this timepoint as well.

4.4 The effects of surgery are responsible for the abrogation of protection against the tumour challenge

In order to validate the presence of a robust anti-tumour immune response on day 19 that would be capable of rejecting the challenge tumour, we established CT26lacZ tumours via transplantation in mice, which subsequently received 4 treatments of VSVΔ51GM-CSF intravenously. Primary CT26lacZ tumours treated with VSVΔ51GM-CSF regressed completely at approximately day 25 (data not shown). Secondary CT26lacZ tumours were inoculated on day 19 and are typically palpable 10 days after inoculation. Mice that received VSVΔ51GM-CSF treatment of the primary CT26lacZ tumour and received no surgery were protected against the secondary CT26lacZ tumour challenge (Figure 9b). In order to determine whether this phenomenon was mediated by an anti-tumour immune response involving T cells, CD1d nude mice were employed.

There was no protection against the CT26lacZ challenge tumour after VSVΔ51GM-CSF treatment of the primary tumour suggesting that the rejection of the CT26lacZ secondary tumour was due to immune-mediated mechanisms in the immunocompetent mice. As seen previously (Figure 8b), mice that received preoperative treatment with PBS or VSVΔ51GM-CSF, were unable to mount a protective response against the CT26lacZ challenge tumour. This result suggested that the surgical intervention was capable of eliminating the protective response against the CT26lacZ challenge tumour. We have previously discussed the importance of TILs and their role in the eradication of tumour cells in patients (42, 47). The removal of these important immune cells could be affecting the efficacy of the immune response in protecting mice against the secondary tumour challenge. We have also previously discussed the numerous effects of surgery on the host immune response (75, 77, 82, 196, 197). Any of these factors or a combination of these factors could be impacting the lack of protection against the secondary tumour.

We hypothesized that the lack of protection to the subsequent tumour challenge was due to surgical effects, and these effects would be transient as previously shown in the literature (188). We assessed the ability of mice treated with VSVΔ51GM-CSF to reject a secondary CT26lacZ challenge tumour after receiving a mock surgery. A mock surgery was performed in order to inflict similar effects, such as anaesthesia and tissue damage, as the surgical tumour resection and to allow the CT26lacZ tumour to remain intact with the TILs undisturbed. It was found that the surgical procedure (anesthesia, wounding, temperature changes, pain, psychological stress, etc.) was responsible for abrogating the protection against the CT26lacZ tumour challenge (Figure 10c). We have also shown that this effect is transient as mice that received the mock surgery were able to reject a CT26lacZ challenge tumour 1 month post-surgery.

We have not established the exact time point at which this surgical effect is reversed. In patients, it has been shown that an abdominal surgical intervention, which induced immunodepression, arose a few hours after surgery and lasted until 15-20 days post-surgery(188). This length of immunosuppression could vary depending on many factors including the invasiveness of the surgery, the anaesthetic used, and the condition of the patient (188, 196).

4.4.1 Facilitation of tumour growth following surgery

The abrogation of immune-mediated protection after surgery could be a result of facilitation of tumour growth or suppression of the immune response or a combination of both. There are a few ways that tumour growth could be facilitated as well as numerous mechanisms that could lead to immunosuppression. In surgical wound healing, there are numerous growth factors involved in the various stages. Many of these growth factors are known to participate in tumourigenesis as well. The growth factors, including EGF, TGF-α, TGF-β, PDGF, and bFGF, mainly have roles in increasing tumourigenicity and metastatic potential in addition to causing neovascularization. During surgery, these growth factors are released locally and systemically. The rate of elimination of cells by the immune system may not be sufficient for the potentially accelerated growth of the challenge tumour.

4.4.2 Immunosuppression following surgery

There have been numerous studies that have shown alterations in immune cell numbers and function after surgery. Suppression of NK cell activity after surgery has been well documented (90, 91, 94, 95). Importantly, the number of lymphocytes has been shown to decrease as well as their capability to proliferate and produce cytokines(98). Reduction in the number of immune effector cells or the impairment of proper function of these cells could lead to the inability to protect against the subsequent challenge tumour.

Although the initial cytokine response to wound injury is pro-inflammatory at the site of injury, a systemic anti-inflammatory response has been shown to follow (100). This systemic anti-inflammatory response causes suppression of the cellular immune system. Even if a specific anti-tumour immune response has been activated by VSVΔ51GM-CSF treatment, the effector cell function could be suppressed by the anti-inflammatory cytokines which are released.

Immunosuppression can be caused by several means such as reducing the number of vital immune cells, causing dysfunction in NK cells and T cells, and by the systemic anti-inflammatory response that would lead to silencing the functioning immune response. Surgery, and the resultant physiological changes, may facilitate tumour growth directly or suppress the immune system in such a way that the mice are no longer able to reject a tumour challenge delivered in the postoperative period.

