# TREATMENT OF MURINE TUMORS WITH RECOMBINANT MYXOMA VIRUSES

BY

ROSALINDA ANN DOTY

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in VMS-Veterinary Pathobiology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2012

Urbana, Illinois

Doctoral Committee:

Clinical Assistant Professor Amy MacNeill, Chair, Director of Research
Professor Matthew Wallig
Associate Professor Timothy Fan
Professor Dongwan Yoo
Professor Mariangela Segre

## ABSTRACT

Oncolytic viruses are designed to be novel cancer treatments that not only specifically target cancer cells, but can act as vectors to express proteins of interest within the tumor bed. Myxoma virus (MYXV), a rabbit-specific poxvirus, was evaluated as an oncolytic viral treatment for cancer in immunocompetent mouse models of cancer. In addition, a recombinant MYXV that expresses the murine gene for interleukin 15 (IL-15) was also used, in order to determine if increased expression of IL-15 within the tumor bed could improve MYXV treatment by recruiting additional anti-tumor immune cells to the tumor. *In vitro* experiments evaluated MYXV infectivity in murine melanoma, glioma, and lymphoma cells using a recombinant MYXV that expresses the fluorescent protein tdTomato (MYXV:Tomato). These studies indicated that B16-F10 melanoma cells were fully-permissive to MYXV infection, while GL261 glioma cells were partially permissive, and EL4 cells were non-permissive. All stages of virus morphology were observed in the permissive cells and there was evidence of cell-to-cell spread of MYXV. Other studies have shown that the susceptibility of human cancer cell lines to MYXV infection depends on the presence of phosphorylated Akt, which was present in the B16-F10 melanoma and GL261 glioma cell lines. Cancer cell death occurred following infection with MYXV and was likely due to cell lysis and necrosis as apoptosis was not evident at 48 hours post infection. Initial *in vivo* studies using immunocompetent mice bearing intracranial melanoma or lymphoma tumors demonstrated that a single inoculation of intratumoral (IT) MYXV:Tomato was safe but did not produce any significant increase in survival, while studies using intracranial GL261 were inconclusive. However, other studies have shown that MYXV treatment of melanoma not located in the brain can increase survival if multiple injections are given. Therefore, weekly IT injections of MYXV or MYXV expressing the gene for murine IL-15 (MYXV:IL15) were administered in an immunocompetent mouse model of subcutaneous melanoma. The injections were safe and MYXV:IL15 did not cause any detectable adverse clinical signs or any significant increase in viral clearance. A statistically significant increase in survival time was obtained in mice given repeated IT injections of MYXV or MYXV:IL15 as compared to controls. The survival time in tumor-bearing mice treated with MYXV expressing IL-15 was not significantly different than mice treated with MYXV. In addition, MYXV-driven expression of IL-15 was evaluated to determine if it would recruit natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) to the tumor. An extensive pathologic study of the mouse model including histopathology and immunohistochemistry (IHC) was performed at various time points to determine the levels of immune cell infiltration in the tumor and the effects of intratumoral virus inoculation. The number of lymphocytes and neutrophils observed histologically to be infiltrating into the tumors as well as into the tissue surrounding the tumors was significantly increased in mice treated with live MYXV or MYXV:IL15 versus PBS treated control mice. However, IHC revealed that a statistically significant increase in the number of infiltrating cytotoxic lymphocytes (NK cells or CTLs) was not present in MYXV:IL15 treated mice versus control mice, although increased levels of IL-15 were detected within the tumors. Serum neutralization assay revealed that some of the mice treated with MYXV, MYXV:IL15, or UV-MYXV, developed an antibody response to MYXV. The presence of an antibody response did not correlate with survival time or the amount of leukocyte response observed by histology or IHC. In conclusion, treatment with weekly IT injections of MYXV or MYXV:IL15 significantly prolonged survival in mice bearing B16-F10 tumors, which correlated with an

increase in the number of neutrophils and lymphocytes infiltrating in and around the tumors, but MYXV:IL15 did not increase survival over MYXV alone.

*To my husband and children*

## ACKNOWLEDGEMENTS

This project would not have been possible without the support of many people. Many thanks to my adviser, Amy MacNeill, who helped with the many revisions needed to complete this thesis. Also thanks to my committee members, Timothy Fan, Mariangela Segre, Matthew Wallig, and Dongwan Yoo, who made time out of there busy schedules to help me finish this thesis. Thanks to Pfizer for awarding me a Veterinary Scholars Fellowship and to the University of Illinois Department of Pathobiology for awarding me a graduate research assistantship, without which I could not have completed this project. And finally, thanks to my husband, parents, and children who endured this long process with me, always offering love and support.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

# CHAPTER 1

# INTRODUCTION

There is a need for novel treatments of cancer that are targeted, effective, and do not cause immunosuppression. We believe that treatments that activate the immune system and recruit it to the tumor can result in an antitumor immune response, increasing survival. To accomplish this, we used myxoma virus and a myxoma virus that expressed murine interleukin-15 as a treatment for murine tumors in immunocompetent mice and monitored survival time as well as the immune response at various time points. This introductory chapter discusses the many considerations that need to be taken into account when using a virus to treat cancer including current cancer treatment results, safety of virus treatment, pathogenesis, anti-tumor mechanism, and tumor biology and immunology.

## Current cancer treatment modalities

For the past several decades, treatment of cancer has centered on surgical tumor removal, chemotherapy, and radiation, with few other treatments utilized. Of those treatments, surgery and radiation are targeted to the tumor by the attending physician based on macroscopic visual clues such as gross visualization of the tumor and medical imaging which cannot detect small metastases. Both surgery and radiation purposefully require involving the adjacent normal tissue in order to make sure that the entire tumor is targeted. Chemotherapy targets rapidly growing cells, which can affect metastases, but also kills rapidly proliferating normal tissues such as the gut epithelium, hair follicles, and the bone marrow, resulting in side effects such as mucositis (nausea, vomiting, diarrhea), hair loss, and immunosuppression, respectively. Immunosuppression can lead to life-threatening secondary infections and is a significant cause of

post cancer treatment complications. In addition, some cancers such as malignant melanoma are not amenable to surgical removal (depending on location), are considered radioresistant, and/or do not respond well to chemotherapy.

Chemotherapy and radiation treatment also predispose the treated patients to increased development of additional cancers due to their mechanism of damaging deoxyribonucleic acid (DNA). A study of over 1500 Hodgkin's lymphoma cancer survivors found that the risk of developing a second cancer was over 5 times that expected for the normal population (Tucker et al. 1988). Some secondary cancers occur at even higher rates, such as breast cancer (20% incidence by 45 years of age) and thyroid carcinoma (36-fold increased rate) (Neglia et al. 2001). Cardiac disease is second to neoplastic disease as a cause of post-treatment death for Hodgkin's disease (Hancock, Donaldson, and Hoppe 1993), and a study of over 14,000 patients found a 2-5 increased risk of heart disease in patients treated with 250mg/$m^2$ of anthracyclines (Mulrooney et al. 2009). Certain chemotherapeutics are also known to cause specific complications, such as irreversible ototoxicity (Einar-Jon et al. 2011).

Long-term risks from radiation exposure are dependent upon the area irradiated, the dosage of radiation received, and the age of the patient. They include: 1) a two- to six-fold increased risk of developing congestive heart failure, myocardial infarction, pericardial disease, and valvular abnormalities if the heart has received 15 gray (Gy) or more (typical treatment doses range from 20-80 Gy) (Mulrooney et al. 2009), and a 45-fold excess mortality risk of acute myocardial infarction in patients treated with more than 30 Gy of mediastinal radiation before the age of 20 (Hancock, Donaldson, and Hoppe 1993), 2) a severely increased risk of hypothyroidism; up to 51% in patients treated with 35-45 Gy to the neck (von der Weid 2008), 3) a 1.8 fold increased risk of developing diabetes mellitus, with even higher odds in patients

who received whole body, abdominal, or cranial irradiation (Meacham et al. 2009), 4) an increased risk of ocular problems such as cataracts, double vision, legal blindness, and dry eye if the ocular area has been irradiated (Whelan et al. 2010) significantly increased rates of stillbirths and neonatal deaths in women who have received 10 Gy or more, and in young girls who received 1 Gy or more, of radiation to the uterus or ovaries (Signorello et al. 2010).

The 2010 annual report on the status of cancer in the United States (an annual collaboration of the American Cancer Society, the Centers for Disease Control and Prevention (CDC), the National Cancer Institute, and the North American Association of Central Cancer Registries) concluded that overall cancer rates are declining by approximately 1% per year due to a combination of prevention, early detection, and treatment (Kohler et al. 2011). However, low survival continues to be a problem for some cancers, such as glioblastoma, in which the 5 year survival rate has only recently exceeded 20% (Kohler et al. 2011). In addition, some cancers were found to have a statistically significant increase in death rates, including melanoma and liver cancer in men, and liver and pancreatic cancer in women (Kohler et al. 2011). Novel treatments are needed to treat these cancers that do not respond well to traditional therapies.

**Cancer resolution secondary to infection**

The first documented indication that viruses could treat cancer occurred just over a century ago. In 1910, Dr. De Pace reported a case at the International Cancer Congress of a woman with a large vegetating cervical carcinoma who experienced complete remission of her tumor after being bitten by a rabid dog and undergoing treatment with the Pasteur-Roux live attenuated rabies vaccine (De Pace 1912). This sparked several studies with intentional treatment of cancer patients with the rabies vaccine, in which partial remissions were observed (Pack 1950). Another case report described remission of untreated chronic lymphocytic

leukemia following revaccination for smallpox (Hansen and Libnoch 1978). Other cases of cancer remission following viral infection have also been reported throughout the last century, including complete remission of lymphocytic lymphosarcoma following viral hepatitis (Weintraub 1969), 5 cases of complete remission of Hodgkin's disease following measles (Taqi et al. 1981), and 4 cases of partial remission of leukemia in children after contracting chickenpox (Bierman et al. 1953) Intentional treatment of various cancers with several different viruses was attempted sporadically, primarily in the early to mid 20$^{th}$ century, with partial remissions typically obtained (Bierman et al. 1953; Okuno et al. 1978; Nemunaitis 1999; Moore 1957; Southam and Moore 1952). Due to the lack of complete remissions, the use of viruses as a treatment of cancer was largely abandoned until recently. Modern day advances in the ability to manipulate the viral genome to attenuate viruses or to add genes of interest, has resulted in the possibility of engineering oncolytic viruses with increased efficacy and safety.

**Viral cancer therapy**

Viruses have shown promise as a way to preferentially kill neoplastic cells by selectively replicating within and lysing them (oncolytic virotherapy) as well recruiting the immune system to the tumor bed. Due to initial concerns about the safety of using viruses in humans, particularly in immunocompromised cancer patients, several engineered viruses were manipulated to make them replication-incompetent mutants. However, the lack of viral replication resulted in minimal effects *in vivo*. Studies now typically use viruses that replicate normally, or use conditionally-replicative viruses. Conditionally replicative viruses are engineered so that they are only able to replicate in neoplastic cells, and not in normal, non-transformed cells. Recently, viral engineering has centered on methods to make the viruses more effective for cancer treatment, often by attempting to recruit the immune system. Recombinant

viruses can be used for targeted delivery of tumor-associated antigens (vaccine virotherapy), cytokines/chemokines (viral immunotherapy), or cancer modulating chemicals (drug virotherapy) to the tumor bed. Viruses can even deliver chemotherapeutics that can increase permissiveness of the neoplastic cells to viral infection, or can decrease clearance of the virus. For example, rapamycin is a drug that can increase the levels of phosphorylated Akt in cells, which preferentially allows some viruses to replicate in those cells, and it is an immunosuppressive drug that decreases the immune response and thus decreases the immune system clearance of the virus (Thomas et al. 2011; Lun et al. 2009). The reason that viruses are useful to specifically target neoplastic cells is because viruses are able to exploit the activated or suppressed cellular mechanisms that also support tumor growth (Parato et al. 2005). Many tumor cells already have acquired mutations in key components of the intracellular signaling pathways, disrupting or involving proteins such as Myc, Ras, Akt, PI3K, and p53. These mutations make them susceptible to viral infection by blocking apoptosis, promoting proliferation, and subverting the host's immune response.

**Recent and current oncolytic virus clinical trials**

There are many viruses that are currently being studied that either naturally target neoplastic cells or have been engineered to selectively target and replicate in neoplastic cells. None of the viruses currently being researched are able to treat every type of cancer, and it is unlikely that there will ever be a single virus that will be able to treat every cancer or be 100% effective and safe. Similar to current chemotherapy protocols, oncolytic virotherapy will likely be tailored to treat the specific cancers for which each virus is most effective. However, there are certain properties that are desirable overall for an oncolytic virus (Table 1.1). They include safety for the patient (even if immunosuppressed) and the patient's contacts, lack of

pathogenicity for livestock (or any other animals), lack of the ability to integrate into the patients DNA, low mutation rate (so the virus cannot mutate into a more virulent form), the ability to enter, replicate within, and kill the cancer cells without harming normal cells, the ability to be given systemically (to reach metastases), and the ability to recruit an immune response (which can also target the cancer cells).

China approved the world's first oncolytic virus, H101, in 2005 for the treatment of head and neck squamous cell carcinoma (HNSCC) (Yu and Fang 2007). The virus is a conditionally-replicative adenovirus (CRAd) which has the gene for E1B-55kD deleted, so that it can only replicate in cells lacking functional p53, a tumor suppressor gene (Yu and Fang 2007). Meanwhile, there are many human clinical trials ongoing in the United States and Europe using different oncolytic viruses to treat various cancers (Appendix B). An overview of recent clinical trials with oncoviruses is given in the following paragraphs.

**Adenovirus:** Adenoviruses are non-enveloped, linear, double-stranded DNA viruses with genomes approximately 38 kilobases (kb). They are one of the most extensively studied viruses that have been genetically engineered for oncolysis. Adenoviruses (Ad) are endemic in the human population and are typically associated with mild respiratory infection so many people already have antibodies to them. In order to limit viral replication to cancer cells (conditionally replicative), adenovirus genes that function to neutralize normal cell defense (e.g. E1A, E1B) are deleted or replaced. H101, the replication-selective oncolytic adenovirus that was approved for cancer treatment in China, has a deletion of the gene encoding the E1B-55-kD protein. E1B-55-kD is a protein that is responsible for inactivating p53 in the host cell after infection, preventing apoptosis (Barker and Berk 1987). Therefore, the deletion mutant should only be able to replicate in tumor cells that are defective in p53, and not in normal cells.

Several other adenoviral treatments for various cancers are in human clinical trials. An adenovirus that expresses interferon gamma (TG1042) just finished a phase II trial for treatment of cutaneous T and B cell lymphomas (Dummer et al. 2010), and was found to cause regression of some distant tumors as well as the injected tumors. A phase 1 trial using a single IT injection of adenovirus that is driven by a telomerase reverse transcriptase (TERT) promoter (Telomelysin) was found safe and resulted in a tumor response (Nemunaitis 1999). The wide use of adenovirus is mainly due to its extensive use in gene therapy; however, the attributes that make it ideal for gene therapy (maintaining the transduced cell, evading the immune system, and preventing viral replication) do not make it ideal for oncolytic virotherapy (Thorne, Bartlett, and Kirn 2005) where increased viral replication and cell death are desired.

**Herpesvirus:** Herpesviruses are enveloped, double-stranded DNA viruses with a genome approximately 152 kb, and are generally neurotropic. Herpes simplex 1 virus is typically used for oncolytic virotherapy, and it is endemic in the human population. It usually causes mucosal ulceration but can rarely cause severe medical illness in immunocompetent adults. OncoVEX$^{GM\text{-}CSF}$ (herpes simplex virus 1 JS1/34.5-/47-/granulocyte-macrophage colony stimulating factor) is an oncolytic herpes simplex type 1 virus that caused local and distant tumor regression and increased survival in patients with metastatic melanoma in a Phase II trial, with a 26% response rate (Senzer et al. 2009; Kaufman et al. 2010). The same virus has also had success in Phase I/II trials for squamous cell carcinoma of the head and neck (SCCHN), with a response rate of 82% (Harrington et al. 2010a). NV1020 (a replication-competent highly attenuated herpes simplex virus) demonstrated 50% stabilization of disease in a phase I/II clinical trial for refractory colorectal cancer metastatic to the liver (Geevarghese et al. 2010). HF101 (a naturally mutated herpesvirus with a high replication rate) just finished a phase I

clinical trial to treat unresectable pancreatic cancer, where it demonstrated a 17% partial response rate and 50% stabilization of disease (Nakao et al. 2011). Treatments using herpesviruses have generally been safe with most toxicities limited to grade 1 or 2, rare dose limiting toxicities (DLT), and seroconversion in most patients.

**Reovirus:** Reovirus is a non-enveloped double-stranded segmented ribonucleic acid (RNA) virus. Most humans have antireovirus antibodies, suggesting a high incidence of subclinical infection (Tai et al. 2005). Reovirus type 3 Dearing (RT3D, Reolysin) is a naturally occurring reovirus isolated from the human respiratory and gastrointestinal tracts (Rosen, Evans, and Spickard 1963). Reolysin naturally targets cancer cells with an activated Ras pathway, whether due to a Ras mutation or due to upregulation of epidermal growth factor receptor signaling. Reolysin therefore, has benefits in that it does not require any additional manipulation to enhance its specificity or to modify it to a less virulent form. A phase 1 study evaluating IT treatment with Reolysin combined with radiation therapy for treatment of advanced solid cancers found only mild toxicity, no DLT, and no viral shedding (Harrington et al. 2010b). In addition, 100% of patients had either a partial response or stable disease, depending on the dose of radiotherapy they received (Harrington et al. 2010b).

**Parvovirus:** Parvoviruses are small nonenveloped single-stranded DNA viruses that are only able to replicate in cells in S phase (actively replicating cells). The first clinical trial using parvovirus to treate glioblastoma multiforme (GBM) is currently underway and is scheduled to be given intratumorally (IT) as well as systemically (IV) (Rommelaere et al. 2010).

**Picornavirus:** Picornaviruses are non-enveloped viruses with single-stranded positive-sense RNA viruses. Picornaviruses include coxsackievirus, poliovirus, and Seneca Valley virus (SVV). A recent phase I trial using IV SVV to treat solid tumors with neuroendocrine features

revealed that the virus is safe with no dose-limiting toxicites (DLT) (Rudin et al. 2011). Intratumoral viral replication occurred and antiviral antibodies were detected and correlated with clearance of the virus (Rudin et al. 2011). However, only 3% (1/30) of patients developed stable disease and there were no tumor regressions (Rudin et al. 2011).

**Paramyxovirus:** These are negative-strand RNA viruses that result in syncytia formation in the cells they infect. The viruses most commonly used for oncolytic virotherapy are Newcastle disease virus (NDV) and measles virus (MV). NDV is an avian virus, and is selective for tumors cells that have defective interferon (IFN) production. PV701, a replication-competent strain of NDV, has been found to cause objective responses in humans but can also cause toxicity. A phase 1 trial of PV701 given IV at various doses and times to patients with advanced solid cancers, resulted in 1.6% CR (1/62), and 1.6% PR (1/62), and 22.6% SD (14/62) (Pecora et al. 2002). The most common side effect was flu-like symptoms observed after treatment that decreased in severity with subsequent treatments. However, toxicity was not uncommon, and included grade 3 (pain, tremors, dehydration, hypoxia, hypoglycemia, fever, fatigue, nausea, vomiting) and grade 4 toxicities (dyspnea, diarrhea) (Pecora et al. 2002). One patient with pre-existing pulmonary compromise died after treatment due to respiratory failure. In addition, virus was shed in the sputum and urine at low levels (Pecora et al. 2002). MV is a human pathogen that can cause serious disease, but most people have been vaccinated against it. MV predominantly enters cells by the CD46 receptor, which is overexpressed in some tumor cells. A phase I trial that administered MV IP every 4 weeks for up to 6 doses found that MV is safe, with no DLT, immunosuppression, or virus shedding (Galanis et al. 2010). There was no significant change in anti-MV antibodies after treatment, despite multiple doses (Galanis et al.

2010). There were no objective clinical responses (regressions) but there was dose-dependent disease stabilization in 67% (14/21) patients (Galanis et al. 2010).

**Poxvirus:** Poxviruses have many attributes that are desirable for oncolytic virotherapy that have been extensively reviewed by Thorne (Thorne, Bartlett, and Kirn 2005). Poxviruses are unique in that they can enter a wide variety of cell types (McFadden 2005), as opposed to most other viruses, which can only enter cells that express a specific cell marker. They also naturally tend to target neoplasms, where new leaky vessels are being formed. Poxviruses have a large genome that allows for the insertion of as much as 25 kb of contiguous DNA, enabling the expression of large eukaryotic genes (Kaufman et al. 2002),. This allows poxviruses to be engineered to express therapeutic genes such as tumor antigens and cytokines that could potentially enhance their therapeutic efficacy. In addition, poxviruses have their own replication machinery and therefore remain in the cytoplasm, so recombination of viral DNA into the host DNA has not been reported as it has with other viral vectors (Thorne, Bartlett, and Kirn 2005). Having their own replication machinery enables poxviruses to start transcription immediately upon entering the host cell, resulting in fast and efficient cell-to-cell spread (Thorne, Bartlett, and Kirn 2005). Additionally poxviruses can release infectious virions from the infected cell before it has lysed the cell (CEVs and EEVs), resulting in the detection of free infectious virions as soon as 6 hours post-infection (HPI), versus adenovirus which remains intracellular until the cell is lysed and free virus is not available to infect adjacent cells for 48-72 HPI (Thorne, Bartlett, and Kirn 2005; Shenk 1996).

Poxviruses are large stable, enveloped, double-stranded DNA viruses with genomes that range from 130-300 kb that replicate with high fidelity. Vaccinia virus (VACV) is the poxvirus that is most often used in oncolytic virotherapy, due to its large genome (200 kb) that allows the

insertion of large genes, and its history of use as a vaccine to eliminate smallpox, which has resulted in extensive characterization of its toxicity. JX-594 is a replication-competent thymidine kinase deletion mutant VACV that expresses human granulocyte macrophage colony-stimulating factor (GM-CSF) and lac-Z and is currently in a phase III clinical trial for treatment of unresectable primary hepatocellular carcinoma (Merrick, Ilett, and Melcher 2009). Trovax (MVA-5T4) is an attenuated VACV expressing the tumor antigen 5T4, which is expressed by most solid tumors (Harrop et al. 2010) and has been studied in 2 phase I/II clinical trials and 7 phase II clinical trials. Increased survival has been associated with high antibody levels to 5T4 (Harrop et al. 2010). Due to the many benefits of poxviruses, we are also studying poxviruses, specifically myxoma virus, as novel oncolytic viral therapeutics.

**Poxvirus biology**

The Poxviridae family is composed of many viruses (Table 1.2) which infect numerous animal species. It has two subfamilies, one which infects vertebrates (Chordopoxvirinae) and one which infects insects (Entomopoxvirinae). Chordopoxvirinae has many virus genera, including Orthopoxvirus, with members including vaccinia virus (the vaccine used to eradicate smallpox) and variola virus (the cause of smallpox), among others, and Leporipoxvirus, whose member myxoma virus (MYXV) is the virus used in this study.

Poxviruses infect cells through an unknown mechanism, as a specific cell surface receptor that the virus binds before cell entry has not been identified. Since they are able to enter many cells it has been suggested that poxviruses can either: 1) use many different types of cell receptors, or 2) use a cell receptor that is ubiquitous (such as surface gylcosaminoglycans), or 3) fuse with the plasma membrane itself (Townsley et al. 2006; Carter et al. 2005). Fusion of VACV to the plasma membrane can occur at neutral pH and is mediated by a multi-protein

poxviral entry fusion complex carried by the virion (Moss 2006). Poxviruses are believed to be restricted to infecting certain cell types in specific animal species through mechanisms that affect their replication after they enter the cell. This is different from most other viruses, which can only infect cells that express their virus-specific cell receptor.

Poxviruses produce three forms of infectious particles (virions): intracellular mature virus (IMV or MV), cell-associated enveloped virus (CEV) and extracellular enveloped virus (EEV). IMV particles are the most abundant form of the virus, and are retained within the cell cytoplasm until cell-lysis. IMVs are more numerous and robust and are therefore believed by some to be important in the spread of poxviruses between individuals (Smith, Vanderplasschen, and Law 2002). They are brick-shaped with a lipoprotein shell (core membrane) surrounding a complex core structure that contains the linear double-stranded DNA genome and several virus-encoded proteins (Figure 1.1). The core is surrounded by lateral bodies and an outer membrane. CEVs and EEVs are similar to IMVs but have an additional lipoprotein envelope. They are believed to be responsible for viral spread throughout the body of an individual, and are important for early virus dissemination. Efficient cell-to-cell spread can occur when actin tails are induced to form underneath the cell surface below CEVs, facilitating virus penetration of the surrounding cells (Hiller et al. 1979). Long-range spread of the virus is accomplished by release of the EEVs, although they only represent a fraction of a percent of the total infectious particles. This is due to their being covered by a membrane that provides resistance to destruction by complement and decreased susceptibility to neutralization by antibodies (Smith, Vanderplasschen, and Law 2002).

The virus-encoded proteins within the core of the infectious virions include RNA polymerase, enzymes for RNA capping, methylation, and polyadenylation, and a transcription

factor. These proteins are packaged within the virus core so as to enable early viral protein synthesis after cell entry, allowing rapid replication. Without these proteins, the poxvirus DNA is itself, noninfectious. Poxviruses are able to remain within the host cell cytoplasm because they provide their own replication machinery. The entire poxvirus life cycle takes place within the cytoplasm of the host, and does not enter the nucleus. This feature distinguishes poxviruses from other DNA viruses, which must enter the nucleus to replicate, and makes viral integration into the host DNA highly unlikely.

**Smallpox**

The most notorious poxvirus is the causative agent of smallpox, the orthopoxvirus variola virus (VARV). Smallpox is a highly contagious disease that was previously endemic in every country. It was unmistakably described as far back as the fourth century A.D, although mummified remains suggest that the disease was prevalent as far back as 3000 B.C. (Fenner et al. 1988). Smallpox changed the course of human history, and at times decimated the human population, possibly accounting for up to 10% of all deaths in the world (Barquet and Domingo 1997). Mortality rates vary among types, but the most common type, variola major, has a mortality rate of approximately 20-30% in adults with an even higher rate in young children (Fenner et al. 1988). Death, when it occurs, is believed to be secondary to toxemia and shock (Martin 2002). Early attempts to decrease the mortality rate and severity of disease were initiated as early as 1000 A.D. in China, thru a process known as variolation. In this process, individuals were intentionally exposed to smallpox material (pus or scabs) either by nasal inhalation or through skin abrasions. Variolation typically resulted in a milder form of the disease. Edward Jenner introduced the concept of vaccination in 1796, when he noticed that milkmaids who developed cowpox lesions were resistant to smallpox. He took material from a

cowpox lesion on a milkmaid and inoculated a young boy, who was subsequently exposed to smallpox and found to be resistant. Jenner repeated this experiment on other children with the same outcome. Initially, vaccination against smallpox was performed with cowpox virus, but over time switched to vaccinia virus (VACV), the origin of which is unknown. By the end of the 19th century, the vaccine was produced by harvesting lymph from infected live animals (typically calves).

Since humans are the only known reservoir of VARV, the potential to eliminate the virus was proposed in 1953 and a worldwide global eradication program against smallpox was begun in 1959. The program involved vaccinating a large portion of the human population. The campaign intensified in 1965 and was eventually successful, with the last naturally occurring case of smallpox reported in Somalia in 1977, and with the WHO declaring in 1980 that smallpox had been eradicated (Wehrle 1980).

**Vaccinia virus**

The most studied poxvirus is VACV, and it is the poxvirus most often used in oncolytic virotherapy. The actual origin of VACV is unknown, as it is distinct from VARV, cowpox virus, and all other poxviruses. It emerged sometime in the 19th century, and soon became the smallpox vaccine of choice due to its ability to be cultivated in many different animals and its ability to provide cross-protective immunity against other Orthopoxviruses (Jacobs et al. 2009). There were many different VACV strains; however, the New York City Board of Health (NYCBH) strain, a 1st generation VACV, was the strain used in the eradication program in the Americas and West Africa (Jacobs et al. 2009). Other strains used include: the EM-63 strain (Russia and India), the Lister strain (United Kingdom), the Paris strain (France), the Copenhagen strain (Denmark), the Bern strain (Switzerland), the Ankara strain (Turkey), the Temple of

Heaven and the Vaccinia Tian Tan strains (China), and the Dairen strain (Japan) (Rosenthal et al. 2001). The strains vary in their safety, with fewer side effects noted in the NYCBH strain, and higher rates of complications noted in the Lister and Copenhagen strain, with the highest rates in the Bern strain (Kretzschmar et al. 2006).

While the adverse events are minimal as compared to smallpox, vaccination with VACV can still cause some serious side effects. The most common adverse event after vaccination is mild pain at the inoculation site (47%), with 2-3% reporting the pain as severe. Fever above 100ºF (37.7ºC) occurs in 5-9% of vaccinees, and fever above 103ºF (38.8ºC) occurs in 3% (Kretzschmar et al. 2006). Mild systemic complications, including headache, myalgia, chills, nausea, fatigue, and difficulty sleeping, occur in over 25% of vaccinees. Serious complications reported after vaccination with VACV include erythema multiforme (164.6/million vaccinees), inadvertent inoculation (25.4-529/million vaccinees), and generalized vaccinia (23.4-241/million vaccinees). Inadvertent inoculation includes autoinoculation, where the virus is spread to sites other than the vaccination site by touching the inoculation area and then another site, the most serious site generally being the eyes (Figure 1.2A). Similarly, the virus can also be spread to people in contact with the vaccinated person. Generalized vaccinia (Figure 1.2B) typically occurs in the young or in immunocompromised vaccinees. Vaccinia virus can also cause very uncommon but severe side effects after vaccination such as vaccinia necrosum (a.k.a. progressive vaccinia, 1.5cases/million people vaccinated, Figure 1.2C), eczema vaccinatum (38.5/million, Figure 1.2D), and postvaccinal encephalitis (12.3/million), which result in a rate of 1 death/million primary vaccinations (Rotz et al. 2001). Eczema vaccinatum occurs in people who have eczema or other skin conditions that result in skin compromise, allowing the virus to spread all over the body. Postvaccinal encephalitis is the most lethal complication, causing

inflammation of the brain that is believed to be due to an immune response to viral infection, rather than to spread of the virus to the brain. Vaccinia necrosum tends to occur in people who lack effective cellular immune responses, while generalized vaccinia is believed to be due to immaturity of the immune system. Therefore, there is already evidence that VACV can be a risk in immunocompromised individuals. Recently, vaccination with VACV has been found to cause myopericarditis at a rate of approximately 100/million vaccinees, with rare cases of myocardial infarction and dilated cardiomyopathy (Mora, Khan, and Sperling 2009). Also, VACV has been shown to commonly infect ovarian tissue in addition to neoplastic cells, *in vivo* (Moss 1996; Whitman et al. 1994). Even in modified, less virulent strains, VACV can often be found in the ovaries of mice whether they are inoculated by intraperitoneal or intravenous injection (Suter et al. 2009; Puhlmann et al. 2000).

Due to the widespread vaccination of people up until the 1970's with VACV, many older individuals have previously been vaccinated with VACV, and it unknown how this will affect the ability of the virus to replicate within the tumor bed, or to spread to other tumors in the body. The presence of a pre-existing antibody response to VACV would theoretically result in rapid clearance of the virus by the immune system, limiting the ability of the virus to infect the neoplastic cells and spread to adjacent cancer cells. Despite this, use of vaccinia virus as an oncolytic virus in clinical trials has not been limited by the presence of pre-existing VACV antibodies, similar to studies using measles, reovirus, or adenovirus.

Importantly, VACV is able to infect cattle, and could therefore get into the livestock population, such as has already occurred in Brazil. In Brazil, VACV primarily infects milking cows and dairy workers, but can also be found in nursing calves and in horses. Infection of dairy cattle has resulted in economic losses as cattle can develop secondary mastitis (Leite et al. 2005).

Milk obtained from infected cows has also been shown to contain virus particles which remain infectious after heat treatment at 65ºC for 30 minutes, suggesting that virus particles would be present in solid curds and cheese whey (de Oliveira et al. 2010). The introduction of such infection into the U.S. dairy population would be economically devastating to the cattle industry, in addition to becoming a source of infection to the human population.

**Myxoma virus**

To avoid complications with VACV, myxoma virus was evaluated as a cancer treatment in this study. MYXV is a rabbit-specific poxvirus that causes a lethal infection, termed myxomatosis, in European rabbits (*Oryctolagus cuniculus*) and a benign infection characterized by a cutaneous fibroma in the North American brush rabbit (*Sylvilagus bruneii*) and the South American tapeti (*Sylvilagus brasilensis*) (Fenner 2000; Fenner 1983). Myxomatosis was first reported in 1896 in South America (as reviewed in Stanford, Werden, and McFadden 2007) as a rapid systemic and lethal infection of European rabbits, and was subsequently found to be endemic in the natural rabbit population. It is mechanically (passively) transmitted by arthropod vectors, resulting in an initial primary skin lesion in wild and European rabbits. In European rabbits, the disease progresses with swelling of the muzzle and anogenital region, blepharoconjunctivitis, and formation of secondary edematous (myxomatous) skin lesions (Fenner and Ratcliffe 1965; Fields, Knipe, and Howley 1996). Death typically occurs in 8-15 days and is believed to be due to multi-organ dysfunction and secondary lung infections (Fenner and Ratcliffe 1965; Fields, Knipe, and Howley 1996). Myxoma virus causes even higher rates of lethal infection in European rabbits (near 100%) than VARV caused in humans.

Due to the high mortality rate, the Lausanne strain of MYXV was released in Australia in 1952 in an attempt to control the millions of rabbits that were devastating the country. The

European rabbit had been intentionally released in Australia in 1859 for hunting and, due to the mild winters and lack of predators, rapidly increased their numbers and spread across the country. Myxoma virus infection resulted in a mortality rate of over 99% of the infected rabbits. However, not all rabbits were infected, and in those that were, co-evolution of the virus and the surviving rabbits resulted in a decreased mortality rate (Best, Collins, and Kerr 2000; Kerr and McFadden 2002; Marshall and Fenner 1958; Marshall and Douglas 1961).

Lack of pathogenicity is a requirement for a successful oncolytic virus therapeutic and makes MYXV (a Leporipoxvirus) an attractive alternative to VACV. Shortly after the release of MYXV in Australia, the public raised concerns about the safety of MYXV to the human population. In order to prove that MYXV was safe, 3 scientists injected themselves with live MYXV virus, and suffered no ill effects. While this small population may not prove that MYXV is safe in all people, MYXV has been found to be non-pathogenic for all non-rabbit vertebrate species tested (Fenner and Ross 1994; Burnet 1968; Pignolet et al. 2008), and despite its widespread infection in Australia, no ill effects to the human population have ever been shown. Due to its restricted host range, MYXV has some advantages when compared to VACV for use as an oncolytic virotherapeutic. Since it does not infect people, treatment with MYXV should not result in any adverse events, should not infect ovarian tissue, and there should not be any pre-existing immunity to MYXV in those treated. It also should not raise any concerns about a potential threat to livestock population, with the exception of rabbitries that use European rabbits. However, even this potential threat can be alleviated by manipulation of the virus, as MYXV:IL15 (Liu et al. 2009) and MYXV that expresses the gene for Interleukin 12 (IL-12) (Stanford et al. 2007a) have both been shown to be non-pathogenic in European rabbits. Despite its strict host range, MYXV has been shown to productively infect cultured cancer cells from

several species *in vitro,* including human (Sypula et al. 2004; Woo et al. 2008; Lun et al. 2007), murine (Thomas et al. 2011; Stanford et al. 2008), racine (Lun et al. 2010), canine (Urbasic et al, accepted AJVR 2011) and feline (Macneill et al. 2011). .

**Myxoma virus infection of human cells**

Myxoma virus was shown to productively infect the majority (15/21) of human tumor cell lines in one study (Sypula et al. 2004). The cell lines tested included melanoma, osteosarcoma, prostate, renal, lung, colon, breast, and ovarian cancer cells. Additional studies have demonstrated that 87.5% (7/8) of human malignant glioma cell lines tested were fully permissive to MYXV infection *in vitro* (Lun et al. 2005) and 83.3% (5/6) of human medulloblastoma cell lines tested were fully permissive *in vitro* (Lun et al. 2007). Infection of human acute myelogenous leukemia (AML) CD34+ cells *in vitro* demonstrated a 65% rate of MYXV:GFP infection, while normal CD34+ human hematopoietic stem and progenitor cells (HSPCs) did not have any evidence of MYXV:GFP infection (Kim et al. 2009).

Susceptibility of human tumor cell lines to infection with MYXV has been found to correlate with the level of endogenous phosphorylated Akt (also known as protein kinase B), within the cell (Werden and McFadden 2008). Akt is a serine/threonine kinase that plays a role in the regulation of proliferation, apoptosis, angiogenesis, and metabolism (Datta et al. 1997; Cardone et al. 1998; Pap and Cooper 1998). Tumor cells with high levels of phosphorylated Akt are fully permissive to MYXV infection, while cells that do not express any phosphorylated Akt are resistant to MYXV infection (Wang et al. 2006). Studies have shown that treatment of some tumors with rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR), can increase levels of phosphorylated Akt, enhancing the oncolytic potential of MYXV (Stanford et al. 2007b).

Conversely, cells that are not permissive to infection with MYXV have been shown to induce anti-viral cytokines upon MYXV infection. Human macrophages that were infected with MYXV rapidly co-induced tumor necrosis factor (TNF) and type 1 interferon (IFN), which then aborted MYXV infection in normal human cells in a paracrine-like manner (Wang et al. 2008). The addition of TNF and IFN-β cytokines to primary human fibroblast cell lines has been shown to make them resistant to MYXV infection (Bartee et al. 2009b). These two cytokines induce a synergistic antiviral state, and it has also been demonstrated that the ability to induce this synergistic antiviral state has been lost in most human cancer cells (Bartee and McFadden 2009a).

**Myxoma virus infection of murine cells**

The presence of phosphorylated Akt may be important for MYXV permissiveness in mouse cells as it is in human cells. The murine melanoma B16F10LacZ cell line has been found to have a high endogenous level of phosphorylated Akt and inhibition of Akt kinase activation drastically inhibited the ability of the MYXV to replicate in the cell line *in vitro* (Stanford et al. 2008).

The ability of MYXV to replicate in mouse cells has also been found to depend on disruption of the Erk-interferon-STAT1 signaling pathway (Wang et al. 2004). Interferons (IFNs) are a family of inducible cytokines that are important in innate antiviral defense and trigger the transcription of hundreds of IFN-stimulated genes (ISG) that have antiviral, as well as antiproliferative/antitumor, and immunomodulatory effects (Fensterl and Sen 2009; Samuel 2001). Type I and III IFNs can be induced in virtually all cell types by the recognition of viral pathogen associated molecular antigens (PAMPs), which are conserved molecular patterns present in pathogens, whereas type II IFNs are only induced in certain immune cells (T cells and

NK cells) upon stimulation by specific cytokines, such as IL-12 (as reviewed by Fensterl and Sen 2009). Type I IFNs (IFN-α and IFN-β) are often induced in normal cells (primarily plasmacytic dendritic cells) during viral infections by activation of Erk 1/2 (extracellular signal-regulated kinase) signaling which operates upstream of IFN regulatory factor 3 (IRF3). STAT1 is a transcription factor that is downstream of IFNs and is essential to mediate antiviral effects. Disruption or deficiency of any of the molecules in this pathway renders normally nonpermissive mouse primary fibroblast cells susceptible to infection with MYXV (Wang et al. 2004). This pathway is often disrupted in cancer cells, presumably because cancer cell growth would be limited by the antiproliferative/antitumor actions of the ISGs.

**Myxoma virus cancer treatment *in vivo***

Myxoma virus has shown oncolytic effects against many different tumor models in mice. When two malignant humanglioma cell lines (U87 and U251) were implanted orthotopically in nude (immunodeficient) mice, a single intratumoral (IT) injection of MYXV "cured" the majority (87.5% and 100%, respectively) of the treated mice (Lun et al. 2005). Intracerebral administration of MYXV to the immunodeficient mice was safe, resulting in minimal inflammation and slight weight loss that was not statistically significant (Lun et al. 2005). The "cured" mice had either no detectable tumor (75%) or had residual tumor cells (25%) detectable by microscopic examination of the tissues (histology). Similar findings occurred with a mouse model of human medulloblastoma, in which 60% (3/5) of mice treated with a single IT injection of MYXV were "cured", with 2 of those having no tumor cells remaining and 1 having a very small residual tumor (Lun et al. 2007). However, initial studies using MYXV in immunodeficient mouse models did not examine the immune response to MYXV treatment.

In a study using MYXV in an immunocompetent mouse model of metastatic (lung)

melanoma (Stanford et al. 2008) it was shown that a single intravenous (IV) injection of MYXV did not significantly reduce the number of lung metastases. The addition of rapamycin or the use of multiple MYXV injections did reduce the size or number (respectively) of lung metastases. The same study showed that multiple (daily) injections of MYXV could significantly decrease the size of subcutaneous melanoma tumors in an immunocompetent mouse model (Stanford et al. 2008). Similarly, a study using an immunocompetent racine model of intracranial glioma demonstrated that a single injection either did not statistically improve survival (RG2 tumors) or only slightly prolonged survival (F98 tumors), but survival was significantly increased when rapamycin (an immunosuppressive drug) was given in combination with MYXV (Lun et al. 2010). In order to improve the efficacy of MYXV treatment without having to resort to immunosuppressants, additional factors can be inserted into the MYXV genome such that they are expressed locally within the tumor as the virus replicates. These can range from antiangiogenic factors to factors that stimulate the immune system, or factors that breakdown the connective tissue of the tumor to enhance spread of the virus.

**Tumor biology and immunology**

When considering designing a recombinant oncolytic virus to treat a tumor, there are many factors to consider, including the tumor microenvironment and the immune system. The tumor microenvironment is composed of tumor cells, immune cells, stromal cells, vessels, and extracellular matrix (ECM), as well as soluble factors (secreted by the normal and neoplastic cells), and areas of edema and necrosis. In this sense, the tumor can almost be regarded as an organ, with its own particular requirements for growth and survival. The tumor environment is a place that must foster tumor cell survival, proliferation, and migration, as well as drive the proliferation of new vessels (angiogenesis) to provide nutrition to the cancer cells. The tumor

microenvironment has increased osmotic pressure due to the presence of these new vessels, which are usually abnormal and leaky, as well as due to pressure produced by overgrowth of the tumor cells. This leads to inefficient perfusion, with subsequent heterogeneous areas of hypoxia and acidosis, both of which contribute to resistance to radiotherapy and many chemotherapeutics. Decreased blood flow has also been found to occur after treatment with oncolytic viruses, and can result in tumor cell necrosis, restricting the ability of the virus to spread (Breitbach et al. 2007).

Another contributor to treatment resistance is the ECM, which is composed of complex secretions of proteins and proteoglycans produced by both the neoplastic cells and the tumor stromal cells. The secretion of ECM is increased in many tumors, contributing to the increased interstitial fluid pressure. In addition, the interwoven meshwork of secreted proteins forms a physical barrier that significantly limits the dispersal of therapeutics within the solid tumor. This has also been a problem with viral oncolytics, with virus observed to be restricted to small focal areas within the tumor in several studies (Sauthoff et al. 2003; Markert et al. 2009; McKee et al. 2006; Cheng et al. 2007). This localization can be decreased by pre- or co-treatment with collagenase, hyaluronidase, metalloproteinases, or other proteins that digest the ECM (McKee et al. 2006; Cheng et al. 2007; Kuriyama et al. 2000; Ganesh et al. 2008; Kim et al. 2006).

