EVALUATION OF ADOPTIVE T CELL THERAPY AND ONCO LY TIC VIRO THERAPY
FOR TREATMENT OF BRAIN TUMORS

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ABSTRACT

Adoptive immunotherapy and oncolytic virotherapy are two promising strategies for treating primary and metastatic malignant brain tumors. We demonstrate the ability of adoptively transferred tumor-specific T cells to rapidly mediate the clearance of established brain tumors in several mouse models. Similar to the clinical situation, tumor recurrences are frequent and result from immune editing of tumors. T cells can eliminate antigen-expressing tumor cells but are not effective against antigen loss variant (ALV) cancer cells that multiply and repopulate a tumor. We show that the level of tumor antigen present affects the success of adoptive T cell therapy. When high levels of antigen are present, tumor stromal cells such as microglia and macrophages present tumor peptide on their surface. As a result, T cells directly eliminate cancer cells and cross-presenting stromal cells and indirectly eliminate ALV cells. We were able to show the first direct evidence of tumor antigen cross-presentation by CD11b+ stromal cells in the brain using soluble, high-affinity T cell receptor monomers. Strategies that target brain tumor stroma or increase antigen shedding from tumor cells leading to increased cross-presentation by stromal cells may improve the clinical success of T cell adoptive therapies.

We evaluated one potential strategy to complement adoptive T cell therapy by characterizing the oncolytic effects of myxoma virus (MYXV) in a syngeneic mouse brain tumor model of metastatic melanoma. MYXV is a rabbit poxvirus with strict species tropism for European rabbits. MYXV can also infect mouse and human cancer cell lines due to signaling defects in innate antiviral mechanisms and hyperphosphorylation of Akt. MYXV kills B16.SIY melanoma cells in vitro, and intratumoral injection of virus leads to robust, selective and transient infection of the tumor. We observed that virus treatment recruits innate immune cells
to the tumor, induces TNFα and IFNβ production in the brain, and results in limited oncolytic effects \textit{in vivo}. To overcome this, we evaluated the safety and efficacy of co-administering 2C T cells, MYXV, and neutralizing antibodies against IFNβ. Mice that received the triple combination therapy survived significantly longer with no apparent side effects, but eventually relapsed. Based on these findings, methods to enhance viral replication in the tumor and limit immune clearance of the virus will be pursued. We conclude that myxoma virus should be further explored as a vector for transient delivery of therapeutic genes to a tumor to enhance T cell responses.
To my grandmother, Lois Annette Meyer
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# TABLE OF CONTENTS

**LIST OF COMMON ABBREVIATIONS**........................................................................ vii

**CHAPTER 1. IMMUNOTHERAPY AND VIROTHERAPY FOR THE TREATMENT OF BRAIN TUMORS** ....................................................................................................................................................................................1

- Brain Tumors: A Clinical Challenge ......................................................................... 1
- The Immune System Response to Brain Tumors ...................................................... 3
- Immune Defects Associated with Brain Tumors ...................................................... 6
- Immunotherapies for Brain Tumors ......................................................................... 9
- Oncolytic Virotreatments for Brain Tumors ............................................................ 19
- Statement of Problem and Research Significance ................................................... 27
- 2C T Cell System and Brain Tumor Models ............................................................ 29
- References ............................................................................................................ 33
- Chapter 1 Figures ................................................................................................. 45

**CHAPTER 2. RECURRENCE OF INTRACRANIAL TUMORS FOLLOWING ADOPTIVE T CELL THERAPY CAN BE PREVENTED BY DIRECT AND INDIRECT KILLING AIDED BY HIGH LEVELS OF TUMOR ANTIGEN CROSS-PRESENTED ON STROMAL CELLS** .........................................................................................................................48

- Abstract ................................................................................................................ 48
- Introduction .......................................................................................................... 49
- Methods ............................................................................................................... 51
- Results .................................................................................................................. 54
- Discussion .......................................................................................................... 63
- References .......................................................................................................... 69
- Chapter 2 Figures ................................................................................................. 73