4.5 After the effects of surgery subside, a CT26lacZ primary tumour is capable of inducing a specific anti-tumour immune response capable of preventing a CT26lacZ tumour challenge

The observation that the effects of surgery are transient, allowed us to test the generation of an anti-tumour immune response with VSVΔ51GM-CSF after these effects had subsided. As we previously found that the effects of surgery are lifted 1 month after surgery (Figure 10c), we preoperatively treated primary tumours with PBS and VSVΔ51GM-CSF, surgically resected the primary tumours, and waited 1 month until challenging the mice. Mice, regardless of PBS or VSVΔ51GM-CSF treatment, were found to be immune to the CT26lacZ tumour challenge (Figure 11b). Importantly, this result was not an issue of poor cell viability or take rate as the naïve mice, which received only the challenge, grew the CT26lacZ tumour. This result suggests that the despite transplanting a solid primary tumour to avoid immune recognition, the CT26lacZ cells were immunogenic enough without the need for OV therapy to elicit a successful anti-tumour immune

response. The growth of the CT26lacZ tumours at an earlier timepoint was very likely due to the effects of surgery – either by tumour growth facilitation or immunosuppression or a combination of both.

4.6 An anti-tumour immune response capable of reducing the number of B16-F10 lung surface metastases was not generated with preoperative VSVΔ51GM-CSF or VSVΔ51 treatment of a B16-F10 tumour

We established a B16-F10 mouse model of surgically resectable cancer and subsequent development of metastatic disease as an alternative to the CT26 model in order to address the impact of neoadjuvant VSVΔ51GM-CSF viral therapy on anti-tumour immunity. We demonstrated that a B16-F10 primary tumour does not protect against a subsequent B16-F10 tumour challenge given intravenously (Figure 12b). As previously discussed, the lung site may have more resident immune cells or may be more accessible to these cells as it is a point of entry for pathogens (192). Lung surface metastases also provide us with a quantifiable method of assessing the immune response. Unlike the CT26lacZ cells, the B16-F10 cells were able to grow in the lung even after the mice had previously grown a primary tumour. This is most likely due to the poor immunogenicity of the B16-F10 cells (198), which are not able to generate an anti-tumour immune response on their own. Preoperative treatment with VSVΔ51GM-CSF or VSVΔ51 did not show a significant decrease in the number of surface lung metastases indicating that a robust anti-tumour immune response was not established (Figure 13b). VSVΔ51 preoperative treatment of the primary B16-F10 tumour showed a slight decrease in surface metastases compared to VSVΔ51GM-CSF treatment.

Upon intravenous (IV) delivery of VSV, the bulk of virus is taken up by the spleen. A minor fraction is delivered to the tumour and self-amplifies (JP unpublished data, (176)). Although the clearance from all organs is quick, preliminary results suggest that a robust anti-viral response to

VSVΔ51 is generated (Figure 13c). Interestingly, the IFN-ɣ production from $CD3^+CD8^+$ T cells in response to VSVΔ51GM-CSF appears to be higher than the production to VSVΔ51. It is possible that the GM-CSF transgene is helping to elicit a higher anti-viral immune response.

Due to the high immunogenicity of viruses and viral vectors encoding tumour antigens, there is often a smaller magnitude of expansion for the tumour antigen. The heterologous prime-boost approach introduced previously aims to skew the expansion of immune cells in favour of the target antigen rather than the viral proteins (106, 199). It may be possible that VSVΔ51GM-CSF given intravenously may be stimulating a robust anti-viral immune response, minimizing the anti-tumour immune response. Similarly, the slightly larger anti-tumour immune response seen with VSVΔ51 treatment may be due to the smaller anti-viral response observed. It would be crucial to evaluate the anti-tumour immune response to address this possibility.

4.7 An anti-tumour immune response capable of reducing the number of B16-F10 lung surface metastases was generated with preoperative JX594mGM-CSF IT treatment of a B16-F10 tumour

In order to assess whether another oncolytic virus, specifically JX594mGM-CSF, could generate an anti-tumour immune response capable of reducing the number of lung metastases when given preoperatively, we used the B16-F10 surgical model. The route of administration in clinical trials with JX594mGM-CSF has been intratumourally (IT) and IV. We assessed both of these routes of administration. Preoperative treatment with JX594mGM-CSF IT showed a significant decrease in the number of surface lung metastases indicating that a robust anti-tumour immune response was established (Figure 14b). Unexpectedly, JX594mGM-CSF treatment IV of the primary tumour showed a slight increase in the number of surface lung metastases. In characterizing this model, B16-F10 cells were found to be poorly susceptible to JX594hGM-CSF infection *in vitro* (Figure 14c). This addresses the question previously discussed – whether robust oncolysis is required for

the generation of a robust anti-tumour immune response. In this case, it appears that robustness of viral replication may have not correlated with subsequent anti-tumour immunity, but this question will need to be further addressed.