An equally important aspect of cancer biology is the ability of the tumor cells to evade the immune system. Leukocyte infiltration into tumors was first observed as far back as 1863 by Rudolf Virchow, the father of pathology, who postulated a possible relationship between inflammatory infiltrates and tumor growth (as reviewed in Zou 2005). In 1957, Burnet and Thomas theorized about the existence of host immunological resistance to the development of cancer (tumor immunosurveillance; as reviewed in Zou 2005). However, these theories were not

given much credence until recently due to the lack of documented spontaneous tumor eradication. Failure of the immune system to recognize and eradicate tumors has been thought to be due to one or more deficiencies: 1) antigen-presenting cells (APCs) are not stimulated enough, 2) there are not enough local effector T or NK cells, 3) there are not enough cytokines, or 4) the tumors do not present enough antigen. Indeed, anti-tumor immunity is believed to cause selective pressures that favor the outgrowth of tumor cells with reduced immunogenicity, a process called immunoediting. For example, a recent study (Thomas et al. 2011) using adoptive transfer of T cells targeted to recognize an antigen on tumor cells found that tumors that recurred in the treated animals had low or absent expression of the antigen recognized by those T cells (termed antigen loss variants). Alternatively there could be tolerizing conditions (cytokines and/or cells) present that limit the immune response. For instance, some human tumors have been found to have increased levels of vascular endothelial growth factor (VEGF), IL-6, M-CSF, transforming growth factor-β (TGF-β), IL-10, cyclooxygenase-2 (COX-2), and prostaglandin E2 (PGE2), while having decreased levels of GM-CSF, IL-12 and IFNγ (as reviewed in (Zou 2005). This aberrant cytokine pattern in the tumor microenvironment blocks dendritic cell (DC) differentiation and maturation, thereby affecting the ability to prime an antitumor immune response to tumor-associated antigens (TAAs). Additionally, there are cells that decrease the immune response, such as regulatory T cells (CD4+CD25+Foxp3+ T cells), which are involved in maintaining peripheral tolerance, and myeloid-derived suppressor cells (MDSC), which are a heterogenous group of myeloid-derived cells that are often found in increased numbers in neoplastic conditions.

Improving the immune response to a tumor can theoretically be achieved by replacing the decreased levels of immune system effector cells and cytokines, or by decreasing inhibitors of

the immune system. In those tumors where the immune response does not appear to be suppressed, the presence of infiltrating leukocytes has been shown to correlate with an improved prognosis. The number of tumor infiltrating NK cells has been found to correlate with survival prognosis in colorectal cancer (CRC), gastric carcinoma, and squamous cell lung cancer (Coca et al. 1997; Villegas et al. 2002; Ishigami et al. 2000). High densities of infiltrating lymphocytes have been found to correlate with a good prognosis in many types of human cancer. High IT memory T-cell density in CRCs has been shown to correlate with decreased metastases to lymph nodes and distal organs (Pagès et al. 2005). In a review of 602 CRCs, the patients with the highest densities of CTLs and memory T cells had an 86.2% survival rate 5 years after diagnosis, versus patients with the lowest densities of these cells, which has a survival rate of 27.5% (Pagès et al. 2009). Staging the tumors according to immune cell infiltration was found to be superior to the traditional staging method, and was one of the few independent prognostic factors (whereas tumor stage was not) (Bindea et al. 2011). Tumors with high density of T cell infiltration in the center and at the infiltrating margin, as well as tumors with low helper T cell infiltration, were found to have a better prognosis than tumors with a heterogeneous distribution of T cells between the center and margin, or in tumors with low numbers of infiltrating T cells or higher proportions of helper T cells (Bindea et al. 2011).

Cytokines within the local tumor environment are modified in such a way as to allow tumor cells to overgrow and avoid detection by the immune system. Such modulations typically include decreased or absent IFN production by the tumor cells, which allow viruses to infect and replicate within the tumor, but not in the healthy tissue outside of the tumor. Since tumorigenesis is a slow process that can resemble a chronic infection, the immune response to a persistent tumor shifts from an acute $T_H1$ dominant response to a $T_H2$ dominant response,

drastically altering T cell responses and cytokine expression (Tsung et al. 1997, and as reviewed in Zou 2005). Since the local expression of cytokines can have a profound effect on the local immune cell response, oncolytic viruses have been engineered to express cytokines that can shift the immune response to one that may recognize the tumor, as a form of immunotherapy.

**Interleukin 2**

Interleukin 2 (IL-2) was the first approved immunotherapy that was based entirely on immunomodulation (as reviewed in Dillman 2011). IL-2 was also approved for the treatment of metastatic renal carcinoma in 1992, for the treatment of melanoma in 1997, and for the treatment of metastatic carcinoma in 1998 (Dillman 2011; Eklund and Kuzel 2004). IL-2 does not directly target cancer cells, but exerts its antitumor effects by stimulating cytotoxic T lymphocytes and NK cells. Immunotherapy with high-doses of adjuvant IL-2 is the current standard of care in high-risk stage II and stage III melanoma. It has been found to increase overall survival from 2.8 to 3.8 years, and result in durable complete remission in 4% of patients (Atkins et al. 1999; Algazi, Soon, and Daud 2010). Localized immune therapy has also been used to treat cutaneous melanoma metastases and has been found to induce high rates of complete and partial regressions in the injected tumors, however, visceral metastases were either not affected or subsequently developed (Weide et al. 2010; Radny et al. 2003).

Unfortunately, IL-2 is toxic at high levels, causing grade 3 and 4 toxicities, including hypotension and vascular leakage reminiscent of septic shock (Eklund and Kuzel 2004). In addition, high-dose IL-2 therapy has been documented to cause myocarditis in approximately 3.5% of treated patients (Eisner, Husain, and Clark 2004).

**Interleukin 15**

Interleukin-2 and IL-15 are cytokines with similar biological properties, despite being

different genetically and antigenically. This is likely due to their shared receptor signaling components (IL-2/15R$\beta\gamma_c$). Specificity of function is provided by their unique α-chain receptors that complete the heterotrimeric receptor complexes (IL-2R$\alpha\beta\gamma$ and IL-15R$\alpha\beta\gamma$). It is the distribution of the distinct IL-15Rα and IL-2Rα chains *in vivo* that determines where each cytokine will bind and activate a signaling pathway via the common $\beta\gamma_c$ receptor. In addition, IL-15 and its α-chain receptor have a much broader tissue distribution than IL-2 or its α-chain receptor. Recently, it has been found that IL-15Rα has high-affinity ($K_a \geq 10^{11}$ $M^{-1}$) for IL-15, even in the absence of other receptor components, but only transduces signals if it binds to IL-2/15R$\beta\gamma_c$. Interleukin 15 may bind the IL-2/15R$\beta\gamma_c$ with intermediate affinity ($K_a \geq 10^{11}$ $M^{-1}$) without binding IL-15 Rα (Giri et al. 1995; Giri et al. 1994).

Interleukin-15 is a cytokine produced by multiple tissues, including monocytes, macrophages, kidney, heart, lung, skeletal muscle, placenta, bone marrow stroma, and thymic epithelium (Grabstein et al. 1994; Leclercq et al. 1996). Interleukin-15 has multiple immune functions (Figure 1.3), including T cell proliferation, activation of cytotoxic effector cells, and activation of monocytes (Perera, Goldman, and Waldmann 2001), as well as costimulatory proliferation of activated B cells (Armitage et al. 1995). Additionally, IL-15 has critical influence on the differentiation, proliferation, maturation, and survival of NK and $CD8^+$ cells which are the cells involved in detecting virally-infected cells and tumor cells (Perera, Goldman, and Waldmann 2001).

Interleukin-15 cancer therapy is more experimental than IL-2 therapy. Nonetheless, IL-15 treatment has had positive effects in several cancer models. Intratumoral administration of syngeneic DCs transduced with IL-15 (using a simian virus 40 viral vector) was associated with a strong antitumor response in colon carcinoma tumors in mice, with 73% complete tumor

remission, and correlated with *in vivo* priming of tumor-specific $CD8^+$ T lymphocytes and NK cells (Vera et al. 2005). The cured mice developed immune memory, with resistance to rechallenge of tumor cells (Vera et al. 2005). Similarly, immunotherapy using a recombinant adenovirus expressing IL-15 was found to significantly inhibit growth of human cervical cancer and human breast cancer in murine models (Yu et al. 2010; Yiang et al. 2009). Mice that overexpress IL-15 are significantly more resistant to tumor growth when challenged with B16-F10 melanoma cells than B6 control mice, while mice that do not express IL-15 have increased susceptibility (Davies et al. 2010). Interleukin-15 has also been demonstrated to have anti-tumor properties independent of NK cell and CTL functions, which is thought to be due to stimulation of macrophages to produce IL-12 and nitric oxide (NO) (Davies et al. 2010). Interleukin-15 (and IL-12) can activate macrophages through the induction of IFNγ and TNFα (Carson et al. 1994; Fehniger, Carson, and Caligiuri 1999a). In addition, IL-15 is a potent stimulus for GM-CSF production by human and murine NK cells (Carson et al. 1994; Fehniger et al. 1999b; Fehniger et al. 2000), which can activate DCs. We hypothesized that the therapeutic efficacy of MYXV could be enhanced using a recombinant MYXV that delivers murine IL-15 directly into the tumor microenvironment. We further theorized that a recombinant MYXV designed to express an immunostimulatory cytokine (IL-15) would be a potent oncolytic therapeutic and would not require combination therapy with a chemotherapeutic drug.

**Natural killer cells**

Natural killer cells are large granular lymphocytes that are a component of the innate immune system response against both tumor cells and virally-infected cells, making them a very important player in oncolytic viral therapy. Natural killer cells develop from the common lymphoid progenitor cells in the bone marrow and circulate to the secondary lymphoid organs

and many other tissues. Natural killer cell precursors respond to IL-15 by maturing into functional NK cells and becoming activated (Leclercq et al. 1996; Yu et al. 1998). Mice that do not have any IL-15 or do not have the IL-15 receptor alpha (IL-15 -/- and IL-15 Rα -/- mice) lack NK cells, suggesting that IL-15 is necessary for the differentiation of mature NK cells (Kennedy et al. 2000). In addition, other cytokines (Figure 1.4) including IL-2, IL-12, IL-21, and IFN-α/β play a role in NK cell maturation or activation (Biron et al. 1999; Smyth et al. 2002; Nutt et al. 2004).

Natural killer cells are predominantly found in the peripheral blood, at sites of inflammation, and in the lymph nodes in humans (Ferlazzo et al. 2004; Freud et al. 2006) and in the spleen of mice. Natural killer cells have abundant cytoplasm that contains granules filled with granzymes and perforin. Upon activation, NK cells exocytose these granules which perforate the targeted cell and cause apoptotic cell death. Natural killer cells also produce a variety of chemokines and cytokines (Figure 1-4), including IFN-γ, TNF, GM-CSF, the C-C chemokines macrophage inflammatory protein (MIP)-1α and MIP-1β, and regulated upon activation, normal T cell-expressed and secreted (RANTES) (Biron et al. 1999; Dorner et al. 2004). Natural killer cells produce MIP-1α and MIP-1β after stimulation with IL-15, which is enhanced by IL-12 (Fehniger, Carson, and Caligiuri 1999a; Bluman et al. 1996). Interferon gamma production is restricted to NK cells and activated T cells, and occurs in response to stimulation by IL-12 and IL-18, not to viral PAMPs (Fensterl and Sen 2009). Interferon gamma produced by NK cells activates macrophages, which produce cytokines, including IL-15, which activates NK cells, and is just one component of the cross-talk that occurs between NK cells and macrophages, as shown in Figure 1-4.

Natural killer cells express a large number of activating and inhibitory receptors that

function to ensure activity against virally-infected cells and tumor cells, while maintaining self-tolerance to non-infected, non-transformed cells. Natural killer cells are able to detect self molecules that are induced in conditions of cell stress, which increases the likelihood that they will be activated during an infection. As part of the innate immune system, once activated, NK cells are able to kill target cells in the absence of antibody (as reviewed in Lanier 2005).

**Immune response to oncolytic viruses**

The ability of the virus to replicate within cancer cells is a key attribute of a successful oncolytic virus; however the primary goal of oncolytic virus therapy is destruction of the neoplastic cells. This can be accomplished by direct cell lysis secondary to viral replication, or by directing the immune response against infected cancer cells. The benefit to involvement of the immune system is the potential to develop immunologic memory against the neoplastic cells. It has been theorized that a vigorous local immune response against virally-infected tumor cells may activate the immune system to recognize tumor cell antigens as well as viral antigens, resulting in tumor clearance locally and systemically (Thorne, Bartlett, and Kirn 2005). $CD8^+$ cytotoxic T lymphocytes (CTLs) have the ability to specifically recognize and kill tumor and virus-infected cells. Therefore, development of an immune response to virally-infected tumor cells would ideally generate a $CD8^+$ CTL response. Poxviruses such as VACV can induce a CTL response against virally-infected cells (Fujiwara et al. 1984), and some animals cleared of tumor by recombinant VACVs are resistant to rechallenge with cells of the same tumor type (Kirn et al. 2007). In one study using VACV expressing GM-CSF delivered intratumorally, patients demonstrated regression of distant metastases, as well as the injected tumor. The uninoculated masses (metastases) did not have any sign of VACV infection, but did have T-

lymphocyte infiltration, suggesting that the immune system had been primed to recognize the neoplastic cells (Mastrangelo et al. 1999).

We hypothesized that MYXV expressing the gene for IL-15 could induce a cell-mediated antitumor immune response and increase survival in immunocompetent mice with tumors. The virus was injected intratumorally, in order to achieve the maximum concentration of virus in the tumor bed. Chapter 3 describes the effects of MYXV and MYXV:IL15 treatment in cancer cell cultures. Chapter 4 reviews the outcome of a single intratumoral injection of MYXV in three murine models of brain cancer. And lastly, in chapter 5 we monitored the response of murine subcutaneous melanomas to treatment by evaluating the tumors histologically at various time points after injection, as well as comparing survival times in virus treated and control animals to multiple injections. We found that multiple injections MYXV and MYXV:IL15 have beneficial effects on survival compared to controls, and recommend further experiments designed to increase the beneficial treatment effects.

## Chapter 1 Tables and Figures

Table 1.1. Properties of an ideal oncolytic virus.

| **Ideal oncolytic virus** | **Myxoma virus** |
|---|---|
| Not a human pathogen but will infect human cancer cells<br>• Avoids pre-existing immunity | Yes |
| Not able to infect livestock | Yes |
| Limited side-effects or toxicity to normal tissues | Yes |
| Large genome capable of transgene insertion<br>• Allows virus to be armed with therapeutic genes | Yes |
| Virus is able to rapidly replicate and cause cytolysis | Yes |
| Does not combine with the host genome<br>• Ideally does not ever enter the host nucleus<br>• Minimizes the risk of virus-host genetic recombination events | Yes |
| Can be given systemically | Yes, in mice |
| Raises a potent inflammatory response<br>• May aid in the establishment of an antitumor immune response | Yes |
| Selectively replicates in tumor cells without infecting normal cells | Yes |
| Can be combined with other cancer treatments | Likely |

Table 1.2. The poxvirus family.

| **Subfamily** | **Genus** | **Species** |
|---|---|---|
| Chordopoxvirinae | Avipoxvirus | canarypox, crowpox, fowlpox, juncopos, mynahpox, pigeonpox, psittacinepox, quailpox, peacockpox, penguinpox, sparrowpox, starlingpox, turkeypox |
| | Capripoxvirus | goatpox, lumpy skin disease, sheeppox |
| | Leporipoxvirus | **myxoma**, hare fibroma, rabbit fibroma, squirrel fibroma |
| | Molluscipoxvirus | molluscum contagiosum |
| | Orthopoxvirus | camelpox, cowpox, ectromelia, monkeypox, raccoonpox, skunkpox, Uasin Gishu, vaccinia, variola, volepox |
| | Parapoxvirus | Auzduk disease, bovine popular stomatitis, chamois contagious ecthyma, orf, pseudocowpox, parapox of red deer, sealpox, squirrelparapox |
| | Suipoxvirus | Swinepox |
| | Yatapoxvirus | tanapox, yaba monkey tumor |
| Entomopoxvirinae | Alphaentomopoxvirus | Melontha melontha |
| | Betaentomopoxvirus | Amsacta morrei, Melanoplus sanguinipes |
| | Gammaentomopoxvirus | Chrionimus luridus |

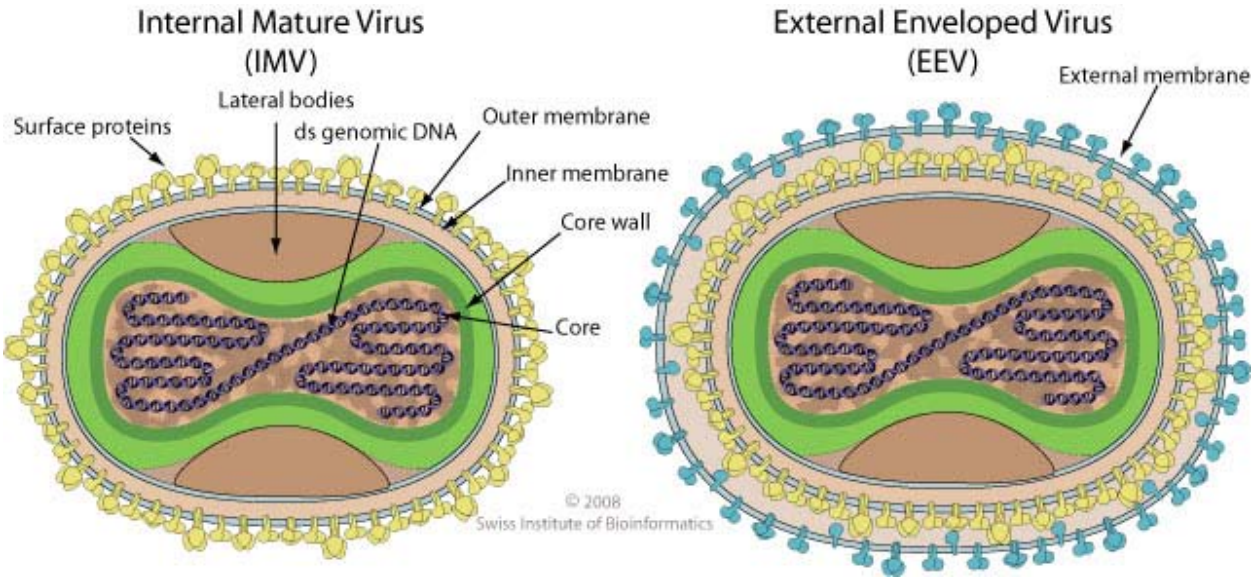



Figure 1.1. Poxvirus structure. Poxvirus intracellular mature viruses (IMVs) are brick-shaped with a lipoprotein core wall (membrane) surrounding a complex core that contains the linear double-stranded DNA genome and several virus-encoded proteins. The core is surrounded by lateral bodies and an outer membrane that contains surface proteins. Cell-associated enveloped viruses (CEVs) and extracellular enveloped viruses (EEVs) are similar to IMVs but have an additional lipoprotein envelope important for early virus dissemination. *Figure source: ViralZone www.expasy.ch/viralzone, Swiss Institute of Bioinformatics

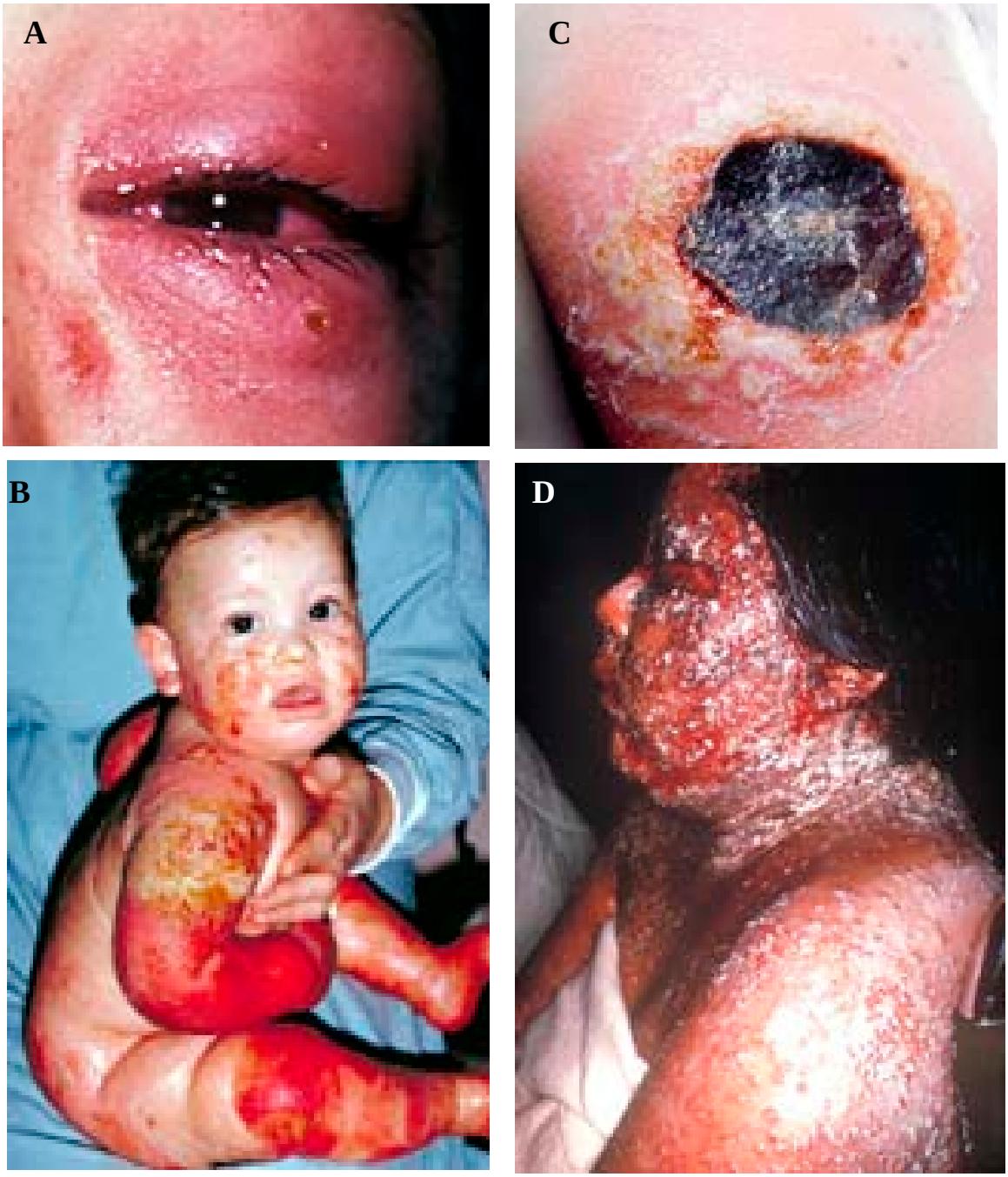



Figure 1.2. Vaccinia vaccination complications. One of the most common complications is autoinoculation (A) the most severe of which can be the eye. Eczema vaccinatum (D) is the next most common complication, while vaccinia necrosum (C) and generalized vaccinia (B) are uncommon. *Pictures are from the CDC and Dr. Weyland (A) and from Dr. Moses Grossman of the California Emergency Preparedness Office – Immunization Branch (B-D), www.gorydetails.net/demo_sites/SmallpoxSite/smpx_vaccine09.html

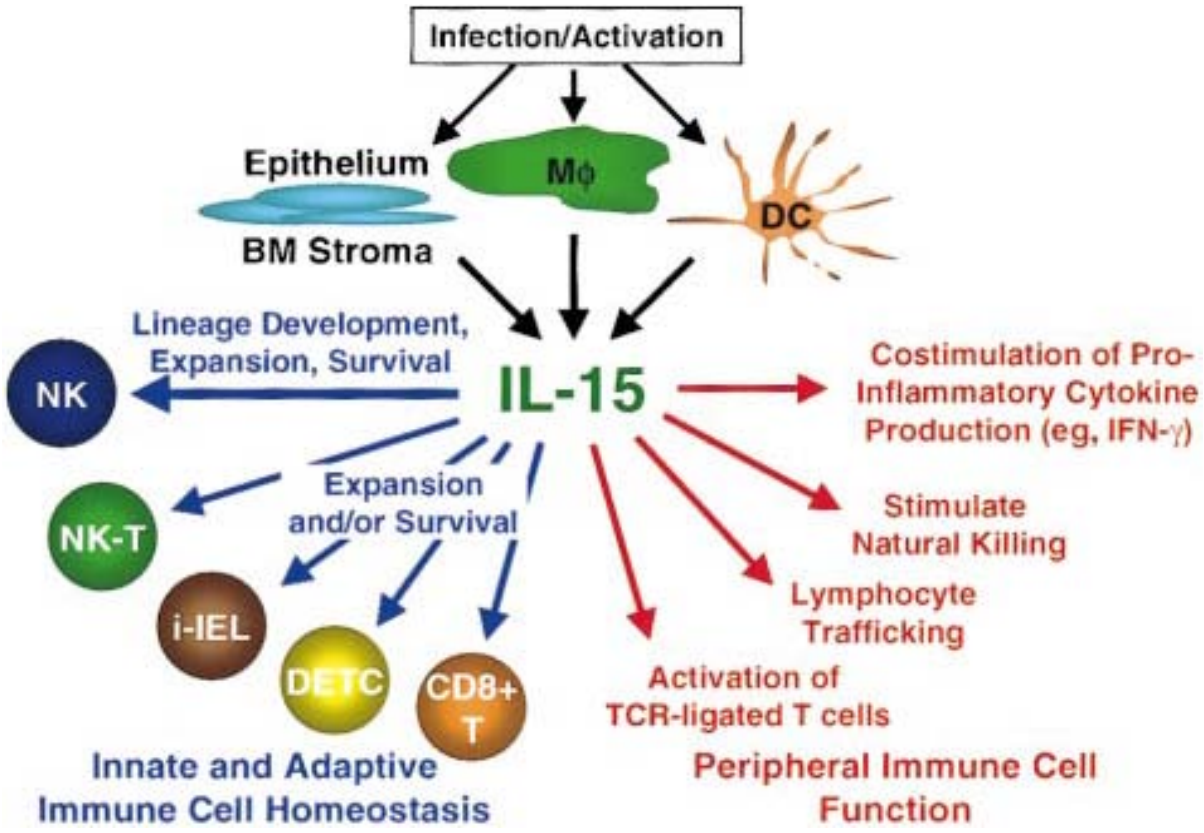



Figure 1.3. IL-15 functions. IL-15 is a pleiotropic cytokine that functions in NK cell development, lymphocyte homeostasis, and peripheral immune functions. Multiple cell types elaborate IL-15, including activated (eg, virus- or bacteria-infected) monocytes/ macrophages, dendritic cells, and epithelium, as well as constitutive production by BM stromal cells. IL-15 has a critical role in the development of the NK lineage, as well as in its survival, expansion, and function. Other innate lymphocytes (NK-T, CD8aa i-IEL, TCRgd DETCs) and memory-phenotype CD81 T cells depend upon IL-15 for survival or expansion. IL-15 also plays multiple roles in peripheral innate and adaptive immune cell function. *Used with permission of American Society of Hematology, from Interleukin-15: Biology and relevance to human disease, Fehniger and Caliguiri, 97, 1, 2001.

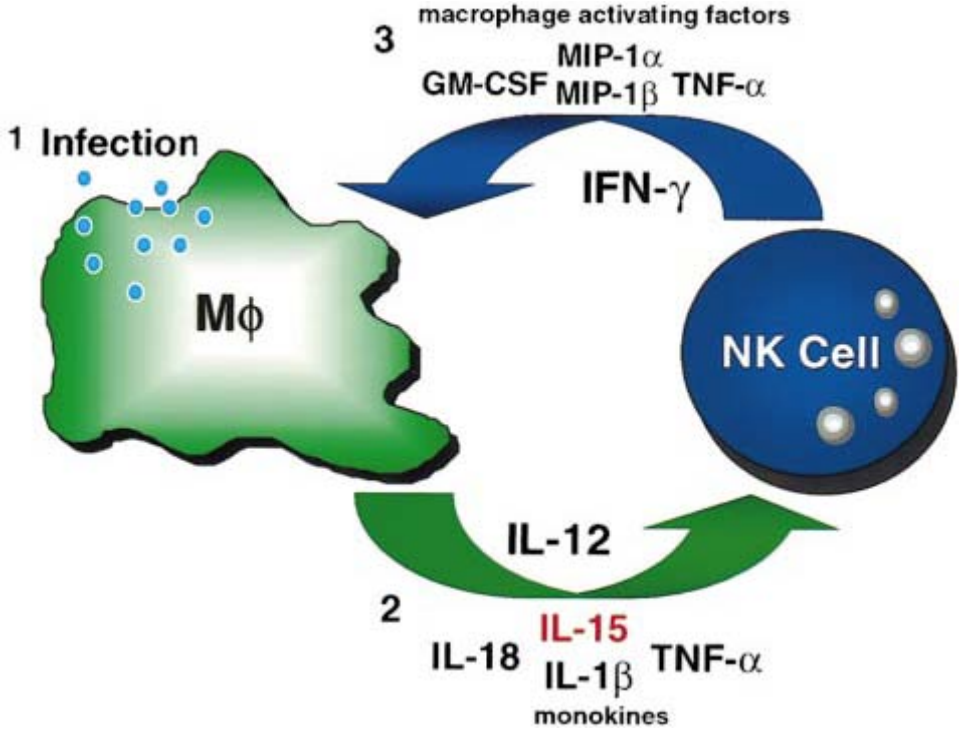



Figure 1.4. NK cell and macrophage interaction. IL-15 participates in innate immune cross-talk between activated monocytes/macrophages and NK cells. After an infectious insult (1), macrophages produce cytokines (2), including IL-15, that bind to constitutively expressed cytokine receptors on NK cells. Stimulated NK cells in turn produce cytokines that activate the macrophage (3), including IFN-γ, allowing the macrophage to clear the offending pathogen and stimulating further cytokine production. *Used with permission of American Society of Hematology, from Interleukin-15: Biology and relevance to human disease, Fehniger and Caliguiri, 97, 1, 2001.

# CHAPTER 2

# MATERIALS AND METHODS

## Cell lines

A murine melanoma cell line, B16-F10, a murine glioma cell line, GL261, and a murine lymphoma cell line, EL4, were obtained from Dr. Ed Roy (University of Illinois, Champaign-Urbana). We also used B16-F10, GL261, and EL4 cells that express the SIY peptide (H-2K$^b$-restricted SIYRYYGL peptide, an exogenous 8 amino acid peptide target for 2C TCR T cells) and a green fluorescent (GFP) marker by retroviral transduction with the pLEGFPSIY vector as previously described (Blank et al. 2004; Spiotto et al. 2002), that are designated B16.SIY, GLSIY, and DP1, respectively. B16.SIY was a gift from Dr. Thomas Gajewski (University of Chicago, IL), while GLSIY and DP1 were obtained from Dr. Ed Roy. An immortalized rabbit kidney epithelial cell line, RK13, (obtained from Dr. Richard Moyer, University of Florida, Gainesville, FL) was used as a positive control cell line for viral infection and titers. Cell lines were maintained using standard cell culture techniques in Eagle's minimum essential medium (MEM; Mediatech, Inc, Manassas, VA) supplemented with 1mM sodium pyruvate, 1× non-essential amino acids, 292 µg/mL L-glutamine, 100 units/mL penicillin,100 µg/mL streptomycin (supplemented MEM) and 10% fetal bovine serum (FBS; Hyclone Laboratories, Inc), except for DP1, which was maintained in Roswell Park Memorial Institute (RPMI) media. All cell lines were incubated at 5% CO2 and 37ºC.

## Viruses

The Lausanne strain of MYXV and recombinant MYXV expressing red tomato fluorescent protein (MYXV:Tomato) with or without expression of IL-15 (MYXV:IL15) were

used to infect the tumor cells. In addition, recombinant MYXV expressing red (MYXV:RFP) or green (MYXV:GFP) fluorescent protein were also used in some of the *in vitro* experiments. The recombinant viruses were created in Dr. McFadden's lab by intergenic insertion of a fluorescent protein cassette driven by a synthetic vaccinia virus early/late promoter between M135R and M136R of the MYXV genome as previously described (Johnston et al. 2003). MYXV:Tomato was originally designated vMyx-tdTr (Liu et al. 2009). MYXV:Tomato is the MYXV that was used in most of our *in vitro* experiments and all of the *in vivo* experiments, and is often simply referred to as MYXV in this paper. MYXV:IL15 virus (originally designated vMyx-IL-15-tdTr) was created by adding an IL-15-expressing cassette containing murine IL-15 driven by a poxvirus p11 late promoter at an intergenic site between M135R and M136R to wild-type myxoma virus, and then adding the tomato red fluorescent cassette downstream as described (Liu et al. 2009). Production of IL-15 from RK13 cells infected with MYXV:IL15 was confirmed by Liu et al. 2009, while no detectable levels of IL-15 were observed in the supernatants of cells infected with MYXV:Tomato. Biological activity of the virus-expressed IL-15 was shown by survival and decreased lesions in all rabbits treated with MYXV:IL15, while all rabbits treated with MYXV:Tomato died (Liu et al 2009). UV-inactivated virus (UV-MYXV) was prepared by exposing MYXV:Tomato to a G30T8 germicidal UV light that produced irradiation at 254 nm for 24 h. Viral inactivity was confirmed by the lack of cytopathic effect, viral plaques, and fluorescence when cultured on RK13 cells. Viruses injected into mice underwent sucrose-pad purification. Briefly, sucrose-pad purified viruses were grown in RK13 monolayers in 150 $cm^2$ dishes until the majority of the cells appeared infected and then cells were scraped into the medium with a sterile rubber policeman. Next, cell-associated virus was isolated by repeatedly lysing pelleted cells in TM buffer (100 mM Tris-HCl pH 8.1 and 100 mM

$MgCl_2$) and physically disrupting cells using a sterile dounce. Cellular debris were pelleted at 400 ×g for 15 min and supernatants were collected. Pooled supernatants were centrifuged at 1000 ×g for 10 min to remove nuclear debris, overlaid onto a 36% sucrose pad, and then ultra-centrifuged at 18,000 rpm for 80 min in order to pellet the purified virus. Virus was resuspended in a small amount of PBS (less than 1 mL per dish of cells collected) and the virus was retitered on RK13 cells.

Titering consisted of placing 10-fold serial dilutions of virus in supplemented MEM without serum on confluent RK13 cells in 6-well plates. The inoculated cells were incubated for 1 hour at 37ºC and 5% CO2, the inoculum was removed, and the RK13 cells were overlaid with 1:1 agarose (Amresco, Ohio):2× MEM (supplemented) with 20% FBS. Plaques were visualized as small white foci (red foci under fluorescent light) and counted at day 7 post-infection (PI).

**Viral infections and mock infections**

Cells were seeded into 6-well plates and allowed to grow in supplemented media until they reached approximately 90-95% confluency. Viral infections were performed with the appropriate multiplicity of infection (MOI, the ratio of infectious virus particles per cell target) of MYXV or recombinant MYXV, which was diluted in supplemented media without serum for a total volume of 400 µl per well. Mock infections consisted of adding 400 µl of supplemented media without serum per well, without the addition of any virus. The cells covered with inoculum (virus diluted in media or media only) were incubated at 37ºC and 5% CO2 for 1 hour, rocking every 15 min. After 1 hour, the inoculum was removed and the cells were overlaid with 1.5 ml of supplemented media with 10% FBS per well.

**Viral growth curves**

Cells were infected as previously described with a MOI of 5 pfu/cell for the single step

growth curves (SSGC) and at a MOI of 0.1 pfu/cell for the multistep growth curves (MSGC). Cells were scraped into the media and collected 0,4,8,12,24,48 HPI for the SSGC and at 0,12,24,48,72,96 HPI for the MSGC. The cells were pelleted at 500 ×g for 5 min, supernatant was removed, and the cells were resuspended in 0.5 ml of phosphate-buffered saline (PBS) and stored at -80ºC. Collected cells were disrupted by 3 freeze/thaw cycles and sonication in order to release virus from the cells. The collected cellular contents underwent titering in duplicate (as previously described). Virus infections of each cell line were performed in triplicate in order to calculate an average pfu/mL.

**Cytopathic effect**

Photomicrographs were taken of the MYXV:Tomato-infected and mock-infected cell lines at 0, 4, 8, 12, 24, 48, 72, and 96 HPI using phase microscopy to evaluate cytopathic effects (CPE) caused by MYXV. Images were acquired using a Leica DMI 4000 B microscope with ImagePro Express software (Media Cybernectics, Bethesda, MD). Cells infected with MYXV:Tomato were imaged using a 560/40 nm bandpass excitation filter and an exposure time of 200 ms, in order to visualize expression of fluorescence by the virus.

**Western immunoblots**

The levels of Akt and phosphorylated Akt in each cell line were determined by performing Western immunoblots. Cells were inoculated with MYXV:Tomato (MOI=5) and collected at 0 and 4 HPI for detection of Akt and phosphorylated Akt. Cells were scraped into media and centrifuged at 500 ×g for 5 min. The supernatant was discarded, the cell pellet washed in PBS, and the pelleting repeated. The cell pellet was resuspended in 50 µl of cell lysis buffer with 2× Halt protease and phosphatase inhibitor (ThermoScientific, Inc). The solution was placed on ice for 20 min, and then nuclei and other insoluble material were pelleted at 5000

×g for 15 min. The cell extract was saved and frozen at -80ºC. The total protein concentration was measured by Bradford analysis. Then 50 µg of cell extract was used for the immunoblot, which was run on PAGEgel dual run and blot vertical mini-gel system (PAGEgel, Inc) using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose membrane. Then, the nitrocellulose membrane was blocked in a solution of milk blocking solution for 1 hour at room temperature (RT). Akt rabbit polyclonal primary antibody and Phospho-Akt (Ser 473) rabbit polyclonal antibody were obtained from Cell Signaling Technology, Inc (Danvers, MA). The primary antibody was diluted 1:100 in antibody dilution solution according to manufacturer's (Cell Signaling Technology, Inc) instructions and was placed on the membrane overnight at 4 ºC. Secondary antibody conjugated to horseradish peroxidase (Goat anti-rabbit IgG Poly-HRP) was obtained from Chemicon (Temecula, CA). After washing the nitrocellulose membrane 3 times in TBS/T (1× Tris Buffered Saline with 0.1% Tween-20), the secondary antibody was diluted 1:200 in milk blocking solution for 1 hour at RT. After incubation, the membrane was washed 3 times in TBS/T. Immunoreactive bands were detected by using Amersham ECL Streptavidin HRP conjugate solution (GE Healthcare) according to manufacturer's instructions and exposed to radiographic film, which was processed.

**Enzyme-linked immunosorbent assays**

The concentrations of type 1 IFN (IFNα and IFNβ), tumor necrosis factor (TNFα), and IL-15 in cell cultures and tissue lysates were determined using commercially available enzyme-linked immunosorbent assays (ELISAs). Samples were prepared by infecting cells with live or UV-inactivated virus (MOI=5 for IFNα, IFNβ, TNFα, and MOI=1 for IL-15), or mock-infecting them, as previously described. Infected cell culture supernatants were collected at 24 and 48 HPI, as well as at 72, 96, and 168 HPI for IL-15. In addition, tumors from mice treated with

MYXV:IL15 were collected and homogenized for detection of IL-15 within the treated tumors. All samples were concentrated using Amicon Ultra-4 centrifugal filter devices (Millipore), from initial sample volumes of 1.5-4 ml, to a final volume of approximately 450 µl, by centrifuging at 3220 ×g at RT for 5-10 min as needed. IFNα and IFNβ ELISAs (PBL Biomedical Laboratories mouse interferon ELISA kits), TNFα ELISA (eBioscience mouse TNF alpha ELISA Ready-SET-Go), and the IL-15 ELISA (eBioscience mouse interleukin-15 ELISA Ready-SET-Go) were performed according to the manufactures instructions. Each kit included a purified protein standard which was used to establish a standard curve. Positive controls for each ELISA were obtained by adding known quantities of standard from the ELISA kits to additional cell lysates from the cell lines, while negative controls were obtained from wells that contained media but did not contain any cells. Negative controls were subtracted from the test samples in order to compensate for background. A Molecular Devices (Sunnyvale, CA) SPECTRAmax *Plus*$^{384}$ Microplate Spectrophotometer was used to detect absorbance at 450 nm.

**Electron microscopy**

All cell lines were infected at a MOI of 10 pfu/cell as previously described. Cells were collected at 24 HPI by scraping into the media, centrifuged at 500 ×g for 5 min, and the pellet was placed in Karnovsky's fixative and shipped on ice to the University of Florida Interdisciplinary Center for Biotechnology Research for processing and imaging using a Hitachi H-7000 with Olympus Soft-Imaging Systems Megaview III digital camera for transmission electron microscopy.

**Trypan blue assay**

Cells were infected with MYXV:RFP (MOI=10) or mock-infected as previously described and collected at 0, 24, and 48 HPI. Cells were stained by combining cells suspended

in media with 0.4% trypan blue solution at a 1:10 ratio. Trypan blue dye is permeable to dead cells but not to intact cells. Clear cells and blue cells were counted on a hemocytometer to quantify the percentage of dead (blue) cells. Data was analyzed with a t-test for independent samples using Statistica software (StatSoft; Tulsa, OK). Any nonparametric data was compared using a Mann-Whitney U test. Significance was given to P-values $\leq 0.05$.

**Cell viability assay**

A Promega CellTiter-Blue Cell Viability Assay (Promega, Madison, WI) was performed for each cell line. Viable cells convert resazurin, a redox dye, into a fluorescent product resorufin, that is detectable a 590 nm. $5 \times 10^4$ cells were added to each well of a 96-well plate, centrifuged at 200 ×g for 5 min, and incubated for 4 h in media with 10% FBS to allow the cells to adhere to the plate. Media was then removed and cells were mock-infected or infected with MYXV (MOI=10) in 50 µl of media without serum as previously described. Infections were done in triplicate. Wild-type MYXV was used so as to avoid interference of viral fluorescence (if MYXV:Tomato, MYXV:RFP, or MYXV:GFP were used) with the fluorescence produced by resorufin. Virus inocula were removed, 100 µl of media with 10% FBS was added, and cells were incubated for 24-48 h. Twenty microliters of cell titer blue reagent was added 3 h before analysis. Analysis was performed using Molecular Devices (Sunnyvale, CA) SpectraMAX GEMINI EM Microplate Spectrofluorometer with excitation at 544 nm and emission at 590 nm. Data was analyzed with a t-test for independent samples using Graph Pad Prism 5 (GraphPad software: La Jolla, CA). Significance was given to P-values $\leq 0.05$.

**Annexin V assay**

Flow cytometry assays were performed to detect cells undergoing the early events associated with apoptosis. Confluent cells were mock-infected or infected with MYXV

(MOI=5) as previously described and collected at 48 HPI. Cell concentration was determined with a hemocytometer and aliquots of $10^5$ cells were incubated with FITC-conjugated annexin V (BD Pharminogen FITC Annexin V Apoptosis Detection Kit 1) and 7-aminoactinomycin D (7-AAD) (BD Pharminogen) according to the instructions provided by BD Biosciences. A positive control was obtained by exposing some cells to staurosporine, an agent that induces apoptosis. The BD Biociences LSR II flow cytometer located in the Roy J. Carver Biotechnology Center at the University of Illinois was utilized to collect the data. Data were analyzed using De Novo FCS Express version 3 software (De Novo Software, Los Angeles, CA). Compensation control samples were evaluated for each cell line. Data was statistically analyzed with a t-test for independent samples using Graph Pad Prism 5 (GraphPad software: La Jolla, CA). Any nonparametric data was compared using a Mann-Whitney U test. Significance was given to P-values $\leq 0.05$.