**CHAPTER 3. EVALUATION OF TUMOR-SPECIFIC ADOPTIVE T CELL THERAPY AND ONCOLYTIC VIROTHERAPY FOR TREATMENT OF METASTATIC MELANOMA BRAIN TUMORS** .........................................................................................................................83

- Abstract ................................................................................................................ 83
- Introduction .......................................................................................................... 84
- Methods ............................................................................................................... 87
- Results .................................................................................................................. 94
- Discussion .......................................................................................................... 102
- References .......................................................................................................... 108
- Chapter 3 Figures ................................................................................................. 111

**CHAPTER 4. SUMMARY AND CONCLUSIONS** .........................................................................................................................124

- References .......................................................................................................... 131
LIST OF COMMON ABBREVIATIONS

Abs: antibodies
ADCC: antibody dependent cellular cytotoxicity
ALV: antigen loss variant cancer cell
APC: antigen presenting cell
BBB: blood-brain barrier
CD: cluster of differentiation antigen
CD4: helper T cell
CD8: cytotoxic T cell
CNS: central nervous system
CTL: cytotoxic T lymphocyte
DC: dendritic cell
EGFR: epidermal growth factor receptor
Fₜ: crystallizable fragment of antibody molecule
GBM: glioblastoma multiforme
GM-CSF: granulocyte-macrophage colony stimulating factor
GFP: green fluorescent protein
HLA: human leukocyte antigen
HSV: herpes simplex virus
IC: intracranial
IDO: indoleamine-2,3-dioxygenase
IFN: interferon
IL: interleukin
IP: intraperitoneal
IT: intratumoral
LAK: lymphokine activated killer
MAGE: melanoma antigen
MHC: major histocompatibility complex
MOI: multiplicity of infection
MYXV: myxoma virus
NDV: Newcastle disease virus
NK: natural killer
NKG2D: NK cell group 2 member D activating receptor
PD-1: programmed death receptor-1
PD-L1: programmed death receptor ligand-1
PFU: plaque forming unit
PGE₂: prostaglandin E2
SART: squamous cell carcinoma-associated reactive antigen for cytotoxic T cells
TCR: T cell receptor
TGF: transforming growth factor
TIL: tumor infiltrating lymphocyte
TNF: tumor necrosis factor
T_{reg}: regulatory T cell
VEGF: vascular endothelial growth factor
VSV: vesicular stomatitis virus
CHAPTER 1. IMMUNOTHERAPY AND VIROTHERAPY FOR THE TREATMENT OF BRAIN TUMORS

Brain Tumors: A Clinical Challenge

Primary and metastatic brain tumors present significant challenges to patients and their physicians. Approximately 50,000 Americans are diagnosed with malignant or non-malignant primary brain tumors annually. Depending on the tumor type, half of these cases are typically treatable with surgery, but the remainder have very poor prognoses. Brain metastases that arise from other cancers (most frequently lung, breast, melanoma, kidney, or colon) are estimated to occur in 170,000 patients annually. If untreated, patients have a median survival of only four weeks. The overall five-year survival rate following diagnosis of a brain tumor is around 30%, but much less for highly malignant tumors.

Most primary central nervous system (CNS) tumors develop from astrocytes, a type of glial cell. Grade IV glioblastoma multiforme (GBM) tumors, the most malignant and most frequently diagnosed astrocytoma, are highly resistant to treatment. Patients with GBM tumors (gliomas) face a poor prognosis with a median survival of 12-15 months post-diagnosis and treatment and a five-year survival rate of only 3%. Standard treatments include surgery followed by chemotherapy and radiation therapy, but even aggressive multimodal treatments only extend survival for several months. The most significant advance for glioma treatment in the past 30 years is the addition of the chemotherapeutic agent temozolomide following surgical resection and fractionated radiation therapy. When complete resection of the tumor is possible, treatment with temozolomide extends the median survival of patients over 55 years of age to 11 months and under 55 years to 18 months.
Gliomas are highly infiltrative and cancer cells can travel along axon tracts, vasculature and around ventricular spaces\textsuperscript{10}. Surgical excision often leaves behind malignant cells that have migrated several centimeters from the primary tumor mass. Due to their heterogeneous makeup and genetically unstable nature\textsuperscript{11}, some tumors become resistant to both irradiation and chemotherapeutic drugs\textsuperscript{12,13}. There is also growing evidence that the elimination of a small population of glioma stem cells that can repopulate a tumor is crucial to preventing tumor recurrence, and a subset of glioma stem cells (CD133\textsuperscript{+}) are both chemoresistant and radioresistant\textsuperscript{14,15}.