JX594mGM-CSF administered IT may have better delivery and replication at the tumour site than JX594mGM-CSF administered IV. Many immunotherapies are administered IT to ensure the arrival of the therapeutic at the tumour site. These IT immunotherapies have also been shown to activate dendritic cells efficiently and elicit robust local inflammation subsequently inducing tumour regression (73, 120, 200). Although we have seen minimal delivery of VSVΔ51 to the tumour site after IV injection, more replication is seen in B16-F10 tumours if the virus is given IT (JP unpublished data). The question of delivery of JX594mGM-CSF administered IV could be addressed by examining the tumours for viral presence or replication by immunohistochemistry or by a plaque assay.

JX594mGM-CSF IT treatment could be causing the local inflammation required to stimulate an immune response, while JX594mGM-CSF IV may not be. A recent study using a recombinant adenovirus vector expressing interferon-α, which has antiviral activity in addition to several anti-tumour properties, was found to be superior at generating anti-tumour immunity through an intratumoural route in comparison to intravenous administration (194). The improved generation of anti-tumour immunity was found to be due to the upregulation of co-stimulatory molecules on DCs isolated from regional lymph nodes when the adenovirus vector expressing IFN-α was given intratumourally. This recent observation comparing an intratumoural route of administration to an intravenous route of administration corroborates the mechanism of efficacy in intratumoural vaccines. Insufficient local inflammation and insufficient activation of draining lymph node DCs may be a factor in our observation that JX594mGM-CSF IV does not generate anti-tumour immunity.

The context of antigen recognition is of crucial importance to the subsequent immune response generated against the foreign antigen *in vivo*. Some of the factors that influence the magnitude and type of response are antigen dose, the physical nature of the antigen, the route of administration or entry of the antigen, and the presence or lack of an adjuvant (201). There have been a large number of studies that have shown that the route of administration of an antigen leads to a differential immune response (202-206). The route an antigen is given through and seen by the host does not only impact what cells will be activated, but the magnitude of that activation as well.

Viruses are highly immunogenic and composed of multiple antigens resulting in an enormous expansion of virus-specific immune cells. A study of murine infection by human immunodeficiency virus (HIV) -1 examined the type of immune response generated by subcutaneous (SQ), intraperitonal (IP), and IV immunization (203). It was observed that SQ and IV injection of antigen resulted in the generation of specific antibodies alone, while IP injection generated both antibody production and antigen-specific T cell proliferation. A recombinant semliki forest virus vaccine showed that the magnitude of T cell response towards the vector varied depending on the route of administration of the vaccine (206). IV and IM delivery elicited higher CTL levels compared to mice that were immunized through the SQ route. In a study of pseudorabies virus, which is an α-herpes virus, the anti-viral response was examined through various routes of delivery. The routes examined were IP, IV, SQ, and in the footpad (FP) (204). CTL activity against the virus was only detected in mice that received injection by IP, IV, and FP. Vaccinia virus injection into mice has been shown to mount massive $CD8^+$ T cell activation and expansion (207, 208). A VV vector has also been shown to induce less vaccinia-specific CTLs when given SQ than when given IV (209). Similar to the SQ injection, IT treatment of JX594mGM-CSF could be stimulating less vaccinia-specific CTLs than when given IV. This smaller expansion of anti-viral CTLs could potentially allow for a larger expansion of an anti-tumour immune response. The IV delivered JX594mGM-CSF could

be stimulating a large CTL response and inversely, a small anti-tumour response may be generated. The magnitudes of the anti-tumour immune response in this setting would be important to examine. It would also be interesting to administer VSVΔ51 and VSVΔ51GM-CSF in the intratumoural route.