**Mice**

C57BL/6 mice, an inbred strain obtained from Jackson Laboratory (Bar Harbor, ME), were used as they are the most frequently used mouse models in immunological studies, and are regarded as a $T_H1$-dominant mouse strain (Rivera and Tessarollo 2008; Watanabe et al. 2004). Mice were 6 weeks old when obtained and were allowed to acclimate for 7 days before tumor inoculation. All mice were female in order to monitor for viral infection of the ovaries. Mice were housed at the University of Illinois at Champaign-Urbana and all procedures were approved by the Institutional Animal Care and Use Committee. Mice were housed up to 5 mice per cage in microisolator cages in a model MD75JU140MVSP 140-position Double-Sided Individually Ventilated Cage Rack (70-bins per side), with a top exhaust pak model EP5000E (Allentown Inc: Allentown, NJ), and set at 60 air changes per hour. The mice were kept in 11” x 6” x 5” clear

plastic bins with stainless steel wire caging (Allentown Inc: Allentown, NJ) that contained corn cob bedding. Mice were allowed free access to Harlan 8604 food pellets (24% protein, 4% fat) and tap water accessed by water bottles. All procedures involving the mice took place in a NuAire biosafety level 2 hood: NU-602-400 series 20 BSC class II type A/B3 (NuAire Laboratory equipment supply; Plymouth MN). Mice were kept in a room set at 72 °F with humidity that varied from 30-70%, 10-15 air changes per hour, with a 12:12 light:dark cycle, and with negative pressure to the hallway (biosafety level-2 ).

**Brain tumor model injection protocol**

Mice were stereotactically injected on one or both sides of the brain in the ventral striatum of the cerebrum (AP 0.5 mm, Lat 2.5 mm, DV 4mm) with $5 \times 10^4$ tumor cells in 0.3 µl PBS (as described in Thomas et al 2011). $5 \times 10^4$ to $1.5 \times 10^6$ pfu's of virus in 0.3-0.6 µl PBS was stereotactically injected into the tumor between days 3 and 14 post tumor inoculation (PTI) depending on the rate of tumor cell growth (Table 2.1), in order to give the tumor cells time to establish. Mice were monitored and weighed daily until a criterion of euthanasia was met. Euthanasia criteria consisted of: lethargy, significant neurological disease signs (e.g.circling, head tilt), or loss of 25% of baseline body weight. Mice were euthanized and tissues were collected for histopathology and IHC as described previously. Survival data was measured from the time of the initial viral injection and plotted using a Kaplan-Meier curve. Survival treatment groups were compared with a Log-rank (Mantel-Cox) test. Significance was given to P-values $\leq$ 0.05.

**Subcutaneous tumor model injection protocol**

Mice were anesthetized with isoflurane gas prior to injection of cells. B16-F10 cells ($5 \times 10^4$ in 100 µl PBS) were injected subcutaneously into the left flank of C57BL/6 mice and tumors

were allowed to establish. Once tumors reached a minimum of 5 mm in diameter, the tumors were directly injected with $1 \times 10^6$ pfu MYXV-IL15:Tomato or MYXV:Tomato in 100 µl PBS. Controls were established by injecting tumors with 100 µl PBS or UV-inactivated MYXV:Tomato. In order to overcome clearance of the virus by the immune system, injections were repeated on a weekly basis for survival mice and the inoculum was injected into several sites in the larger tumors in order to spread the virus. Six mice from each treatment group were collected at 24 h, 48 h, 96 h, and 1 week after virus treatment, and 12 mice from each treatment group were allowed to reach a criterion of euthanasia. Mice were observed and weighed every three days until tumors established, and then weighed daily. Tumors were measured with a micrometer until a criterion of euthanasia was met. The criteria (designated in the University of Illinois Institutional Animal Care and Use Committee, approved protocol # 10074) consisted of: tumors $\geq$ 3 cm in diameter, lethargy, significant dermal ulceration over the tumor site, or 25% baseline body weight loss. Survival data was measured from the time of the initial viral injection and plotted using a Kaplan-Meier curve. Survival treatment groups were compared with a Log-rank (Mantel-Cox) test or a Gehan-Breslow-Wilcoxon (GBW) test using Graph Pad Prism 5 (GraphPad Software; La Jolla, CA). Significance was given to P-values $\leq 0.05$.

**Tissue collection**

Mice were anesthetized with isoflurane gas prior to euthanasia with an intraperitoneal injection of 0.5 ml pentobarbital. All major internal organs were grossly examined on all mice in order to check for metastases, evidence of viral infection, or other pathology. All major organs and the tumors were collected and placed in 10% buffered formalin for examination by light microscopy (histopathology). A section of tumor from most mice and a section of the major internal organs (brain, eye, salivary gland, lung, thymus, heart, liver, kidney, spleen,

gastrointestinal tract, genital tract, bladder) from at least one mouse per treatment group were also frozen and stored in a liquid nitrogen tank (approximately -170ºC) for viral titers. A section of the tumor from most mice was also placed in OTC (optimal cutting temperature, Ted Pella, Inc) compound, flash frozen, and then stored a liquid nitrogen tank (approximately -170ºC) for immunohistochemistry (IHC).

**Histopathology**

Tumors and major organs (brain, eye, heart, lung, liver, kidney, spleen, uterus, and ovary) were trimmed into cassettes, processed, paraffinized, and evaluated by light microscopy. Tumors were rated on percent necrosis, neutrophil infiltration, and lymphocyte infiltration (Table 2.2). Representative photomicrographs of each grade were taken (Figure 2.1). For the inflammatory cell infiltrates, all fields were looked at and the 400× field with the highest number of inflammatory cells was used for grading. Since the presence of virus within the tumor was likely highly localized due to the focal nature of the injection, evaluation of the most severely affected area is a more appropriate method to gauge the immune response to the virus, as averaging multiple fields (where virus may not be present) would underestimate the immune response. The site of cellular infiltration (surrounding the mass, within the mass, within areas of necrosis, perivascular) was also noted. Organs were examined for evidence of metastases or inflammation.

**Immunohistochemistry**

Tissues flash-frozen in OTC compound were trimmed to 6 μm sections on a cryostat, placed on a slide, rinsed with PBS, and blocked with 10 % goat serum for 1 hour at room temperature. Serial sections were analyzed for infiltrating leukocytes. Tissues were incubated with either rabbit anti-mouse granzyme B polyclonal antibody (Abcam Inc) to detect NK and

CTL cells, rat anti-mouse MHC Class II 1A + 1E monoclonal antibody (Abcam Inc) to detect APCs including macrophages, rat anti-mouse CD3 monoclonal antibody (Abcam Inc) to detect T cells, rat anti-mouse CD4 monoclonal antibody (BD Biosciences) to detect helper T cells, rat anti-mouse CD8a monoclonal antibody (BD Biosciences) to detect cytotoxic lymphocytes, or rat anti-mouse Ly-6G (Gr-1) monoclonal antibodies (eBioscience) to detect neutrophils. The antibodies were diluted in PBS and placed on the tissues for 1 h at RT. All tissues were washed with PBS and amplified with a secondary antibody that had a fluorescent (fluorescein isothiocyanate, FITC) marker: goat polyclonal antibody to rat IgG-H&L FITC (Abcam Inc); except for the rabbit anti-mouse granzyme B polyclonal antibody treated tissues, which were amplified with a goat anti-rabbit FITC (Jackson Lab) secondary antibody. Slides were rinsed with PBS, covered with Vectashield mounting medium with fluorescence with DAPI (Vector Labortatory, Inc), and coverslipped. IHC was performed on at least 3 tumors per treatment group. The entire tumor was reviewed and the 400× field with the highest number of positive cells was used for evaluation of the immune response. The number of positive cells per 400× field was recorded, and representative photomicrographs were taken.

**MYXV red fluorescence**

Evidence of MYXV expressing red fluorescence protein was examined in all tumors on frozen (IHC) sections that had not been covered with Vectashield mounting medium. The presence of red fluorescence was recorded as a 0 or 1, indicating absence or presence of the virus, respectively. Localization with any foci of inflammation observed on similar IHC tissues was noted and photomicrographs were taken. Treatment groups were analyzed by a Fisher's exact test. Significance was given to P-values $\leq 0.05$.

**Antibody production**

Plaque reduction neutralization tests (PRNTs) were performed to quantify anti-MYXV antibody production in mice after treatment with UV-MYXV, MYXV:Tomato, and MYXV:IL15. $PRNT_{50}$ values represent the reciprocal of the serum dilution that reduced the viral titer by 50%. The 50% endpoint titer was defined as ½ of the average titer recovered from virus inoculum incubated with serum from PBS-treated mice.

To collect serum, blood was removed from mice by cardiac puncture at the time of euthanasia, placed into red-topped tubes, and centrifuged. Murine serum was added to an equal volume of diluted MYXV:GFP (1:1) in order to make a 1:2 dilution of the serum and inoculate RK13 cells with a known number of viral particles (100 pfu/well). In addition, serum from virally treated survival mice was also serially diluted (1:4, 1:8, 1:16, 1:32) with supplemented MEM without serum. Serum did not have complement proteins heat-inactivated prior to use because it has been found that complement is essential for *in vitro* neutralization of (and *in vivo* protection against) the extracellular enveloped virion produced by poxviruses (Benhnia et al. 2009).

PRNTs were performed by incubating RK13 cells in 24-well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) with serum and virus for 1 h. Cells were then overlaid with 50:50 2× supplemented MEM with 20% FBS:1% agarose and evaluated by fluorescent microscopy for viral plaques at 7 days PI. Virus titers (pfu/mL of diluted sample) were plotted versus the reciprocal of the sample dilution of serum from UV-MYXV, MYXV:Tomato, and MYXV:IL15-treated mice. Using Microsoft Excel 2010, the sample dilution of the 50% endpoint was determined by linear interpolation of the dilutions above and below the 50% endpoint titer. Samples were compared for statistical significance using unpaired t-tests with GraphPad Prism software.

**Viral detection *in vivo***

Tumors and organs were individually weighed and homogenized in 400 µl of supplemented MEM without serum. All tumors from MYXV:IL15, MYXV:Tomato, UV-MYXV, and PBS treated mice were homogenized and those of mice treated with live virus were serially diluted. The tissue homogenates were placed on 6-well plates of RK13 cells for 1 h at 37ºC, at which time they were washed with PBS and the cells overlaid with 50:50 2× supplemented MEM complete with 20% FBS:1% agarose. Six-well culture plates were examined by fluorescent microscopy for red fluorescent foci at 1 week and the number of foci was recorded in order to determine the amount of virus per gram of tissue.

## Chapter 2 Tables and Figures

Table 2.1. Intracranial murine tumor experiments.

| Tumor cells | Cell # and volume | Virus | PFU's and volume | Days PTI |
|---|---|---|---|---|
| B16.SIY | $5 \times 10^4$ in 0.3μ l | MYXV:RFP | $5 \times 10^4$ in 0.3μ l | 5 |
| B16.SIY | $5 \times 10^4$ in 0.3μ l | MYXV:Tomato | $5 \times 10^4$ in 0.3μ l | 3 |
| B16.SIY | $5 \times 10^4$ in 0.3μ l | MYXV:Tomato | $5 \times 10^4$ in 0.3μ l | 3 |
| DP1 | $5 \times 10^4$ in 0.3μ l | MYXV:Tomato | $7.5 \times 10^5$ in 0.6μ l | 5 |
| GL261 | $5 \times 10^4$ in 0.3μ l | MYXV:Tomato | $1.5 \times 10^6$ in 0.5μ l | 11 |
| GL261 | $5 \times 10^4$ in 0.3μ l | MYXV:Tomato | $1.5 \times 10^6$ in 0.5μ l | 11 |
| GL261 | $5 \times 10^4$ in 0.3μ l | MYXV:Tomato | $1.5 \times 10^6$ in 0.5μ l | 14 |

PFU = plaque-forming units. PTI = post-tumor inoculation.

Table 2.2. Histopathology grading of tumors.

| | Grade 0 | Grade 1 | Grade 2 | Grade 3 | Grade 4 |
|---|---|---|---|---|---|
| Percent Necrosis | 0% | 1-15% | 16-49% | 50-74% | 75-100% |
| Neutrophil Infiltration | 0-10 | 11-25 | 26-49 | >50 | TNTC |
| Lymphocyte Infiltration | 0-5 | 6-15 | 16-30 | >30 | TNTC |

TNTC = too numerous to count, and had >150 cells per 400× field.

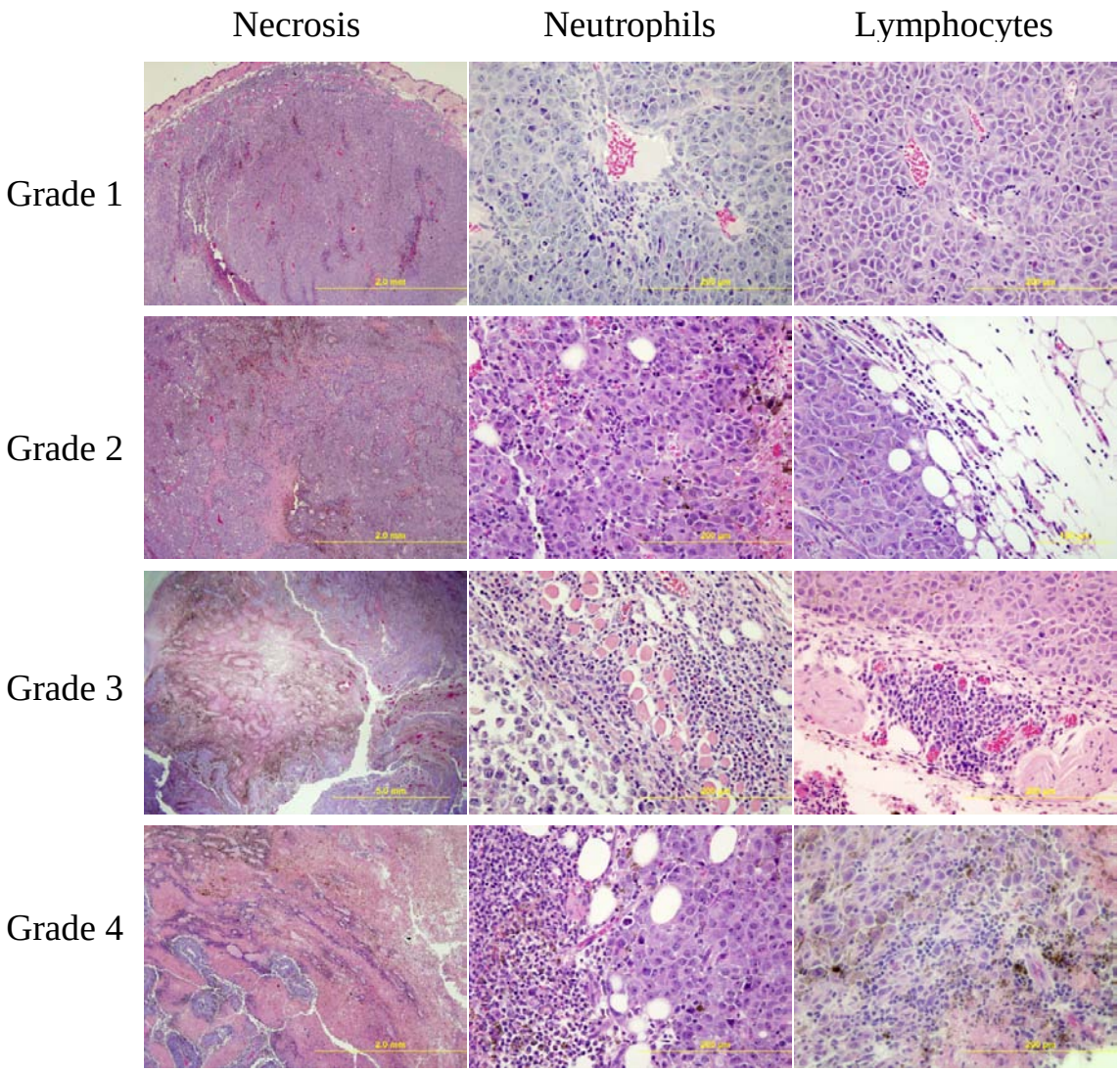



Figure 2.1. Histopathology grading of tumors. Images are representative examples of Grade 1-4 for degree of tumor necrosis, neutrophil infiltration, and lymphocyte infiltration. Necrosis images at are 4× magnification, while neutrophil and lymphocyte images are at 400× magnification.

# CHAPTER 3

## *IN VITRO* MYXOMA VIRUS INFECTION OF MURINE TUMOR CELL LINES

In order to determine if our murine cancer cell lines were susceptible to infection with MYXV, we tested several cell lines for evidence of viral replication and cell death. We also evaluated cellular production of phosphorylated Akt and IFNs, two mechanisms known to affect MYXV replication. We hypothesized that MYXV would infect, replicate within, and cause cell death in murine tumor cells lines that contained activated Akt and failed to produce antiviral Type 1 IFNs. .

### Cytopathic effects were observed in all murine tumor cell lines *in vitro*

In order to characterize the ability of MYXV to infect B16-F10, B16.SIY, GL261, GLSIY, EL4, and DP1 cell lines *in vitro*, the cells were inoculated with a high (MOI=5) or low (MOI=0.1) multiplicity of infection. Then the cells were observed at various time points post-inoculation and compared to mock-infected cells. RK13 cells were also inoculated as a positive control for viral growth. Inoculation resulted in significant cytopathic effects (CPE) in the rabbit control cell line (RK13) the melanoma cell lines (B16-F10, B16.SIY) and the glioma cell lines (GL261, GLSIY), and mild CPE in the lymphoma lines (EL4, DP1) as demonstrated in Figure 3.1. CPE caused by MYXV:Tomato inoculation included rounding up and hyalinization of the cells, with eventual detachment from the culture plate of the adherent cell lines, while the nonadherent lymphoma cell lines (EL4, DP1) showed hyalinization and granulation. Red fluorescence was observed in the virus-inoculated cell lines using fluorescence microscopy (Figure 3.1). Since the tomato red protein is expressed by an early-late poxviral promoter, red fluorescence by the cells confirms early translation of the MYXV genome. CPE were first

evident in B16-F10, B16.SIY, GL261, and GLSIY cells between 8 and 12 HPI at high MOI (5). CPE were first evident in B16-F10 and B16.SIY cells between 12 and 48 HPI, while they were not evident in GL261 and GLSIY cells until 24 to 48 HPI at low MOI (0.1). CPE were significantly more severe after inoculation with MYXV:Tomato at MOI=5 as compared to MOI=0.1 and were more evident in melanoma (B16-F10, B16.SIY) cells than in glioma (GL261, GLSIY) cells. CPE was mild in the lymphoma cell lines and red fluorescence of the cells was not evident. In summary, while CPE was evident in all of our cell lines treated with MYXV, the degree of CPE present varied according to cell type and virus concentration used, with more severe CPE in the melanoma cell line and in cell lines treated with a higher MOI

**The B16-F10 melanoma and GL261 glioma cell lines contain phosphorylated Akt**

Previous studies have demonstrated that MYXV infects human and murine cells with phosphorylated Akt (Werden and McFadden 2008; Stanford et al. 2008). Western immunoblot analysis confirmed that the B16-F10 and GL261 murine cell lines and those transformed with the SIYGFP peptide have phosphorylated Akt at the time of inoculation of the virus, as well as during the early stages of infection (Figure 3.2). As expected, given the lack of severe CPE in the lymphoma cell lines, EL4 and DP1 were not found to have any significant amount of phosphorylated Akt before or after infection (data not shown).

**The B16-F10 melanoma and GL261 glioma cell lines do not produce IFNα and TNFα in response to infection with MYXV, but GL261 produced IFNβ after infection with MYXV or UV-MYXV**

Previous studies have demonstrated that MYXV is able to infect murine cell lines that do not produce Type I IFNs in response to infection (Wang et al. 2004). We assessed the murine melanoma and glioma cell lines for production of Type I IFNs and TNFα. None of the cell lines

produced significant concentrations of IFNα (data not shown) or TNFα (Figure 3.3A), whether mock-infected or infected with live or UV-inactivated MYXV, at early (4 h) or late (24 h) time points after inoculation. Most cell lines also failed to produce IFNβ in response to infection; however, the GL261 parental cell line produced IFNβ in response to infection with UV-inactivated and live virus at 24 HPI, and was highest in the cells infected with UV-inactivated MYX (Figure 3.3B). Positive controls for each ELISA were obtained by adding known quantities of standard to additional cell lysates from the cell lines, while negative controls consisted of wells that contained media but did not contain any cells.

**Myxoma virus productively infects RK13, B16-F10, and GL261 cell lines**

We next performed SSGC and MSGC of all cell lines to demonstrate the ability of MYXV:Tomato to replicate in the tumor cell lines (SSGC) as well as its ability to spread to adjacent cells (MSGC). Viral titers were high in the B16-F10 and B16.SIY melanoma cell lines, similar to the positive control (RK13) cells in both SSGC and MSGC (Figure 3.4). Viral titers were also robust but slightly lower in the GL261 and GLSIY glioma cell lines tested (Figure 3.4). Viral titers mildly increased in the EL4 and DP1 lymphoma cell lines in the SSGC but the titer did not logarithmically increase in the EL4 cell line in the MSGC, and the DP1 titer was very low and was undetectable by the 96 h time point in the MSGC (Figure 3.4B). Logarithmic increases in viral titers suggest virus replication and production of infectious viral progeny occurs in the melanoma and glioma cells. Growth curves were repeated using MYXV:IL15 and showed similar logarithmic viral growth (Figure 3.5).

**Melanoma and glioma cell lines supported all stages of viral morphogenesis**

To confirm that orderly and complete maturation of MYXV occurs within murine tumor cells, electron microscope (EM) analysis of all cell lines was performed and images were

compared to previous studies of poxvirus morphology (Resch, Weisberg, and Moss 2009; Bisht et al. 2009; Roberts and Smith 2008; Chichon et al. 2009; Duteyrat, Gelfi, and Bertagnoli 2006). No previous studies have examined MYXV morphology in tumor cells to our knowledge. Since a complete cycle of MYXV replication has been estimated to occur within 16 HPI (Duteyrat, Gelfi, and Bertagnoli 2006), permissive cell lines should contain all stages of viral morphology by 24 HPI. All stages (crescent, immature virions, immature virions with a nucleoid, intracellular mature virions, wrapped virions, cell-associated virions, and enveloped virions) of MYXV virus maturation were observed in B16-F10, B16.SIY, GL261, and GLSIY as well as in the positive control RK13 cells at 24 HPI (Figure 3.6). Subjectively, virions were more abundant within the B16-F10 and B16.SIY melanoma cell lines than in the GL261 and GLSIY glioma cell lines. No immature or mature virions were observed within the EL4 or DP1 lymphoma cell lines at 24 HPI, and most of these cells were lysed (data not shown). This data confirms the virus growth curve data findings.

**Myxoma virus infection causes significant cell death of murine neoplastic cells**

One of the main goals of viral therapy of cancer is to induce significant cell death of tumor cells (oncolysis). We evaluated the ability of MYXV to cause cell death of murine tumor cell lines *in vitro*. Cell death was analyzed utilizing trypan blue exclusion of cells infected with MYXV:RFP (MOI=10) versus mock-infected cells. Significant cell death was observed in all cell lines infected with MYXV:RFP, and was calculated as a percentage of dead cells (trypan blue positive cells) compared to mock-infected controls (Figure 3.7A-C). When infected cells were compared to mock-infected cells, significant cell death was observed by 0 HPI in the RK13 control cell line and continued at 24 and 48 HPI (Table 3.1; Figure 3.7A-C). Significant cell death was observed in the B16-F10 and B16.SIY melanoma cells at 24 and 48 HPI but not at 0

HPI (Table 3.1; Figure 3.7A-C). Significant cell death was observed in the GL261 glioma cells at 48 HPI, but was not significant at 0 or 24 HPI (Table 3.1; Figure 3.7A-C). Significant cell death was observed in the GLSIY glioma cells at 0 HPI, 24 HPI, and 48 HPI (Table 3.1, Figure 3.7A-C), similar to the RK13 cells (Figure 3.7A-C). Significant cell death was observed in the EL4 and DP1 lymphoma cells at 0 and 24 HPI, but the degree of cell death of the inoculated cells versus the mock-infected cells was not significant at 48 HPI (Table 3.1; Figure 3.7A-C) because most of the remaining cells were trypan blue positive (dead) at 48 HPI, regardless of whether they were infected with virus or were mock-infected. Due to the questionable infectivity of the lymphoma (EL4 and DP1) cell lines as evidenced by CPE, growth curves, and ultrastructural examination, and evidence from trypan blue assay that the cells do not survive past 48 hours (*in vitro*) to support MYXV infection, further *in vitro* studies utilizing the lymphoma cell lines were discontinued.

Due to the fact that many of the cells killed by MYXV infection are lysed and would not be detectable by the trypan blue assay, the effect of viral infection on cell viability and proliferation was also examined using a CellTiter blue cell viability assay. A statistically significant decrease in the number of live cells was observed in all of the cell lines (RK13 $p<0.0001$, B16-F10 $p<0.0001$, B16.SIY $p=0.0026$, GL261 $p=0.0099$, GLSIY $p<0.0001$) infected with live MYXV, versus mock-infected cells (Figure 3.7D). However, viable cells were still present at 24 and 48 HPI with MYXV despite using a high MOI of 10.

To evaluate whether cell death caused by MYXV infection was driven by apoptotic cell pathways, B16-F10 and GL261 cells were mock-infected or infected with MYXV:Tomato (MOI=5) and labeled with annexin V and 7-amino-actinomycin D (7-AAD) at 48 HPI, then detected using flow cytometry. Annexin V is a protein that is able to detect the presence of

apoptotic cells by binding to the negatively-charged phospholipid, phosphatidylserine, which is expressed on the surface of apoptotic cells but not on live cells. 7-AAD is a fluorescent protein that has a high affinity for DNA, but does not readily pass through intact cell membranes, and is therefore useful for detecting necrotic cells. The percent of apoptotic cells was not significantly different between infected and mock-infected cells (Figure 3.7E), suggesting that infected cells were dying by necrosis.

**Discussion**

*In vitro* experiments showed that both of the murine melanoma (B16-F10 and B16.SIY) cell lines are highly permissive to infection with MYXV, with evidence of cytopathic effect, production of phosphoryated Akt, lack of production of Type 1 IFNs, complete intracellular viral morphogenesis, significant cell death, and production of high virus titers. The permissiveness of B16-F10 cells to a recombinant MYXV (vMYXlac) was previously reported by McFadden (Stanford et al. 2008). That study revealed similar viral growth curves and classified B16-F10 cells as highly permissive to infection with vMYXVlac.

Murine glioma (GL261 and GLSIY) cells were also shown to be permissive to infection with MYXV in our *in vitro* experiments, but not to the degree that the melanoma cells were. McFadden's group also looked at the permissiveness of GL261 to infection with MYXV, but did not consider the cells to be permissive, with a minimal decrease in GL261 cell viability at 72 HPI with 1 MOI, and just over a 20% decrease in cell viability at 72 HPI when infected with 10 MOI of MYXV (Lun et al. 2009). While our lab also found that the number of GL261 cells do not decrease much after MYXV infection (the least of all the cell lines studied in our lab), the difference was still found to be statistically significant. Combined with the presence of virions in the cell by EM, the positive CPE observed when cells were infected, and the increase in the

number of trypan blue positive cells after infection, we classified the GL261 cells as semi-permissive to MYXV infection. While the difference between our findings and those of the McFadden group are possibly due to differences in the criteria used to classify the cells as permissive or nonpermissive, there are a number of other reasons why our findings may differ from theirs. For one, the McFadden lab titers their virus on BGMK (baby green monkey kidney) cells, while our MYXV is titered on RK13 cells. Since RK13 cells originate from the naturally affected host, they may support a different level of viral growth than the BGMK cells at different time points after viral inoculation. Alternatively, it may be that the passage number of our GL261 cells was likely different than the passage number of McFadden's GL261 cells. This may indicate that one of the cell lines changed to have different properties than the original cell line. For example, studies have shown that that IFN levels may increase (Johnston et al. 2005, 235-248) or decrease (Wang et al. 2009) as cells undergo progressive passaging. We did find that infection of the GL261 cell line resulted in the production of IFNβ. While this cytokine was not produced when the cells were infected with live virus, all poxviruses employ at least 1 mechanism to disrupt interferon responses (Seet et al. 2003), and MYXV is predicted to encode 2 proteins that disrupt interferon responses (Barrett et al. 2001). We think that UV-inactivation of MXV prevented expression of the proteins that would have suppressed interferon production in the GL261 cells, while the mere presence of the inactivated virus particle was enough to trigger production of IFNβ in these cells.

The murine lymphoma cells (EL4 and DP1) did not appear to support viral growth in our *in vitro* experiments, although the virus did cause a significant increase in cell death. This may be due to the fact that in rabbits, apoptosis of lymphocytes within the lymph nodes is a major feature of MYXV infection (Best, Collins, and Kerr 2000). Detectable viral antigen is not found

within these apoptotic lymphocytes, but the apoptotic lymphocytes are often adjacent to infected cells (Best, Collins, and Kerr 2000). This is similar to our *in vitro* findings, in which murine lymphocytes had evidence of cell death but viral growth curves and EM did not indicate that the cells were able to support MYXV infection. Myxoma virus encodes at least 4 immunomodulatory proteins (M-T2, M-T4, M-T5, and M11L) that act as intracellular apoptosis inhibitors in rabbit lymphocytes. Myxoma virus mutants in which one or more of these immunomodulatory genes have been deleted have been shown to induce rapid apoptosis in infected rabbit lymphocytes that aborts the viral infection, while infection occurs normally in rabbit fibroblasts (Barry et al. 1997; Macen et al. 1996). It may be that these viral proteins do not function effectively in EL4 or DP1 murine lymphocytes, and therefore any MYXV may result in lymphocyte apoptosis and nonproductive infection in these cells.

While all of the cell lines had statistically significant cell death rates after infection with MYXV:RFP as compared to controls (mock-infected) by trypan blue assay, the degree of cell death measured was not as significant as what was visually observed by CPE. This was believed to be due to the large amount of cell death by cell lysis, which would not be measurable as blue cells by trypan blue assay. Therefore we compared the number of viable cells remaining after infection with MYXV versus controls by CellTiter blue viability assay. Comparing the number of live cells per well in MYXV-infected wells versus the number in mock-infected wells would ideally represent the impact of viral infection on the cell population. While we observed a statistically significant decrease in the number of cells in the infected wells, there were still neoplastic cells remaining, even after infection with 10 MOI. One confounding factor is that while the virus is infecting and lysing cells, the remaining uninfected cells are able to continue to grow and proliferate, increasing their numbers. Proliferation of the remaining cells may occur at

an even higher rate after viral infection than mock infection, as there is less cell overgrowth present to restrict the ability of the cells to replicate, and there would be more nutrients remaining in the media. However, this likely represents what will occur *in vivo*, where uninfected cells will continue to proliferate and replace the cells killed by the virus. The turnover rate of the neoplastic cells versus the replicative capacity of the oncolytic virus used is, therefore, an important thing to consider, as it is critical that the wave-front of viral replication be able to overtake that of the neoplastic cells. In order for this to occur, the virus used for treatment will need to be able to replicate rapidly and produce large numbers of infectious virions that are released rapidly. This need is why poxviruses are possibly superior to other oncolytic viruses, since they replicate quickly, produce many infectious progeny, and CEV's can infect neighboring cells before the cell is lysed. The infectious virions will then be able to infect adjacent cells, ideally before those cells are able to replicate. The rate of virus spread has been found to be a critical factor in the success of tumor virotherapy using mathematical modeling, as has treating smaller tumors (small starting number of neoplastic cells) (Wein, Wu, and Kirn 2003).

All of the *in vitro* results indicated that MYXV productively infects the murine melanoma (B16.F10) and the murine glioma (GL261) cell lines, with evidence of cytopathic effect, complete viral replication, and increased cell death, allowing us to procede with *in vivo* experiments.

## Chapter 3 Tables and Figures

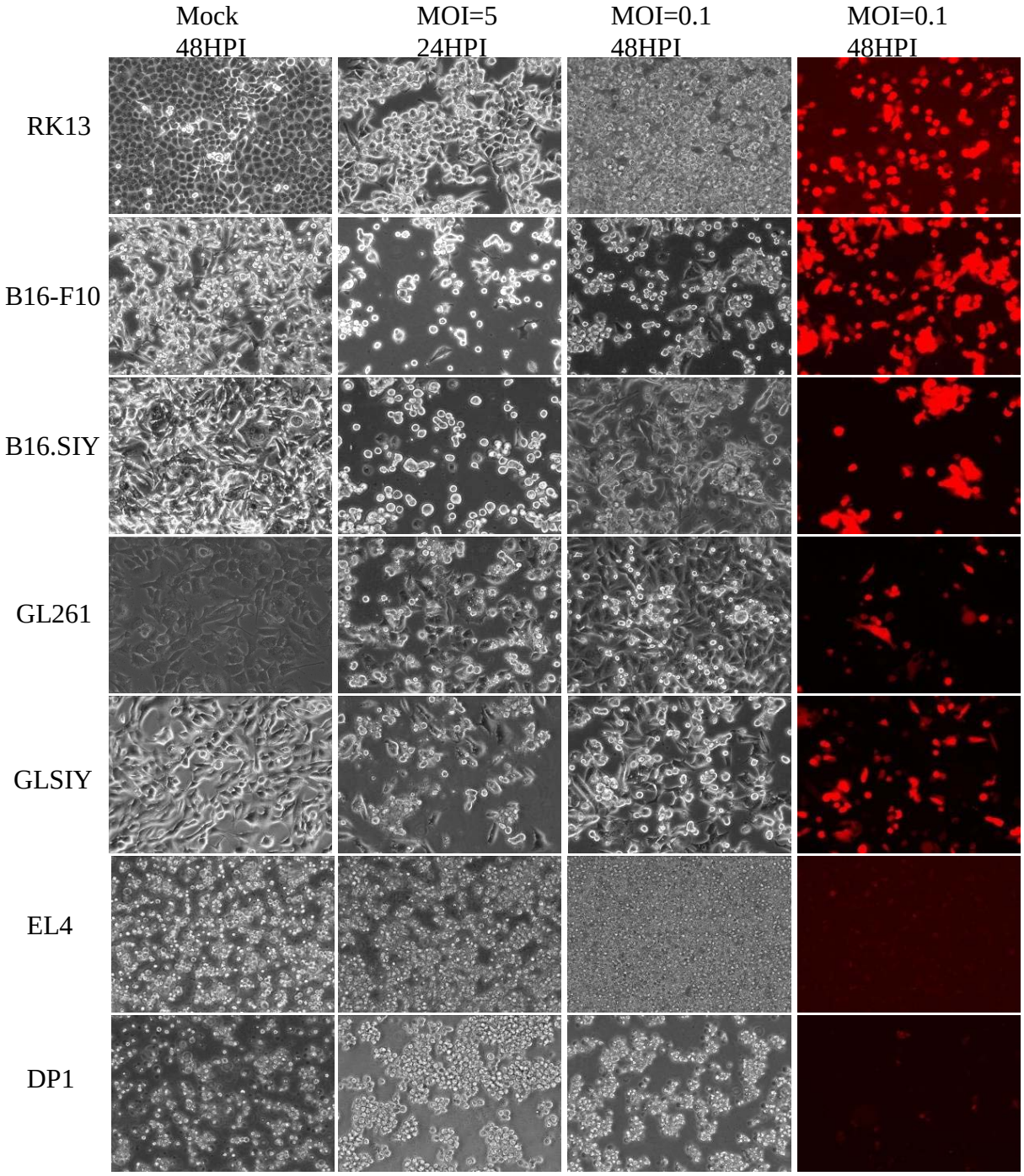



Figure 3.1. Cytopathic effect. Cell lines were mock-infected or infected with MYXV:Tomato. Rabbit control (RK13), murine melanoma (B16-F10, B16.SIY) and murine glioma (GL261, GLSIY) cell lines show significant CPE by 24 HPI when infected with 5 MOI of virus, and by 48 HPI when infected with 0.1 MOI. Lymphoma cell lines (EL4, DP1) had only mild CPE. Phase microscopy photomicrographs were obtained using an exposure time of 5 ms. Fluorescent photomicrographs were obtained with a 560/40 nm bandpass excitation filter and an exposure time of 200 ms and depict fluorescent virus present in cells in the third column.

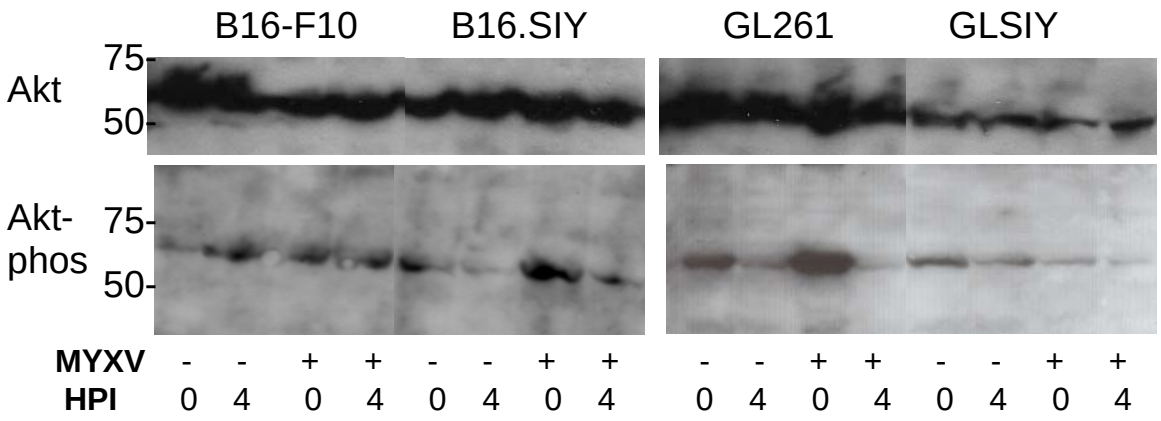
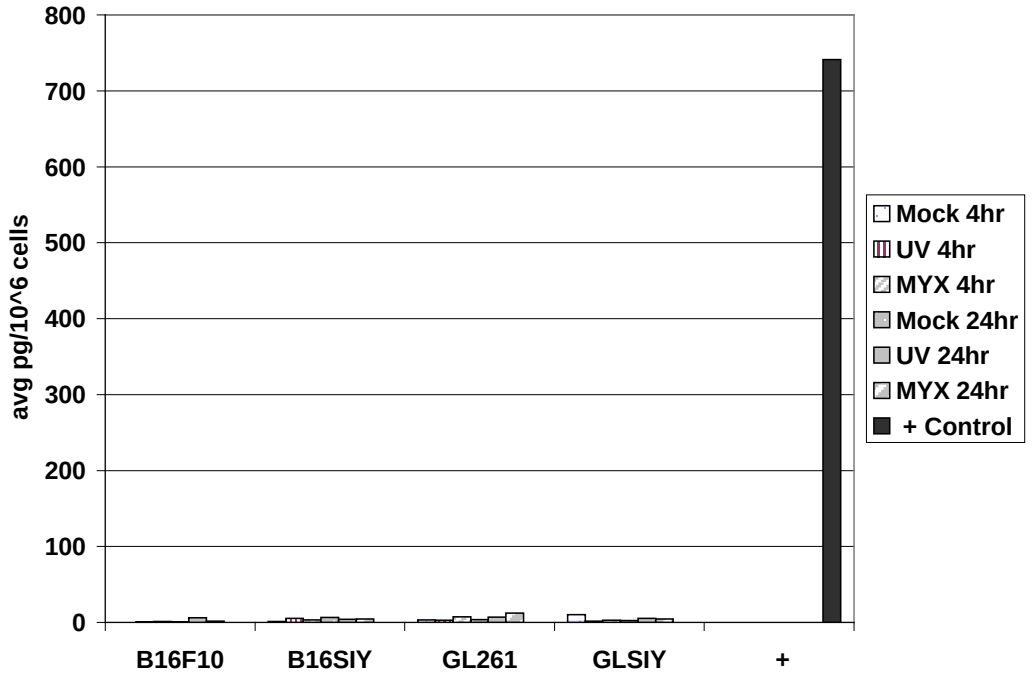



Figure 3.2. Detection of phosphorylated Akt. Melanoma and glioma cell lines were infected with MYXV:Tomato or mock–infected with supplemented MEM without serum and allowed to incubate for 1 hour. The inoculum was removed and cells were either collected immediatedly (0 HPI), or overlaid with supplemented MEM with serum and collected 4 hours later (4 HPI). Cell lysates were evaluated for the presence of activated Akt (Akt-phos) and total Akt by western immunoblot.

A




B

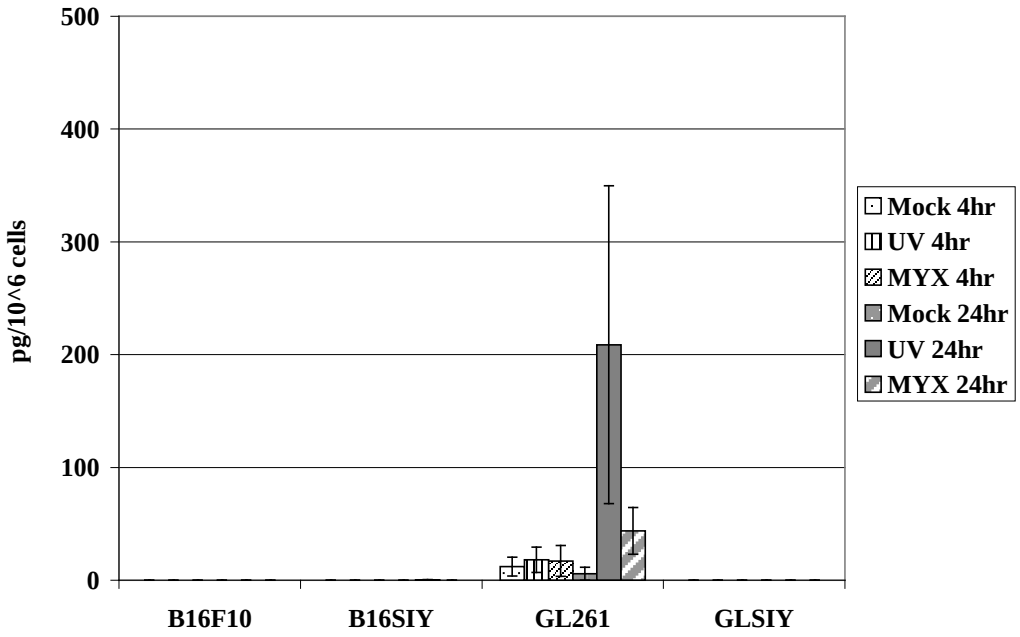
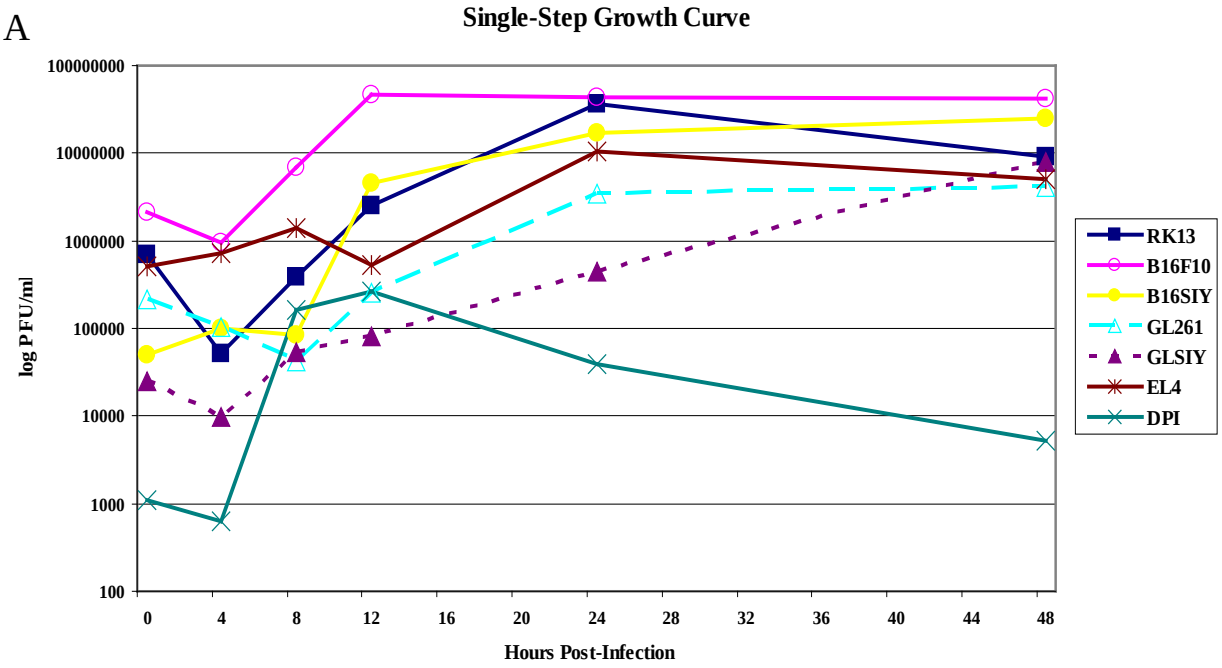



Figure 3.3. Cytokine ELISAs. Cells were infected with MYXV:Tomato (MOI=5). Supernatants were collected at 4 or 24 HPI, concentrated, and TNFα (A) and IFNβ (B) levels were detected as described in materials and methods. Error bars represent standard error of the mean of results from three experiments. The positive control in A consisted of standard mixed with cell lysates.