The incidence of secondary, or metastatic, CNS tumors is thought to be rising due to the improving effectiveness of cancer treatment in general. Patients are surviving long enough to develop complications from metastatic tumors that arise when cancer cells spread via the blood or lymphatics. 15-40\% of all patients with cancer, particularly lung and breast carcinomas and melanomas, develop metastatic brain tumors\textsuperscript{2,3}. Melanomas are most likely to metastasize to the brain over other sites, and patients with CNS involvement have very poor prognoses. 17-39\% of patients with disseminated cutaneous melanoma develop multiple CNS metastases and without therapy, survive only 1-2 months\textsuperscript{16}. Whole brain radiation therapy can extend survival to four months, and combined with surgery to ten months. However, even with the addition of systemic chemotherapy, these treatment combinations have not improved the overall outcome. Due to the improved clinical responses observed in patients with GBM tumors, temozolomide is now being evaluated for treatment of patients with metastatic melanoma brain tumors\textsuperscript{17}. 
The Immune System Response to Brain Tumors

The immune system has evolved to defend against invading pathogens and altered cells, including cancer cells. However, CNS tumors are a challenging target. Both innate and adaptive immune cells should prevent tumor development by recognizing and eliminating mutated cells, yet in most types of cancer there are subtle distinctions at the molecular level between normal and malignant ‘self’ cells. Immune cells such as T cells that react strongly with ‘self’ peptides are typically deleted in the thymus during development to prevent autoimmunity. Furthermore, most immune cells must also migrate to the brain to locate malignant cells and then overcome local immunosuppressive mechanisms. Circulating immune cells have limited access to the brain under normal conditions and inflammation is actively suppressed, leading to the historically misleading description of the brain as immune-privileged\textsuperscript{18}. In non-pathological states, or in early stages of tumor development, the brain is better characterized as quiescent. The blood-brain barrier (BBB) generally limits circulating antibodies and unactivated lymphocytes from entering the CNS\textsuperscript{19}. Inflammation is actively suppressed in normal brain tissue by soluble and cellular mediators including TGF-β, IL-10, prostaglandins (PGE\textsubscript{2}) and gangliosides, resulting in an environment that hinders or delays immune reactivity\textsuperscript{19}. Despite its normal quiescence, both innate and adaptive immune responses do occur in the brain during infection and injury, in autoimmune diseases such as multiple sclerosis, and in brain tumors. However, the suppressive environment of the CNS and tumor-induced immune defects often result in tumor escape.
Innate Immune Responses to Brain Tumors

Over the lifespan of an individual, the frequency at which transformed or malignant cells are quickly eliminated by the immune system before a tumor can establish is unknown, but likely numerous. Innate immune cells are considered the first responders to developing tumors. NK cells and macrophages can respond quickly to stress-induced proteins expressed on tumor cells. Upregulated ligands such as MHC class I chain-related molecules A and B (MICA/B) and UL16-binding proteins are recognized by NKG2D receptors on NK cells and macrophages, making glioma cells susceptible to killing\textsuperscript{20,21}. NK cells can also detect the loss of class I major histocompatibility complex (MHC) expression on transformed cells and kill them either directly by releasing granules containing perforin and granzymes, or indirectly by secreting cytokines such as interferon-\(\gamma\) (IFN\(\gamma\)) to activate other innate immune cells and sensitize the cancer cells for apoptosis or killing\textsuperscript{22}. Macrophages kill tumor cells by releasing nitric oxide and TNF-\(\alpha\)\textsuperscript{19,23}, phagocytose dead and dying tumor cells, and secrete cytokines such as IL-12 that recruit and activate monocytes and T cells.