4.8 Conclusions

The overarching hypothesis of this project was that neoadjuvant therapy with oncolytic viruses could generate an anti-tumour immune response capable of protecting the host against the development of metastatic disease. We have generated several murine models of surgically resectable cancer and subsequent development of metastatic disease, such that, it would mimic the clinical model and provide us with the ability to assess our hypothesis. In the CT26lacZ surgical model, we developed a model with consistent tumour challenge outgrowth by minimizing the immune recognition of the primary tumour by using a tumour implant. In this model, we showed that preoperative VSVΔ51GM-CSF treatment of the primary tumour was unable to generate an anti-tumour immune response capable of rejecting the challenge tumour. Immunity capable of rejecting the challenge tumour was seen with VSVΔ51GM-CSF treatment of a primary tumour with no surgical intervention. The abrogation of immunity against the challenge tumour was found to be due to the effects of surgery, although this effect was found to be transient. When the effects of surgery were reversed or had diminished, the CT26lacZ cells were determined to generate a specific immune response capable of rejecting a tumour challenge, without the use of an OV treatment. Although VSVΔ51GM-CSF and VSVΔ51 preoperative treatment of a B16-F10 primary tumour were unable to cause a statistically significant reduction in the number of lung metastases upon post-surgical challenge, we conclude that JX594GM-CSF preoperative intratumoural treatment is effective at reducing the number of lung metastases resulting from a post-surgical challenge.

Overall, this suggests that neo-adjuvant administration of OV with surgery is a feasible approach given appropriate selection of the OV strain used.

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Contributions of Collaborators

Chantal Lemay cloned and rescued VSVΔ51 expressing the transgene GM-CSF.

Lisa Ferreira and Theresa Falls performed all intravenous and intratumoural injections and aided me with subcutaneous injections and surgeries.

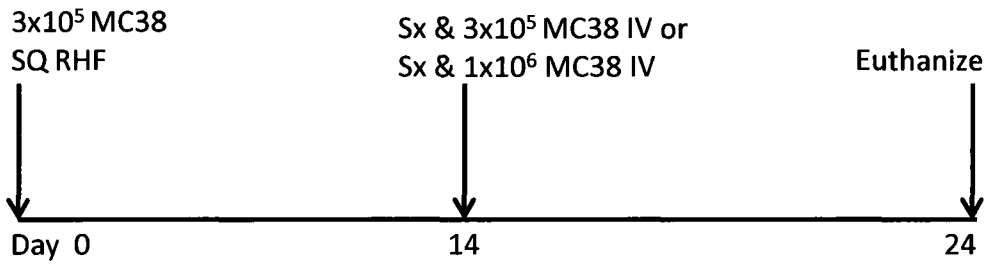
Christiano de Souza aided me with subcutaneous injections and euthanizing C57/Bl6 mice.

Dr. Fabrice LeBoeuf provided the JX594mGMCSF that was used in Figure 14.

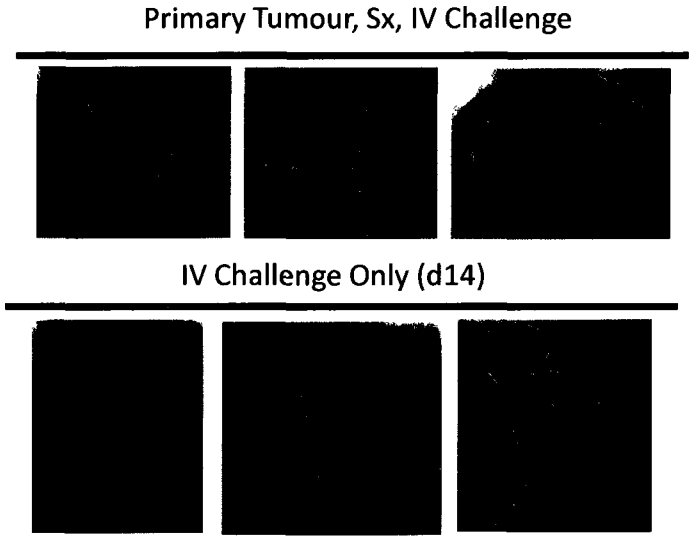
Dominique Vaillant provided the JX594hGMCSFgfp that was used in Figure 14.

Appendix I. **Assessment of MC38 tumour growth in the lung after MC38 primary tumour resection.** C57/Bl6 mice were injected with $3x10^5$ MC38 cells SQ in the right hind flank. On day 14, MC38 tumours were surgically resected from the right hind flank and the mice were injected with $3x10^5$ or $1x10^6$ MC38 cells IV. Control naïve C57/Bl6 mice were injected with $3x10^5$ or $1x10^6$ MC38 cells IV only. On day 24, mice were euthanized, their lungs removed, and examined for lung tumours by visual inspection. (A) Experimental setup (B) Images of lungs taken out of mice on day 24 that received $3x10^5$ MC38 cells IV (C) Images of lungs taken out of mice on day 24 that received $1x10^6$ MC38 cells IV.