A




B

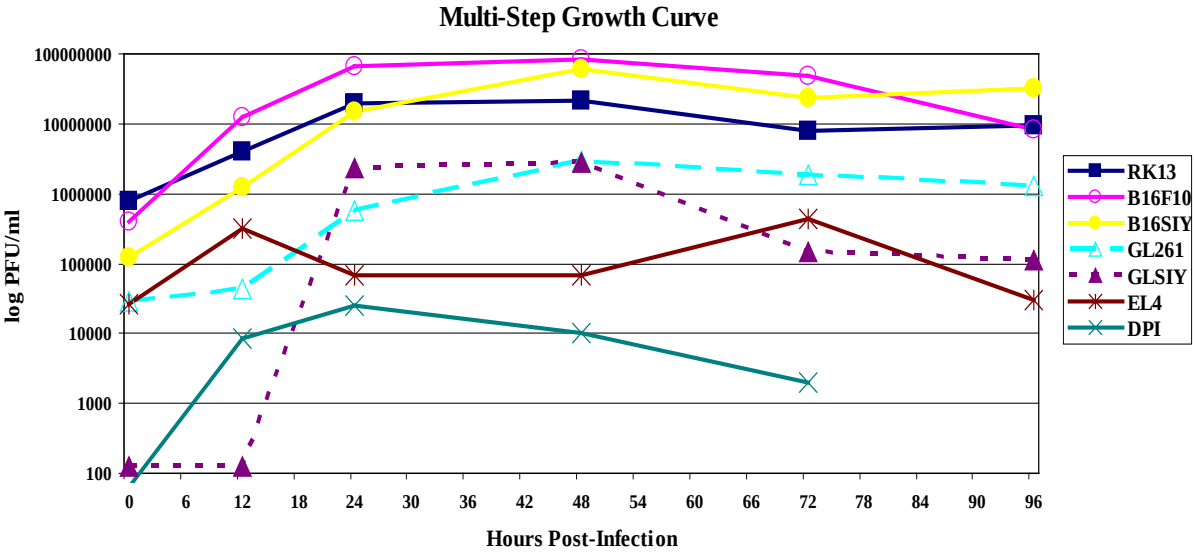
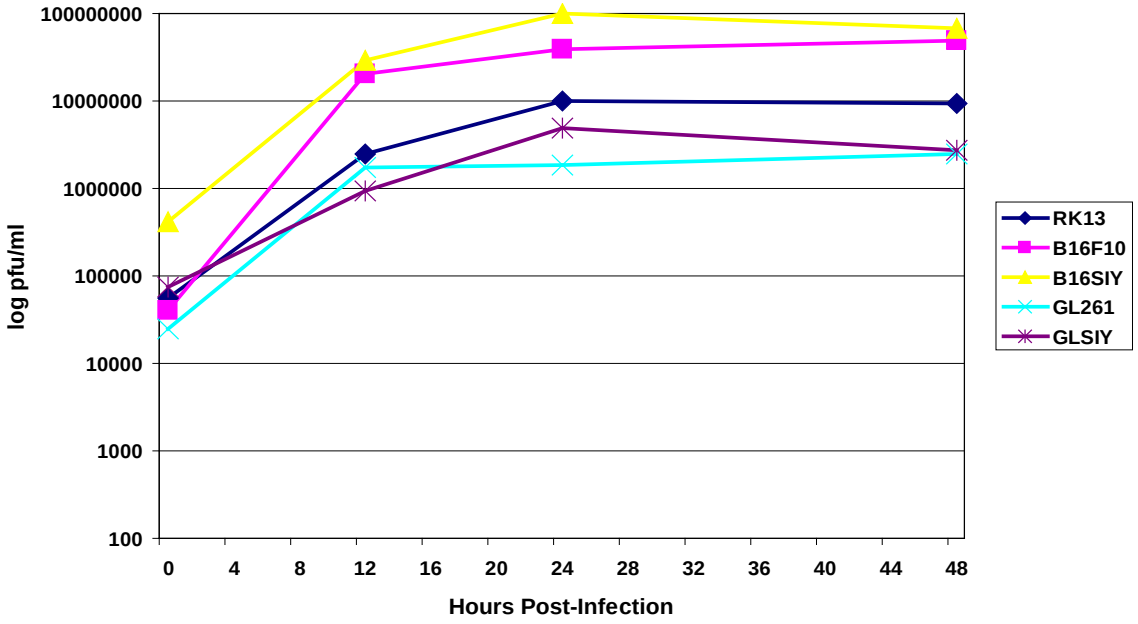



Figure 3.4. MYXV:Tomato viral growth kinetics. Cell lines were infected with MYXV:Tomato at a MOI=5 (A) or MOI=0.1 (B). Cells were collected at various time points, frozen and thawed 3 times, sonicated to release virus, and then titered on RK13 cells. Foci were counted after 7 days. The rabbit control (RK13), murine melanoma (B16-F10, B16.SIY) and murine glioma (GL261, GLSIY) cell lines demonstrated a logarithmic increase in viral titer.

A




B

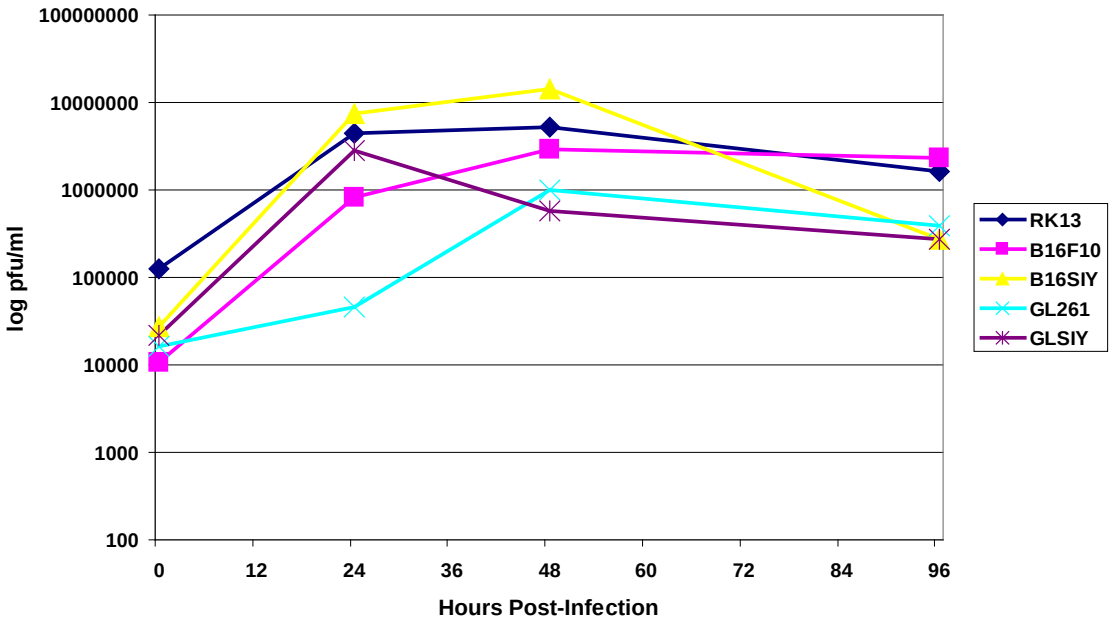



Figure 3.5. MYXV:IL15 viral growth kinetics. Cell lines were infected with MYXV:IL15 at a MOI=5 (A) or MOI=0.1 (B). Cells were collected at various time points, frozen and thawed 3 times, sonicated to release virus, and then titered on RK13 cells. Foci were counted after 7 days. The rabbit control (RK13), murine melanoma (B16-F10, B16.SIY) and murine glioma (GL261, GLSIY) cell lines demonstrated a logarithmic increase in viral titer

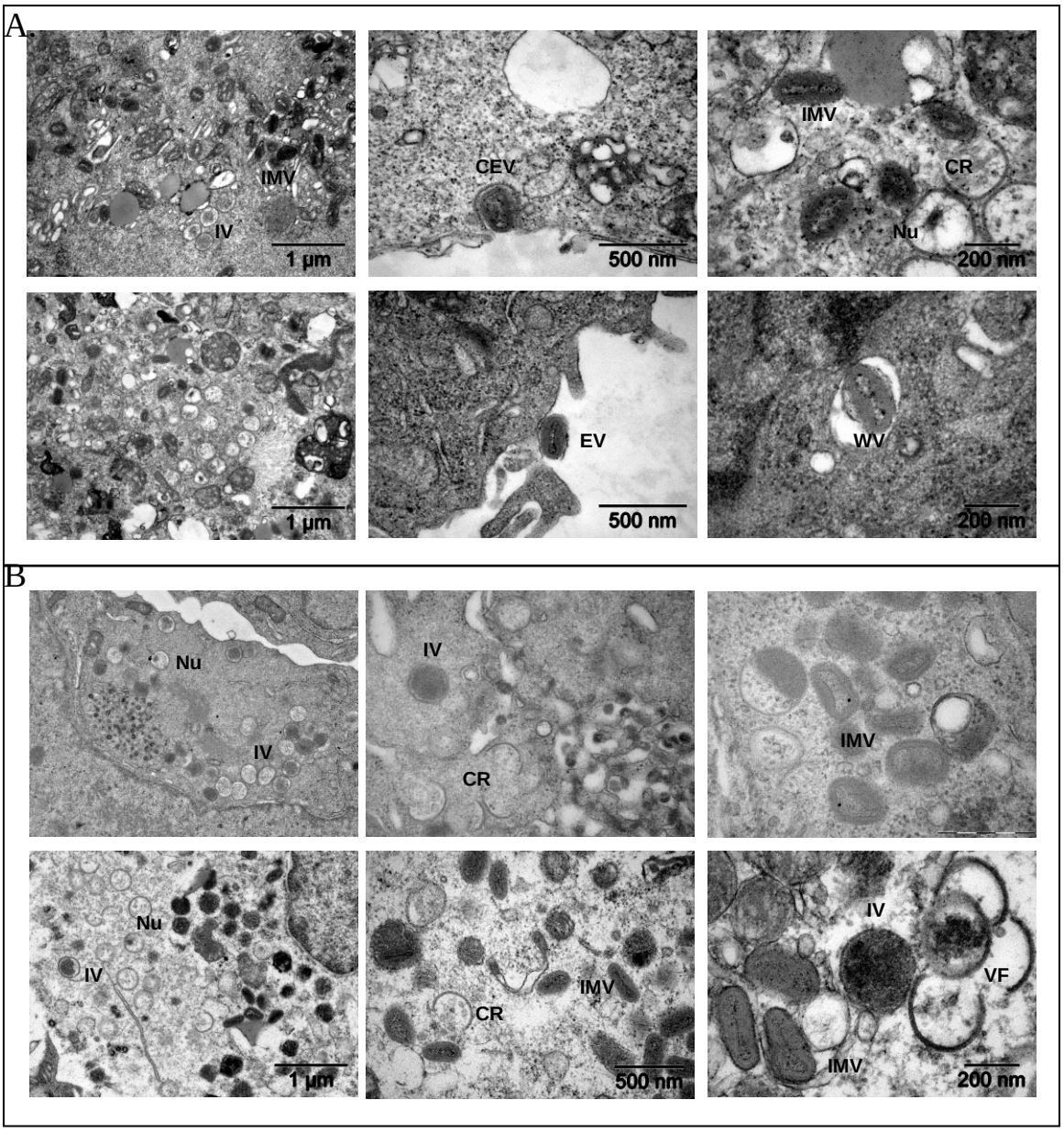



Figure 3.6. Electron microscopy. RK13 (A), B16-F10 (B top row), B16.SIY (B bottom row), GL261 (C top row), and GLSIY (C bottom row) cells 24 HPI with MYXV (MOI=5). Magnification at 17,000-30,000x (left column), 40,000-60,000x (middle column), and 80,000-100,000x (right column). Crescents (CR), Immature virions (IV), Immature virions with nucleoid (Nu), Intracellular mature virions (IMV), Wrapped virions (WV), Cell-associated enveloped virions (CEV), and Enveloped virions (EV) are all present.

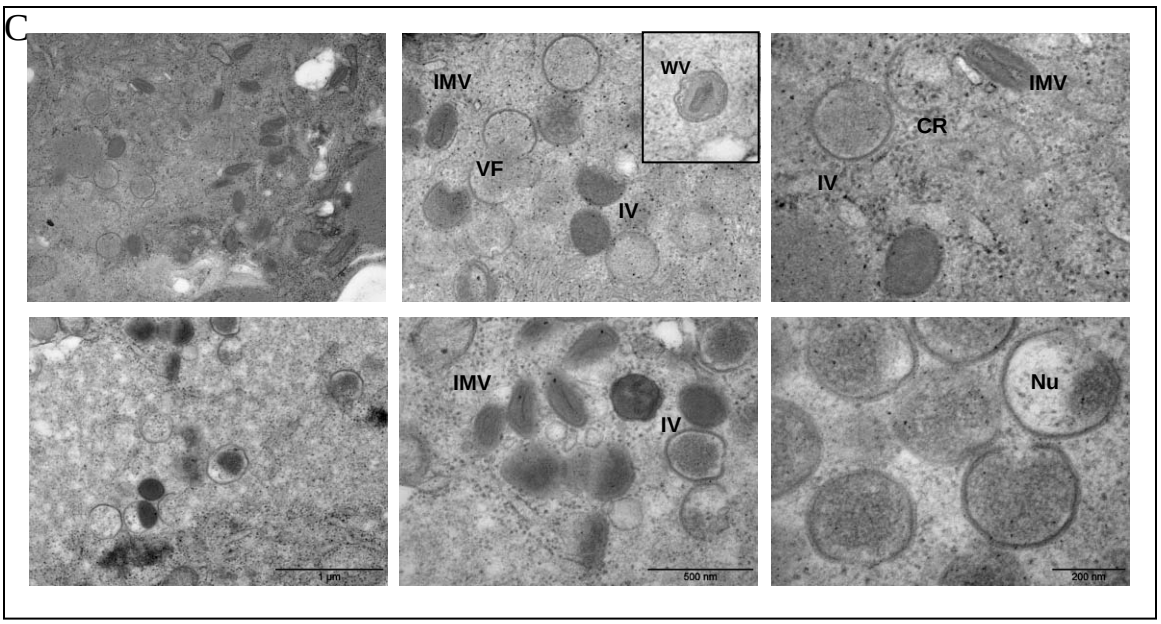



Figure 3.6. Electron microscopy continued.

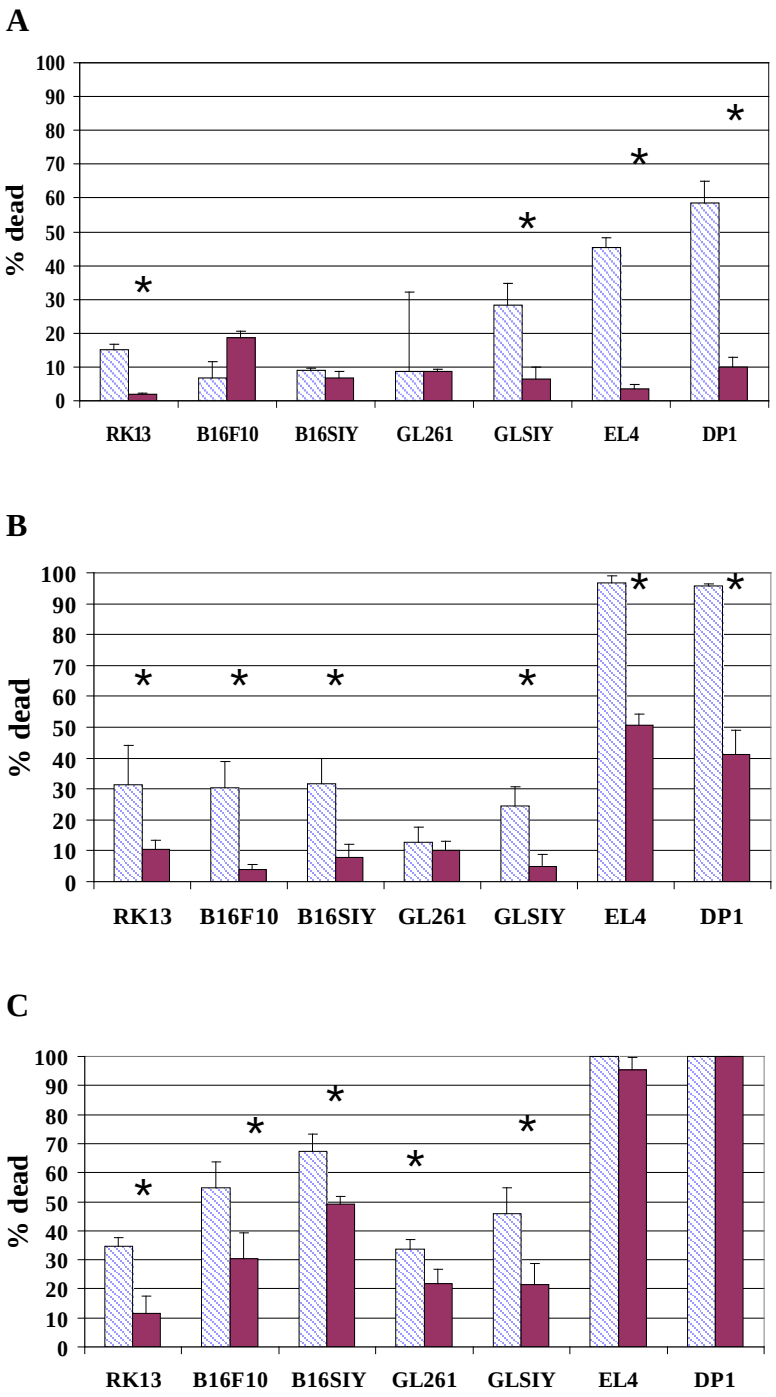
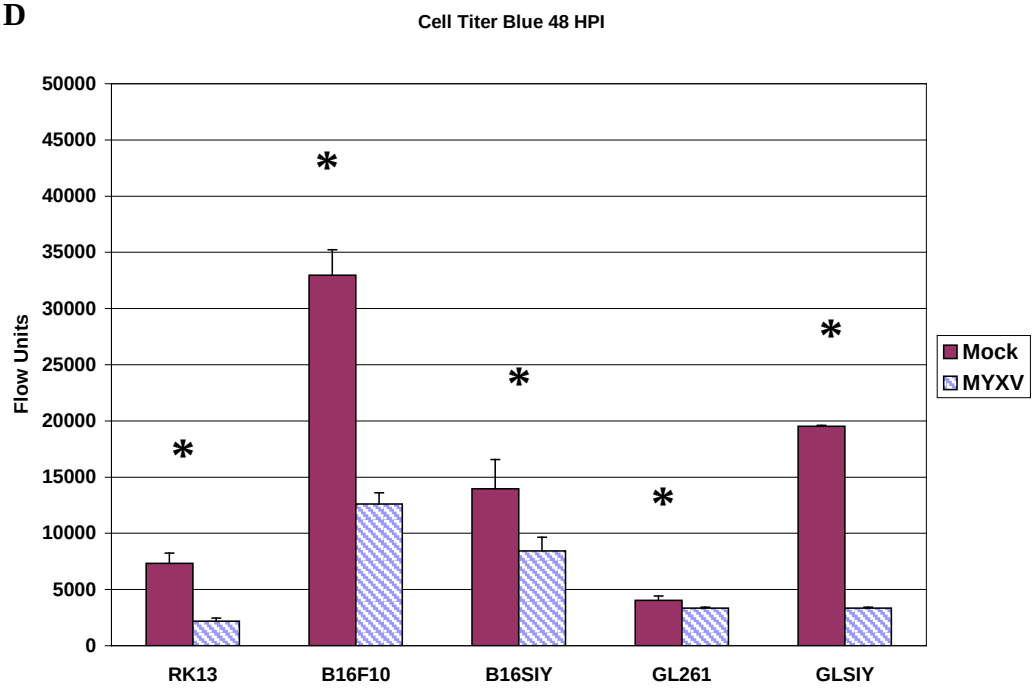



Figure 3.7. Cell viability assays. Trypan blue assays were performed using cells infected with recombinant MYXV:RFP (MOI=10) or mock-infected. At 0h (A), 24h (B), and 48h (C) PI, cells were stained 1:10 in trypan blue solution and counted on a hemocytometer. Data was calculated as the percent of dead cells versus total remaining intact cells for both infected cells (striped bar), and mock-infected cells (solid bar).

**D**




**E**

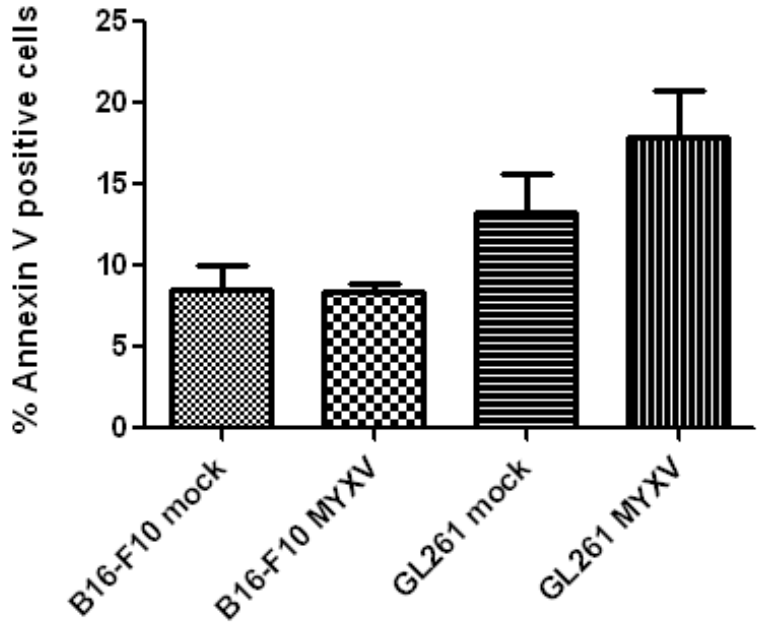



Figure 3.7. Cell viability assays continued. CellTiter-Blue Assays were performed at 24 h (D light bar) and 48 h (D dark bar) PI. Data was calculated as a percent of fluorescence produced in MYXV-infected cells (MOI=10) versus that produced in mock-infected cells. Apoptotic cells (E) were detected 48 HPI with an Annexin V/7-ADD flow cytometry assay. * Indicates statistical significance ($p<0.05$).

Table 3.1. P values from statistical comparison of cell death. Different hours post-inoculation (HPI) in MYXV-infected (multiplicity of infection = 10) and mock-infected cell lines as measured by Trypan blue exclusion assays.

| | RK13 | B16-F10 | B16.SIY | GL261 | GLSIY | EL4 | DP1 |
|---|---|---|---|---|---|---|---|
| 0 HPI | 0.0078 | 0.0037 | 0.2699 | 0.9037 | 0.0009 | <0.0001 | <0.0001 |
| 24 HPI | 0.0183 | 0.0007 | 0.0024 | 0.4258 | 0.0016 | <0.0001 | <0.0001 |
| 48 HPI | 0.0004 | 0.0091 | 0.0013 | 0.0068 | 0.0060 | 0.0725 | 0.00 |

# CHAPTER 4

# TREATMENT OF MURINE BRAIN TUMORS WITH RECOMBINANT MYXOMA VIRUSES

The purpose of these experiments was to create murine models of primary and secondary brain cancer in order to test a novel cancer treatment using an oncolytic virus, MYXV. We hypothesized that treating murine brain tumors with live IT MYXV would be safe and would result in increased survival compared to controls (PBS or dead MYXV).

## Introduction

Metastatic brain tumors are the most common intracranial neoplasms in adults and their incidence is rising, possibly secondary to increased survival time after cancer diagnosis. Metastasis to the brain is considered one of the worst sequela to most cancers, typically resulting in the cancer stage being upgraded to stage IV, which has a worse prognosis. The incidence of aggressive primary brain tumors, such as glioblastoma, anaplastic astrocytoma, and lymphoma are also increasing, particularly in the elderly and in certain areas of the world. The increased incidence of these aggressive tumors is histology specific, and not simply due to improved diagnostic techniques (Werner, Phuphanich, and Lyman 1995; Surawicz et al. 1999; Kohler et al. 2011). Brain tumors can be very difficult to treat depending on their location and degree of invasiveness due to the critical structures present, the limited space in the cranial vault, the presence of the blood brain barrier, and the possibility of neurotoxicity. New highly effective treatment modalities that can specifically target tumors while sparing the normal vital brain tissue are needed. To determine the safety and efficacy of using MYXV to treat brain tumors, we performed preliminary studies using MYXV in immunocompetent mice to treat three types of

tumors that have potential to affect the brain; melanoma, lymphoma, and glioma.

Malignant melanoma was chosen as a model because it is the second most common cause of brain metastasis (behind lung cancer) with an incidence proportion of 6.9% (Sloan, Nock, and Einstein 2009). Melanoma has a high propensity in adults to metastasize to the brain, with nearly 37% of patients with stage IV melanoma eventually developing clinically apparent brain metastasis. However, autopsy results indicate that this rate may actually be much higher, with 55-75% of patients dying of melanoma found to have brain metastasis (as reviewed in Sloan, Nock, and Einstein 2009). Of those patients with known melanoma metastases to the brain, approximately 95% of these patients die of due to their brain tumors, with a median survival of less than 1 year despite treatment (Sampson et al. 1998). While patients may be treated with surgery if the tumor is resectable, systemic treatment is rarely beneficial since most chemotherapeutic agents do not penetrate well into the CNS.

Lymphoma was chosen as a model because it may occur as either a primary brain tumor or as a metastatic (secondary) tumor, and its incidence is markedly increasing. While lymphoma is not a common brain tumor, non-Hodgkin lymphoma accounts for approximately 4% of all primary brain tumors (Surawicz et al. 1999), and 4-8% of patients with non-Hodgkin lymphoma (Kim and Glantz 2001) are diagnosed with CNS metastases. Primary CNS lymphoma is more frequent than secondary cerebral spread of a systemic lymphoma, and it is being diagnosed with increasing frequency in most parts of the world, with a 10-fold increase in incidence rate between 1973 and 1992 (Werner, Phuphanich, and Lyman 1995; Corn et al. 1997; Dubuisson et al. 2004). Primary CNS lymphoma is highly aggressive with a prognosis only slightly better than that of glioblastoma and a median survival time of only a few months when untreated or treated with surgery alone (as reviewed in Commins 2006). Treating the tumors with radiation

and chemotherapy improves survival (13.9-60 months) depending upon the treatment used, but some treatment regimens, are associated with a high incidence of neurotoxicity (Dubuisson et al. 2004; Abrey, Yahalom, and DeAngelis 2000). Two of the most commonly used treatments, whole brain radiation and methotrexate chemotherapy, are neurotoxic and act synergistically (Blay et al. 1998). This neurotoxicity can be progressive and functionally devastating, and include symptoms of memory impairment and ataxia (Abrey, Yahalom, and DeAngelis 2000). In one study, neurotoxicity developed in more than 80% of patients aged 60 or more (Blay et al. 1998). Review of 5 patients who died after combination treatment showed that all had evidence of cortical atrophy and leukoencephalopathy and 3 had cerebral infarcts (necrosis) (Abrey, DeAngelis, and Yahalom 1998; Lai et al. 2004). It is clear that novel treatments that are less toxic are needed to treat patients with cerebral lymphoma.

We choose glioma as our primary brain tumor model because gliomas are the most frequently occurring primary brain tumors. Gliomas are a heterogeneous group of tumors that resemble glial cells, which include astrocytes and oligodendrocytes. The most common types of gliomas are: astrocytomas, oligodendrogliomas, and mixed gliomas. Astrocytomas are the most common, and include low grades (grades I and II) and high grades (III and IV) (Louis et al. 2007). Glioblastoma multiforme (GBM) is a grade IV glioma and is one of the most aggressive and lethal forms of cancer. It has a poor median survival rate of 14 months when treated with combination chemotherapy and radiation therapy (Stupp, van den Bent, and Hegi 2005), and the survival rate has not improved much in the last few decades. Malignant gliomas do not metastasize systemically, but are extremely invasive within the CNS, and widespread early migration of tumor cells has typically occurred by the time of clinical diagnosis. There are no curative treatments for malignant glioma at present, and survival has remained essentially the

same in the last three decades despite advancements in surgical technology and medical treatments. The overall incidence rate of primary brain tumors of adults in the United States is 24.55 per 100,000 persons, with 33% of those diagnosed as malignant gliomas (Kohler et al. 2011).

Murine brain tumor models of melanoma, lymphoma, and giloma were treated with recombinant myxoma virus (MYXV:Tomato) to determine if viral treatment could increase survival. Tumors were induced by intracranial injection of murine cancer cells into mice. GL261 cells were used to model primary glioblastoma, B16-F10 cells modeled metastatic melanoma, and EL4 cells modeled lymphoma. The use of transfected tumor cell lines that could be recognized by a specific set of $CD8^+$ T cells was also explored to track the tumor-specific T cell response to infected tumors. In preparation of these experiments, GL261, B16-F10 and EL4 cell lines were transfected with an SIY peptide (that could be recognized by 2C T cells) and a green fluorescent (GFP) marker: designated GLSIY, B16.SIY, and DP1, respectively. The results of our *in vivo* experiments utilizing some of these cell lines to model brain cancer are described in the following parargraphs.

**Intratumoral MYXV treatment safety**

Cancer cells expressing tomato red protein (viral infection) could be found up to 8 days post-injection with live MYXV:Tomato, but peaked at 48-72 HPI (Thomas et al. 2011). Tomato red expression was not observed in tumors treated with UV-inactivated MYXV (dead MYXV) confirming that the virus was inactivated by UV treatment. Tomato red expression was also not observed in untreated contralateral brain tumors (tumors on the opposite side of the brain) following IT MYXV:Tomato injection of mice with bilateral tumors. In order to determine MYXV safety if injected outside of the tumor, mice that did not have any brain tumors were

injected with MYXV directly into the brain parenchyma. These mice did not develop any adverse clinical signs. In addition, while a small focus of neutrophils could be observed in the brain parenchyma at the site of MYXV injection, necrosis was not observed in the normal brain parenchyma (France et al. 2010). Transient expression of red fluorescent protein was observed in some ependymal cells if the virus was injected near/into the ventricle; however, expression was not associated with any necrosis (France et al. 2010). Live virus was not detected in the blood, spleen, lymph nodes, liver, kidney, lung, heart, intestine, or reproductive tract from any mouse (data not shown). Mice eventually experienced clinical signs such as weight loss, hunched posture, rough haircoat, head tilt, or circling that were attributed to growth of the tumor rather than viral infection. This was due to the observation that the clinical signs did not occur directly after injection, but occurred 1 or more weeks later, in addition to the fact that no virus was detected in the tissues at the time when the mice showed clinical signs.

**Intratumoral treatment with live or dead MYXV resulted in neutrophilic infiltration and necrosis of B16.SIY brain tumors**

Mice were stereotactically injected on one side of the brain with B16.SIY melanoma cells and then, 3-5 days later, tumors were injected with PBS, recombinant MYXV, or UV-inactivated MYXV as described in the methods (Table 2.1). Histopathology revealed that both live and UV-inactivated MYXV induced a neutrophilic response at the site of injection within B16.SIY tumors in mice (Figure 4.1). The neutrophils were visible at 24 and 48 h after intracranial injection of MYXV:Tomato or UV-MYXV, while injection of PBS did not result in any inflammatory response. Tumors injected with PBS also did not have any necrosis (Figure 4.1A), while necrosis was minimal in UV-MYXV treated tumors (Figure 4.1B), and was signficiant in tumors injected with MYXV:Tomato (Figure 4.1C). By 1 week after treatment with

MYXV:Tomato the neoplastic cells had proliferated to the point where the tumor was very large and distorting the brain (Figure 4.2A), and neither neutrophils nor necrosis were observed (Figure 4.2B). While mice treated with MYXV:Tomato were observed to survive slightly longer than mice treated with UV-MYXV or PBS (Figure 4.3), the difference was not statistically significant.

**Intratumoral treatment with live or dead MYXV resulted in neutrophilic infiltration and necrosis of DP1 brain tumors**

Mice were stereotactically injected on one side of the brain with DP1 lymphoma cells and then the tumors were injected with PBS, UV-MYXV, or MYXV:Tomato, 5 days later as described in the methods (Table 2.1). On subgross examination, DP1 tumors (Figure 4.4A) were much smaller than the B16.SIY tumors. Histopathology revealed that both live and UV-inactivated MYXV induced a neutrophilic response at the site of injection within DP1 tumors in mice (Figure 4.4B). The neutrophils were visible at 24 and 48 HPI after intracranial injection of MYXV:Tomato or UV-inactivated MYXV, while injection of PBS did not result in any inflammatory response. Necrosis was minimal in UV-MYXV treated tumors and was not observed in PBS treated tumors (data not shown), while tumors injected with live MYXV:Tomato had significant necrosis (Figure 4.4B). Mice treated with MYXV:Tomato survived for a slightly shorter time than mice treated with UV-MYXV, but the difference was not statistically significant (Figure 4.5).

**Intratumoral treatment with MYXV:Tomato resulted in neutrophilic infiltration and necrosis of GL261 brain tumors**

Mice were stereotactically injected on one or both sides of the brain with GL261 glioma cells and then the tumors were injected with PBS or MYXV:Tomato, 11-14 days later as

described in the methods (Table 2.1). Subgross examination showed that these tumors were also small (Figure 4.6A, dashed circles), similar to the DP1 tumors. Histopathology revealed that while injection of PBS into GL261 tumors did not result in any inflammatory response (Figure 4.6B) at 24 HPI, injection with live MYXV:Tomato induced a neutrophilic response at the site of injection (Figure 4.6C). Mild lymphocytic perivascular cuffing of cerebral vessels adjacent to MYXV:Tomato treated tumors was observed 24 HPI (Figure 4.6C inset), but was not present in PBS treated mice (Figure 4.6B inset). These observations are consistent with recruitment of an immune response to the area of the brain that was treated with MYXV:Tomato. A single IT injection of MYXV:Tomato increased survival in mice with GL261 brain tumors (Figure 4.7A), with one mouse surviving to 100 days post tumor inoculation. An inflammatory infiltrate was not visible at the time of death in the GL261 survival mouse, nor was there any trace of neoplastic cells, or evidence of necrosis (Figures 4.7B and 4.7C). However, GL261 cells have been classified in the literature as mildly immunogenic, and spontaneous cures of mice injected with GL261 cells were observed. Subsequent studies using GLSIY cells resulted in spontaneous rejection of most of the tumors, suggesting that transfection with the SIYRGFP peptide made the cells even more immunogenic. This made evaluation of the statistical significance of survival rate in the GL261 and GLSIY tumor models difficult and would have required utilization of very large numbers of mice. This prohibited further use of the GL261 and GLSIY brain tumor models for this study.

**Discussion**

Treatment with MYXV:Tomato by intracranial injection was shown to be safe in all of the tumor models, without detection of virus in any other organs, or any evidence of encephalitis, or neurological signs after injection. However, MYXV tomato red fluorescence was transiently

detected in ependymal and choroid plexus cells within the brain at early time points (France et al. 2010). Tomato red expression was absent by 11 days post-infection and there was no evidence of necrosis or disruption of the ependymal lining (France et al. 2010). Experiments were also performed on RAG mice (Thomas et al. 2011), with no evidence of viral replication or pathology in other organs, indicating that MYXV treatment is also safe in immunodeficient hosts, as has been documented in other studies (Lun et al. 2005; Kim et al. 2009).

Initial experiments using recombinant MYXV to treat intracranial murine tumors suggested that treatment of tumors with MYXV:Tomato or UV-inactivated MYXV resulted in a focus of neutrophilic inflammation and necrosis in the tumor at the site of injection at 24 and 48 HPI that was no longer present at 1 week PI or thereafter. The neutrophilic inflammation was observed whether live (MYXV:Tomato) or dead (UV-MYXV) virus was injected into the tumor, or into the brain parenchyma itself; however, injection of virus into the brain parenchyma did not result is visible necrosis. A neutrophilic response to oncolytic viral infection has been documented in other studies (Best, Collins, and Kerr 2000; Fu et al. 2011; Grote, Cattaneo, and Fielding 2003). Using standard laboratory strains of MYXV to infect European rabbits, one study observed a neutrophilic inflammatory response in the deep dermis of infected rabbits, whereas the same virus in wild rabbits, or an attenuated strain (Uriarra) in laboratory rabbits, produced a more intense mononuclear inflammatory response (Best, Collins, and Kerr 2000). That study may indicate that effective viral clearance depends on recruitment of mononuclear (likely T lymphocyte) cellular responses.

Despite evidence of tumor necrosis and an immune response to the virus, B16.SIY and DP1 tumors treated with a single injection of MYXV did not have any statistically significant increase in survival. The lack of a statistically significant increase in survival in mice with

B16.SIY tumors treated with MYXV:Tomato was unexpected. Due to the high viral titers that were obtained in B16.SIY cells *in vitro*, rapid infection and proliferation of the virus within the tumor was expected *in vivo*. However, trypan blue and CellTiter blue analyses of B16.SIY cells after MYXV infection had indicated that despite high rates of cell infection and visibly impressive CPE, there are still many tumor cells that survive. Tumor cells that survive continue to proliferate, and since the doubling time of B16.SIY cells *in vivo* is approximately 20 hours, it is likely that the tumor is rapidly able to recover from the necrosis caused by a single injection of MYXV:Tomato.

Treatment of GL261 tumors with live MYXV did result in increased survival in 2 mice; however, rare apparent spontaneous cures were also observed in GL261-bearing untreated mice, making these results questionable. Spontaneous cures of GLSIY tumors were also often observed, indicating that this tumor cell line is too immunogenic to use as a tumor model. The GL261 cell line has previously been shown to be mildly immunogenic, possibly due to elevated MHC class I expression and the constitutive expression of low levels of B7-1 and B7-2 (Szatmari et al. 2006). This may be due to the fact that GL261 is a chemically-induced tumor, rather than a spontaneous tumor. The tumor was originally induced by intracranial injection of 3-methylcholantrene into C57BL/6 mice, and was propagated by serial intracranial and subcutaneous transplantations. It has mutated p53, K-ras, and c-myc, but does not express glial differentiation markers (glial fibrillary acidic protein: GFAP), which is unexpected if it is truly derived from astrocytes. However, it is similar to human gliomas in that it is an aggressive tumor with slightly invasive growth but does not metastasize.

The GL261 and GLSIY murine glioma cell lines in our hands were determined to be too immunogenic to use as a tumor model. This was likely at least partially due to the multiple

passages that both cell lines underwent in our laboratory, which could have resulted in the evolution of more immunogenic cell lines. Since the glioma cell lines were immunogenic and only moderately permissive to MYXV infection, and since the lymphoma cell lines did not support MYXV infection, the melanoma model was the only model that was pursued further, and experiments utilizing GL261, GLSIY, EL4, and DP1 cells were discontinued.

A timely publication reported that multiple injections of MYXV at high titer resulted in increased survival times and decreased tumor size in either lung or subcutaneous B16-F10 tumors in C57BL/6 mice (Stanford et al. 2008). Since administration of multiple intracranial injections of virus was not practical, and experiments using the glioma and lymphoma cell lines were no longer being pursued, it was determined that the intracranial model was no longer necessary. Subcutaneous tumor models allow for daily monitoring of tumor volume as well as direct and repeatable injection of cancer treatments. Subcutaneous tumor growth can be followed for a longer period of time than intracranial tumor growth, as vital organs are not affected by tumor mass effect. Also, swelling of the tumor due to inflammation and necrosis (a potential complication of viral treatment), could have been a limiting factor in the intracranial tumor model, but was not an issue with the subcutaneous tumor model. Additionally, melanoma tumors typically originate in the dermis, and can metastasize to many locations, so using a subcutaneous site for melanoma tumor initiation was a logical choice.

## Chapter 4 Figures

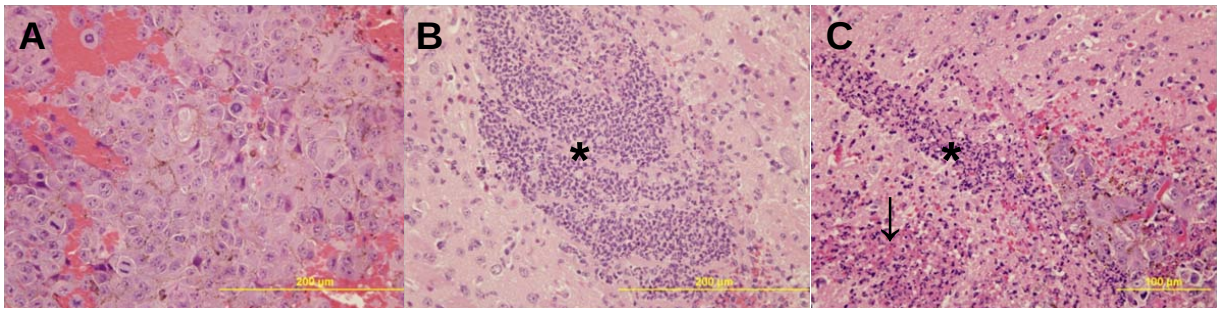



Figure 4.1. B16.SIY tumors 48 HPI. IT PBS (A), UV-MYXV (B), or MYXV:Tomato (C). Dead or live MYXV results in a focus of neutrophils* at the site of injection. Necrosis is indicated with an arrow. Images were taken at a magnification of 400x.

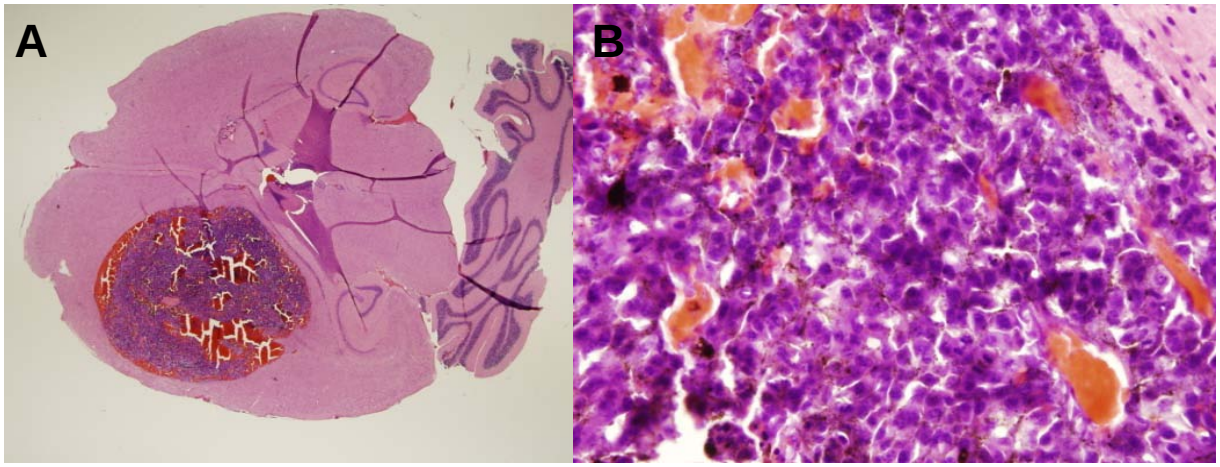



Figure 4.2. Intracranial B16.SIY MYXV-treated survival mouse 7 days post virus injection. A. Subgross (12.5x) examination revealed that the melanoma tumor was large and distorting the brain, pushing the midline to one side. B. Neither inflammatory infiltrates nor areas of necrosis were noted within or surrounding the tumor, but there were many dilated blood vessels and multifocal areas of hemorrhage (400x).