Microglia, the resident phagocytic macrophage-like cells of the brain, accumulate in brain tumors in large numbers in response to chemokines secreted by the tumor\textsuperscript{24} and have both anti-tumor and pro-tumor functions. Cells with macrophage and microglia surface markers represent approximately one-third of all cells in glioma biopsies\textsuperscript{25,26} and between 5-35\% of the total tumor burden in experimental rodent glioma models\textsuperscript{24,27}. In their resting state, microglia survey the brain parenchyma and become activated in response to CNS damage\textsuperscript{28,29}. In their activated state, microglia differentiate into effector cells and phagocytose dead or dying cells, secrete pro- and anti-inflammatory cytokines and neurotrophic factors, and induce a non-specific inflammatory state\textsuperscript{29-33}. Microglia also have the ability to act as antigen presenting cells (APCs)
and can activate CD4+ helper T cells by expressing tumor peptides bound to MHC class II molecules and costimulatory molecules such as CD80 and CD86. Macrophages/microglia also cross-present tumor peptides on MHC class I to activate cytotoxic CD8+ T cells.

*Adaptive immune responses to brain tumors*

In general, tumor rejection is largely dependent on activated T cells and their secreted products, and some studies indicate the presence of tumor-infiltrating T cells in malignant gliomas is associated with longer survival. The CNS lacks traditional lymphatics, but cervical lymph nodes collect CNS antigens that drain along cranial nerves and subarachnoid space and through the cribiform plate. T cells circulate between blood and lymph until they are activated by APCs presenting CNS antigens in cervical and possibly other peripheral lymph nodes. Upon co-stimulation by APCs, T cells respond to their antigen by acquiring an activated phenotype and proliferating. Activated T cells can traffic to the brain and adhere to endothelium to extravasate across the BBB into brain tissue. Once activated, CD8+ T cells are capable of recognizing and killing cancer cells expressing their cognate antigen bound to MHC class I molecules by direct contact mechanisms (e.g., perforin, granzyme, FasL, TRAIL). Activated CD4+ helper T cells also traffic to brain tumors and play an important role in anti-tumor immunity by recognizing antigen presented by APCs on MHC class II molecules, secreting cytokines (e.g., IFNγ), and expressing surface molecules (e.g., CD40L) to directly activate APCs and indirectly activate CD8+ T cells. In contrast to their classical characterization as support cells for CD8+ T cells and APCs, a new appreciation for CD4+ T cell orchestration of the overall anti-tumor immune response and their direct cytotoxic effects on tumor cells is emerging.
T cell responses are highly antigen-specific and MHC-restricted. Within a polyclonal population of CD8+ T cells, each clone will only recognize and kill cancer cells expressing peptide/MHC antigens recognized by their unique T cell receptor (TCR). Effector CD8+ T cells, also called cytotoxic T lymphocytes (CTLs), require presentation of their antigen on class I MHC molecules. Brain tumor cells express low levels of MHC I, in part due to normally low expression by brain cells50, but also from presentation pathway defects in cancer cells51. CD8+ T cells may also recognize antigen presented by phagocytic cells that internalize and process tumor proteins, and cross-present them on MHC I. CTLs can kill tumor cells and cross-presenting APCs directly or indirectly by secreting cytokines to recruit other cells (macrophages, NK cells, etc.). A number of human glioma- and melanoma-associated antigens recognized by CD8+ TCRs have now been identified including MHC I (HLA-A02 allele) restricted IL-13Rα, HER-2, gp100, MAGE-1, EGFRvIII, and others (reviewed in 7).

**Immune Defects Associated with Brain Tumors**

Immune surveillance mechanisms may be intact, but the effector response to brain tumors is typically insufficient, at least at the point of clinical detection. A large percentage (11-72%) of glioma specimens are reported to have T cell infiltrates42,52,53, and this finding is not unique to gliomas. For example, patients with progressively growing metastatic melanomas have had frequencies of Melan-A/MART-1-specific CD8+ T cells as high as 1% in peripheral blood, but are unable to reject established tumors54.