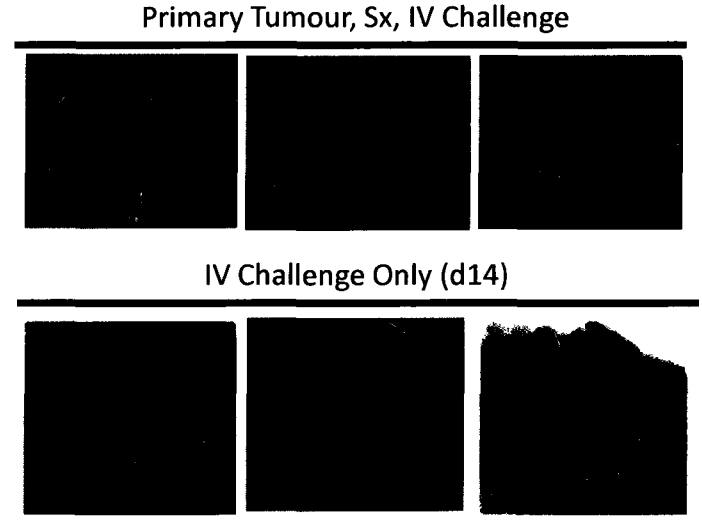
A


3x10^5 MC38
SQ RHF
Sx & 3x10^5 MC38 IV or
Sx & 1x10^6 MC38 IV
Euthanize
Day 0
14
24

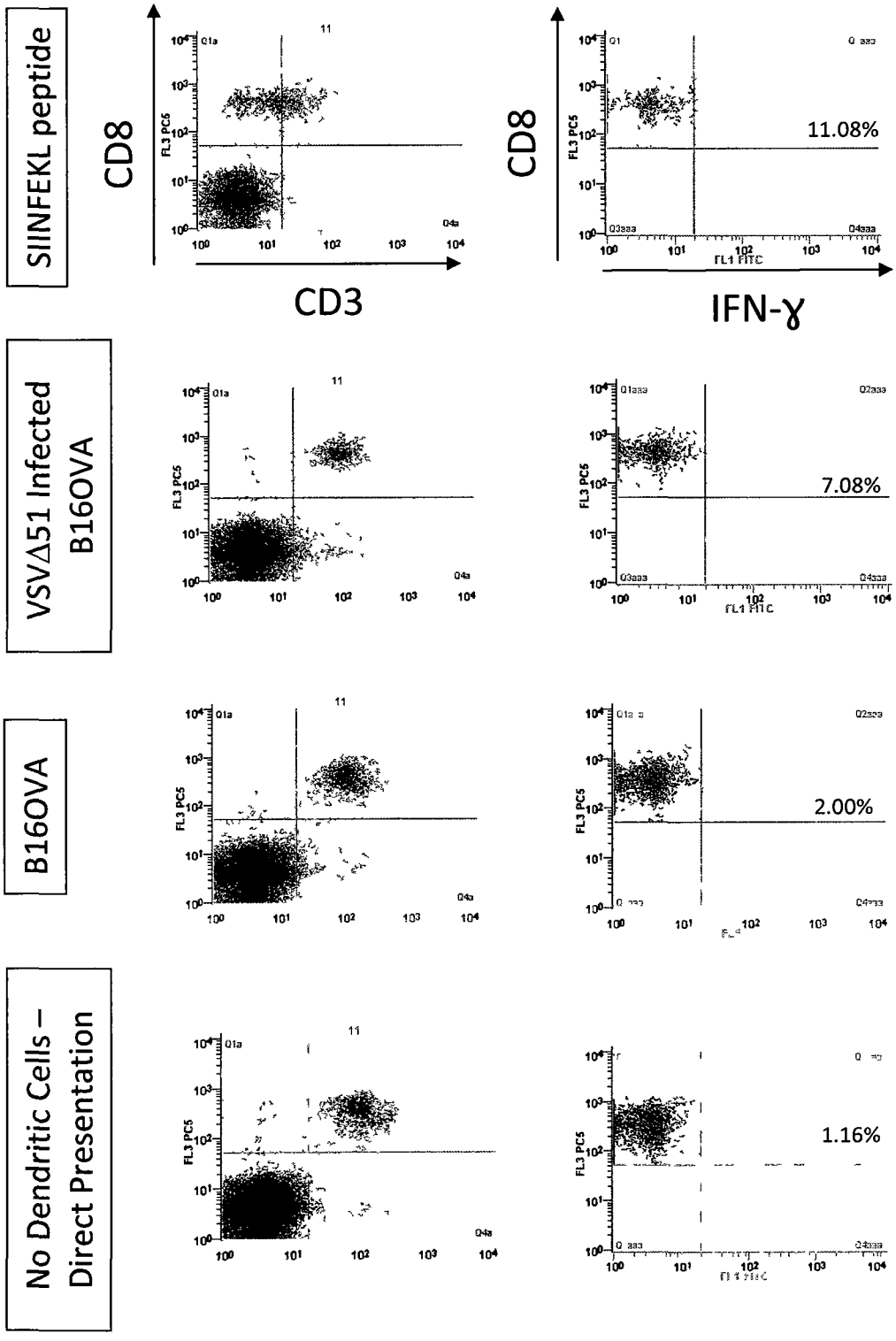
B


Primary Tumour, Sx, IV Challenge
IV Challenge Only (d14)