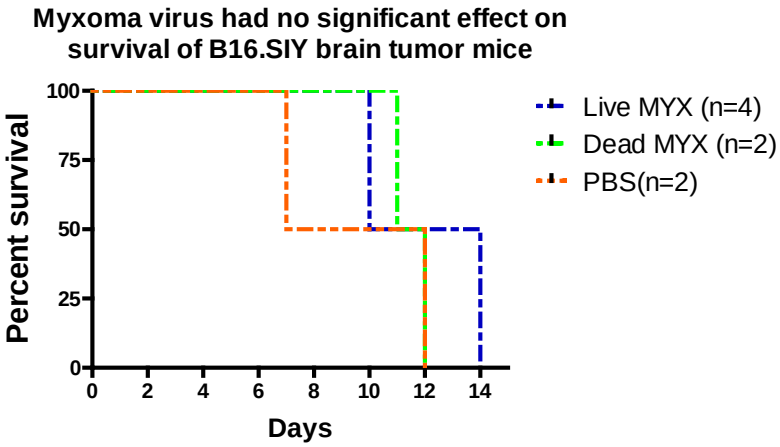



Figure 4.3. Intracranial B16.SIY Kaplain-Meier survival curve. Intratumoral injection with live MYXV did not significantly increase survival in B16.SIY intracranial tumor models over PBS or dead (UV-inactivated) MYXV treated controls.

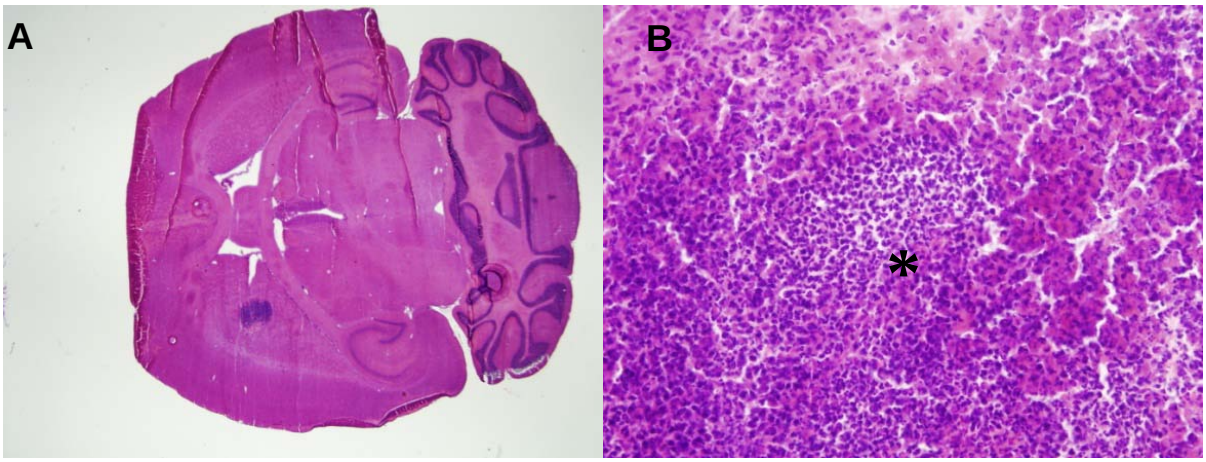



Figure 4.4. Intracranial DP1 tumor 24 HPI with MYXV:Tomato. A. Subgross (12.5x, coronal section) examination revealed that the lymphoma tumor was very small. B. An intense focus of neutrophil infiltration* and necrosis was observed within the MYXV:Tomato treated tumor (400x).

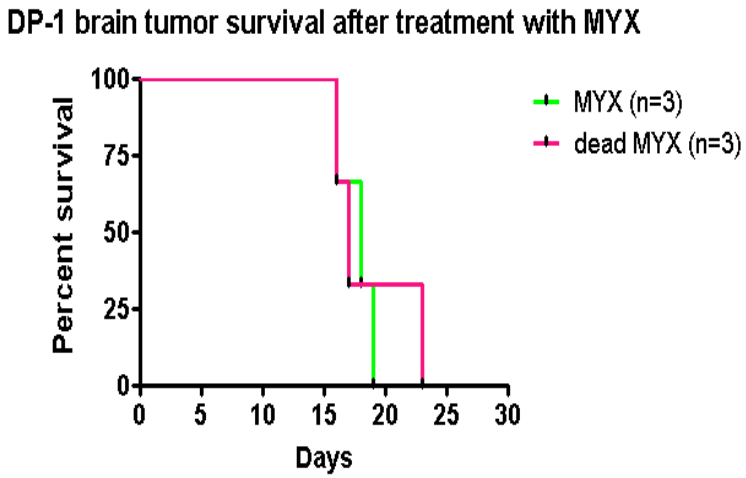



Figure 4.5. Intracranial DP1 Kaplain-Meier survival curve. Intratumoral injection with live MYXV did not significantly increase survival in DP1 intracranial tumor models over PBS or dead (UV-inactivated) MYXV treated controls.

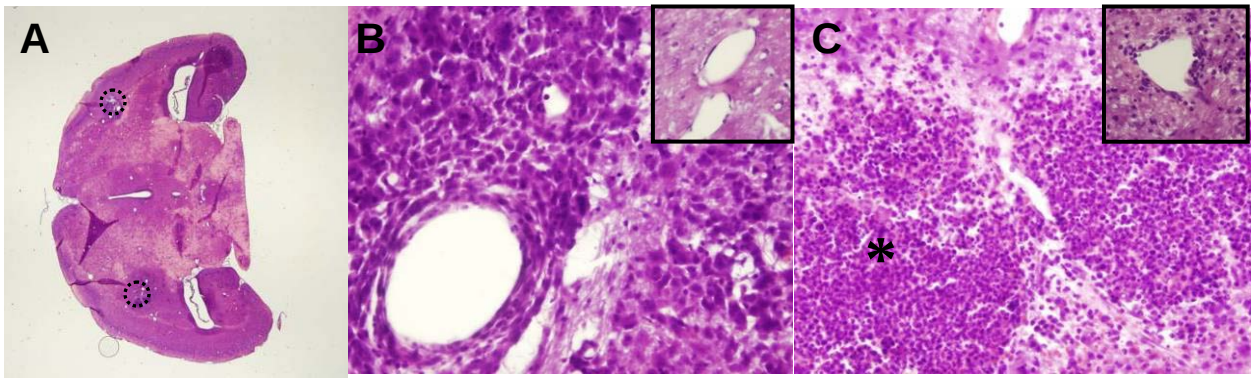



Figure 4.6. Bilateral intracranial GL261 tumors 24 HPI with MYXV:Tomato. A. Subgross (x, coronal section) examination revealed small tumor size (A, dashed circles). One tumor was injected with PBS (B) and the other with MYXV (C). At 24 HPI the PBS tumor does not have a neutrophilic infiltrate and does not have any vascular response (B and B inset), while the MYXV tumor has an intense focus of infiltrating neutrophils*, increased glial cells in the parenchyma, and mild perivascular cuffing (C and C inset).

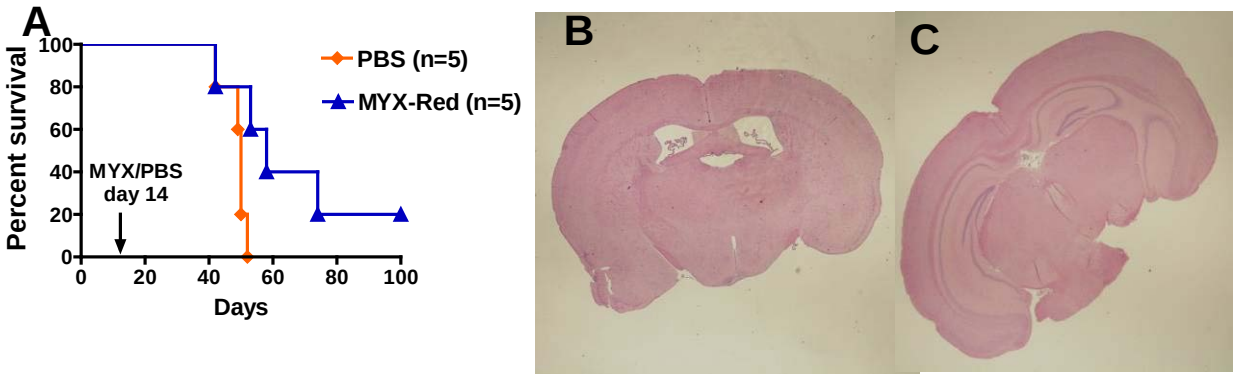



Figure 4.7. Intracranial GL261 survival study. Mice treated with MYXV:Tomato survived significantly longer than mice treated with PBS (A). Examination of the brain from a mouse that survived to 100 days (B and C) revealed no detectable tumor, and no focus of necrosis, inflammation, scarring, or detectable parenchymal loss. Subgross images were photographed at 12.5 x magnification (cross-section).

# CHAPTER 5

# TREATMENT OF MURINE SUBCUTANEOUS MELANOMA TUMORS WITH RECOMBINANT MYXOMA VIRUSES

In order to test the effectiveness of MYXV and MYXV:IL15 for the treatment of murine melanoma, we injected immunocompetent C57BL/6 mice subcutaneously with B16.F10 cells, and then injected the tumors IT with MYXV. The tumors were injected when they reached 5 mm in diameter and injections were repeated on a weekly basis, as our previous studies indicated that the virus titer decreased between 96 hours and 1 week PI. UV-MYXV and PBS were also injected into the tumors of some mice as controls. Mice were monitored for survival and for viral titer, IL-15 levels, and leukocyte infiltration at 5 different time points. Our hypothesis was that treatment of murine melanomas IT with MYXV:IL15 would result in increased numbers of tumor- infiltrating leukocytes and increased survival over treatment with MYXV.

## Introduction

Skin cancer is the most common form of cancer in the United States (U.S. Cancer Statistics Working Group 2010). Melanoma is the third most common skin cancer (after basal cell carcinoma and squamous cell carcinoma), but is more deadly, and is currently ranked as the fifth most common cancer in men, and the seventh most common cancer in women (U.S. Cancer Statistics Working Group 2010). The incidence is statistically significantly increasing every year by an average annual percent change of a 2.4% increase in men and a 2.2% increase in women (Kohler et al. 2011). This increase is despite an average annual percent decrease in the overall cancer rate of 0.8% in the general population (Kohler et al. 2011).

In order to explore new treatments for melanoma, animal models of melanoma are

initially utilized. The original B16 melanoma cell line was derived from a spontaneous melanoma in a C57BL/6 mouse. B16 murine melanoma is an aggressive model in which treatment is notoriously difficult, and it is believed by some that if a treatment works in this mouse model, it is also likely to work in human melanomas. Therefore, inoculation of B16 cells into C57BL/6 mice is a well-established and widely-used spontaneous mouse tumor model for human melanoma. Melanomas grow rapidly in C57BL/6 mice after injection with B16 murine melanoma cells, allowing research to be performed in an immunocompetent animal model. This has benefits over treating a human xenograft tumor model in immunodeficient mice, because the immune system can play a large role in cancer development, metastasis, and treatment. It has been observed with many virotherapeutics that while a treatment may have good anti-tumor effects in a xenograft model, treatment benefit is often lost or reduced when used in an immunocompetent animal model or when used in people with cancer. This is likely due to a combination of factors. For one, a xenograft is a foreign tumor from a different species, so there is an increased likelihood that the host animal will reject those cells, even when the animal is immunodeficient. These foreign cells are coated with foreign antigens, which can act as TAAs to the host immune system. Even in immunodeficient animal models the host immune system still possesses functional NK cells, the very cells responsible for monitoring the body for abnormal cells. Another reason xenograft models are not ideal is that the immune system can affect the ability of the tumor to establish and spread, as well affect the body's response to the cancer treatment. This is especially relevant in the case of oncolytic viruses, which are an infectious agent and have a large effect on the immune system.

Our results using recombinant myxoma virus as a treatment for melanoma in immunocompetent mice follow. We assessed treatment safety, characterized the tumor

histologically, including necrosis and cellular infiltrates, and evaluated survival in the different treatment groups.

**Virus treatment did not cause any deleterious effects in the mice**

The most important consideration for an oncolytic virus is that it is safe. In these experiments, we noted that mice did not have any observable adverse effects after IT injection of the any of the viral treatments or control treatments. Mice continued to eat and drink after treatment, maintained their weight without even a transient drop, and continued to act normally.

Portions of organs collected at the time of euthanasia were evaluated histologically for pathological changes that might be associated with treatment. Review of the organs did not reveal any significant pathological changes that could be attributed to treatment. In addition, portions of multiple organs were homogenized and plated on RK13s to detect the presence of virus. Viral infection of organs was generally absent, but 2-4 viral colonies (106-108 pfu/gram) were detected in lung homogenates from 2 mice at 24 HPI, and one viral colony (53 pfu/gram tissue) was detected in an eye homogenate at 96 HPI (data not shown).

**Characterization of the subcutaneous B16-F10 melanoma tumor after IT treatment**

Tumors collected at various time points were examined histologically. Subgross examination (Figure 5.1A) revealed an unencapsulated, moderately well-demarcated, invasive, and highly cellular tumor within the dermis that occasionally invaded the deep skeletal muscle (Figure 5.1B and 5.2A). The tumor cells were large round cells with distinct cell margins and abundant basophilic cytoplasm that occasionally had a fine to moderate dusting with brown melanin granules. The nuclei were large and round to oval with marginated chromatin and 1-3 large nucleoli. Anisocytosis and anisokaryosis were marked. Mitotic figures averaged 6 per 400x field. Individual or small rafts of neoplastic cells could be observed within surrounding

lymphatics in some tumors (Figure 5.2B). The tumors contained multifocal to coalescing areas of necrosis (Figure 5.1A) that ranged from 5% to 85% of the tumor, and tended to be located centrally. The multifocal necrosis was due to remaining viable blood vessels, which were surrounded by a thick cuff of viable neoplastic cells, while cells further away from the blood vessel were necrotic (Figure 5.1C). When large areas of necrosis were present, the vessels in the area were also necrotic (Figure 5.1C, upper right). Tumors from the 24, 48 and 96 HPI time points were often surrounded by mild to marked dermal edema that contained mixed leukocytes, including lymphocytes, macrophages, and neutrophils (Figure 5.1D). The outer edge of the tumors was lined by a thin band of necrosis (Figure 5.1E) and was surrounded by a band of granulation tissue (Figure 5.1F). The epidermis overlying the tumors was occasionally absent (ulcerated) but was more often hypereosinophilic with loss of cellular detail (necrotic) and variably infiltrated by neutrophils (Figure 5.1G).

**B16-F10 melanoma cells tend to metastasize to the draining (renal) lymph node and the lungs**

Mice were examined after euthanasia for grossly apparent metastases, and the organs (brain, eye, lung, heart, liver, kidney, adrenal, lymph node, spleen, genital tract, and bladder) were examined microscopically for micrometastases. Metastases were most often located in the renal lymph node (Figure 5.2A-B) which is located medial to the kidney, but were also rarely found in the axillary lymph nodes (which drain the trunk) consistent with previous studies (Van den Broeck, Derore, and Simoens 2006; Tilney 1971). The draining lymph node in this subcutaneous flank model appeared to be the renal lymph node (Figure 5.2A, yellow arrow), likely due to the proximity of the tumor to the retroperitoneal space (Figure 5.2A, white arrow). The tumors were observed histologically to invade the surrounding lymphatics (Figure 5.2C), the

deep muscles (lumbar and gluteal muscles) (Figure 5.2D), and grossly the melanotic tumor could be observed through the thin muscle of the abdominal wall (Figure 5.2A, white arrow); however, the tumors did not infiltrate all of the way through the wall. The renal lymph node has been documented to drain the lumbar area, as well as the kidney and perirenal area in rats (Tilney 1971). The renal lymph node also receives drainage from the iliac and para-aortic lymph nodes, both of which drain the gluteal area (Tilney 1971). In fact, in rats, the renal lymph node ultimately receives lymph from all pelvic and retroperitoneal viscera, hind limbs, and the tail (Tilney 1971). Similarly, in humans, injection of dye into the perirenal space has been found to track into the posterior pararenal space and the fascial planes of the lumbar and sacral areas (Thornton et al. 2001).

The second most common area to find metastases was the lungs. Tumors could be observed grossly (Figure 5.2E-F), but sometimes were only detectable by light microscopy (Figure 5.2G-H). Microscopic metastases were most often adjacent to small vessels (Figure 5.2G) in the lungs or along the pleural surface (Figure 5.2H). Melanoma cells likely spread to the lungs by the hematogenous route since micrometastases were often adjacent to small vessels (Figure 5.2G). Additionally, melanoma cells were also rarely observed in blood vessels within the lung (Figure 5.2I).

Invasion of the renal lymph node by melanoma cells was observed in 27% of survival mice, while metastases were detected in the lungs of 25% of survival mice. Metastases were more common in the renal lymph node and lung of MYXV:IL15 (5/12 and 5/12, respectively) and MYXV (5/12 and 3/12, respectively) treated mice than in UV-MYXV (1/12 and 2/12, respectively) and PBS (2/12 and 2/12, respectively) mice, possibly due to the longer survival times obtained by the mice treated with live virus. Most metastases were present in mice that

survived at least 14 days PI, and the average survival times of PBS and UV-MYXV treated mice were only 11 days and 14.5 days, respectively, while MYXV:IL15 treated mice survived an average of 17 days PI, and MYXV mice survived an average of 19 days PI. However, invasion of the renal lymph node was detected in one mouse treated with MYXV:IL15 at 4 days PI and invasion of the lung was found in one mouse treated with MYXV:IL15 at 7 days PI, and in the lung of a mouse treated with MYXV at 8 days PI. Other organs where metastases were found include the spleen (1 mouse) and the diaphragm (1 mouse).

**Repeated injections of MYXV:IL15 or MYXV resulted in significantly increased survival compared to controls**

Injections of virus were repeated weekly in survival mice until the mice exhibited criterion of euthanasia, resulting in survival mice receiving between 1 and 4 injections of virus. Both groups of mice that were treated with live virus (MYXV:IL15 or MYXV) survived statistically significantly longer than did control mice (UV-MYXV or PBS) (Figure 5.3, Table 5.1). This was an encouraging finding, confirming the idea that repeated injections of the virus can result in clinically significant improvement, without the need to employ immunosuppressants. However, there was not a significant difference between the treated groups, indicating that the expression of IL15 by MYXV did not result in any increased survival. In fact, the average survival of the mice treated with MYXV:IL15 was slightly shorter (17 days) than the mice treated with MYXV (19 days).

**Virus remained at high levels within the tumor until 96 HPI, but drastically declined by 1 week PI**

In order to determine how long virus persisted in the subcutaneous melanoma tumors, the presence of virus within the treated tumors was detected by plaque assay and by detecting

Tomato red protein expression by fluorescent microscopy. Sections of tumors from mice that were treated with MYXV:IL15, MYXV:Tomato, UV-MYXV, or PBS were homogenized, serially diluted, and plated on RK13 cells to detect and quantitate the amount of virus within the treated tumors. Virus was detected from most of the tumors treated with either MYXV:IL15 or MYXV:Tomato that were collected at 24, 48, and 96 HPI (Figure 5.4), but the frequency of detection as well as the viral titer significantly decreased by 1 week PI, and was similar in tumors collected from survival mice. By 1 week PI, only 33.3% (2/6) of mice treated with MYXV:IL15 had virus detectable by plaque assay, and only 42.9% (3/7) of mice treated with MYXV:Tomato had detectable virus (Figure 5.4A). In survival mice, 42.9% (3/7) of MYXV:IL15 mice and 20% (1/5) of MYXV:Tomato treated mice had detectable virus by plaque assay. Tumors from UV-MYXV and PBS treated mice did not have any detectable virus by plaque assay. Observation of the tumors for viral tomato red expression had similar results, but was not as sensitive (Figure 5.4B-C). Tomato red fluorescence was observed in 66% of tumors collected at 24 HPI and in 100% of tumors collected at 48 HPI in either MYXV:IL15 (2/3 and 3/3, respectively) and MYXV:Tomato (2/3 and 4/4, respectively) treated mice (Figure 5.4B). Only 33% of MYXV:IL15 (1/3) and MYXV:Tomato (1/3) treated mice had observable viral fluorescence by 96 HPI, and while 20% of MYXV:Tomato (1/5) treated mice had viral fluorescence at 1 week PI, none of the MYXV:IL15 (0/3) treated mice had detectable virus (Figures 5.4B-C). None of the MYXV:IL15 (0/3) or MYXV (0/3) survival mice had virus observed (Figure 5.4B-C), and no viral fluorescence was observed from tumors of UV-MYXV or PBS treated mice (data not shown). These results indicate that while virus can persist for 1 week PI, virus is present in higher amounts at earlier (24, 48, and 96 HPI) timepoints. In addition, plaque assay appears to be a more sensitive test for detecting virus in tumors than fluorescent microscopy.

**Interleukin-15 was detected in tumors collected at 24, 48, and 96 HPI, as well as at survival time points, but dropped to very low levels by 1 week PI**

In order to confirm production of IL-15 and in order to determine how long IL-15 was present after treatment, levels of IL-15 were determined by ELISA after *in vitro* and *in vivo* treatment with MYXV:IL15. Tumors collected from MYXV:IL15 mice were homogenized. Both *in vivo* and *in vitro* collected cells were lysed by multiple freeze-thaw cycles and sonication. IL-15 was detected for a shorter period of time *in vitro* (Figure 5.5A) than *in vivo* (Figure 5.5B). This is presumed due to the ability of the virus to continue to spread to adjacent viable cells *in vivo* and produce more virus and IL-15, while the amount of virus produced (and therefore the amount of IL-15) is limited by the number of cells in the well *in vitro*. If most of the cells are infected and die with the first round of viral infection, then there are fewer cells left for the virus to spread to in order to replicate and produce virus and IL-15. This may be what occurred *in vitro*, as the IL-15 level was high at 24 hours after infection but then rapidly declined to near the detection limit by 48 hours (Figure 5.5A). Levels of IL-15 present in cells infected with MYXV:Tomato were below the minimal detection limit of the ELISA, similar to the study by Liu et al 2009. Similarly, IL-15 levels were below the detection limit for cells mock-infected with media without virus, or in wells with only media and no cells. Positive controls were also run and contained samples of MYXV:IL15 treated cells combined with the company standard.

**The degree of tumor necrosis between treatment groups was not significant**

Tumors were evaluated histologically for the degree of tumor necrosis present in order to determine if the degree of necrosis correlated with treatment or survival time. Tumors from various time points and treatment groups were graded according to the degree of necrosis as

described in the methods (Table 2.2, Figure 2.1) and the results were statistically compared. While the degree of necrosis tended to increase over time in all treatment groups, there was not a statistically significant difference in the amount of necrosis between treatment groups and control groups (Figure 5.6), and therefore, not a statistically significant correlation between necrosis and survival.

**The number of lymphocytes surrounding and within the tumors was significantly different between treatment groups**

In order to determine if MYXV:IL15 recruited more lymphocytes to the tumor than MYXV that does not express IL-15, or UV-MYXV or PBS controls, tumors were examined for infiltrating lymphocytes. All collected tumors from each treatment group and time point were graded histologically on the number of lymphocytes observed per 400x high powered field as described in the methods (Table 2.2, Figure 2.1). The average lymphocyte grades surrounding the tumor or within the tumor were compared between treatment groups and at various time points (24h, 48h, 96h, 1w, survival). Overall, lymphocytes grade was found to be statistically significantly different between treatment groups ($p<0.0001$) and at different time points ($p=0.0358$), with more lymphocytes present in groups treated with live MYXV and at earlier time points than at survival (Figure 5.7, Table 5.2). MYXV:IL15 treated tumors were found to have more lymphocytes surrounding and within the tumors than PBS ($p=0.0008$ and 0.0001, respectively) and UV-MYXV treated control mice ($p<0.0001$ and 0.0020, respectively) as well as MYXV ($p=0.0436$ and 0.0179, respectively) treated mice. MYXV treated tumors also had more lymphocytes surrounding and within the tumors than PBS treated control mice ($p=0.0398$ and 0.0025, respectively) but only had significantly more lymphocytes surrounding the tumors ($p=0.0001$) of UV-MYXV treated mice, not within the tumors ($p=0.1716$). UV-MYXV and PBS

treated control mice did not significantly differ in the number of lymphocytes surrounding or within the tumors (p=0.2937 and 0.2389, respectively).

**The number of immunohistochemically labeled mononuclear leukocytes was rarely significantly different between treatment groups**

In order to determine which type of leukocytes were infiltrating into the tumors after treatment, we used immunohistochemistry for several different cell types. We anticipated increased numbers of cytotoxic lymphocytes and NK cells in tumors treated with MYXV:IL15. At least three tumors from each treatment group and time point were evaluated for the number of lymphocytes per 400x high powered field that labeled with anti-CD3 (all T cells), CD4 (helper T cells), and CD8 (CTLs) antibodies, as well as for cells that labeled with anti-granzyme B (GrB; NK cells and CTLs) antibodies and anti-MHCII antibodies (APCs).

CD3 (cluster of differentiation 3) is a glycoprotein that is part of the T cell receptor complex present on all mature T cells. Overall, the number of CD3+ lymphocytes present at the junction of the tumor and the surrounding tissue was not statistically significantly different between treatment groups (p=0.5046), but was found to be more prevalent at early time points (24, 48 and 96 HPI) than at later time points (p=0.0285). The MYXV:IL15 treated tumors were found to have more lymphocytes surrounding tumors, at the tumor junction, or within the tumors than PBS and UV-MYXV treated control mice as well as MYXV treated mice but none of the numbers were statistically significant (Table 5.3). MYXV treated tumors were found to have more lymphocytes surrounding tumors or within the tumors than PBS and UV-MYXV treated control mice, but the only statistically significant difference was the number of CD3+ cells within the tumor of MYXV treated tumors compared to PBS treated tumors (p=0.0356). The number of CD3+ cells at the tumor junction of MYXV mice was not much different than that

present in PBS or UV-MYXV treated mice (Table 5.3). UV-MYXV and PBS treated control mice did not significantly differ in the number of lymphocytes surrounding tumors or at the tumor junction (Table 5.3). More CD3+ lymphocytes were present within UV-MYXV treated tumors than in PBS treated tumors but the number was not statistically significant (Table 5.3).

CD4 and CD8 are glycoproteins present on the surface of the 2 main subsets of T cells: helper and cytotoxic T cells, respectively. Overall, the number of CD4+ or CD8+ cells present was not statistically significantly different between treatment groups ($p=0.6743$ and 0.1895, respectively), or at different time points ($p=0.7592$ and 0.4874, respectively). The MYXV:IL15 treated mice did not have significantly more CD4+ lymphocytes than PBS, UV-MYXV, or MYXV treated mice (Table 5.4). The MYXV:IL15 treated mice were found to have more CD8+ lymphocytes than PBS or UV-MYXV treated control mice as well as MYXV treated mice but none of the numbers were statistically significant (Table 5.5). MYXV treated mice did not differ significantly in the number of CD4+ or CD8+ lymphocytes from PBS treated mice. MYXV treated mice had more CD4+ and CD8+ cells than UV-MYXV treated mice but not significantly more. UV-MYXV and PBS treated control mice did not significantly differ in the number of CD4+ and CD8+ lymphocytes (Table 5.5).

Granzyme B is a serine protease present in CTLs and NK cells. The number of cells that were positively labeled with anti-GrB antibodies was observed by fluorescent microscopy (Table 5.6). Overall, the number of GrB+ cells present was not statistically significantly different between treatment groups ($p=0.2121$), or at different time points ($p=0.3763$). Rare GrB+ cells were observed in MYXV:IL15 or MYXV treated mice, but were not observed at all in UV-MYXV or PBS treated mice. The MYXV:IL15 treated mice did not have significantly more GrB+ cells than PBS ($p=0.2209$), UV-MYXV ($p=0.2209$), or MYXV ($p=0.2393$) treated mice.

MYXV treated mice also did not have significantly more GrB+ cells than PBS (p=0.3055) or UV-MYXV (p=0.3055) treated mice. UV-MYXV and PBS treated control mice did not significantly differ in the number of GrB+ cells (p=1.0).

Cells that express MHCII are APCs, and include dendritic cells, macrophages, and B lymphocytes. Overall, the number of MHCII+ cells present surrounding or within the tumors, or in areas of tumor necrosis, were not statistically significantly different between treatment groups (p=0.6288, 0.5341, and 0.3376, respectively). Significantly more MHCII+ cells were present at early time points surrounding the tumor (p=0.0347) than at later time points, but the number of MHCII+ cells within the tumor or in areas of tumor necrosis (p=0.8449 and 0.0934, respectively) were not significantly different between time points (Table 5.7). The MYXV:IL15 treated tumors were found to have more MHCII+ cells surrounding them than tumors treated with PBS but the difference was not significant (Table 5.7). There was also not a significant difference between the MYXV:IL15 and PBS treated mice in the number of MHCII positive cells within the tumors and in areas of tumor necrosis. MYXV:IL15 treated mice did not differ significantly in the number of MHCII positive cells surrounding the tumor, within the tumor, or in areas of tumor necrosis than UV-MYXV or MYXV treated mice (Table 5.7). The MYXV treated tumors were found to have more MHCII+ cells within areas of tumor necrosis than tumors treated with PBS but the difference was not significant (Table 5.7). There was also not a significant difference between the MYXV and PBS treated mice in the number of MHCII positive cells surrounding the tumors or within the tumors (Table 5.7). However, if the number of MHCII+ cells were assessed in all areas, there was a statistically significant difference between the MYXV treated mice and PBS treated mice (p=0.0470). MYXV treated mice did not differ significantly from UV-MYXV treated mice in the number of MHCII positive cells in any area of

the tumor (Table 5.7). UV-MYXV and PBS treated control mice did not significantly differ in the number of MHCII+ cells in any area, although there were generally more in the UV-treated mice than the PBS treated mice (Table 5.7).

**The number of neutrophils surrounding and within the tumors was significantly different between MYXV treated tumors and PBS treated control tumors**

Tumors were observed histologically to determine if neutrophils significantly varied between treatment groups. Tumors were graded histologically on the number of neutrophils observed per 400x high powered field as described in the methods (Table 2.2, Figure 2.1). The average neutrophil grade surrounding the tumor, within viable areas of the tumor, and within areas of tumor necrosis were compared between treatment groups and at various time points (24h, 48h, 96h, 1w, survival). Overall, neutrophil grade was found to be statistically significantly different surrounding the tumor, within viable areas of the tumor, and within areas of tumor necrosis, between treatment groups ($p=0.0280$, 0.0016, and $<0.0001$, respectively). Neutrophil grade was also found to be statistically significantly different surrounding the tumor ($p<0.0001$), and within viable areas of the tumor ($p=0.0015$) at different time points, but not within areas of tumor necrosis ($p=0.4422$). Overall, neutrophils were more prevalent in areas of tumor necrosis and surrounding the tumor than in viable areas of the tumors, which only had neutrophils during early time points (Figure 5.8, Table 5.8).

MYXV:IL15 treated tumors were found to have statistically more neutrophils surrounding, within the tumors, and within areas of necrosis than PBS control mice ($p=0.0448$, 0.0050, and 0.0040, respectively), which tended to have few neutrophils, but not statistically more than UV-MYXV or MYXV treated mice (Table 5.8). MYXV treated tumors, however, had significantly more neutrophils surrounding, within tumors, and within areas of necrosis than

PBS control mice (p=0.0049, 0.0001, and <0.001, respectively) and UV-MYXV treated mice (p=0.0500, 0.0291, and 0.0017, respectively). UV-MYXV and PBS treated control mice significantly differed in the number of neutrophils present in viable areas of the tumors (p=0.0083), with UV-MYXV mice having more neutrophils present than PBS treated mice. UV-MYXV treated mice also had more neutrophils histologically than PBS treated mice had surrounding the tumors or in area of necrosis, but the numbers were not statistically significant (Table 5.8).

In summary, while MYXV:IL15 and MYXV treated tumors were both observed to have more neutrophils in all areas than PBS treated tumors, and MYXV treated tumors also had more neutrophils in all areas than UV-MYXV treated tumors; MYXV:IL15 treated tumor did not have significantly more neutrophils than MYXV treated tumors, indicating that the IL-15 expressed by the virus did not significantly affect neutrophil infiltration.

**The number of immunohistochemically labeled neutrophils (Gr-1 positive cells) was not significantly different between most treatment groups**

Since neutrophils were observed histologically, we used immunohistochemistry to further evaluate the numbers of neutrophils present in different areas of the tumor to determine if neutrophils significantly varied between treatment groups. At least three tumors from each treatment group and time point were evaluated for the number of neutrophils per 400x high powered field that labeled with anti-Gr-1 antibodies. Overall, the number of Gr-1+ cells present surrounding tumors, within tumors and within areas of tumor necrosis did not statistically significantly differ between treatment groups (p=0.4763, 0.2370, and 0.1023, respectively), or between different time points (p=0.7776, 0.05089, and 0.5902).

The MYXV:IL15 treated tumors were found to have more Gr-1 positive cells in areas of

tumor necrosis (0.0781) than PBS treated cells, but not surrounding or within the tumors (Table 5.9). MYXV:IL15 treated tumors did not differ significantly in the number of Gr-1 positive cells in any area than UV-MYXV or MYXV treated mice (Table 5.9). The MYXV treated tumors were found to have significantly more Gr-1 positive cells in areas of tumor necrosis (0.0305) than PBS treated cells, but not surrounding or within the tumors (Table 5.9). MYXV treated tumors did not significantly differ from UV-treated tumors in the number of Gr-1 positive cells present in any areas (Table 5.9). UV-MYXV and PBS treated control mice did not significantly differ in the number of neutrophils in any area (Table 5.9). In summary, our IHC evaluation of neutrophils only found significant differences between MYXV:IL15 or MYXV treated tumors and PBS treated tumors in areas of tumor necrosis.

**Antibody response to the virus was present in some treated mice**

In order to evaluate the humoral antibody response to viral treatment, blood was collected at the time of euthanasia of all mice and the ability of the serum to neutralize the virus was evaluated. An antibody response capable of reducing the formation of MYXV:Tomato plaques by more than 50% (when compared to serum from control mice inoculated with PBS) was observed in some survival mice treated with MYXV:Tomato (4/5), MYXV:IL15 (4/5), and UV-MYXV (1/4). Since some of the treated mice did not develop an antibody response detectable by plaque reduction neutralization titer (PRNT) 50 (as described in materials and methods), the results were not statistically significant between groups (Figure 5.9). However, variability in the formation of a detectable antibody response to vaccination with vaccinia virus has been documented (Harrop et al. 2006); therefore, these results are not unexpected with IT treatment with MYXV.

**Discussion**

A statistically significant increase in survival was achieved in the B16 aggressive murine melanoma model by repeating intratumoral injections of MYXV or MYXV:IL15 every week. This is similar to other studies with oncolytic viruses, including MYXV, which have shown that while a single injection with an oncolytic virus often does not have beneficial results, multiple injections with the same virus can have positive results (Stanford et al. 2008). For this experiment, weekly injections were chosen because previous experiments had demonstrated that MYXV was usually no longer detectable at 1 week PI. This experiment further confirmed that virus titer markedly decreased in tumors at one week PI, but was still high at 96 HPI. Visualization of virus tomato red expression within the tumor by fluorescent microscopy was similarly absent at 1 week PI in all mice, but was visualized in some mice at 96 HPI. These findings support that MYXV should be administered at least weekly, but also indicates that boostering the injections every 5-6 days may be beneficial. Some studies with oncolytic viruses have utilized daily boostering of virus in an attempt to infect and kill as many cancer cells as possible (Stanford et al. 2008). This approach is feasible due to the fact that oncolytic viruses have a very wide safety margin, with most only causing mild flu-like symptoms, and with clinical trials generally unable to find a maximum tolerated dose (MTD). Due to the rapid course of disease with B16-F10 melanomas in mice, survival mice in this study only received between 1 and 4 injections before they met criteria of euthanasia. The use of multiple treatment doses is common when treating cancer, especially with chemotherapy and radiation therapy, so it should be no surprise that multiple treatments may also be necessary for oncolytic virotherapy. Additional studies may find that daily injections or a short series of daily injections that are repeated periodically (e.g. every 1-2 weeks) may provide even better results than those seen in this study.

The average survival of the mice treated with MYXV:IL15 was shorter (17 days) than the mice treated with MYXV (19 days). However, the difference was not statistically significant. This is also an important observation, as there is always a concern that any treatment that will increase the immune response during treatment with an oncolytic virus will result in faster clearance of the virus, with subsequent decreased tumor cell lysis and shorter survival times. In addition, there is also concern when using cytokines that the patient may experience adverse events, as has been observed in some people treated with IL-2 (Eisner, Husain, and Clark 2004). Treatment with cytokines can result in adverse events that could decrease survival, up to and including rapid death due to a cytokine storm. However, the mice that were injected with MYXV:IL15 did not have any observable adverse effects, with no evidence of weight loss or decreased appetite, and it does not seem likely that the slight decrease in survival time is due to IL-15 treatment, since the mice appeared normal and the difference is not statistically significant.

One of the goals of this study was to characterize the response of the immune system to IT injection of the virus as well as to IL-15. Histological analysis of the tumors revealed that the initial response at the site of tumor injections was an infiltration of neutrophils and an area of necrosis. Overall, neutrophils were more prevalent in areas of tumor necrosis and surrounding the tumor than in viable areas of the tumors, which only had neutrophils during early time points (Table 5.7, Figure 5.8). Large areas of necrosis were often associated with necrotic blood vessels, whereas tumor cells remaining in areas of multifocal necrosis were surrounding viable vessels. Neutrophils have been shown to inhibit blood flow in some pathological conditions, and this has been documented to occur following oncolytic virotherapy with VACV (Breitbach et al. 2007). Depletion of neutrophils in that study, resulted in improved blood flow with subsequent decreased tumor cell apoptosis in uninfected cells, as well as improved viral replication, spread,

and persistence, over mice whose neutrophils were not depleted (Breitbach et al. 2007). This suggests that treatments that target the vasculature may or may not be of benefit if given in combination with MYXV oncolytic therapy, since they may increase tumor cell death but may limit the spread of the virus.

Macrophages were also present in the areas of necrosis, but not lymphocytes. This is expected as neutrophils and macrophages are commonly found in areas of necrosis, but lymphocytes are not. Lymphocytes were most prevalent at the junction of the tumor and the dermis, with some present at the outer edge of the mass, and many in the dermis surrounding the tumor. Lymphocytes were also rarely surrounding vessels in tumors treated with virus. A rim of necrosis and granulation tissue surrounded the tumors at early time points, and the surrounding dermis was edematous and contained increased numbers of lymphocytes in all tumors. This was present at early time points regardless of treatment, and was most likely a response to growth of the tumor.

The statistically significant increase in the number of lymphocytes in mice treated with live MYXV indicates that MYXV results in an influx of lymphocytes to the tumor bed and to the dermis surrounding the tumor that would be minimally present in control mice that are not treated with the virus. Additionally, treatment with MYXV:IL15 resulted in a greater influx of lymphocytes than MYXV without IL-15, suggesting that IL-15 induced increased infiltration of the tumor bed and surrounding area by lymphocytes. While dead MYXV resulted in a mild increase in lymphocytes over PBS treated control mice, the mere presence of these virus particles was not enough to result in more than a minimal increase in lymphocytes.

Intratumoral treatment with virus was found to be safe, with no adverse effects noted, and rare viral spread. When compared to the viral titers detected in the treated tumors, which

averaged over 1,000,000 pfu/gram between 24 and 96 HPI, the titers detected in the lung (106-108 pfu/gram) from 2 mice at 24 HPI and eye (53 pfu/gram tissue) of 1 mouse at 96 HPI, are miniscule. It is likely that the virus detected in the lung was due to hematogenous spread that occurred right after injection, since the 2 positive mice were collected at 24 HPI. Due to the increased blood supply that is commonly found in tumors, it is possible that the IT injection resulted in some virus being injected into a vessel. Alternatively, the virus may have gained access to the circulation through leaky vessels in the tumor or by infecting cells that entered the blood stream and circulated to the lung. Indeed, detection of virus in the blood after IT injection has been documented with adenovirus and vaccinia virus, with one study showing viral DNA in the blood of 18/21 patients treated with adenovirus (Nokisalmi et al. 2010) and in 14/14 patients treated with vaccinia virus (Park et al. 2008). In the vaccinia study, all patients had viral genomes detected in the blood immediately after IT injection, but concentrations decreased by 90% within the first 4-6 hours (Park et al. 2008). However, there was also delayed re-emergence of circulating viral DNA consistent with viral replication (Park et al. 2008). In this experiment, we were able to detect higher levels of MYXV than the original injected dose, also consistent with viral replication. The fact that virus was not detected in the lungs of mice at later time points in our experiments indicates that the virus was not able to productively infect the normal cells in the lung. The one viral colony from the eye was likely on the exterior of the eye, and was possibly secondary due to autoinoculation or contact with another mouse with an ulcerated mass.

Some of the treated survival mice in each treatment group that received virus developed an antibody response, whether they were treated with live or dead virus. Meanwhile, some mice that received the same treatment did not develop an antibody response detectable by PRNT. This

is common with poxviruses, and has been documented in patients receiving vaccinia treatments. In one study, only 82% (14/17) of patients developed a neutralizing antibody response to VACV (modified vaccinia Ankara), despite multiple intratumoral injections (Harrop et al. 2006). This is in contrast to some other virotherapeutics, such as adenovirus, in which most if not all patients treated with the virus develop a strong antibody response (Kimball et al. 2010). The development of an antibody response in the mice in this study did not correlate with any significant difference in the length of survival or in the number of infiltrating leukocytes. A lack of significant correlation between an antiviral antibody response and clinical responses is not uncommon, and has been documented in several studies (Park et al. 2008; Harrop et al. 2006; Dummer et al. 2010). Antibodies can be produced as early as 3-5 days post-infection with some viruses (Park et al. 2008; Liang et al. 1999), and levels rise over time and may or may not be detected depending on the virus and the method of antibody detection. In addition, titers rise significantly after repeated exposure, which is why vaccine regimens against most viruses typically require booster inoculations. The fact that an antiviral antibody response is not necessary in order to achieve a treatment benefit with oncolytic virotherapy lends support to the theory that it is the development of cell-mediated immune responses that may be important in tumor regression.

Intratumoral treatment with MYXV:IL15 was found to be safe, with no discernable deleterious effects to the mice and no statistically signficant decrease in survival time compared to mice treated with MYXV. MYXV:IL15 treatment did result in a statistically significant increase in survival when compared to control mice, but not when compared to mice treated with MYXV that does not express IL-15. Additionally, MYXV:IL15 treatment did not result in a

decreased rate of metastases compared to MYXV treated mice, nor was there a difference in the rate of formation of a humoral immune response to the virus.