Brain tumors escape detection, attack, and elimination by activated T cells through a variety of soluble and cell-associated mechanisms present in both draining lymph nodes and locally in the tumor7,55. Activated T cells that traffic to a glioma or melanoma brain tumor
encounter a local suppressive environment characterized by expression of TGF-β, IL-10, 
prostaglandin E2, indoleamine-2,3-dioxygenase (IDO) and other inhibitory molecules that 
imhibit proliferation and cytotoxic activity and can induce anergy or apoptosis. Tumor-
infiltrating CD4+ lymphocytes often adopt a Th2 phenotype, produce IL-4 and GM-CSF rather 
than IL-2 and IFN-γ, dampening the CTL response. Glioma and metastatic melanoma brain tumors also possess mechanisms to avoid T cell 
cytotoxicity by expressing apoptosis or tolerance-inducing ligands (e.g., FasL, PD-L1), 
inducing anergy through lack of co-stimulatory molecule expression (e.g., B7), and 
downregulating or expressing altered MHC. Notably, engagement of the inhibitory 
programmed death (PD-1) receptor on T cells by its ligand PD-L1 expressed by many human 
cancers including melanoma appears to have a significant inhibitory effect on T cell effector 
function.

T cells can be suboptimal for executing tumor cytotoxicity even without tumor-induced 
suppression. Most tumor antigens are derived from altered or overexpressed normal proteins. If 
endogenous T cells recognize overexpressed ‘self’ tumor antigens via their TCR and become 
activated, it is usually via a low affinity interaction, as highly avid TCR-bearing T cells are 
deleted during development to prevent autoimmunity. In addition, afferent immune responses 
from the brain occur through drainage of antigens to cervical lymph nodes, which tend to bias 
CD4+ T cell responses towards a Th2, or immunosuppressive phenotype. Tumor-specific T 
cells found in patients and animal models also exhibit signaling defects involved in activation 
and are more likely to undergo apoptosis.

CD4+CD25+ regulatory T cells (Treg), a subset of CD4+ T cells, are major contributors to 
maintaining peripheral tolerance by immune cells to normal tissues, but also contribute to the
immune evasion of brain tumors by suppressing effector T cells through secretion of TGFβ and IL-10 and direct cytolysis\textsuperscript{75}. \textit{T}	extsubscript{reg} cells are increased in the blood and tumors of patients with gliomas and melanomas\textsuperscript{76,77}, and in animal models\textsuperscript{64,78-80}. Depletion of \textit{T}	extsubscript{reg} cells leads to prolonged survival in animal models\textsuperscript{80,81}.

In cases where T cell effector functions are intact and effective, tumor heterogeneity due to genetic instability or immune editing (elimination of antigen-positive but not antigen-negative cancer cells) can lead to outgrowth of antigen loss variant (ALV) cancer cells which are no longer recognized by CTLs\textsuperscript{82,83}. ALVs are a significant cause for concern in all cases where a single tumor antigen is targeted.

Defects in innate immunity against brain tumors also play a role in immune escape. Glioma cells avoid cytolysis by NKG2D receptor-expressing NK cells and macrophages by secreting TGF-β and matrix metalloproteinases that downregulate MICA andUL16 binding proteins\textsuperscript{84}. One immunohistological study showed that only 9\% of malignant gliomas contained NK cells\textsuperscript{46}, perhaps due to this ligand downregulation.

Microglia and macrophages are recruited to brain tumors to participate in the repair of local tissue damage and the anti-tumor immune response\textsuperscript{85}, but can also support the growing tumor\textsuperscript{58,86}. Microglia are known to suppress T cells through multiple mechanisms including FasL expression, prostaglandin E\textsubscript{2}, and IL-10 production\textsuperscript{55,87,88}. In fact, microglia have been shown to be the major source of IL-10 in tumors\textsuperscript{87}. IL-10 supports enhanced proliferation and infiltration of tumor cells\textsuperscript{58} and promotes MHC downregulation and reduced antigen presentation by microglia\textsuperscript{89}. Microglia also produce the cysteine protease cathepsin B and vascular endothelial growth factor (VEGF) that promote tumor invasion and angiogenesis\textsuperscript{90,91}.
The evolution and accumulation of many selective advantages for immune escape by tumors suggest that the immune cells can initially be sufficient for controlling tumor growth. However, once tumors reach a certain size and degree of heterogeneity (correlating with diagnostic grade), various mechanisms have tipped the balance in favor of tumor escape. Our growing understanding of these mechanisms of immune suppression and methods to overcome them, especially within the brain, support the pursuit of therapies that can tip the balance back in favor of immune control.