C

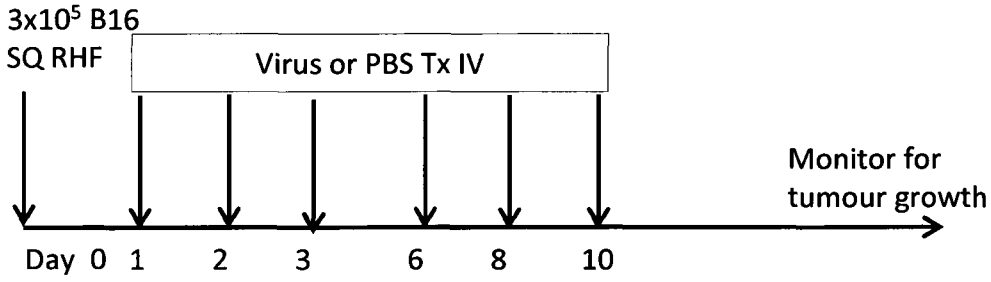

Primary Tumour, Sx, IV Challenge
IV Challenge Only (d14)

Appendix II. **Comparison of IFN-γ production by $CD8^+CD3^+$ cells after incubation with B16OVA cells or VSVΔ51 infected B16OVA cells.**
$5x10^5$ B16OVA cells were infected with VSVΔ51 at a MOI of 10. $1x10^5$ JAWS II cells were incubated with B16OVA cells for 18 hours. $1x10^5$ splenocytes from an OT-I mouse were incubated with JAWS II and B16OVA for 5 hours. Surface and intracellular cytokine staining was then performed for CD3, CD8, and IFN-γ.

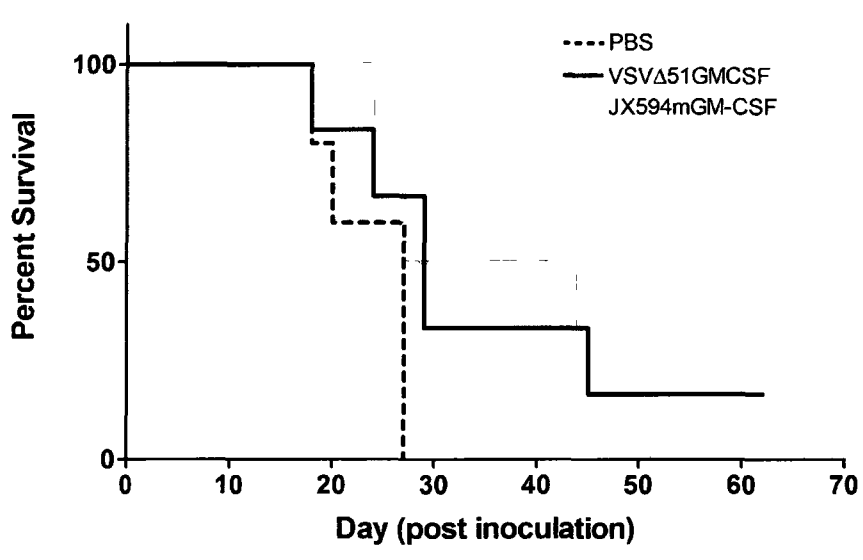

SIINFEKL peptide
CD8
CD3
CD8
IFN-γ
11.08%
VSVΔ51 Infected B16OVA
7.08%
B16OVA
2.00%
No Dendritic Cells – Direct Presentation
1.16%

Appendix III. **Comparison of B16-F10 tumour growth SQ in C57/Bl6 mice with JX594mGM-CSF or VSVΔ51GM-CSF treatment IV**. C57/Bl6 mice were injected with $3x10^5$ B16-F10 SQ in the right hind flank. On day 1, 2, 3, 6, 8, and 10, mice were treated with $5x10^8$ pfu VSVΔ51GM-CSF or $1x10^7$ pfu JX594mGM-CSF IV. Tumour growth was monitored and mice were euthanized when tumour size exceeded $750mm^3$. (A) Experimental setup (B) The percent survival is plotted in a Kaplan Meier Survival Plot.