Infection with MYXV:IL15 resulted in the production of IL-15 within the melanoma tumors as well as in cell culture, and IL-15 was able to be detected for a longer period of time *in vivo* (96 h, Figure 5.5B) than *in vitro* (24 h, Figure 5.5A). This is presumed due to the ability of MYXV:IL15 to spread and infect adjacent cells within the tumor, while infection of cells *in vitro* is limited by the number of cells within the well, many of which were dead by 48 h (Figure 3.1 and 3.7C). Treatment with MYXV:IL15 resulted in a statistically significant increase in infiltrating lymphocytes in all areas of the tumors versus treatment with MYXV that does not express IL-15, as well as controls, which was one of the goals of this study. However, expression of IL-15 and increased numbers of lymphocytes did not correlate with an increase in survival as was expected. It may be that the level of IL-15 expressed was not sufficient to overcome tolerance to the tumor. The presence of higher numbers of lymphocytes at earlier time points (48-96 HPI), with fewer present at 1 week PI and at survival time points suggests that the immune response was dampened over time.

Treatment with MYXV:IL15 did not result in statistically significant increases in CTL lymphocytes or NK cells in this model. Similarly, there was not a significant increase in the numbers of helper T cells. In fact, when the numbers of CD4+, CD8+, and GrB+ cells detected by IHC was compared to the number of CD3+ cells, there were much fewer CD4+, CD8+ and GrB+ cell than CD3+ cells. This is likely due to the increased sensitivity of the CD3+ IHC, compared to the other IHC's, which displayed much weaker fluorescence even when using much higher amounts of primary antibody. It is possible that some CD4+ Tcells, CD8+ T cells, and NK cells that labeled weakly were not able to be differentiated. MHCII labeled cells very well

but was detected in all tumors treated with either virus or control, with no statisitically significant differences. While there was an increase in infiltrating neutrophils in mice treated with MYXV:IL15 over PBS treated tumors, the number of neutrophils was not increased over MYXV treated tumors, indicating that IL-15 expression does not recruit neutrophils, as expected.

In summary, multiple IT treatments with MYXV or MYXV:IL15 were able to provide a small but statistically significant increase in survival over control treated mice without the necessity of using immunosuppressants. While IL-15 did not provide a survival advantage compared to MYXV treatment alone, it did result in a statistically significant increase in lymphocytes within the tumor and in the dermis surrounding the tumor. Additional modification of treatment (e.g. co-expression of IL-15 receptor alpha, IL-12, or a tumor antigen) or of the tumor model (e.g. different tumor, daily injections) may provide a survival benefit.

## Chapter 5 Tables and Figures

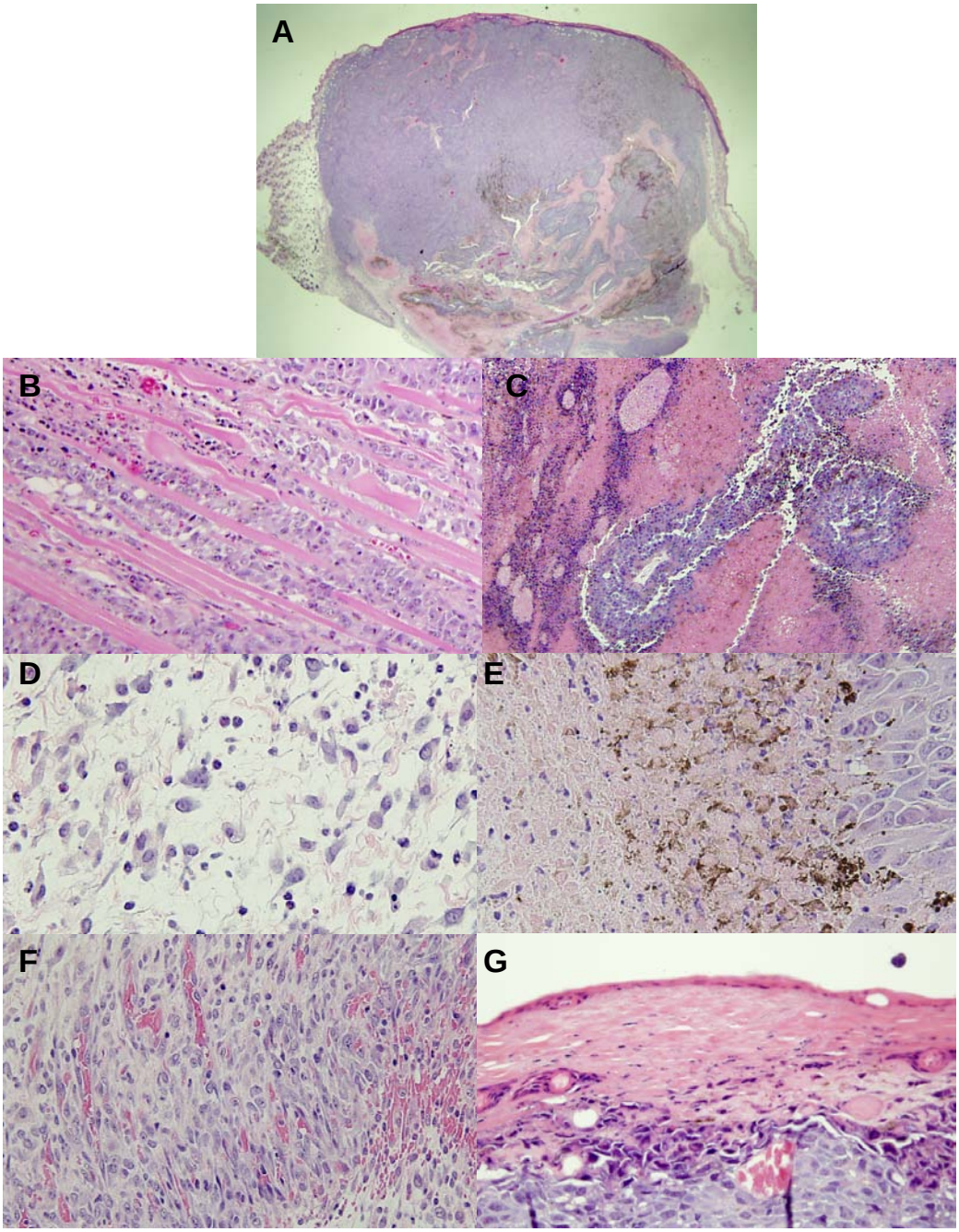



Figure 5.1. Diagram of a B16-F10 subcutaneous tumor. Tumors occasionally infiltrated the deep muscle (B). Tumors had a large central area of necrosis or multifocal to coalescing areas of necrosis (C). When necrosis was multifocal, thick bands of viable neoplastic cells were typically remaining surrounding vessels (C). Most tumors at the early stages were covered by dermis that was markedly thickened by edema (D), multifocally had a necrotic edge (E), and were surrounded by a rim of granulation tissue (F). Some tumors were covered by an area of ulceration or an area of necrotic epithelium (G).

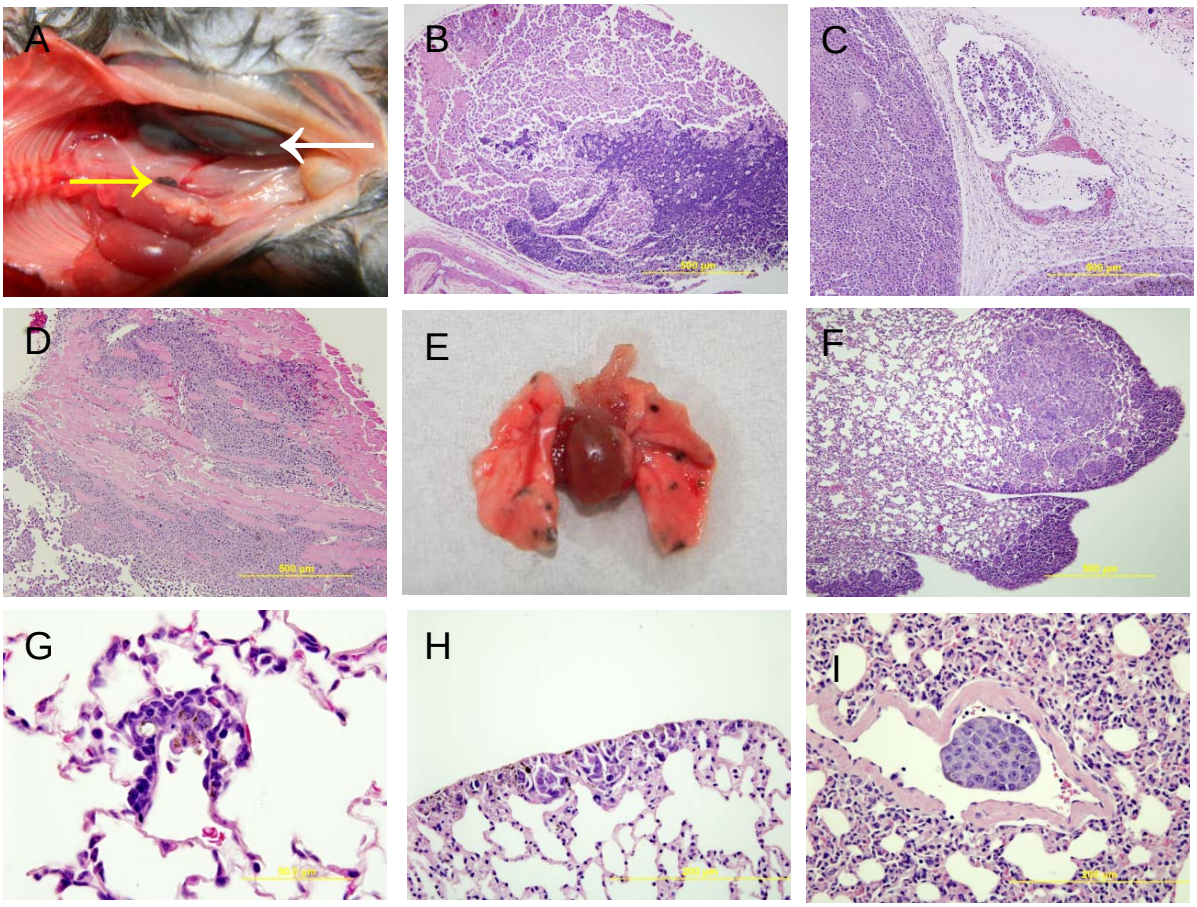



Figure 5.2. Metastases and invasion. Metastases were most commonly found in the renal lymph node (A, yellow arrow, and B). Adjacent skeletal muscle was commonly invaded with B16-F10 neoplastic cells (D), which could also be found in the surrounding lymphatic vessels (C). Metastases in the lung ranged from grossly visible masses (E-F) to small micrometastases that were only visible with a light microscope (G-H). Metastases in the lung most often surrounded a small vessel (G) or were along the pleural surface (F and H). Neoplastic cells were also observed in the blood vessels (I).

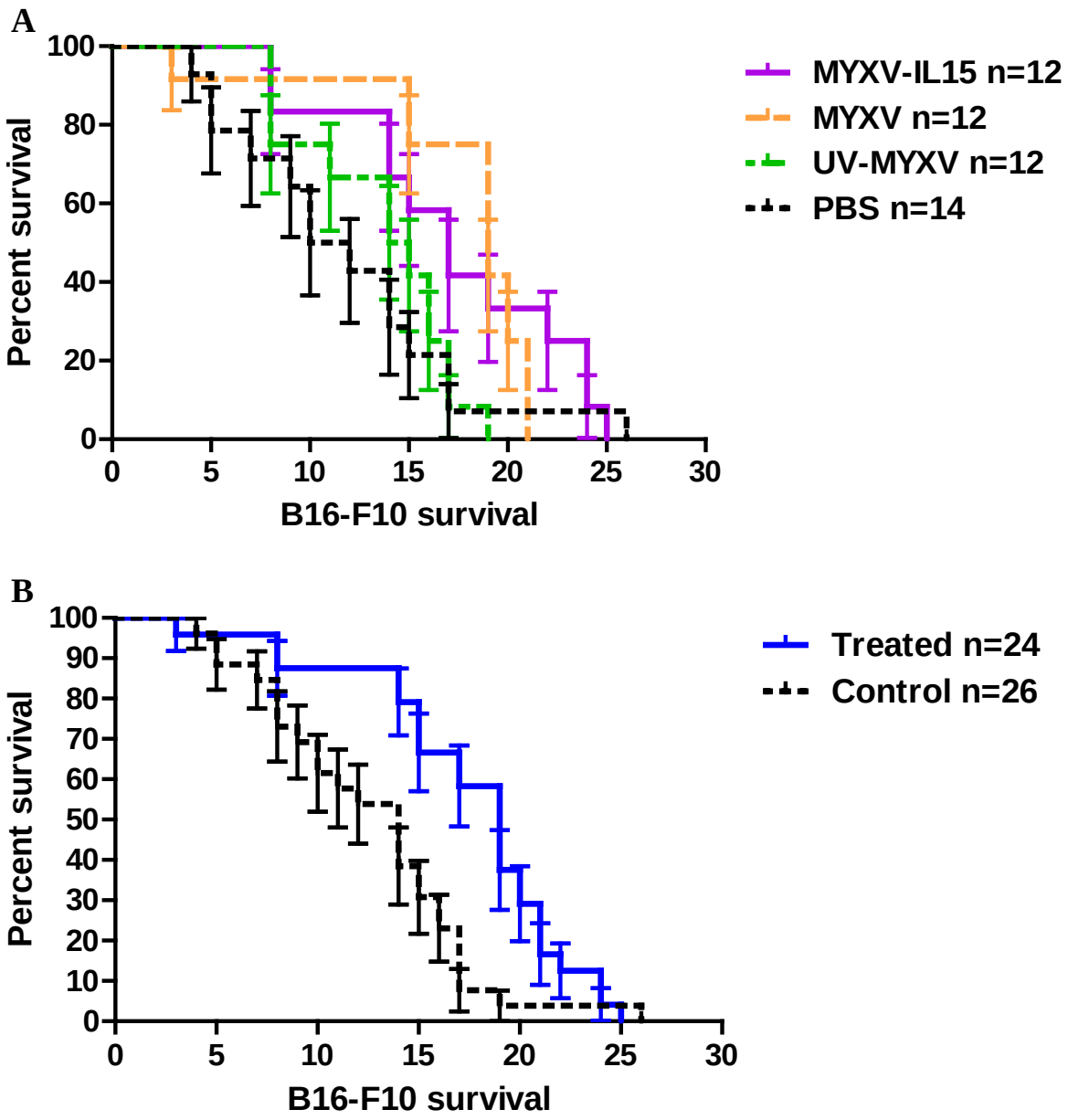



Figure 5.3. Subcutaneous B16-F10 Kaplan-Meier survival curves. Mice were inoculated intratumorally with MYXV:Tomato, MYXV-IL15:Tomato, UV-MYXV, or PBS once their B16-F10 tumors reached ≥ 5 mm in diameter. The day of virus inoculation was designated day 0, and mice were monitored daily until they reached a criterion of euthanasia. A statistically significant increase in survival was present between the treated and control mice, but not between the MYXV:Tomato and MYXV-IL15:Tomato treated mice, or between the UV-MYXV or PBS treated mice.

Table 5.1. Survival statistics. Treatment groups were compared by log-rank and Gehan-Breslow-Wilcoxon tests. * significant *p*-values less than 0.05

| Treatment Groups | Log-rank *P*-value | Gehan-Breslow-Wilcoxon *P*-value |
|---|---|---|
| MYXV:IL15 vs. MYXV | 0.6102 | 0.6826 |
| MYXV:IL15 vs. UV-MYXV | 0.0382* | 0.1136 |
| MYXV:IL15 vs. PBS | 0.1209 | 0.0314* |
| MYXV vs. UV-MYXV | 0.0011* | 0.0046* |
| MYXV vs. PBS | 0.0124* | 0.0041* |
| UV-MYXV vs. PBS | 0.5396 | 0.2480 |
| Treated vs. Control | 0.0028* | 0.0008* |

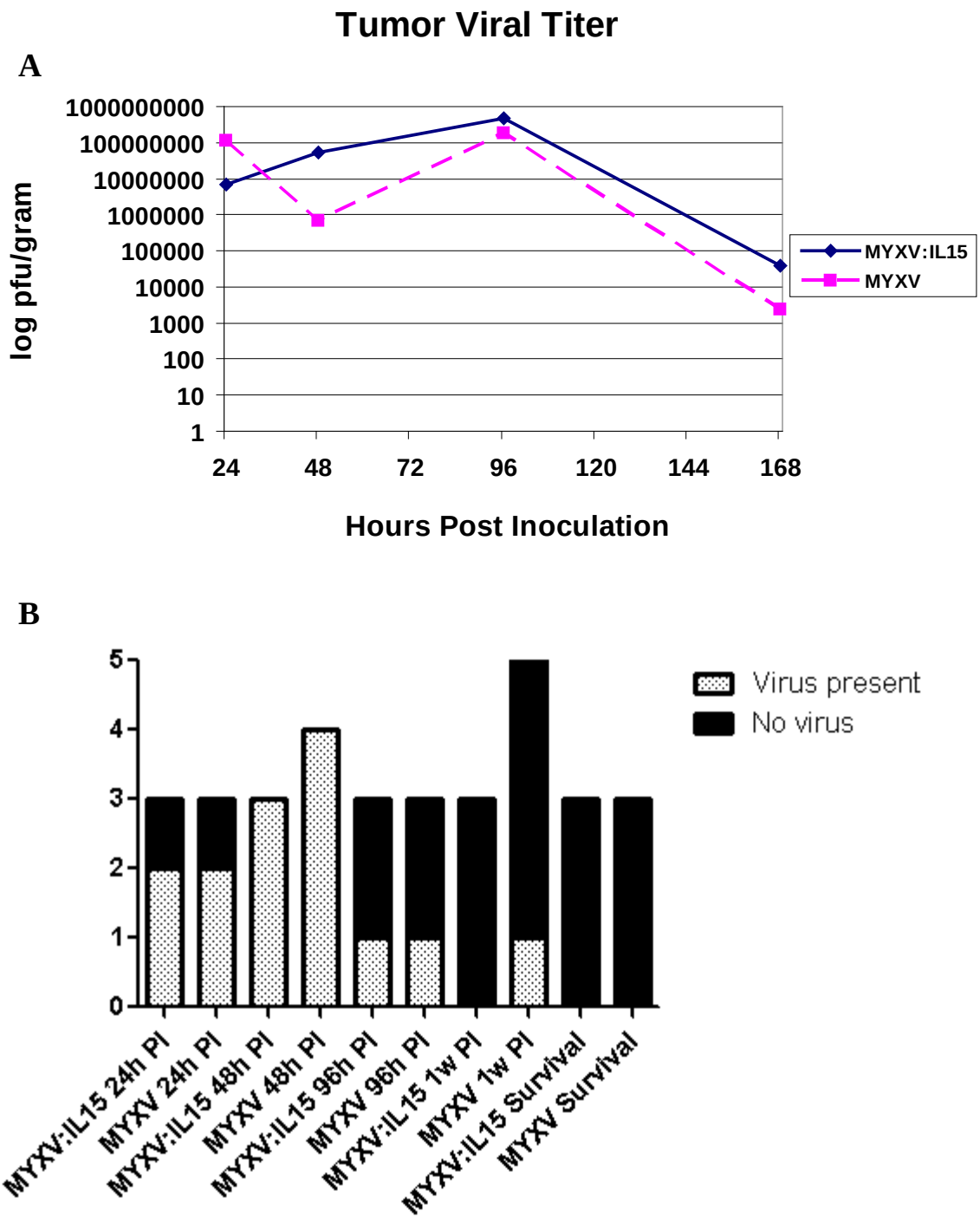



Figure 5.4. Myxoma virus in tumors. A) Sections of tumors were collected at various time points, homogenized, serially diluted, and plated on RK13s to determine pfu's of virus per gram of tissue. B and C) Tumors were analyzed by fluorescent microscopy for the presence of red fluorescence, which was considered indicative of the presence of MYXV:Tomato (or MYXV:IL15). Red fluorescence was not observed in any UV-MYXV or PBS treated tumors.

C

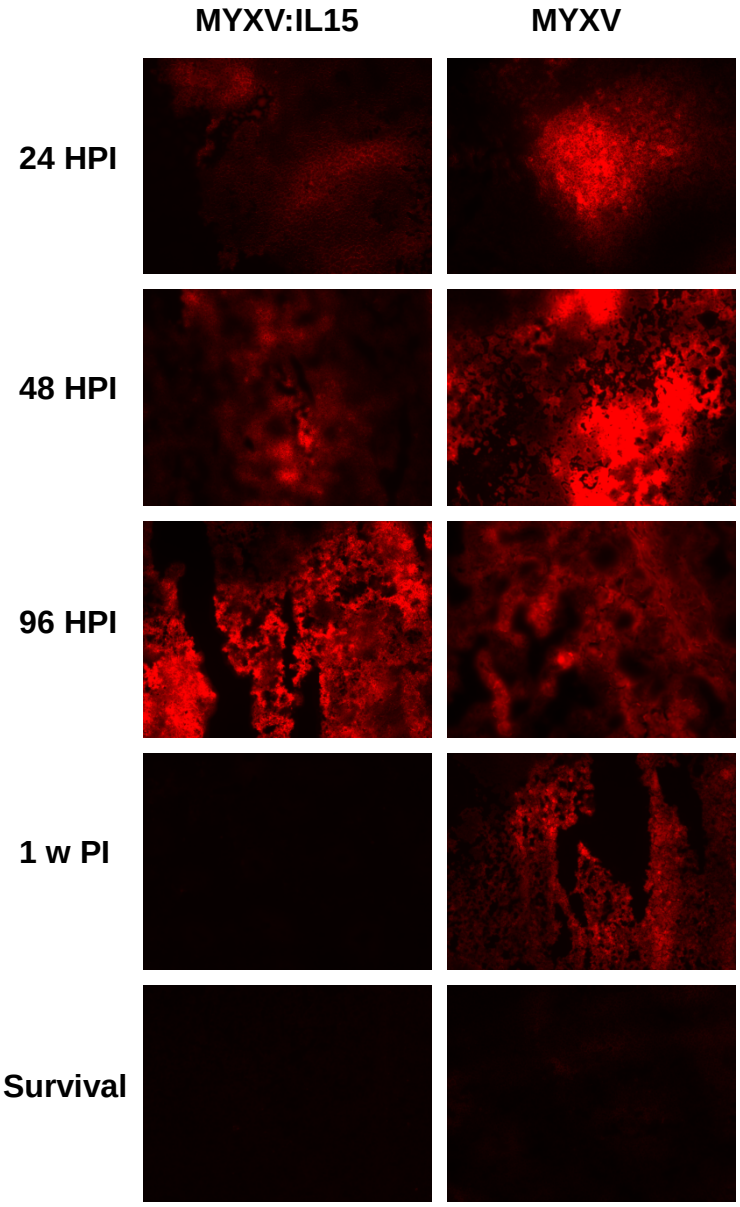



Figure 5.4. Myxoma virus in tumors, continued.

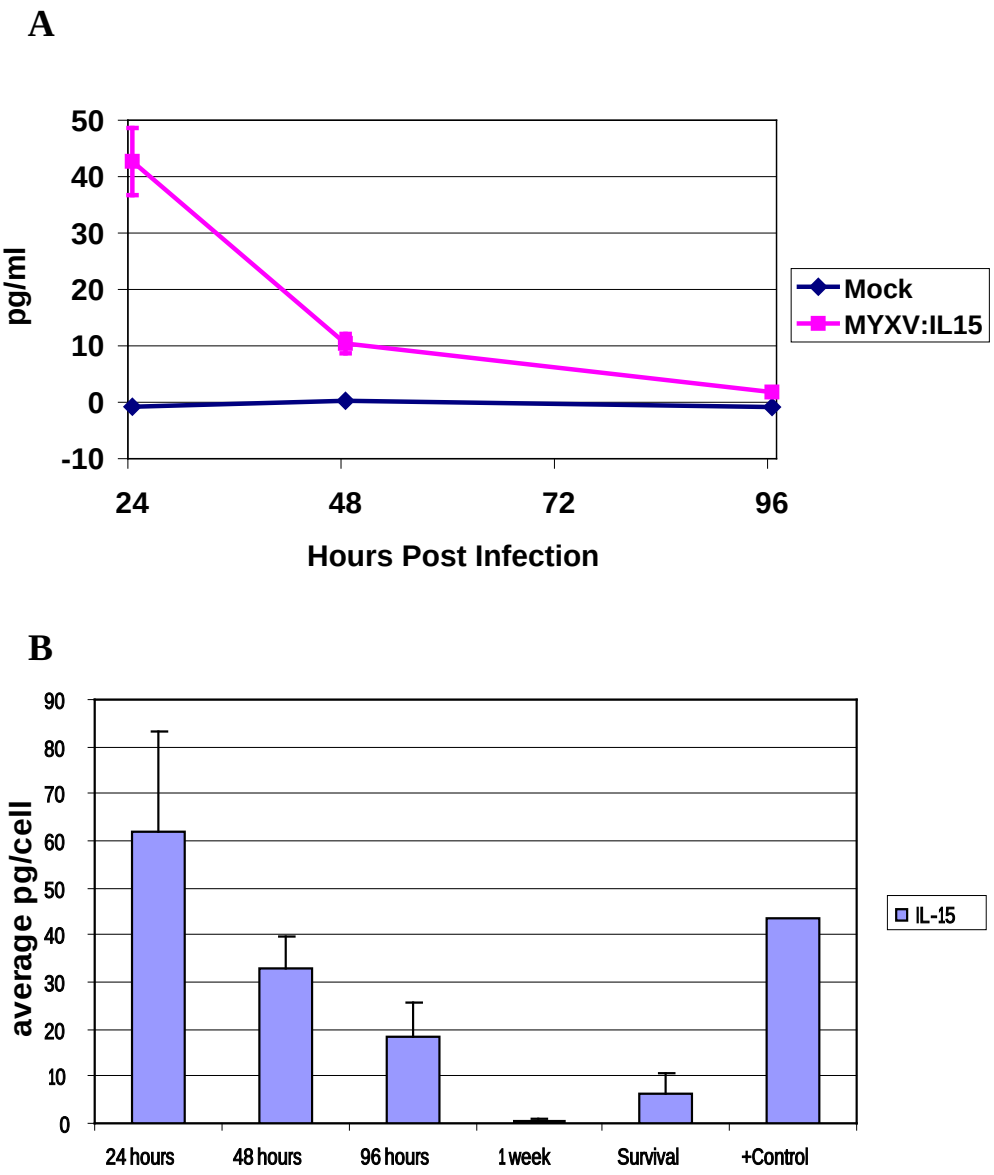



Figure 5.5. IL-15 levels in B16-F10 cells and tumors. B16-F10 cells (A) were infected with MYXV:IL15 and collected at 24, 48, and 96 HPI. Tumors (B) inoculated IT with MYXV:IL15 were collected at euthanasia (24h, 48h, 96h, or 1 w after inoculation or at survival). IL-15 levels were detected by ELISA. Positive control consisted of adding 62.5 pg of IL-15 standard to lysates from a 1 week PI treated tumor.

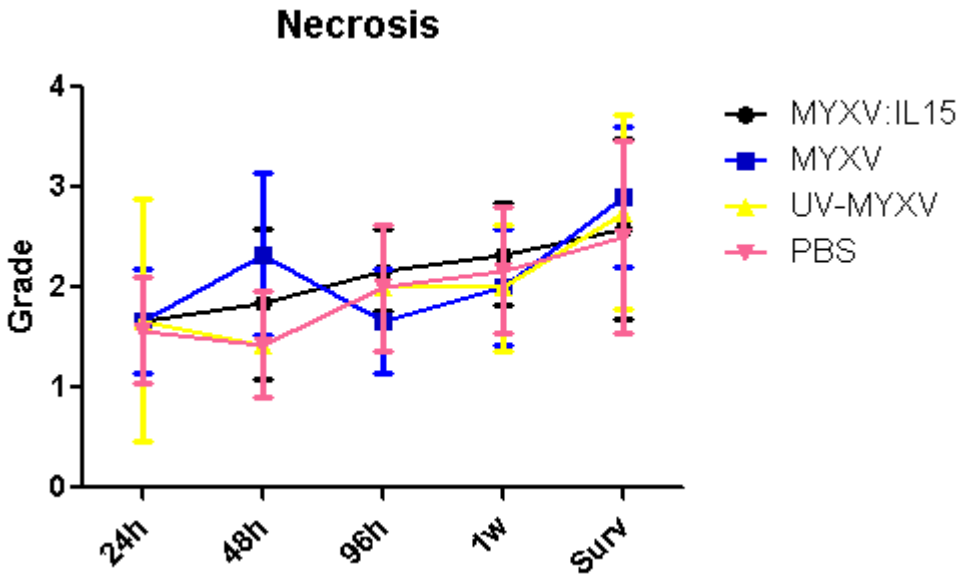



Figure 5.6. Average grade of subcutaneous tumor necrosis. The amount of tumor necrosis as graded histologically did not differ significantly between treatment groups.

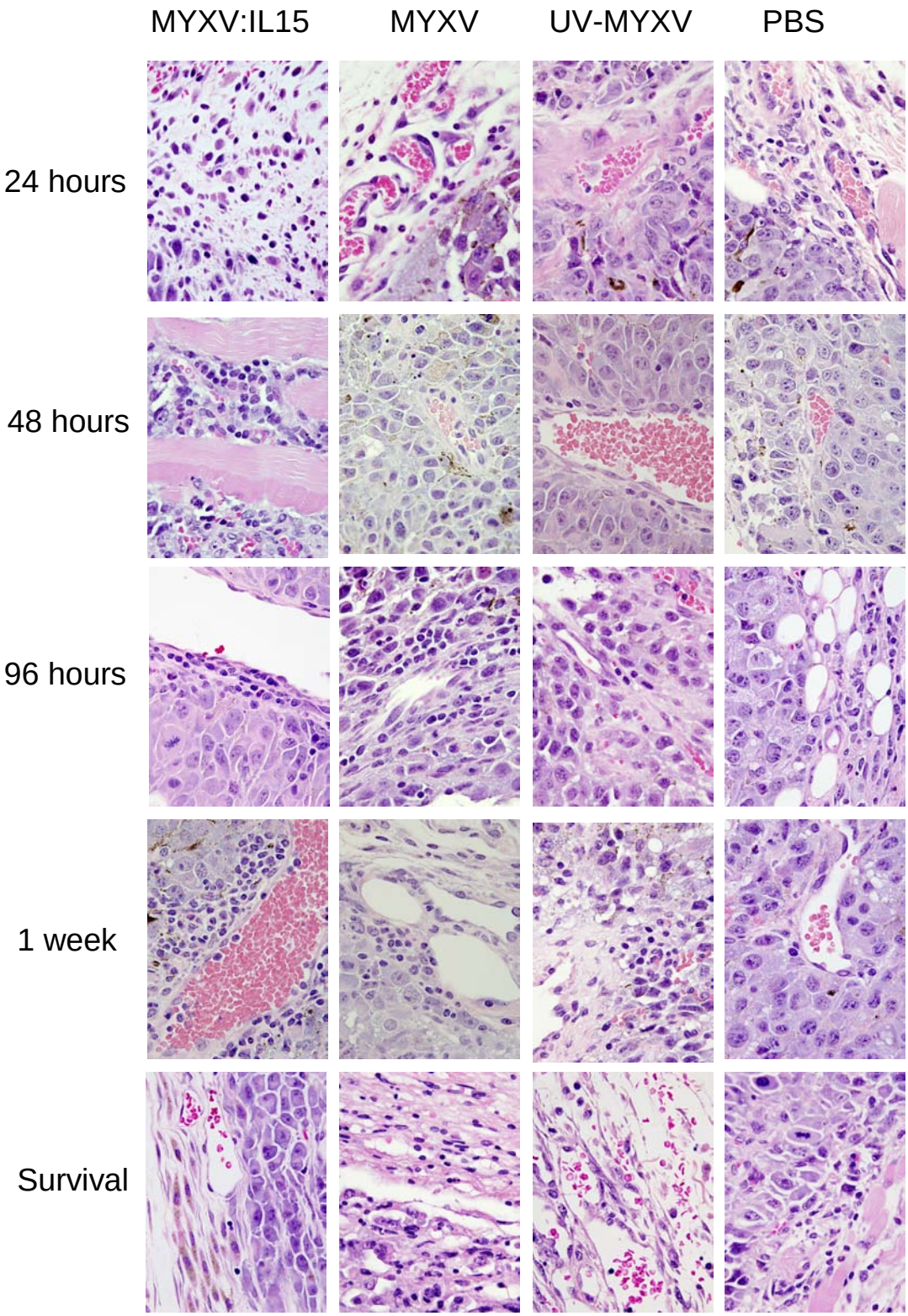



Figure 5.7. Lymphocytes surrounding and within subcutaneous melanoma tumors. A few scattered lymphocytes surrounded most tumors regardless of treatment, but were more prevalent in virally treated tumors and at early time points. Lymphocytes often surrounded vessels, and were more prevalent in MYXV:IL15 treated tumors and at early time points.

Table 5.2. Average lymphocyte grades.

| Lymphocyte | MYXV:IL15 | | MYXV | | UV-MYXV | | PBS | |
|---|---|---|---|---|---|---|---|---|
| | Surr | Tum | Surr | Tum | Surr | Tum | Surr | Tum |
| 24 hours | 0.50 ±0.34 | 0.17 ±0.17 | 0.33 ±0.33 | 0.00 ±0.00 | 0.00 ±0.00 | 0.50 ±0.50 | 0.14 ±0.14 | 0.00 ±0.00 |
| 48 hours | 1.67 ±0.49 | 0.83 ±0.65 | 0.17 ±0.17 | 0.17 ±0.17 | 0.14 ±0.14 | 0.00 ±0.00 | 0.71 ±0.57 | 0.00 ±0.00 |
| 96 hours | 1.67 ±0.49 | 2.00 ±0.68 | 0.83 ±0.17 | 0.17 ±0.17 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 |
| 1 week | 0.67 ±0.49 | 0.83 ±0.54 | 1.14 ±0.40 | 1.00 ±0.38 | 0.17 ±0.17 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 |
| Survival | 0.50 ±0.34 | 0.00 ±0.00 | 0.18 ±0.12 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.08 ±0.08 | 0.00 ±0.00 |

Surr = tissue surrounding the tumor, Tum = tumor. ± standard error of the mean

Table 5.3. Average numbers of CD3 positive cells.

| CD3 | MYXV:IL15 | | | MYXV | | | UV-MYXV | | | PBS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Surr | Tum | TDJ | Surr | Tum | TDJ | Surr | Tum | TDJ | Surr | Tum | TDJ |
| 24 hours | 5.33 ±2.91 | 7.00 ±4.36 | 16.00 ±13.58 | 4.00 ±4.00 | 1.33 ±1.33 | 3.00 ±3.00 | 1.67 ±0.67 | 7.67 ±5.36 | 12.33 ±12.33 | 2.67 ±2.67 | 8.33 ±4.41 | 7.00 ±7.00 |
| 48 hours | 71.33 ±64.34 | 32.67 ±24.91 | 66.67 ±66.67 | 3.33 ±1.76 | 14.33 ±6.53 | 37.00 ±17.20 | 0.00 ±0.00 | 5.33 ±5.33 | 22.67 ±13.92 | 0.00 ±0.00 | 0.00 ±0.00 | 22.33 ±12.85 |
| 96 hours | 38.67 ±21.98 | 12.33 ±7.84 | 23.33 ±6.01 | 10.00 ±6.43 | 6.00 ±3.46 | 6.00 ±6.00 | 1.67 ±0.67 | 3.33 ±2.40 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 |
| 1 week | 0.00 ±0.00 | 4.00 ±4.00 | 1.33 ±1.33 | 0.00 ±0.00 | 38.20 ±14.45 | 0.00 ±0.00 | 0.00 ±0.00 | 14.00 ±7.81 | 1.33 ±1.33 | 0.00 ±0.00 | 0.00 ±0.00 | 16.00 ±16.00 |
| Survival | 0.00 ±0.00 | 2.00 ±2.00 | 1.67 ±1.67 | 0.00 ±0.00 | 16.33 ±16.33 | 0.00 ±0.00 | 0.00 ±0.00 | 0.67 ±0.67 | 0.33 ±0.33 | 1.00 ±1.00 | 0.00 ±0.00 | 0.00 ±0.00 |

Surr = tissue surrounding the tumor, Tum = tumor, TDJ = tumor dermis junction. ± standard error of the mean

Table 5.4. Average numbers of CD4 positive cells.

| CD4 | MYXV:IL15 | | MYXV | | UV-MYXV | | PBS | |
|---|---|---|---|---|---|---|---|---|
| | Surr | Tum | Surr | Tum | Surr | Tum | Surr | Tum |
| 24 hours | 0.00 ±0.00 | 7.00 ±7.00 | 0.00 ±0.00 | 0.00 ±0.00 | 1.33 ±1.33 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 |
| 48 hours | 1.33 ±1.33 | 0.00 ±0.00 | 0.00 ±0.00 | 13.25 ±10.75 | 0.00 ±0.00 | 2.67 ±2.67 | 0.00 ±0.00 | 0.00 ±0.00 |
| 96 hours | 0.00 ±0.00 | 1.33 ±1.33 | 4.00 ±4.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 |
| 1 week | 0.00 ±0.00 | 4.67 ±4.67 | 0.00 ±0.00 | 0.80 ±0.80 | 1.33 ±1.33 | 0.00 ±0.00 | 0.00 ±0.00 | 2.00 ±2.00 |
| Survival | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 14.00 ±14.00 |

Surr = tissue surrounding the tumor, Tum = tumor. ± standard error of the mean

Table 5.5. Average numbers of CD8 positive cells.

| CD8 | MYXV:IL15 | | MYXV | | UV-MYXV | | PBS | |
|---|---|---|---|---|---|---|---|---|
| | Surr | Tum | Surr | Tum | Surr | Tum | Surr | Tum |
| 24 hours | 0.00 ±0.00 | 4.33 ±2.60 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 |
| 48 hours | 0.00 ±0.00 | 4.67 ±4.67 | 0.00 ±0.00 | 0.00 ±0.00 | 1.33 ±1.33 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 |
| 96 hours | 0.00 ±0.00 | 6.67 ±6.67 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 |
| 1 week | 0.00 ±0.00 | 17.33 ±17.33 | 0.00 ±0.00 | 3.40 ±2.14 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 5.00 ±5.00 |
| Survival | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 9.00 ±9.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.67 ±0.67 |

Surr = tissue surrounding the tumor, Tum = tumor. ± standard error of the mean

Table 5.6. Average numbers of GrB positive cells.

| CD8 | MYXV:IL15 | | MYXV | | UV-MYXV | | PBS | |
|---|---|---|---|---|---|---|---|---|
| | Surr | Tum/n | Surr | Tum/n | Surr | Tum/n | Surr | Tum/n |
| 24 hours | 4.67 ±2.40 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 |
| 48 hours | 2.00 ±2.00 | 0.00 ±0.00 | 0.00 ±0.00 | 2.50 ±2.50 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 |
| 96 hours | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 |
| 1 week | 0.00 ±0.00 | 24.33 ±24.33 | 0.00 ±0.00 | 1.40 ±1.17 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 |
| Survival | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 |

Surr = tissue surrounding the tumor, Tum/n = tumor including areas of necrosis. ± standard error of the mean

Table 5.7. Average numbers of MHCII positive cells.

| MHCII | MYXV:IL15 | | | MYXV | | | UV-MYXV | | | PBS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Surr | Tum | Tnec | Surr | Tum | Tnec | Surr | Tum | Tnec | Surr | Tum | Tnec |
| 24 h | 30.33 ±2.96 | 3.33 ±3.33 | 138.00 ±62.00 | 12.33 ±6.49 | 11.00 ±9.54 | 133.33 ±66.67 | 29.00 ±3.79 | 0.67 ±0.67 | 81.67 ±59.23 | 21.67 ±7.22 | 16.67 ±8.17 | 70.67 ±64.76 |
| 48 h | 17.00 ±5.00 | 0.00 ±0.00 | 76.33 ±62.40 | 13.50 ±9.43 | 100.00 ±57.74 | 5.00 ±2.20 | 15.00 ±11.68 | 18.33 ±4.48 | 31.33 ±31.33 | 5.67 ±2.96 | 2.00 ±2.00 | 49.00 ±15.78 |
| 96 h | 15.33 ±8.19 | 66.67 ±66.67 | 4.00 ±2.00 | 24.33 ±7.54 | 0.00 ±0.00 | 98.67 ±55.21 | 22.33 ±4.98 | 12.33 ±11.35 | 79.67 ±37.30 | 5.67 ±4.25 | 0.00 ±0.00 | 9.00 ±4.58 |
| 1 w | 2.67 ±2.67 | 10.00 ±10.00 | 18.33 ±14.08 | 19.00 ±16.61 | 45.20 ±39.03 | 81.40 ±48.43 | 12.67 ±2.19 | 16.00 ±16.00 | 67.33 ±24.88 | 11.67 ±11.67 | 7.00 ±7.00 | 16.67 ±0.88 |
| Surv | 7.67 ±7.67 | 31.33 ±15.33 | 4.33 ±4.33 | 1.33 ±1.33 | 0.00 ±0.00 | 67.67 ±17.37 | 1.00 ±1.00 | 7.33 ±4.67 | 38.33 ±22.84 | 0.00 ±0.00 | 17.00 ±11.15 | 18.00 ±10.12 |

Surr = tissue surrounding the tumor, Tum = tumor, TDJ = tumor dermis junction. ± standard error of the mean

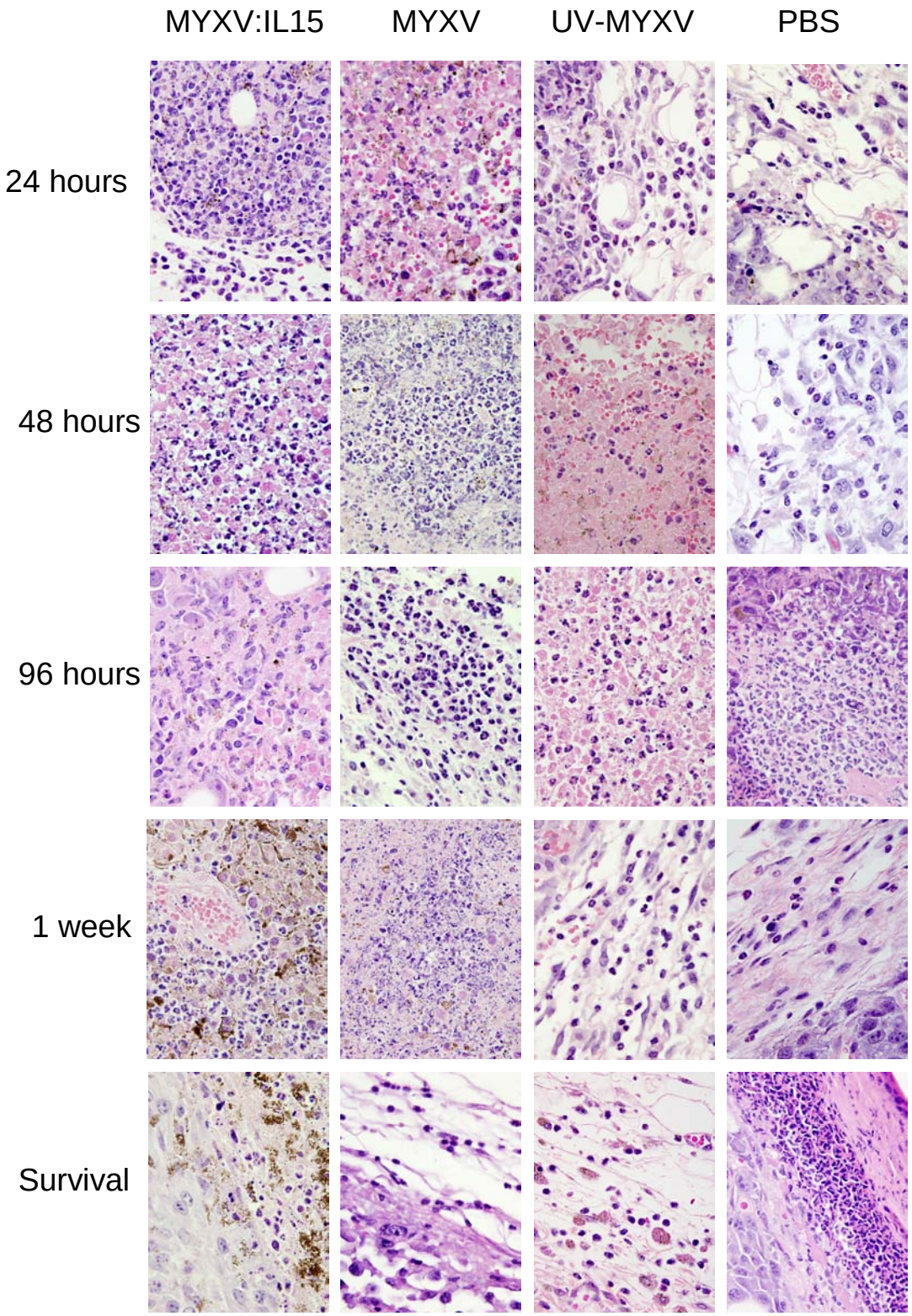



Figure 5.8. Neutrophils surrounding and within subcutaneous melanoma tumors. Neutrophils were most numerous in areas of necrosis in tumors treated with MYXV. Neutrophils were most typically associated with ulceration and crusts in PBS mice. Small numbers of neutrophils were surrounding tumors or in areas of necrosis in most mice.