A

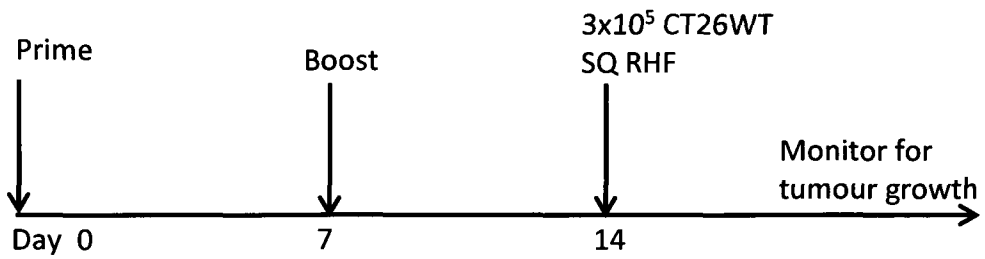

3x10^5 B16
SQ RHF
Virus or PBS Tx IV
Monitor for
tumour growth
Day 0 1 2 3 6 8 10

B

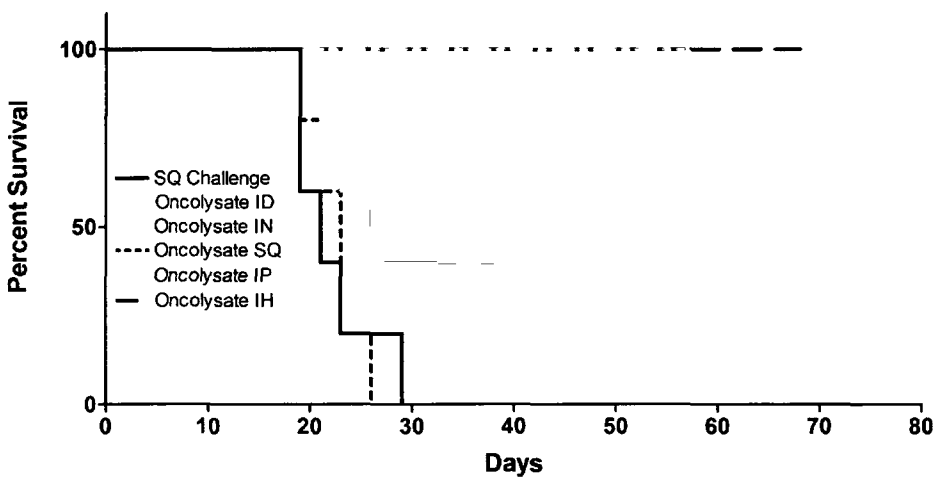

PBS
VSVΔ51GMCSF
JX594mGM-CSF
Percent Survival
100
50
0
0 10 20 30 40 50 60 70
Day (post inoculation)

Appendix IV. **Comparison of vaccine routes of administration on efficacy against a subsequent tumour challenge.**
$5x10^6$ CT26WT cells were gamma irradiated (60 Gy) and subsequently infected with $5x10^7$pfu VSVΔ51GM-CSF for 2 hours. This oncolysate was given to Balb/c mice intraperitoneally, intrahepatically, intranodally, subcutaneously, or intradermally on days 0 and 7. $3x10^5$ CT26WT cells were given SQ on the right hind flank on day 14. Tumour growth was monitored and mice were euthanized when tumour size exceeded $750mm^3$. (A) Experimental setup (B) The percent survival is plotted in a Kaplan Meier Survival Plot.

A


Prime
Boost
3x10^5 CT26WT
SQ RHF
Monitor for
tumour growth
Day 0
7
14

B


Percent Survival
100
50
0
SQ Challenge
Oncolysate ID
Oncolysate IN
Oncolysate SQ
Oncolysate IP
Oncolysate IH
0
10
20
30
40
50
60
70
80
Days

Curriculum Vitae

Education

2008 – present: University of Ottawa, Ottawa Ontario
M.Sc. Biochemistry, Specialization in Human and Molecular Genetics

2004 – 2008: Queen's University, Kingston Ontario
B.Sc. Honours, Subject of Specialization in Life Sciences

Research Experience

September 2008 – present: Thesis Based Masters at the University of Ottawa, Department of Biochemistry
Supervisors: Dr. John Bell & Dr. Rebecca Auer
Project Title: Neoadjuvant Oncolytic Virus Therapy in a Murine Model of Cancer Surgery

September 2007 – May 2008: 4th Year Research Project at Queen's University, Department of Microbiology and Immunology
Supervisor: Dr. Sameh Basta
Project Title: Relationship of macrophage maturation on the ability to present virus antigens

Summer 2007: Summer Employment at Queen's University, Department of Microbiology and Immunology
Supervisor: Dr. Sameh Basta
Project Title: The role combined toll-like receptor ligation plays on APC activation

Publications

Cross, but not direct, presentation of cell-associated virus antigens by spleen macrophages is influenced by their differentiation state. Alatery A, Siddiqui S, Chan M, **Kus A**, Petrof EO, Basta S. Immunology and Cell Biology. 2010 Jan; 88(1):3-12.