Table 5.8. Average neutrophil grades.

| Neuts | MYXV:IL15 | | | | MYXV | | | | UV-MYXV | | | | PBS | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Surr | Tum | Tnec | Ulc | Surr | Tum | Tnec | Ulc | Surr | Tum | Tnec | Ulc | Surr | Tum | Tnec | Ulc |
| 24 h | 2.33 ±0.21 | 0.83 ±0.54 | 2.33 ±0.80 | 1.33 ±0.84 | 1.67 ±0.21 | 0.67 ±0.49 | 2.67 ±0.72 | 0.50 ±0.50 | 2.50 ±0.50 | 0.50 ±0.34 | 1.83 ±0.70 | 0.00 ±0.00 | 1.71 ±0.42 | 0.14 ±0.14 | 0.00 ±0.00 | 0.00 ±0.00 |
| 48 h | 2.17 ±0.31 | 0.67 ±0.49 | 2.50 ±0.81 | 0.67 ±0.67 | 3.17 ±0.17 | 1.83 ±0.60 | 3.50 ±0.22 | 0.00 ±0.00 | 1.71 ±0.36 | 0.29 ±0.18 | 0.71 ±0.56 | 1.14 ±0.74 | 1.57 ±0.53 | 0.00 ±0.00 | 0.71 ±0.42 | 1.00 ±0.65 |
| 96 h | 1.17 ±0.48 | 0.67 ±0.49 | 2.00 ±0.89 | 0.00 ±0.00 | 2.17 ±0.40 | 0.17 ±0.17 | 2.50 ±0.62 | 0.67 ±0.67 | 1.33 ±0.33 | 0.17 ±0.17 | 1.00 ±0.63 | 0.50 ±0.50 | 1.20 ±0.73 | 0.00 ±0.00 | 0.00 ±0.00 | 3.20 ±0.80 |
| 1 w | 2.33 ±0.49 | 0.50 ±0.50 | 0.67 ±0.49 | 0.67 ±0.67 | 3.00 ±0.31 | 0.86 ±0.46 | 1.57 ±0.53 | 0.00 ±0.00 | 3.00 ±0.45 | 0.50 ±0.34 | 1.33 ±0.71 | 0.00 ±0.00 | 1.67 ±0.61 | 0.00 ±0.00 | 1.83 ±0.83 | 0.00 ±0.00 |
| Surv | 1.83 ±0.37 | 0.00 ±0.00 | 2.17 ±0.51 | 0.58 ±0.40 | 0.82 ±0.35 | 0.00 ±0.00 | 1.64 ±0.59 | 1.09 ±0.56 | 1.17 ±0.42 | 0.00 ±0.00 | 0.83 ±0.37 | 1.00 ±0.52 | 0.67 ±0.33 | 0.00 ±0.00 | 0.25 ±0.25 | 1.67 ±0.59 |

Surr = tissue surrounding the tumor, Tum = tumor, Tnec = areas of necrosis within the tumor, Ulc = ulcerated area. ± standard error of the mean

Table 5.9. Average numbers of Gr-1 positive cells.

| Gr-1 | MYXV:IL15 | | | MYXV | | | UV-MYXV | | | PBS | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Surr | Tum | Tnec | Surr | Tum | Tnec | Surr | Tum | Tnec | Surr | Tum | Tnec |
| 24 h | 13.67 ±10.81 | 0.00 ±0.00 | 71.67 ±64.20 | 4.33 ±4.33 | 0.00 ±0.00 | 133.33 ±66.67 | 1.67 ±1.67 | 0.00 ±0.00 | 6.33 ±4.48 | 11.00 ±6.08 | 0.00 ±0.00 | 0.00 ±0.00 |
| 48 h | 0.00 ±0.00 | 0.00 ±0.00 | 66.67 ±66.67 | 35.00 ±35.00 | 50.00 ±50.00 | 53.75 ±48.88 | 1.67 ±1.67 | 1.33 ±1.33 | 6.33 ±6.33 | 0.00 ±0.00 | 0.00 ±0.00 | 16.67 ±8.96 |
| 96 h | 2.00 ±2.00 | 4.00 ±4.00 | 133.33 ±66.67 | 8.67 ±4.33 | 0.00 ±0.00 | 133.33 ±66.67 | 6.67 ±2.67 | 5.00 ±5.00 | 80.00 ±60.80 | 0.00 ±0.00 | 0.00 ±0.00 | 0.00 ±0.00 |
| 1 w | 0.00 ±0.00 | 0.00 ±0.00 | 4.67 ±2.40 | 3.20 ±3.20 | 1.00 ±0.80 | 133.33 ±39.34 | 11.00 ±4.16 | 3.67 ±3.67 | 85.00 ±57.82 | 0.00 ±0.00 | 0.00 ±0.00 | 66.67 ±66.67 |
| Surv | 12.00 ±12.00 | 0.00 ±0.00 | 78.33 ±61.67 | 0.00 ±0.00 | 24.33 ±24.33 | 66.67 ±66.67 | 2.67 ±2.67 | 1.33 ±1.33 | 3.67 ±1.86 | 0.00 ±0.00 | 12.00 ±12.00 | 0.00 ±0.00 |

Surr = tissue surrounding the tumor, Tum = tumor, Tnec = areas of necrosis within the tumor. ± standard error of the mean

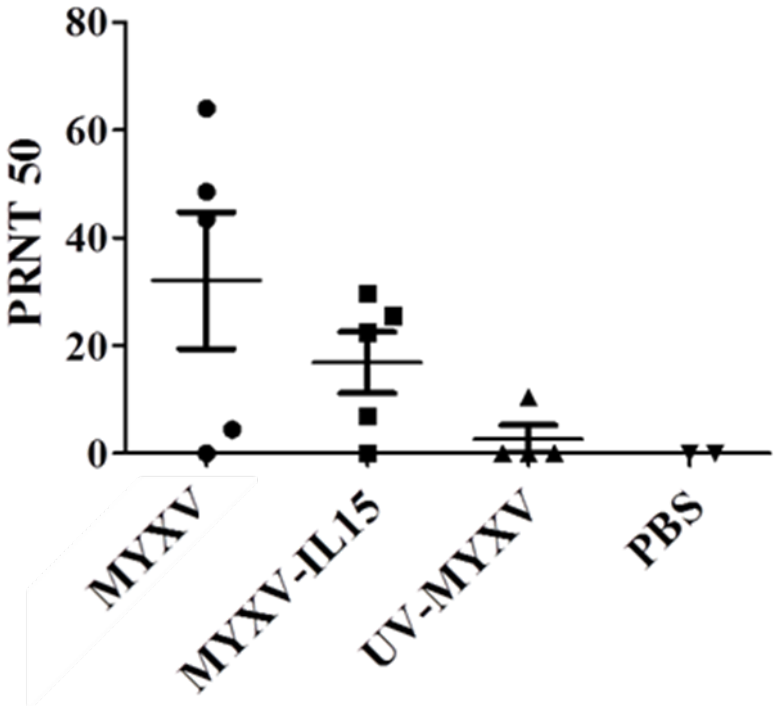



Figure 5.9. Plaque reduction neutralization titer 50%. Some mice treated with either live MYXV (MYXV, MYXV:IL15) or dead MYXV (UV-MYXV) developed a neutralizing antibody response to MYXV.

# CHAPTER 6

# CONCLUSIONS

Oncolytic viruses are multi-modal therapeutics that cause tumor-specific necrosis and a local immune response, which ideally may target the neoplastic cells in addition to the virus. They can also be armed to act as vehicles to deliver any number of targeted combinations to the tumor by inserting one or more genes that encode inflammatory cytokines, antiangiogenic factors, proteases, costimulatory molecules, tumor antigens, or prodrugs. We evaluated MYXV for its ability to infect murine tumor cell lines, and for safety and effectiveness in a brain tumor model and a subcutaneous tumor model. In addition, we evaluated MYXV that expressed the gene for murine IL-15 in order to determine if the cellular immune response could be enhanced and survival subsequently increased. We chose MYXV for the oncolytic virus in this series of experiments for its many beneficial attributes, which include safety, rapid replication, and a large maniputable genome. Immunocompetant mice were utilized, as was a murine tumor cell line and murine IL-15, in order to best evaluate the immune response without potential complications from xenobiotics. Notably, we performed a detailed histological analysis of the tumor during treatment and documented the immune cell response, which to our knowledge is the first time this has been reported following MYXV oncolytic treatment.

Susceptibility of tumor cell lines to viral replication and necrosis after infection with MYXV *in vitro* was initially evaluated in order to determine which cell lines would be most successful when used in an *in vivo* model. We then evaluated histology and survival in mice with glioma, lymphoma, or melanoma brain tumors that were treated with MYXV versus control treatment (UV-MYXV or PBS). However, the brain tumor model revealed a problem with

increased immunogenicity in the glioma cell lines. Additionally, the rapid growth of the melanoma cells resulted in rapid death due to neurological compromise, which made evaluation of survival time difficult. Also, the rapid growth of the melanoma cells results in rapid repopulation of the tumor cells after MYXV treatment. This made switching to a subcutaneous model that could be treated repeatedly imperative when using this tumor model. Additional attributes of tumor cell lines, may also affect the success of an *in vivo* model include the presence of TAAs, the ability of the cells to express cytokines after viral infection, predilection to invade and metastasize early, ability to suppress immune responses, and ability to initiate angiogenesis.

In both the brain tumor model and the subcutaneous tumor model, neutrophils were observed to infiltrate the tumors the tumors and surrounding area in the first 48 hours after IT injection of MYXV. In the brain tumor model, initial experiments indicated that both live and UV-inactivated MYXV induced a neutrophilic response at the site of injection within B16.SIY tumors in mice (Figure 4.1). Neutrophil infiltration is not unexpected, since neutrophils are one of the first cell types recruited to sites of infection (Nathan 2006). In our experiment, infiltration was likely due to an initial innate immune response against the physical virus. Histological analysis of the number of infiltrating neutrophils in the subcutaneous model revealed a statistically significant increase in mice treated with MYXV or MYXV:IL15 vs. PBS control mice in all areas, but not a difference in the number of neutrophils between MYXV and MYXV:IL15. This suggests that neutrophils infiltrate as a response to the presence of virus and that the expression of IL-15 does not play a significant role in the recruitment of neutrophils. Indeed, it is IL-8, IL-17, and GMCSF, rather than IL-15 that have been found to be associated with neutrophil responses. At higher doses of IL-15, it may be possible for IL-15 to indirectly affect neutrophils through the release of GM-CSF from stimulated NK cells.

An influx of neutrophils has been documented in other studies using oncolytic viruses (Best, Collins, and Kerr 2000; Fu et al. 2011; Grote, Cattaneo, and Fielding 2003; Breitbach et al. 2007). In rabbits, a neutrophilic response to virus has been documented to occur in rabbits susceptible to MYXV infection, while rabbits that are resistant to infection respond with a monocytic infiltrate (Best, Collins, and Kerr 2000). This suggests that the inability to adequately respond to a virus can result in a neutrophilic rather than a monocytic inflammatory response. Since interferons and other cytokines are often not induced in these situations, T lymphocytes and NK cells are not recruited to the area. Production of IL-15 *in vitro* and *in vivo* after MYXV:IL15 infection was confirmed; and as expected, there was not a significant difference in the number of neutrophils observed histologically in MYXV:IL15 treated tumors compared to MYXV treated tumors, but there was an increased number of lymphocytes suggesting that IL-15 is associated with increased infiltration of lymphocytes, but not of neutrophils, as would be expected.

Most of the survival mice treated with MYXV developed an antiviral antibody response; however, there was not see a correlation between an antiviral antibody response and survival. This has been documented in other cases. In a study using VACV that encoded a tumor antigen (5T4), antibody response to the tumor antigen correlated with an increase in survival, but an antibody response to VACV did not correlate with increased survival (Harrop et al. 2006). While an antiviral cellular and antibody response typically developed after the first treatment, a 5T4-specific (tumor targeting) immune response usually only occurred following 2-3 treatments (Harrop et al. 2006). This suggests that repeated exposure is required to overcome the tolerance to this tumor (self) antigen. In addition, immune responses were relatively short-lived, indicating that repeated boosters are likely needed for a successful antitumor treatment. For future

experiments using MYXV, increasing the number of boosters by giving injections more frequently, combined with engineering MYXV to express a tumor antigen, would likely produce a significant increase in treatment efficacy by promoting an antitumor response.

The absence of "danger signals", such as inflammatory cytokines, and the lack of cellular and molecular T cell activation have been considered the main causes of poor tumor immunity (Matzinger 2002; Fuchs and Matzinger 1996). Immunotherapy, which stimulates the immune system in an attempt to overcome tolerance to tumor cells, has already become a state of the art treatment for some cancers. Superficial bladder cancer is currently treated with BCG (bacillus Calmette-Guerin), which creates an acute local inflammatory response (Herr and Morales 2008). Similarly, interleukin-2 (which stimulates T cells) is approved for the treatment of renal carcinoma and melanoma, (Eklund and Kuzel 2004; Dillman 2011) despite its serious side effects at high doses (Eklund and Kuzel 2004; Eisner, Husain, and Clark 2004). Oncolytic viruses have the potential to act as immunotherapeutics by causing a severe acute inflammatory response (danger signals) in the tumor microenvironment. Combined with lysis of the cells, which should result in the release of tumor antigens, oncolytic viruses have the potential to stimulate the immune system to mount an immune response to the tumor itself, through cross-presentation of antigens. This can be greatly enhanced if the virus has been engineered to express the tumor antigen, as has been shown in other studies (Harrop et al. 2006). In addition, viruses can be engineered to express genes that enhance immunity, such as IL-2 or IL-15. For example, a herpesvirus which contains the coding sequence for GM-CSF resulted in regression not only of injected tumors but also of distant metastases, consistent with an antitumor immune response (Senzer et al. 2009). Analysis revealed a significant decrease in immunosuppressive cell types (Park et al. 2008). Similarly, an adenovirus encoding IFN-γ had regression of

noninjected lesions in 27% of patients (Dummer et al. 2010).

The results of our study indicate that oncolytic viral expression of IL-15 delivered intratumorally is safe and is effective at increasing the number of lymphocytes infiltrating the tumor. Delivery of the gene for IL-15 directly into the tumor via a virus has the benefit of providing continuous production of IL-15 every time the virus replicates, which is important for effective treatment since cytokines have a short half-life. However, while viral production of IL-15 results in higher levels of IL-15 within the tumor for a longer period of time, boostering of the virus is necessary since viral titer eventually decreases (between 4 and 7 days post-infection in our experiment, Figure 5.4). Boostering the tumors with virus more often, perhaps every 2 days, or with higher concentration of virus, would result in even higher levels of IL-15 within the tumor, which could result in a more dramatic infiltration of leukocytes. Additionally, the IL-15 could be placed under a stronger promoter which could result in higher levels of secretion of IL-15. This has been done in another study that used vesicular stomatitis virus, and IL-15 levels were detected that were many times the levels detected in this experiment (Stephenson, et al. 2012).

Our finding that expression of IL-15 by MYXV correlated with increased infiltrating lymphocytes histologically is important as increased numbers of infiltrating leukocytes has been correlated with improved clinical outcome in some tumors (Pagès et al. 2009). While increased survival was not observed in mice treated with MYXV:IL15 over mice treated with MYXV alone, treatment of a less aggressive tumor, or combination with another therapeutic (chemotherapy), or adding another gene to MYXV:IL15 (such as IL-15Rα, IL-12, or GM-CSF) could potentially result in improved efficacy and increased survival.

In tumors injected with either MYXV or MYXV:IL15, levels of virus were detected that

were higher than the initial input dose ($1 \times 10^6$ pfu), indicating viral replication. This was especially evident in tumors during the 96 hours after injection, with lower levels detected thereafter. Detection of viral titer within the tumor that is higher than the input dose has been documented in several studies using oncolytic virues and is considered to be evidence of productive viral infection of the cancer cells (Harrington et al. 2010a; Nemunaitis et al. 2010). While the viral dose is not always correlated with improved therapeutic results, in general, the more virus within the tumor, whether due to initial dose, viral replication, or repeated dosing, the better the clinical results. For example, in a study with HSV, 100% of patients treated with the lowest dose (3 x 10^6) showed steady progression of their cancer, while those treated at higher doses (3 x 10^7 and 1 x 10^8) showed disease stabilization (75% and 100%, respectively) (Geevarghese et al. 2010). In our experiment, replication of the virus was also important for the continued production of IL-15 that could act locally within the tumor. In future experiments, it would likely be beneficial to give injections that contain a much higher concentration of virus, and to booster the injections more often.

While there are many oncolytic viruses currently or recently in clinical trials (Appendix B), virotherapy is typically used as an experimental last resort treatment in patients that have received multiple rounds of treatment and have become refractory (Park et al. 2008; Geevarghese et al. 2010; Nokisalmi et al. 2010; Galanis et al. 2010; Pecora et al. 2002). Therefore, any improvement in the clinical course that occurs after oncolytic virotherapy is significant, but is also much less likely to occur. Due to the limited responses obtained thus far, oncolytic viruses are increasingly being tested as a component of combination therapy. Oncolytic viral treatment has been repeatedly shown to be safe, with minimal side effects, and can be combined with other commonly used cancer treatments without decreasing their efficacy. In cases where oncolytic

virotherapy has been used in previously untreated cancers, better responses are typically observed, especially if given with concurrent chemotherapy and radiotherapy (Harrington et al. 2010a). Due to the complexities of tumor biology and the heterogeneity of tumors, it is unlikely that one kind of virus or one targeting strategy will be sufficient to treat tumor types. Similar to chemotherapy, there is a wide range of oncolytic viruses (Appendix B) that can be utilized for treatment of different types of cancer, and each may deliver one or more cytokines, chemokines, costimulatory molecules, tumor antigens, or prodrugs to the tumor. Analyzing patients for clinical biomarkers and their tumors for mutations could help select the most appropriate treatment regimen for each individual patient. Even if these therapies do not provide a cure for cancer, in advanced terminal cancers, treatments that result in modest increases in survival (1-2 months) are considered useful as long as they do not significantly decrease quality of life.

These experiments demonstrated that MYXV and MYXV:IL15 are safe and result in significantly increased survival, and are therefore suitable for further study as oncolytic viruses. MYXV:IL15 also resulted in the local production of IL-15, which resulted in increased numbers of leukocytes within and surrounding the tumor. Despite the lack of improved survival with MYXV:IL15 over MYXV without IL-15, there was also not a significant decrease in survival, which is essential to consider, since immunotherapy combined with virotherapy always has the potential to target the virus and clear it early, negating the virus' oncolytic effects. Additional manipulation of the virus to express other cytokines or tumor antigens, use in a different model, or administration of higher levels of virus and/or more frequent dosing may greatly improve results with this viral therapy.

## CHAPTER 7

## REFERENCES


Abrey, L. E., L. M. DeAngelis, and J. Yahalom. 1998. Long-term survival in primary CNS lymphoma. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* 16 (3): 859-863.

Abrey, L. E., J. Yahalom, and L. M. DeAngelis. 2000. Treatment for primary CNS lymphoma: The next step. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* 18 (17): 3144-3150.

Algazi, A. P., C. W. Soon, and A. I. Daud. 2010. Treatment of cutaneous melanoma: Current approaches and future prospects. *Cancer Management and Research* 2: 197-211.

Amato, R. J., R. E. Hawkins, H. L. Kaufman, J. A. Thompson, P. Tomczak, C. Szczylik, M. McDonald, et al. 2010. Vaccination of metastatic renal cancer patients with MVA-5T4: A randomized, double-blind, placebo-controlled phase III study. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 16 (22): 5539-5547.

Armitage, R. J., B. M. Macduff, J. Eisenman, R. Paxton, and K. H. Grabstein. 1995. IL-15 has stimulatory activity for the induction of B cell proliferation and differentiation. *The Journal of Immunology* 154 (2): 483-490.

Atkins, M. B., M. T. Lotze, J. P. Dutcher, R. I. Fisher, G. Weiss, K. Margolin, J. Abrams, et al. 1999. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: Analysis of 270 patients treated between 1985 and 1993. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* 17 (7): 2105-2016.

Barker, D. D., and A. J. Berk. 1987. Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection. *Virology* 156 (1): 107-121.

Barquet, N., and P. Domingo. 1997. Smallpox: The triumph over the most terrible of the ministers of death. *Annals of Internal Medicine* 127 (8 Pt 1): 635-642.

Barrett, J. W., J. X. Cao, S. Hota-Mitchell, and G. McFadden. 2001. Immunomodulatory proteins of myxoma virus. *Seminars in Immunology* 13 (1): 73-84.

Barry, M., S. Hnatiuk, K. Mossman, S. F. Lee, L. Boshkov, and G. McFadden. 1997. The myxoma virus M-T4 gene encodes a novel RDEL-containing protein that is retained within the endoplasmic reticulum and is important for the productive infection of lymphocytes. *Virology* 239 (2): 360-377.

Bartee, E., and G. McFadden. 2009a. Human cancer cells have specifically lost the ability to induce the synergistic state caused by tumor necrosis factor plus interferon-β. *Cytokine* 47 (3): 199-205.

Bartee, E., M. R. Mohamed, M. C. Lopez, H. V. Baker, and G. McFadden. 2009b. The addition of tumor necrosis factor plus beta interferon induces a novel synergistic antiviral state against poxviruses in primary human fibroblasts. *Journal of Virology* 83 (2): 498-511.

Benhnia, M. R., M. M. McCausland, J. Moyron, J. Laudenslager, S. Granger, S. Rickert, L. Koriazova, R. Kubo, S. Kato, and S. Crotty. 2009. Vaccinia virus extracellular enveloped virion neutralization in vitro and protection in vivo depend on complement. *Journal of Virology* 83 (3): 1201-1215.

Best, S. M., S. V. Collins, and P. J. Kerr. 2000. Coevolution of host and virus: Cellular localization of virus in myxoma virus infection of resistant and susceptible European rabbits. *Virology* 277 (1): 76-91.

Bierman, H. R., D. M. Crile, K. S. Dod, K. H. Kelly, N. L. Petrakis, L. P. White, and M. B. Shimkin. 1953. Remissions in leukemia of childhood following acute infectious disease: Staphylococcus and streptococcus, varicella, and feline panleukopenia. *Cancer* 6 (3): 591-605.

Bindea, G., B. Mlecnik, W. H. Fridman, and J. Galon. 2011. The prognostic impact of anti-cancer immune response: A novel classification of cancer patients. *Seminars in Immunopathology* 4: 335-340.

Biron, C. A., K. B. Nguyen, G. C. Pien, L. P. Cousens, and T. P. Salazar-Mather. 1999. Natural killer cells in antiviral defense: Function and regulation by innate cytokines. *Annual Review of Immunology* 17: 189-220.

Bisht, H., A. S. Weisberg, P. Szajner, and B. Moss. 2009. Assembly and disassembly of the capsid-like external scaffold of immature virions during vaccinia virus morphogenesis. *Journal of Virology* 83 (18): 9140-9150.

Blank, C., I. Brown, A. C. Peterson, M. Spiotto, Y. Iwai, T. Honjo, and T. F. Gajewski. 2004. PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic CD8+ T cells. *Cancer Research* 64 (3): 1140-1145.

Blay, J. Y., T. Conroy, C. Chevreau, A. Thyss, N. Quesnel, H. Eghbali, R. Bouabdallah, et al. 1998. High-dose methotrexate for the treatment of primary cerebral lymphomas: Analysis of survival and late neurologic toxicity in a retrospective series. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* 16 (3): 864-871.

Bluman, E. M., K. J. Bartynski, B. R. Avalos, and M. A. Caligiuri. 1996. Human natural killer cells produce abundant macrophage inflammatory protein-1 alpha in response to monocyte-derived cytokines. *The Journal of Clinical Investigation* 97 (12): 2722-2727.

Breitbach, C. J., J. M. Paterson, C. G. Lemay, T. J. Falls, A. McGuire, K. A. Parato, D. F. Stojdl, et al. 2007. Targeted inflammation during oncolytic virus therapy severely compromises tumor blood flow. *Molecular Therapy : The Journal of the American Society of Gene Therapy* 15 (9): 1686-1693.

Burnet, F. M. 1968. *Changing patterns: An atypical autobiography.* Melbourne: Heinemann.

Cardone, M. H., N. Roy, H. R. Stennicke, G. S. Salvesen, T. F. Franke, E. Stanbridge, S. Frisch, and J. C. Reed. 1998. Regulation of cell death protease caspase-9 by phosphorylation. *Science (New York, N.Y.)* 282 (5392): 1318-1321.

Carson, W. E., J. G. Giri, M. J. Lindemann, M. L. Linett, M. Ahdieh, R. Paxton, D. Anderson, J. Eisenmann, K. Grabstein, and M. A. Caligiuri. 1994. Interleukin (IL) 15 is a novel cytokine that activates human natural killer cells via components of the IL-2 receptor. *The Journal of Experimental Medicine* 180 (4): 1395-1403.

Carter, G. C., M. Law, M. Hollinshead, and G. L. Smith. 2005. Entry of the vaccinia virus intracellular mature virion and its interactions with glycosaminoglycans. *The Journal of General Virology* 86 (Pt 5): 1279-1290.

Chang, J., X. Zhao, X. Wu, Y. Guo, H. Guo, J. Cao, Y. Guo, D. Lou, D. Yu, and J. Li. 2009. A phase I study of KH901, a conditionally replicating granulocyte-macrophage colony-stimulating factor: Armed oncolytic adenovirus for the treatment of head and neck cancers. *Cancer Biology & Therapy* 8 (8): 676-682.

Cheng, J., H. Sauthoff, Y. Huang, D. I. Kutler, S. Bajwa, W. N. Rom, and J. G. Hay. 2007. Human matrix metalloproteinase-8 gene delivery increases the oncolytic activity of a replicating adenovirus. *Molecular Therapy* 15 (11): 1982-1990.

Chichon, F. J., M. J. Rodriguez, C. Risco, A. Fraile-Ramos, J. J. Fernandez, M. Esteban, and J. L. Carrascosa. 2009. Membrane remodelling during vaccinia virus morphogenesis. *Biology of the Cell / Under the Auspices of the European Cell Biology Organization* 101 (7): 401-414.

Coca, S., J. Perez-Piqueras, D. Martinez, A. Colmenarejo, M. A. Saez, C. Vallejo, J. A. Martos, and M. Moreno. 1997. The prognostic significance of intratumoral natural killer cells in patients with colorectal carcinoma. *Cancer* 79 (12): 2320-2328.

Commins, D. L. 2006. Pathology of primary central nervous system lymphoma. *Neurosurgical Focus* 21 (5): 1-10.

Corn, B. W., S. M. Marcus, A. Topham, W. Hauck, and W. J. Curran Jr. 1997. Will primary central nervous system lymphoma be the most frequent brain tumor diagnosed in the year 2000? *Cancer* 79 (12): 2409-2413.

Datta, S. R., H. Dudek, X. Tao, S. Masters, H. Fu, Y. Gotoh, and M. E. Greenberg. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 91 (2): 231-241.

Davies, E., S. Reid, M. F. Medina, B. Lichty, and A. A. Ashkar. 2010. IL-15 has innate anti-tumor activity independent of NK and CD8 T cells. *Journal of Leukocyte Biology* 88(3): 529-536.

de Oliveira, T. M., I. S. Rehfeld, J. M. Siqueira, J. S. Abrahão, R. K. Campos, A. K. Dos Santos, M. M. Cerqueira, E. G. Kroon, and Z. I. Lobato. 2010. Vaccinia virus is not inactivated after thermal treatment and cheese production using experimentally contaminated milk. *Foodborne Pathogens and Disease* 7 (12): 1491-1496.

De Pace, N. G. 1912. Sulla scomparsa di un enorme cancro vegetante del collo dell'utero senza cura chirurgica. *Ginecologia* 9: 82--88.

Dillman, R. O. 2011. Cancer immunotherapy. *Cancer Biotherapy & Radiopharmaceuticals* 26 (1): 1-64.

Dorner, B. G., H. R. Smith, A. R. French, S. Kim, J. Poursine-Laurent, D. L. Beckman, J. T. Pingel, R. A. Kroczek, and W. M. Yokoyama. 2004. Coordinate expression of cytokines and chemokines by NK cells during murine cytomegalovirus infection. *Journal of Immunology* 172 (5): 3119-3131.

Dubuisson, A., B. Kaschten, J. Lénelle, D. Martin, P. Robe, M. Fassotte, I. Rutten, M. Deprez, and A. Stevenaert. 2004. Primary central nervous system lymphoma: Report of 32 cases and review of the literature. *Clinical Neurology and Neurosurgery* 107 (1): 55-63.

Dummer, R., S. Eichmuller, S. Gellrich, C. Assaf, B. Dreno, M. Schiller, O. Dereure, et al. 2010. Phase II clinical trial of intratumoral application of TG1042 (adenovirus-interferon-gamma) in patients with advanced cutaneous T-cell lymphomas and multilesional cutaneous B-cell lymphomas. *Molecular Therapy : The Journal of the American Society of Gene Therapy* 18 (6): 1244-1247.

Duteyrat, J. L., J. Gelfi, and S. Bertagnoli. 2006. Ultrastructural study of myxoma virus morphogenesis. *Archives of Virology* 151 (11): 2161-2180.

Einar-Jon, E., O. Trausti, H. Asgeir, M. Christian, W. Thomas, M. Mans, K. Jon, and P. Hannes. 2011. Hearing impairment after platinum-based chemotherapy in childhood. *Pediatric Blood & Cancer* 56 (4): 631-637.

Eisner, R. M., A. Husain, and J. I. Clark. 2004. Case report and brief review: IL-2 induced myocarditis. *Cancer Investigation.* 22 (3):401-404.
Eklund, J. W., and T. M. Kuzel. 2004. A review of recent findings involving interleukin-2-based cancer therapy. *Current Opinion in Oncology* 16 (6): 542-546.
Fehniger, T. A., W. E. Carson, and M. A. Caligiuri. 1999a. Costimulation of human natural killer cells is required for interferon gamma production. *Transplantation Proceedings* 31 (3): 1476-1478.
Fehniger, T. A., M. H. Shah, M. J. Turner, J. B. VanDeusen, S. P. Whitman, M. A. Cooper, K. Suzuki, M. Wechser, F. Goodsaid, and M. A. Caligiuri. 1999b. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: Implications for the innate immune response. *Journal of Immunology*162 (8): 4511-4520.
Fehniger, T. A., H. Yu, M. A. Cooper, K. Suzuki, M. H. Shah, and M. A. Caligiuri. 2000. Cutting edge: IL-15 costimulates the generalized shwartzman reaction and innate immune IFN-gamma production in vivo. *Journal of Immunology* 164 (4): 1643-1647.
Fenner, F., D. A. Henderson, I. Arita, Z. Jezek, and I. D. Ladnyi. 1988. *Smallpox and its eradication*. Geneva: World Health Orgination.
Fenner, F., and F. N. Ratcliffe. 1965. *Myxomatosis*. London: Cambridge University Press.
Fenner, F., and J. Ross. 1994. *Myxomatosis.InThe European Rabbit.the History and Biology of a Successful Colonizer*.
Fenner, F. 1983. The Florey lecture, 1983. Biological control, as exemplified by smallpox eradication and myxomatosis. *Proceedings of the Royal Society of London.Series B, Containing Papers of a Biological Character.Royal Society (Great Britain)* 218 (1212): 259-285.
Fenner, F. 2000. Adventures with poxviruses of vertebrates. *FEMS Microbiology Reviews* 24 (2): 123-133.
Fensterl, V., and G. C. Sen. 2009. Interferons and viral infections. *Biofactors* 35 (1): 14-20.
Ferlazzo, G., D. Thomas, S. L. Lin, K. Goodman, B. Morandi, W. A. Muller, A. Moretta, and C. Münz. 2004. The abundant NK cells in human secondary lymphoid tissues require activation to express killer cell ig-like receptors and become cytolytic. *Journal of Immunology* 172 (3): 1455-1462.
Fields, B. N., D. M. Knipe, and P. M. Howley, eds. 1996. *Fields virology*. 3rd ed. Vol. 2. Philadelphia: Lippencott-Raven.
France, M. R., D. L. Thomas, J. Liu, G. McFadden, A. L. Macneill, and E. J. Roy. 2010. Intraventricular injection of myxoma virus results in transient expression of viral protein in mouse brain ependymal and subventricular cells. *Journal of General Virology* 92 (1): 195-199.
Freud, A. G., A. Yokohama, B. Becknell, M. T. Lee, H. C. Mao, A. K. Ferketich, and M. A. Caligiuri. 2006. Evidence for discrete stages of human natural killer cell differentiation in vivo. *The Journal of Experimental Medicine* 203 (4): 1033-1043.
Fu, X., L. Tao, A. Rivera, H. Xu, and X. Zhang. 2011. Virotherapy induces massive infiltration of neutrophils in a subset of tumors defined by a strong endogenous interferon response activity. *Cancer Gene Therapy* 18 (11): 785-794.
Fuchs, E. J., and P. Matzinger. 1996. Is cancer dangerous to the immune system? *Seminars in Immunology* 8 (5): 271-280.

Fujiwara, H., Y. Shimizu, Y. Takai, N. Wakamiya, S. Ueda, S. Kato, and T. Hamaoka. 1984. The augmentation of tumor-specific immunity by virus help. I. Demonstration of vaccinia virus-reactive helper T cell activity involved in enhanced induction of cytotoxic T lymphocyte and antibody responses. *European Journal of Immunology* 14 (2): 171-175.

Galanis, E., L. C. Hartmann, W. A. Cliby, H. J. Long, P. P. Peethambaram, B. A. Barrette, J. S. Kaur, et al. 2010. Phase I trial of intraperitoneal administration of an oncolytic measles virus strain engineered to express carcinoembryonic antigen for recurrent ovarian cancer. *Cancer Research* 70 (3): 875-882.

Galanis, E., S. H. Okuno, A. G. Nascimento, B. D. Lewis, R. A. Lee, A. M. Oliveira, J. A. Sloan, et al. 2005. Phase I-II trial of ONYX-015 in combination with MAP chemotherapy in patients with advanced sarcomas. *Gene Therapy* 12 (5): 437-445.

Ganly, I., D. Kirn, G. Eckhardt, G. I. Rodriguez, D. S. Soutar, R. Otto, A. G. Robertson, et al. 2000. A phase I study of onyx-015, an E1B attenuated adenovirus, administered intratumorally to patients with recurrent head and neck cancer. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 6 (3): 798-806.

Ganesh, S., M. Gonzalez-Edick, D. Gibbons, M. Van Roey, and K. Jooss. 2008. Intratumoral coadministration of hyaluronidase enzyme and oncolytic adenoviruses enhances virus potency in metastatic tumor models. *Clinical Cancer Research* 14 (12): 3933-3941.

Geevarghese, S. K., D. A. Geller, H. A. de Haan, M. Horer, A. E. Knoll, A. Mescheder, J. Nemunaitis, et al. 2010. Phase I/II study of oncolytic herpes simplex virus NV1020 in patients with extensively pretreated refractory colorectal cancer metastatic to the liver. *Human Gene Therapy* 21 (9) (Sep): 1119-1128.

Giri, J. G., M. Ahdieh, J. Eisenman, K. Shanebeck, K. Grabstein, S. Kumaki, A. Namen, L. S. Park, D. Cosman, and D. Anderson. 1994. Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. *The EMBO Journal* 13 (12) : 2822-2830.

Giri, J. G., S. Kumaki, M. Ahdieh, D. J. Friend, A. Loomis, K. Shanebeck, R. DuBose, D. Cosman, L. S. Park, and D. M. Anderson. 1995. Identification and cloning of a novel IL-15 binding protein that is structurally related to the alpha chain of the IL-2 receptor. *The EMBO Journal* 14 (15): 3654-3663.

Grabstein, K. H., J. Eisenman, K. Shanebeck, C. Rauch, S. Srinivasan, V. Fung, C. Beers, J. Richardson, M. A. Schoenborn, and M. Ahdieh. 1994. Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. *Science (New York, N.Y.)* 264 (5161): 965-968.

Grote, D., R. Cattaneo, and A. K. Fielding. 2003. Neutrophils contribute to the measles virus-induced antitumor effect: Enhancement by granulocyte macrophage colony-stimulating factor expression. *Cancer Research* 63 (19): 6463-6468.

Hancock, S. L., S. S. Donaldson, and R. T. Hoppe. 1993. Cardiac disease following treatment of hodgkin's disease in children and adolescents. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* 11 (7): 1208-1215.

Hansen, R. M., and J. A. Libnoch. 1978. Remission of chronic lymphocytic leukemia after smallpox vaccination. *Archives of Internal Medicine* 138 (7): 1137-1138.

Harrington, K. J., M. Hingorani, M. A. Tanay, J. Hickey, S. A. Bhide, P. M. Clarke, L. C. Renouf, et al. 2010a. Phase I/II study of oncolytic HSV GM-CSF in combination with radiotherapy and cisplatin in untreated stage III/IV squamous cell cancer of the head and neck. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 16 (15): 4005-4015.

Harrington, K. J., E. M. Karapanagiotou, V. Roulstone, K. R. Twigger, C. L. White, L. Vidal, D. Beirne, et al. 2010b. Two-stage phase I dose-escalation study of intratumoral reovirus type 3 dearing and palliative radiotherapy in patients with advanced cancers. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 16 (11): 3067-3077.

Harrop, R., N. Connolly, I. Redchenko, J. Valle, M. Saunders, M. G. Ryan, K. A. Myers, et al. 2006. Vaccination of colorectal cancer patients with modified vaccinia ankara delivering the tumor antigen 5T4 (TroVax) induces immune responses which correlate with disease control: A phase I/II trial. *Clinical Cancer Research* 12 (11): 3416-3424.

Harrop, R., W. Shingler, M. Kelleher, J. de Belin, and P. Treasure. 2010. Cross-trial analysis of immunologic and clinical data resulting from phase I and II trials of MVA-5T4 (TroVax) in colorectal, renal, and prostate cancer patients. *Journal of Immunotherapy* 33 (9): 999-1005.

Herr, H. W., and A. Morales. 2008. History of bacillus calmette-guerin and bladder cancer: An immunotherapy success story. *Journal of Urology* 179 (1): 53-56.

Hiller, G., K. Weber, L. Schneider, C. Parajsz, and C. Jungwirth. 1979. Interaction of assembled progeny pox viruses with the cellular cytoskeleton. *Virology* 98 (1): 142-153.

Hu, J. C., R. S. Coffin, C. J. Davis, N. J. Graham, N. Groves, P. J. Guest, K. J. Harrington, et al. 2006. A phase I study of OncoVEXGM-CSF, a second-generation oncolytic herpes simplex virus expressing granulocyte macrophage colony-stimulating factor. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 12 (22): 6737-6747.

Ishigami, S., S. Natsugoe, K. Tokuda, A. Nakajo, X. Che, H. Iwashige, K. Aridome, S. Hokita, and T. Aikou. 2000. Prognostic value of intratumoral natural killer cells in gastric carcinoma. *Cancer* 88 (3): 577-583.

Jacobs, B. L., J. O. Langland, K. V. Kibler, K. L. Denzler, S. D. White, S. A. Holechek, S. Wong, T. Huynh, and C. R. Baskin. 2009. Vaccinia virus vaccines: Past, present and future. *Antiviral Research* 84 (1): 1-13.

Johnston, J. B., J. W. Barrett, W. Chang, C. Chung, W. Zeng, J. Masters, M. Mann, F. Wang, J. Cao, and G. McFadden. 2003. Role of the serine-threonine kinase PAK-1 in myxoma virus replication. *Journal of Virology* 77 (10): 5877-88.

Johnston, J. B., S. H. Nazarian, R. Natale, and G. McFadden. 2005. Myxoma virus infection of primary human fibroblasts varies with cellular age and is regulated by host interferon responses. *Virology* 332 (1): 235-248.

Kaufman, H. L., D. W. Kim, G. DeRaffele, J. Mitcham, R. S. Coffin, and S. Kim-Schulze. 2010. Local and distant immunity induced by intralesional vaccination with an oncolytic herpes virus encoding GM-CSF in patients with stage IIIc and IV melanoma. *Annals of Surgical Oncology* 17 (3): 718-730.

Kaufman, H. L., K. Flanagan, C. S. Lee, D. J. Perretta, and H. Horig. 2002. Insertion of interleukin-2 (IL-2) and interleukin-12 (IL-12) genes into vaccinia virus results in effective anti-tumor responses without toxicity. *Vaccine* 20 (13-14): 1862-1869.

Kaufman, H. L., B. Taback, W. Sherman, D. W. Kim, W. H. Shingler, D. Moroziewicz, G. DeRaffele, et al. 2009. Phase II trial of modified vaccinia ankara (MVA) virus expressing 5T4 and high dose interleukin-2 (IL-2) in patients with metastatic renal cell carcinoma. *Journal of Translational Medicine* 7: 2.

Kennedy, M. K., M. Glaccum, S. N. Brown, E. A. Butz, J. L. Viney, M. Embers, N. Matsuki, et al. 2000. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *The Journal of Experimental Medicine* 191 (5): 771-780.

Kerr, P., and G. McFadden. 2002. Immune responses to myxoma virus. *Viral Immunology* 15 (2): 229-246.

Khuri, F. R., J. Nemunaitis, I. Ganly, J. Arseneau, I. F. Tannock, L. Romel, M. Gore, et al. 2000. A controlled trial of intratumoral ONYX-015, a selectively-replicating adenovirus, in combination with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer. *Nature Medicine* 6 (8): 879-885.

Kim, L., and M. J. Glantz. 2001. Neoplastic meningitis. *Current Treatment Options in Oncology* 2 (6): 517-527.

Kim, J. H., J. Y. Oh, B. H. Park, D. E. Lee, J. S. Kim, H. E. Park, M. S. Roh, et al. 2006. Systemic armed oncolytic and immunologic therapy for cancer with JX-594, a targeted poxvirus expressing GM-CSF. *Molecular Therapy : The Journal of the American Society of Gene Therapy* 14 (3): 361-370.

Kim, M., G. J. Madlambayan, M. M. Rahman, S. E. Smallwood, A. M. Meacham, K. Hosaka, E. W. Scott, C. R. Cogle, and G. McFadden. 2009. Myxoma virus targets primary human leukemic stem and progenitor cells while sparing normal hematopoietic stem and progenitor cells. *Leukemia* 23 (12): 2313-2317.

Kimball, K. J., M. A. Preuss, M. N. Barnes, M. Wang, G. P. Siegal, W. Wan, H. Kuo, et al. 2010. A phase I study of a tropism-modified conditionally replicative adenovirus for recurrent malignant gynecologic diseases. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 16 (21) (Nov 1): 5277-5287.

Kirn, D. H., Y. Wang, F. Le Boeuf, J. Bell, and S. H. Thorne. 2007. Targeting of interferon-beta to produce a specific, multi-mechanistic oncolytic vaccinia virus. *PLoS Medicine* 4 (12): e353.

Kohler, B. A., E. Ward, B. J. McCarthy, M. J. Schymura, L. A. Ries, C. Eheman, A. Jemal, R. N. Anderson, U. A. Ajani, and B. K. Edwards. 2011. Annual report to the nation on the status of cancer, 1975-2007, featuring tumors of the brain and other nervous system. *Journal of the National Cancer Institute* 103 (9): 714-736.

Kretzschmar, M., J. Wallinga, P. Teunis, S. Xing, and R. Mikolajczyk. 2006. Frequency of adverse events after vaccination with different vaccinia strains. *PLoS Medicine* 3 (8): e272.

Kuriyama, N., H. Kuriyama, C. M. Julin, K. Lamborn, and M. A. Israel. 2000. Pretreatment with protease is a useful experimental strategy for enhancing adenovirus-mediated cancer gene therapy. *Human Gene Therapy* 11 (16): 2219-2230.

Lai, R., L. E. Abrey, M. K. Rosenblum, and L. M. DeAngelis. 2004. Treatment-induced leukoencephalopathy in primary CNS lymphoma: A clinical and autopsy study. *Neurology* 62 (3): 451-456.

Lanier, L. L. 2005. NK cell recognition. *Annual Review of Immunology* 23: 225-274.

Leclercq, G., V. Debacker, M. de Smedt, and J. Plum. 1996. Differential effects of interleukin-15 and interleukin-2 on differentiation of bipotential T/natural killer progenitor cells. *The Journal of Experimental Medicine* 184 (2): 325-336.

Leite, J. A., B. P. Drumond, G. S. Trindade, Z. I. Lobato, F. G. da Fonseca, S. J. dos, M. C. Madureira, et al. 2005. Passatempo virus, a vaccinia virus strain, brazil. *Emerging Infectious Diseases* 11 (12): 1935-1938.

Liang, S. C, J. W. Simecka, J. R. Lindsey, G. H. Cassell, and J. K. Davis. 1999. Antibody responses after sendai virus infection and their role in upper and lower respiratory tract disease in rats. *Laboratory Animal Science* 49 (4): 385-394.

Liu, J., S. Wennier, M. Reinhard, E. Roy, A. MacNeill, and G. McFadden. 2009. Myxoma virus expressing interleukin-15 fails to cause lethal myxomatosis in European rabbits. *Journal of Virology* 83 (11): 5933-5938.

Louis, D. N., H. Ohgaki, O. D. Wiestler, W. K. Cavenee, P. C. Burger, A. Jouvet, B. W. Scheithauer, and P. Kleihues. 2007. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathologica* 114 (2): 97-109.

Lun, X., T. Alain, F. J. Zemp, H. Zhou, M. M. Rahman, M. G. Hamilton, G. McFadden, J. Bell, D. L. Senger, and P. A. Forsyth. 2010. Myxoma virus virotherapy for glioma in immunocompetent animal models: Optimizing administration routes and synergy with rapamycin. *Cancer Research* 70 (2): 598-608.

Lun, X., W. Yang, T. Alain, Z. Q. Shi, H. Muzik, J. W. Barrett, G. McFadden, et al. 2005. Myxoma virus is a novel oncolytic virus with significant antitumor activity against experimental human gliomas. *Cancer Research* 65 (21): 9982-9990.

Lun, X. Q., J. H. Jang, N. Tang, H. Deng, R. Head, J. C. Bell, D. F. Stojdl, et al. 2009. Efficacy of systemically administered oncolytic vaccinia virotherapy for malignant gliomas is enhanced by combination therapy with rapamycin or cyclophosphamide. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 15 (8): 2777-2788.

Lun, X. Q., H. Zhou, T. Alain, B. Sun, L. Wang, J. W. Barrett, M. M. Stanford, et al. 2007. Targeting human medulloblastoma: Oncolytic virotherapy with myxoma virus is enhanced by rapamycin. *Cancer Research* 67 (18): 8818-8827.

Macen, J. L., K. A. Graham, S. F. Lee, M. Schreiber, L. K. Boshkov, and G. McFadden. 1996. Expression of the myxoma virus tumor necrosis factor receptor homologue and M11L genes is required to prevent virus-induced apoptosis in infected rabbit T lymphocytes. *Virology* 218 (1): 232-237.

MacNeill, A. L., T. Moldenhauer, R. Doty, and T. Mann. 2011. Myxoma virus induces apoptosis in cultured feline carcinoma cells. *Research in Veterinary Science*.

Markert, J. M., P. G. Liechty, W. Wang, S. Gaston, E. Braz, M. Karrasch, L. B. Nabors, et al. 2009. Phase Ib trial of mutant herpes simplex virus G207 inoculated pre-and post-tumor resection for recurrent GBM. *Molecular Therapy : The Journal of the American Society of Gene Therapy* 17 (1): 199-207.

Markert, J. M., M. D. Medlock, S. D. Rabkin, G. Y. Gillespie, T. Todo, W. D. Hunter, C. A. Palmer, et al. 2000. Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: Results of a phase I trial. *Gene Therapy* 7 (10): 867-874.

Marshall, I. D., and F. Fenner. 1958. Studies in the epidemiology of infectious myxomatosis in rabbits. V. changes in the innate resistance of australian wild rabbits exposed to myxomatosis. *Journal of Hygeine* 56 : 288.

Marshall, I. G., and G. W. Douglas. 1961. Studies in the epidemiology of infectious myxomatosis in rabbits. VIII. further observations on changes in the innate resistance of australian rabbits exposed to myxomatosis. *Journal of Hygeine* 59 : 117.

Martin, D. B. 2002. The cause of death in smallpox: An examination of the pathology record. *Military Medicine* 167 (7): 546-551.

Mastrangelo, M. J., H. C. Maguire Jr, L. C. Eisenlohr, C. E. Laughlin, C. E. Monken, P. A. McCue, A. J. Kovatich, and E. C. Lattime. 1999. Intratumoral recombinant GM-CSF-encoding virus as gene therapy in patients with cutaneous melanoma. *Cancer Gene Therapy* 6 (5): 409-422.

Matzinger, P. 2002. The danger model: A renewed sense of self. *Science* 296 (5): 301-305.

McFadden, G. 2005. Poxvirus tropism. *Nature Reviews.Microbiology* 3 (3): 201-213.

McKee, T. D., P. Grandi, W. Mok, G. Alexandrakis, N. Insin, J. P. Zimmer, M. G. Bawendi, Y. Boucher, X. O. Breakefield, and R. K. Jain. 2006. Degradation of fibrillar collagen in a human melanoma xenograft improves the efficacy of an oncolytic herpes simplex virus vector. *Cancer Research* 66 (5): 2509-2513.

Meacham, L. R., C. A. Sklar, S. Li, Q. Liu, N. Gimpel, Y. Yasui, J. A. Whitton, M. Stovall, L. L. Robison, and K. C. Oeffinger. 2009. Diabetes mellitus in long-term survivors of childhood cancer. Increased risk associated with radiation therapy: A report for the childhood cancer survivor study. *Archives of Internal Medicine* 169 (15): 1381-1388.

Merrick, A. E., E. J. Ilett, and A. A. Melcher. 2009. JX-594, a targeted oncolytic poxvirus for the treatment of cancer. *Current Opinion in Investigational Drugs (London, England : 2000)* 10 (12): 1372-1382.

Moore, A. E. 1957. Oncolytic properties of viruses. *Texas Reports on Biology and Medicine* 15 (3): 588-602.

Mora, L. F., A. H. Khan, and L. S. Sperling. 2009. Cardiac complications after smallpox vaccination. *Southern Medical Journal* 102 (6): 615-619.

Moss, B. 1996. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. *Proceedings of the National Academy of Sciences of the United States of America* 93 (21): 11341-11348.

Moss, B. 2006. Poxvirus entry and membrane fusion. *Virology* 344 (1): 48-54.

Mulrooney, D. A., M. W. Yeazel, T. Kawashima, A. C. Mertens, P. Mitby, M. Stovall, S. S. Donaldson, et al. 2009. Cardiac outcomes in a cohort of adult survivors of childhood and adolescent cancer: Retrospective analysis of the childhood cancer survivor study cohort. *BMJ (Clinical Research Ed.)* 339 : b4606.

Mulvihill, S., R. Warren, A. Venook, A. Adler, B. Randlev, C. Heise, and D. Kirn. 2001. Safety and feasibility of injection with an E1B-55 kDa gene-deleted, replication-selective adenovirus (ONYX-015) into primary carcinomas of the pancreas: A phase I trial. *Gene Therapy* 8 (4): 308-315.

Nakao, A., H. Kasuya, T. T. Sahin, N. Nomura, A. Kanzaki, M. Misawa, T. Shirota, et al. 2011. A phase I dose-escalation clinical trial of intraoperative direct intratumoral injection of HF10 oncolytic virus in non-resectable patients with advanced pancreatic cancer. *Cancer Gene Therapy* 18 (3): 167-175.

Nathan, C. 2006. Neutrophils and immunity: Challenges and opportunities. *Nature Reviews.Immunology* 6 (3): 173-182.

Neglia, J. P., D. L. Friedman, Y. Yasui, A. C. Mertens, S. Hammond, M. Stovall, S. S. Donaldson, A. T. Meadows, and L. L. Robison. 2001. Second malignant neoplasms in five-year survivors of childhood cancer: Childhood cancer survivor study. *Journal of the National Cancer Institute* 93 (8): 618-629.

Nemunaitis, J. 1999. Oncolytic viruses. *Investigational New Drugs* 17 (4): 375-386.

Nemunaitis, J., N. Senzer, S. Sarmiento, Y. A. Zhang, R. Arzaga, B. Sands, P. Maples, and A. W. Tong. 2007. A phase I trial of intravenous infusion of ONYX-015 and enbrel in solid tumor patients. *Cancer Gene Therapy* 14 (11): 885-893.

Nemunaitis, J., A. W. Tong, M. Nemunaitis, N. Senzer, A. P. Phadke, C. Bedell, N. Adams, et al. 2010. A phase I study of telomerase-specific replication competent oncolytic adenovirus (telomelysin) for various solid tumors. *Molecular Therapy : The Journal of the American Society of Gene Therapy* 18 (2): 429-434.

Nokisalmi, P., S. Pesonen, S. Escutenaire, M. Sarkioja, M. Raki, V. Cerullo, L. Laasonen, et al. 2010. Oncolytic adenovirus ICOVIR-7 in patients with advanced and refractory solid tumors. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 16 (11): 3035-3043.

Nutt, S. L., J. Brady, Y. Hayakawa, and M. J. Smyth. 2004. Interleukin 21: A key player in lymphocyte maturation. *Critical Reviews in Immunology* 24 (4): 239-250.

Okuno, Y., T. Asada, K. Yamanishi, T. Otsuka, M. Takahashi, T. Tanioka, H. Aoyama, et al. 1978. Studies on the use of mumps virus for treatment of human cancer. *Biken Journal* 21 (2): 37-49.

Pack, G. T. 1950. Note on the experimental use of rabies vaccine for melanomatosis. *A.M.A.Archives of Dermatology and Syphilology* 62 (5): 694-695.

Pagès, F., A. Berger, M. Camus, F. Sanchez-Cabo, A. Costes, R. Molidor, B. Mlecnik, et al. 2005. Effector memory T cells, early metastasis, and survival in colorectal cancer. *New England Journal of Medicine* 353 (25): 2654-2666.

Pagès, F., A. Kirilovsky, B. Mlecnik, M. Asslaber, M. Tosolini, G. Bindea, C. Lagorce, et al. 2009. In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. *Journal of Clinical Oncology* 27 (35): 5944-5951.

Pap, M., and G. M. Cooper. 1998. Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. *The Journal of Biological Chemistry* 273 (32): 19929-19932.

Parato, K. A., D. Senger, P. A. Forsyth, and J. C. Bell. 2005. Recent progress in the battle between oncolytic viruses and tumours. *Nature Reviews.Cancer* 5 (12): 965-976.

Park, B., T. Hwang, T. Liu, D. Y. Sze, J. Kim, H. Kwon, S. Y. Oh, et al. 2008. Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: A phase I trial. *The Lancet Oncology,* 9 (6): 533-542.

Pecora, A. L., N. Rizvi, G. I. Cohen, N. J. Meropol, D. Sterman, J. L. Marshall, S. Goldberg, et al. 2002. Phase I trial of intravenous administration of PV701, an oncolytic virus, in patients with advanced solid cancers. *Journal of Clinical Oncology* 20 (9): 2251-2266.

Perera, L. P., C. K. Goldman, and T. A. Waldmann. 2001. Comparative assessment of virulence of recombinant vaccinia viruses expressing IL-2 and IL-15 in immunodeficient mice. *Proceedings of the National Academy of Sciences of the United States of America* 98 (9): 5146-5151.

Pignolet, B., S. Boullier, J. Gelfi, M. Bozzetti, P. Russo, E. Foulon, G. Meyer, M. Delverdier, G. Foucras, and S. Bertagnoli. 2008. Safety and immunogenicity of myxoma virus as a new viral vector for small ruminants. *Journal of General Virology* 89: 1371-1379.

Puhlmann, M., C. K. Brown, M. Gnant, J. Huang, S. K. Libutti, H. R. Alexander, and D. L. Bartlett. 2000. Vaccinia as a vector for tumor-directed gene therapy: Biodistribution of a thymidine kinase-deleted mutant. *Cancer Gene Therapy* 7 (1): 66-73.

Radny, P., U. M. Caroli, J. Bauer, T. Paul, C. Schlegel, T. K. Eigentler, B. Weide, M. Schwarz, and C. Garbe. 2003. Phase II trial of intralesional therapy with interleukin-2 in soft-tissue melanoma metastases. *British Journal of Cancer* 89 (9): 1620-1626.

Resch, W., A. S. Weisberg, and B. Moss. 2009. Expression of the highly conserved vaccinia virus E6 protein is required for virion morphogenesis. *Virology* 386 (2): 478-485.

Rivera, J., and L. Tessarollo. 2008. Genetic background and the dilemma of translating mouse studies to humans. *Immunity* 28 (1): 1-4.

Roberts, K. L., and G. L. Smith. 2008. Vaccinia virus morphogenesis and dissemination. *Trends in Microbiology* 16 (10): 472-479.

Rommelaere, J., K. Geletneky, A. L. Angelova, L. Daeffler, C. Dinsart, I. Kiprianova, J. R. Schlehofer, and Z. Raykov. 2010. Oncolytic parvoviruses as cancer therapeutics. *Cytokine & Growth Factor Reviews* 21 (2-3): 185-195.

Rosen, L., H. E. Evans, and A. Spickard. 1963. Reovirus infections in human volunteers. *American Journal of Hygiene* 77: 29-37.

Rosenthal, S. R., M. Merchlinsky, C. Kleppinger, and K. L. Goldenthal. 2001. Developing new smallpox vaccines. *Emerging Infectious Diseases* 7 (6) : 920-926.

Rotz, L. D., D. A. Dotson, I. K. Damon, J. A. Becher, and Immunization Practices Advisory Committee on. 2001. Vaccinia (smallpox) vaccine: Recommendations of the advisory committee on immunization practices (ACIP), 2001. *Morbidity and Mortality Weekly Report Recommendations and Reports* 50 (RR-10): 1-7.

Rudin, C. M., J. T. Poirier, N. N. Senzer, J. Stephenson Jr, D. Loesch, K. D. Burroughs, P. S. Reddy, C. L. Hann, and P. L. Hallenbeck. 2011. Phase I clinical study of seneca valley virus (SVV-001), a replication-competent picornavirus, in advanced solid tumors with neuroendocrine features. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 17 (4): 888-895.

Sampson, J. H., J. H. Carter Jr, A. H. Friedman, and H. F. Seigler. 1998. Demographics, prognosis, and therapy in 702 patients with brain metastases from malignant melanoma. *Journal of Neurosurgery* 88 (1): 11-20.

Samuel, C. E. 2001. Antiviral actions of interferons. *Clinical Microbiology Reviews* 14 (4): 778-809.

Sauthoff, H., J. Hu, C. Maca, M. Goldman, S. Heitner, H. Yee, T. Pipiya, W. N. Rom, and J. G. Hay. 2003. Intratumoral spread of wild-type adenovirus is limited after local injection of human xenograft tumors: Virus persists and spreads systemically at late time points. *Human Gene Therapy* 14 (5): 425-433.

Seet, B. T., J. B. Johnston, C. R. Brunetti, J. W. Barrett, H. Everett, C. Cameron, J. Sypula, S. H. Nazarian, A. Lucas, and G. McFadden. 2003. Poxviruses and immune evasion. *Annual Review of Immunology* 21 (1): 377-423.

Senzer, N. N., H. L. Kaufman, T. Amatruda, M. Nemunaitis, T. Reid, G. Daniels, R. Gonzalez, et al. 2009. Phase II clinical trial of a granulocyte-macrophage colony-stimulating factor-encoding, second-generation oncolytic herpesvirus in patients with unresectable metastatic melanoma. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* 27 (34): 5763-5771.

Shenk, T. 1996. Adenoviridae: The viruses and their replication. In *Fields virology.*, eds. DM Knip, BN Fields and PM Howle. Third Edition ed., 2111-2148. Philadelphia: Lippincott-Raven.

Signorello, L. B., J. J. Mulvihill, D. M. Green, H. M. Munro, M. Stovall, R. E. Weathers, A. C. Mertens, J. A. Whitton, L. L. Robison, and J. D. Boice Jr. 2010. Stillbirth and neonatal death in relation to radiation exposure before conception: A retrospective cohort study. *Lancet* 376 (9741): 624-630.

Sloan, A. E., C. J. Nock, and D. B. Einstein. 2009. Diagnosis and treatment of melanoma brain metastasis: A literature review. *Cancer Control : Journal of the Moffitt Cancer Center* 16 (3): 248-255.

Smith, G. L., A. Vanderplasschen, and M. Law. 2002. The formation and function of extracellular enveloped vaccinia virus. *The Journal of General Virology* 83 (Pt 12): 2915-2931.

Smyth, M. J., N. Y. Crowe, D. G. Pellicci, K. Kyparissoudis, J. M. Kelly, K. Takeda, H. Yagita, and D. I. Godfrey. 2002. Sequential production of interferon-gamma by NK1.1(+) T cells and natural killer cells is essential for the antimetastatic effect of alpha-galactosylceramide. *Blood* 99 (4): 1259-1266.

Southam, C. M., and A. E. Moore. 1952. Clinical studies of viruses as antineoplastic agents with particular reference to egypt 101 virus. *Cancer* 5 (5): 1025-1034.

Spiotto, M. T., P. Yu, D. A. Rowley, M. I. Nishimura, S. C. Meredith, T. F. Gajewski, Y. X. Fu, and H. Schreiber. 2002. Increasing tumor antigen expression overcomes "ignorance" to solid tumors via crosspresentation by bone marrow-derived stromal cells. *Immunity* 17 (6): 737-747.

Stanford, M. M., J. W. Barrett, P. A. Gilbert, R. Bankert, and G. McFadden. 2007a. Myxoma virus expressing human interleukin-12 does not induce myxomatosis in European rabbits. *Journal of Virology* 81 (22): 12704-12708.

Stanford, M. M., M. Shaban, J. W. Barrett, S. J. Werden, P. A. Gilbert, J. Bondy-Denomy, L. Mackenzie, K. C. Graham, A. F. Chambers, and G. McFadden. 2008. Myxoma virus oncolysis of primary and metastatic B16F10 mouse tumors in vivo. *Molecular Therapy : The Journal of the American Society of Gene Therapy* 16 (1): 52-59.

Stanford, M. M., S. J. Werden, and G. McFadden. 2007. Myxoma virus in the European rabbit: Interactions between the virus and its susceptible host. *Veterinary Research* 38 (2): 299-318.

Stanford, M. M., J. W. Barrett, S. H. Nazarian, S. Werden, and G. McFadden. 2007b. Oncolytic virotherapy synergism with signaling inhibitors: Rapamycin increases myxoma virus tropism for human tumor cells. *Journal of Virology* 81 (3): 1251-1260.

Stephenson, K.B., N. G. Barra, E. Davies, A. A. Ashkar, B. D. Lichty. 2012. Expressing human interleukin-15 from oncolytic vesicular stomatitis virus improves survival in a murine metastatic colon adenocarcinoma model through the enhancement of anti-tumor immunity. *Cancer Gene Therapy* 19 (4): 238-246.

Stupp, R., M. J. van den Bent, and M. E. Hegi. 2005. Optimal role of temozolomide in the treatment of malignant gliomas. *Current Neurology and Neuroscience Reports* 5 (3): 198-206.

Surawicz, T. S., B. J. McCarthy, V. Kupelian, P. J. Jukich, J. M. Bruner, and F. G. Davis. 1999. Descriptive epidemiology of primary brain and CNS tumors: Results from the central brain tumor registry of the United States, 1990-1994. *Neuro-Oncology* 1 (1): 14-25.

Suter, M., C. Meisinger-Henschel, M. Tzatzaris, V. Hülsemann, S. Lukassen, N. H. Wulff, J. Hausmann, P. Howley, and P. Chaplin. 2009. Modified vaccinia ankara strains with identical coding sequences actually represent complex mixtures of viruses that determine the biological properties of each strain. *Vaccine* 27 (52): 7442-7450.

Sypula, J., F. Wang, Y. Ma, J. Bell, and G. McFadden. 2004. Myxoma virus tropism in human tumor cells. *Gene Therapy & Molecular Biology* 8 : 103-114.

Szatmari, T., K. Lumniczky, S. Desaknai, S. Trajcevski, E. J. Hidvegi, H. Hamada, and G. Safrany. 2006. Detailed characterization of the mouse glioma 261 tumor model for experimental glioblastoma therapy. *Cancer Science* 97: 546-553.

Tai, J. H., J. V. Williams, K. M. Edwards, P. F. Wright, J. E. Crowe Jr, and T. S. Dermody. 2005. Prevalence of reovirus-specific antibodies in young children in Nashville, Tennessee. *The Journal of Infectious Diseases* 191 (8): 1221-1224.

Taqi, A. M., M. B. Abdurrahman, A. M. Yakubu, and A. F. Fleming. 1981. Regression of Hodgkin's disease after measles. *The Lancet* 317 (8229): 1112.

Thomas, D. L., R. Doty, V. Tosic, J. Liu, D. M. Kranz, G. McFadden, A. L. Macneill, and E. J. Roy. 2011. Myxoma virus combined with rapamycin treatment enhances adoptive T cell therapy for murine melanoma brain tumors. *Cancer Immunology, Immunotherapy : CII* 60 (10):1461-1472.

Thorne, S. H., D. L. Bartlett, and D. H. Kirn. 2005. The use of oncolytic vaccinia viruses in the treatment of cancer: A new role for an old ally? *Current Gene Therapy* 5 (08): 429-443.

Thornton, F. J., S. S. Kandiah, W. S. Monkhouse, and M. J. Lee. 2001. Helical CT evaluation of the perirenal space and its boundaries: A cadaveric study. *Radiology* 218 (3): 659-663.

Tilney, N. L. 1971. Patterns of lymphatic drainage in the adult laboratory rat. *Journal of Anatomy* 109 : 369-383.

Townsley, A. C., A. S. Weisberg, T. R. Wagenaar, and B. Moss. 2006. Vaccinia virus entry into cells via a low-pH-dependent endosomal pathway. *Journal of Virology* 80 (18): 8899-8908.

Tsung, K., J. B. Meko, G. R. Peplinski, Y. L. Tsung, and J. A. Norton. 1997. IL-12 induces T helper 1-directed antitumor response. *Journal of Immunology (Baltimore, Md.: 1950)* 158 (7): 3359-3365.

Tucker, M. A., C. N. Coleman, R. S. Cox, A. Varghese, and S. A. Rosenberg. 1988. Risk of second cancers after treatment for hodgkin's disease. *The New England Journal of Medicine* 318 (2): 76-81.

U.S. Cancer Statistics Working Group. *United states cancer statistics: 1999–2007 incidence and mortality web-based report*. in U.S. Department of Health and Human Services, Centers for Disease Control and Prevention and National Cancer Institute [database online]. Atlanta, GA, 2010.

Van den Broeck, W., A. Derore, and P. Simoens. 2006. Anatomy and nomenclature of murine lymph nodes: Descriptive study and nomenclatory standardization in BALB/cAnNCrl mice. *Journal of Immunological Methods* 312 (1-2): 12-19.

Vasey, P. A., L. N. Shulman, S. Campos, J. Davis, M. Gore, S. Johnston, D. H. Kirn, et al. 2002. Phase I trial of intraperitoneal injection of the E1B-55-kd-gene-deleted adenovirus ONYX-015 (dl1520) given on days 1 through 5 every 3 weeks in patients with recurrent/refractory epithelial ovarian cancer. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* 20 (6): 1562-1569.

Vera, M., N. Razquin, J. Prieto, I. Melero, P. Fortes, and G. Gonzalez-Aseguinolaza. 2005. Intratumoral injection of dendritic cells transduced by an SV40-based vector expressing interleukin-15 induces curative immunity mediated by CD8+ T lymphocytes and NK cells. *Molecular Therapy : The Journal of the American Society of Gene Therapy* 12 (5): 950-959.

Villegas, F. R., S. Coca, V. G. Villarrubia, R. Jiménez, M. J. Chillón, J. Jareño, M. Zuil, and L. Callol. 2002. Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. *Lung Cancer* 35 (1): 23-28.

von der Weid, N. X. 2008. Adult life after surviving lymphoma in childhood. *Supportive Care in Cancer : Official Journal of the Multinational Association of Supportive Care in Cancer* 16 (4): 339-345.

Wang, F., J. W. Barrett, Y. Ma, G. A. Dekaban, and G. McFadden. 2009. Induction of alpha/beta interferon by myxoma virus is selectively abrogated when primary mouse embryo fibroblasts become immortalized. *Journal of Virology* 83 (11): 5928-5932.

Wang, F., X. Gao, J. W. Barrett, Q. Shao, E. Bartee, M. R. Mohamed, M. Rahman, et al. 2008. RIG-I mediates the co-induction of tumor necrosis factor and type I interferon elicited by myxoma virus in primary human macrophages. *PLoS Pathogens* 4 (7): e1000099.

Wang, F., Y. Ma, J. W. Barrett, X. Gao, J. Loh, E. Barton, H. W. Virgin, and G. McFadden. 2004. Disruption of erk-dependent type I interferon induction breaks the myxoma virus species barrier. *Nature Immunology* 5 (12): 1266-1274.

Wang, G., J. W. Barrett, M. Stanford, S. J. Werden, J. B. Johnston, X. Gao, M. Suns, J. Q. Cheng, and G. McFadden. 2006. Infection of human cancer cells with myxoma virus requires akt activation via interaction with a viral ankyrin-repeat host range factor. *Proceedings of the National Academy of Sciences of the United States of America* 103 (12): 4640-4645.

Watanabe, H., K. Numata, T. Ito, K. Takagi, and A. Matsukawa. 2004. Innate immune response in Th1- and Th2-dominant mouse strains. *Shock* 22 (5): 460-466.

Wehrle, P. F. 1980. A reality in our time: Certification of the global eradication of smallpox. *The Journal of Infectious Diseases* 142 (4): 636-638.

Weide, B., E. Derhovanessian, A. Pflugfelder, T. K. Eigentler, P. Radny, H. Zelba, C. Pfohler, G. Pawelec, and C. Garbe. 2010. High response rate after intratumoral treatment with interleukin-2: Results from a phase 2 study in 51 patients with metastasized melanoma. *Cancer* 116 (17): 4139-4146.

Wein, L. M., J. T. Wu, and D. H. Kirn. 2003. Validation and analysis of a mathematical model of a replication-competent oncolytic virus for cancer treatment: Implications for virus design and delivery. *Cancer Research* 63 (6): 1317-1324.

Weintraub, L. R. 1969. Lymphosarcoma. *JAMA: The Journal of the American Medical Association* 210 (8): 1590-1591.

Werden, S. J., and G. McFadden. 2008. The role of cell signaling in poxvirus tropism: The case of the M-T5 host range protein of myxoma virus. *Biochimica Et Biophysica Acta* 1784 (1): 228-237.

Werner, M. H., S. Phuphanich, and G. H. Lyman. 1995. The increasing incidence of malignant gliomas and primary central nervous system lymphoma in the elderly. *Cancer* 76 (9): 1634-1642.

Whelan, K. F., K. Stratton, T. Kawashima, J. W. Waterbor, R. P. Castleberry, M. Stovall, C. A. Sklar, et al. 2010. Ocular late effects in childhood and adolescent cancer survivors: A report from the childhood cancer survivor study. *Pediatric Blood & Cancer* 54 (1): 103-109.

Whitman, E. D., K. Tsung, J. Paxson, and J. A. Norton. 1994. In vitro and in vivo kinetics of recombinant vaccinia virus cancer-gene therapy. *Surgery* 116 (2): 183-188.

Wolf, J. K., D. C. Bodurka, J. B. Gano, M. Deavers, L. Ramondetta, P. T. Ramirez, C. Levenback, and D. M. Gershenson. 2004. A phase I study of Adp53 (INGN 201;

ADVEXIN) for patients with platinum- and paclitaxel-resistant epithelial ovarian cancer. *Gynecologic Oncology* 94 (2): 442-448.

Woo, Y., K. J. Kelly, M. M. Stanford, C. Galanis, Y. Shin Chun, Y. Fong, and G. McFadden. 2008. Myxoma virus is oncolytic for human pancreatic adenocarcinoma cells. *Annals of Surgical Oncology* 15 (8): 2329-2335.

Xia, Z. J., J. H. Chang, L. Zhang, W. Q. Jiang, Z. Z. Guan, J. W. Liu, Y. Zhang, et al. 2004. Phase III randomized clinical trial of intratumoral injection of E1B gene-deleted adenovirus (H101) combined with cisplatin-based chemotherapy in treating squamous cell cancer of head and neck or esophagus. *Ai Zheng = Aizheng = Chinese Journal of Cancer* 23 (12): 1666-1670.

Yiang, G. T., H. J. Harn, Y. L. Yu, S. C. Hu, Y. T. Hung, C. J. Hsieh, S. Z. Lin, and C. W. Wei. 2009. Immunotherapy: RAAV2 expressing interleukin-15 inhibits HeLa cell tumor growth in mice. *Journal of Biomedical Science* 16 (47): 1-8.

Yu, H., T. A. Fehniger, P. Fuchshuber, K. S. Thiel, E. Vivier, W. E. Carson, and M. A. Caligiuri. 1998. Flt3 ligand promotes the generation of a distinct CD34(+) human natural killer cell progenitor that responds to interleukin-15. *Blood* 92 (10): 3647-3657.

Yu, W., and H. Fang. 2007. Clinical trials with oncolytic adenovirus in china. *Current Cancer Drug Targets* 7 (2): 141-148.

Yu, Y. L., C. W. Wei, Y. L. Chen, M. H. Chen, and G. T. Yiang. 2010. Immunotherapy of breast cancer by single delivery with rAAV2-mediated interleukin-15 expression. *International Journal of Oncology* 36 (2): 365-370.

Zou, W. 2005. Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nature Reviews.Cancer* 5 (4): 263-274.

## APPENDIX A:

## ABBREVIATIONS AND ACRONYMS

Ab: antibody
Ad: Adenovirus
Ag: antigen
Akt: a serine/threonine protein kinase also called protein kinase B
APC: antigen-presenting cells
B16-F10: murine melanoma cell line
B16.SIY: B16-F10 cell line that expresses the SIY peptide and GFP protein
C57BL/6: immunocompetent strain of mice
CEA: carcinoembryonic antigen
CEV: cell-associated enveloped virus
CNS: central nervous system
COX2: cyclooxygenase-2
CPE: cytopathic effect
CR: complete response
CRAd: conditionally-replicative adenovirus
CRC: colorectal carcinoma
CTL: cytotoxic T lymphocytes
d: day
DC: dendritic cells
DLT: dose-limiting toxicity
DNA: deoxyribonucleic acid
DP1: EL4 cell line that expresses the SIY peptide and GFP protein
EEV: extracellular enveloped virus
EL4: murine thymic lymphoma cell line
ELISA: enzyme-linked immunosorbent assay
EM: electron microscopy
ERK1/2: extracellular signal-regulated kinase
FITC: fluorescein isothiocyanate
FBS: fetal bovine serum
GBM: glioblastoma multiforme
GFP: green fluorescent protein
GL261: murine glioma cell line
GLSIY: GL261 cell line that expresses the SIY peptide and GFP protein
GM-CSF: granulocyte macrophage colony stimulating factor
Gy: Gray, the international unit of absorbed radiation dose (1 joule of ionizing radiation absorbed by 1 kilogram of matter)
h: hour
HBSS: Hanks balanced salt solution
HNC: head and neck cancer
HPI: hours post-infection
HSV: Herpesvirus

ID: intradermal
IFN: interferon
IHC: immunohistochemistry
IL-12: interleukin 12
IL-15: interleukin 15
IM: intramuscular
IMV: intracellular mature virus
Inj: injection
IP: intraperitoneal
ISG: interferon-stimulated genes
IT: intratumoral
IV: intravenous
kb: kilobases
MEM: Eagle's minimum essential medium
ml: milliliter
min: minute
MIP-1α: macrophage inflammatory protein-1α
MIP-1β: macrophage inflammatory protein-1β
MOI: multiplicity of infection
MTD: maximum tolerated dose
mTOR: mammalian target of rapamycin
MV: Measles virus
MYXV: Myxoma virus, a rabbit poxvirus
MYXV:GFP: recombinant MYXV expressing green fluorescent protein
MYXV:IL15: recombinant MYXV expressing red tomato fluorescent protein and IL-15
MYXV:RFP: recombinant MYXV expressing red fluorescent protein
MYXV:Tomato: recombinant MYXV expressing red tomato fluorescent protein
MV: Measles virus, a paramyxovirus
NDV: Newcastle disease virus, a paramyxovirus
NK: natural killer cells
nm: nanometer
ORR: objective response rate
PAMPs: pathogen-associated molecular patterns
PBS: phosphate buffered saline
PFU: plaque-forming units
PGE2: prostaglandin E2
PI: post-infection
PMV: Paramyxovirus
PR: partial response
PRNT: plaque-reduction neutralization test
PTI: post-tumor inoculation
PV: Picornavirus
q: every (typically used in dosing)
RANTES: regulated upon activation, normal T cell-expressed and secreted
RCC: renal cell carcinoma
RNA: ribonucleic acid

RT: room temperature
RT3D: Reovirus type 3 Dearing (also Reolysin)
SCC: squamous cell carcinoma
SCCHN: squamous cell carcinoma of the head and neck
SD: stable disease
SQ: subcutaneous (also SC)
SVV: Seneca valley virus, a picornavirus
sx: surgery
TAAs: tumor-associated antigens
TBS/T: 1× Tris Buffered Saline with 0.1% Tween-20
TGF-β: transforming growth factor-β
TNF: tumor necrosis factor
TNTC: too numerous to count
tx: treatment
µl: microliter
UV-MYXV: Myxoma virus (MYXV:Tomato) that has been UV-inactivated
VACV: Vaccinia virus, the virus used to vaccinate against smallpox
VARV: Variola virus, the causative agent of smallpox
VEGF: vascular endothelial growth factor
w: week
XRT: radiation therapy

## APPENDIX B:

## RECENT ONCOLYTIC VIRUS CLINICAL TRIALS IN HUMAN CANCER PATIENTS

| Viral Agent | Tumor | Phase | Injection | Additional treatments | Response | Comments |
|---|---|---|---|---|---|---|
| ADVEXIN (replication-deficient Ad containing p53 cDNA (Wolf et al. 2004) | Epithelial ovarian cancer<br><br>Refractory | I | IP daily × 5d, q 3w | None | 0% CR<br>0% PR<br>55% SD | No DLT |
| Ad5-Δ24-RGD (CRAd) (Kimball et al. 2010) | Malignant gynecologic cancer | I | IP, daily × 3 | None | 0% CR<br>0% PR<br>71% SD | No DLT, 100% developed antiviral Abs |
| H101 (CRAd) (Xia et al. 2004) | SCCHN | III | IT daily × 5d, q 3w | Cisplatin + 5-FU (PF) or Adriamycin + 5-FU (AF) | PF + H101= 78% ORR, PF alone = 40% ORR, AF +/- H101 = 50% ORR | |
| ICOVIR-7 (Ad) (Nokisalmi et al. 2010) | Solid tumors Refractory | I | IT | Cyclophos-phamide | 0% CR<br>6% PR<br>24% SD | No DLT<br>Virus detected in blood (86%) |
| KH901 (CRAd + GM-CSF) (Chang et al. 2009) | HNC | I | IT | None | Not evaluated | No DLT<br>GM-CSF in blood 12 HPI, increased Ab titer |
| ONYX-015 (CRAd) (Vasey et al. 2002) | Epithelial ovarian cancer | I | IP, daily × 5d | None | 0% CR<br>0% PR<br>0% SD | Dose-limiting toxicity, viral DNA in blood |
| ONYX-015 (CRAd) (Mulvihill et al. 2001) | Pancreatic cancer | I | IT q 4w | None | 0% CR<br>0% PR<br>0% SD | No DLT, no viral replication |
| ONYX-015 (CRAd) (Ganly et al. 2000) | HNC recurrent | I | IT | None | 0% CR<br>0% PR<br>0% SD | No DLT |
| ONYX-015 (CRAd) (Nemunaitis et al. 2007) | Advanced solid tumors | I | IV weekly up to 4 doses | Enbrel | 0% CR<br>0% PR<br>44% SD | No DLT, no adverse effects with drug |

| | | | | | | |
|---|---|---|---|---|---|---|
| ONYX-015 (CRAd) (Galanis et al. 2005) | Advanced sarcomas | I/II | IT daily × 5d, q month | Mitomycin-C, doxorubicin, cisplatin | 17% CR<br>0% PR<br>67% SD | No DLT, viral replication |
| ONYX-015 (CRAd) (Khuri et al. 2000) | SCCHN | II | IT daily × 5d, q 3w | Cis-platinum, 5-FU | 27% CR<br>36% PR | Grade 3 and 4 toxicities. No correlation between Ab's and tx results |
| Telomelysin (Ad-TERT) (Nemunaitis et al. 2010) | Solid tumors | I | IT | None | 0% CR<br>6% PR<br>25% SD | No DLT<br>Viral replication |
| TG1042 (Ad-IFNγ) (Dummer et al. 2010) | Cutaneous Lymphoma | II | IT | None | Local:<br>20% CR<br>33% PR<br>27% SD<br>Global:<br>20% CR<br>7% PR<br>53% SD | Tumor and antiviral Abs produced, not correlated with response |
| G207 (HSV) (Markert et al. 2000) | Malignant glioma | I | IT 1-5 doses Reccurence post-sx and post-XRT | None | 0% CR<br>0% PR<br>40% SD | No DLT<br>Not all patients seroconverted |
| G207 (HSV) (Markert et al. 2009) | GBM Recurrent | Ib | IT pre-sx<br>IC post-sx | None | 0% CR<br>0% PR | No DLT |
| HF10 (HSV) (Nakao et al. 2011) | Pancreatic cancer Un-resectable | I | IT at sx, IT by catheter daily × 2d | None | 0% CR<br>17% PR<br>50% SD | No DLT<br>Blood NK cells increased after injection |
| OncoVEX$^{GM-CSF}$ (HSV+GM-CSF) (Hu et al. 2006) | Various cancers | I | IT, 1-3 tx | None | 0% CR<br>0% PR<br>12% SD | Local reaction was dose-limiting in sero-negative patients |
| OncoVEX$^{GM-CSF}$ (HSV+GM-CSF) (Harrington et al. 2010a) | Stage III/IV SCCHN Untreated | I/II | IT on days 1, 22, 43 and 64 | XRT and cisplatin | 24% CR<br>59% PR | No DLT, Viral replication |

| | | | | | | |
|---|---|---|---|---|---|---|
| OncoVEX$^{GM-CSF}$ (HSV-GMCSF) (Senzer et al. 2009) | Melanoma Un-resectable | II | IT, repeated in 3w, then q 2w up to 24 tx | None | 16% CR<br>10% PR<br>20% SD | No DLT<br>Regression of injected and distant lesions<br>Doubled 1 year survival rate |
| NV1020 (attenuated HSV) (Geevarghese et al. 2010) | CRC metastatic to liver Refractory | I/II | In hepatic artery, weekly × 4 | Chemo-therapy after tx | 50% SD after virus tx<br>5% PR and 64% SD after chemo | Mild toxicity, no DLT,<br>No viral shedding |
| PV701 (PMV) (Pecora et al. 2002) | Advanced solid cancers Refractory | I | IV, various tx regimens | None<br>Heavily pre-tx | 2% CR<br>2% PR<br>19% SD | Grade 1-4 toxicities, viral shedding |
| MV-CEA (PMV) (Galanis et al. 2010) | Recurrent ovarian cancer (refractory) | I | IP q 4w up to 6 tx | None<br>Pre-tx | 0% CR<br>0%PR<br>67% SD | No DLT, no increased antiviral Ab's |
| SVV-001 (PV) (Rudin et al. 2011) | Advanced solid tumor with Neuro-endocrine features | I | IV one dose | None | 0% CR<br>0% PR<br>3% SD | No DLT, viral clearance correlated with antiviral Ab's |
| RT3D=Reolysin (Reovirus) (Harrington et al. 2010b) | Advanced solid cancers | I | 2 IT inj then 2-6 IT inj | Concurrent XRT | Low-dose XRT: 29% PR 71% SD<br>High-dose XRT: 71% PR 29% SD | No DLT, no viral shedding, no exacerbation of radiation reaction |
| Trovax (VACV+5T4) (Harrop et al. 2006) | CRC | I/II | IM or ID at 0,4, 8w, +/- 14, 20 w | None | 29% CR | Increased survival with Ab response to TAA but not virus |
| Trovax (VACV + 5T4) (Kaufman et al. 2009) | RCC | II | IM q 3w × 3 | IL-2 | 0% CR<br>0% PR<br>48% SD | Significant increase in CD8+ T cells in patients with SD |
| Trovax (VACV + 5T4) (Amato et al. 2010) | RCC | III | IM at weeks 1,3,6,9,13, 17 21, 25, 33, 41, 49, 57, 65 | With or without IL-2, or INFα, or sunitinib | No significant difference | No significant increase in toxicities over controls |

| | | | | | | |
|---|---|---|---|---|---|---|
| JX-594 (VACV+ GMCSF) (Park et al. 2008) | Primary and metastatic liver cancer Refractory | I | IT q 3w × 4 tx | None Heavily pre-tx | 0% CR 21% PR 43% SD | DLT due to hyperbilirubinemia. Viral DNA in blood. Response in tx and untreated tumors |

Ab= antibody, Ad= adenovirus, Ag= antigen, CR= complete response, CRAd= conditionally replicative adenovirus, CRC= colorectal carcinoma, d= day, DLT= dose-limiting toxicity, GBM= glioblastoma multiforme, GM-CSF= granulocyte macrophage coloncy sitmulating factor, HNC= head and neck cancer, HSV= herpes simplex virus, IC= intracranial, IM= intramuscular, IP=intraperitoneal, IT= intratumoral, MV= measles virus, NDV= Newcastle disease virus, NK= natural killer cells, ORR= objective response rate, PMV= paramyxovirus, PR= partial response, PV= picornavirus, q= every, RCC= renal cell carcinoma, RT3D= reovirus type 3 Dearing, SCCHN= squamous cell carcinoma of the head and neck, SD= stable disease, SVV= Seneca valley virus, sx= surgery, tx= treatment, VACV= vaccinia virus, w= week, XRT= radiation therapy, 5T4= a tumor antigen