Combined, non-simultaneous, TLR engagement prior to virus infection alters nitric oxide production influencing antigen presentation in an epitope dependent manner. Siddiqui S, Alatery A, **Kus A**, Basta S. Journal of Leukocyte Biology. Under Revision.

Developing a Neoadjuvant Murine Surgical Model of Cancer using Vesicular Stomatitis Virus. **Kus A**, Mackenzie L, Lemay C, Parato K, Atkins H, Bell J, Auer R. Abstract Accepted and Published in the Journal of Immunotherapy. September 23, 2010.

Scholarships & Awards

November 2010: 2nd Place Poster Presentation (MSc Category) at OHRI Research Day
September 2009 – August 2010: Ontario Graduate Scholarship in Science & Technology
September 2009 – August 2010: University of Ottawa's Excellence Award
September 2009 – August 2010: University of Ottawa's Excellence Award
December 2009: University of Ottawa's Cash with Admission
March 2008: 2nd Place Poster Presentation at Queen's Microbiology & Immunology Department Poster Day
2007 – 2008: Dean's Honour List

Poster Presentations

First Author Presentations:

- Poster Entitled: Developing a Neoadjuvant Murine Surgical Model of Cancer using Vesicular Stomatitis Virus.
  - Ottawa Hospital Research Institute Research Day, *November 18, 2010*, Ottawa, ON.
  - International Society for Biological Therapy of Cancer Annual Scientific Meeting, *September 30 – October 4, 2010*, Washington DC.
  - Ontario Institute for Cancer Research Annual Scientific Meeting, *February 28 – March 2, 2010*, Alliston ON.
  - Ottawa Hospital Research Institute Research Day, *November 27, 2009*, Ottawa, ON.
- Poster Entitled: Anti-tumour Immunity Generated by Preoperative Oncolytic Virus Therapy in a Murine Surgical Cancer Model
  - University of Ottawa, Biochemistry, Microbiology, and Immunology Poster Symposium, *May 17, 2009*, Ottawa, ON.
  - 5th International Meeting on Replicating Oncolytic Virus Therapeutics, *March 18 – 22, 2009*, Banff, AB.
- Poster Entitled: Effect of Macrophage Maturation on their Ability to Present Virus Antigens
  - Microbiology & Immunology Poster Symposium, *March 2008*, Queen's University, Kingston, ON.

Co-Author Presentations:

- Keystone Symposia: Mobilizing Cellular Immunity for Cancer Therapy, *January 11-16, 2009*, Snowbird, Utah.
- Canadian Society of Immunology Meeting, *April 11 – 14, 2008*, Mont Tremblant, Quebec.

Employment History

Summer 2008: Ottawa Health Research Institute, Supervised by Dr. John Bell & Dr. Rebecca Auer
September 2007 – April 2008: Common Ground Café, Server
Summer 2007: Queen's University, Department of Microbiology and Immunology, Supervised by Dr. Sameh Basta
September 2006 – April 2007: Beauty Bar, Customer Service
Summer 2006: Stoney's Restaurant, Server
Summer 2005: Calculus Tutor
Summer 2005: La Casa Ristorante, Server
Summer 2005: 29 Park Nightclub, Server

Selected Extracurricular Activities

2010 – Present: Children's Hospital of Eastern Ontario: Child Life Program: Playroom Monitor (Oncology, In-patient medicine, & Emergency Department)

- Engaged patients and siblings in recreational play activities
- Planned, prepared, and adapted activities according to patients needs
- Aimed to alleviate stress associated with hospitalization and illness

2008 - Present: Let's Talk Science

- Let's Talk Science is a volunteer program to engage children, youth, and adults in learning and improve scientific literacy

- Prepared lectures and carried out demonstrations for elementary and secondary school students in various topics: The Ethics of Research Involving Animals, The Hallmarks of Cancer, Skeletal System, Digestive System, Butterfly Biology
- Judged elementary school science fairs

2006 – 2008: Queen's University Model Parliament

- Participated in Model Parliament by drafting mock legislation and engaging in parliamentary debate
- Served as Minister of Fisheries and Oceans, 2006-07
- Served as Critic for Economic Development (Atlantic Canada), 2007-08

2006 – 2008: Exposure Arts Festival Chair

- Exposure is an annual week-long arts festival organized by students at Queen's University to enrich students' university experience through music, fine art, literature, dance, and drama
- Head organizer of the festival which consisted of 12 committee members
- Drafted a budget of $14,000, and was responsible for financial record keeping and ensuring the organization remained on budget
- Generated revenue through fundraising and ticket sales