**Immunotherapies for Brain Tumors**

Important developments over the past several decades include improved brain imaging and surgical methods, targeted radiation treatment, and better chemotherapeutic drugs with fewer side effects. Yet even with an aggressive approach of removing substantial amounts of brain tissue, these therapies have failed to significantly improve the survival outcome for patients with gliomas and metastatic brain tumors, and often lead to impaired cognition, sensory and motor deficits, seizures, and other serious complications. Following treatment, relapse and death are attributed to progressive growth of minimal residual disease. Thus, complementary treatments that target minimal residual disease are urgently needed. Strategies that harness the sensitivity, specificity and cytotoxicity of immune cells by tipping the balance in favor of an effective immune response remain very attractive.

Active, passive and adoptive immunotherapy strategies are potentially useful approaches and have been successful in preclinical studies, but have only modestly improved survival in clinical trials thus far. Further developments using animal tumor models are improving our
understanding of the requirements for successful immunotherapy, gene therapy, oncolytic virotherapy, and other novels forms of treatment for brain tumors.

**Active Immunotherapy**

Active immunotherapy involves generating a host immune response against known or unknown tumor antigens through vaccination. By exploiting the efficient antigen presentation capabilities of dendritic cells (DCs), due in part to their abundant expression of co-stimulatory molecules, tumor vaccines can effectively prime CD4+ and CD8+ effector T cells. Vaccination is typically accomplished by injecting (often with cytokines) irradiated tumor cells, tumor peptides, or DCs pulsed with acid-eluted tumor peptides or tumor lysate. Sampson and colleagues showed that systemic vaccination of mice with irradiated, cytokine-producing (IL-3, IL-6, or GM-CSF) melanoma cells followed by intracranial challenge with melanoma cells protected mice and led to improved survival that was CD8+ T cell dependent. Soon after, Ashley et al. were the first to show that DCs pulsed with melanoma cell extract or melanoma RNA and injected into mice with established intracranial melanoma tumors were able to prime cytotoxic T cells and extend survival significantly. Similar results have also been observed in a mouse glioma model after mice were injected with DCs pulsed with tumor homogenate. Treatment improved the median survival and memory T cells were generated that protected against later intracranial challenge in mice that were cured.

Vaccination has shown good efficacy in mouse brain tumor models but smaller successes in patients thus far. Two strategies currently in clinical trials include intradermal injections of tumor cell lysate-pulsed DCs (DCVax, Phase I/II) or DCs loaded with tumor-associated peptides derived from EGFR variant III (CDX-110, Phase II/III). Recent data reported by
Northwest Biotherapeutics, Inc. on the DC Vax™ trial are very impressive: the median survival of GBM patients was over 36 months and 22% of patients have survived greater than six years. The CDX-110™ vaccination protocol uses a peptide derived from an overexpressed mutant epidermal growth factor receptor (EGFRvIII) found on 24-67% of glioblastomas and shows strong objective responses in an ongoing Phase II trial. All patients had EGFRvIII-specific immune responses and 18 of the 23 recurrent tumors that were studied no longer expressed EGFRvIII, indicating the need to target additional or multiple tumor-specific antigens.

An important consideration regarding vaccination is the potential to induce autoimmunity if vaccine peptides are not tumor-specific and induce T cell responses against normal tissue. In a non-CNS mouse tumor model, vaccination with tumor-associated peptides that were also expressed on normal tissue (pancreas, smooth muscle, cardiomyocytes) resulted in tumor cures but also severe autoimmunity (diabetes, arteritis, cardiomyopathy). For CNS antigens, Bigner and colleagues showed that lethal experimental autoimmune encephalomyelitis (EAE) was induced in nonhuman primates after injection of human glioma tissue and complete Freund’s adjuvant, an immune booster comprised of mycobacterial components.

Many preclinical and clinical vaccination strategies are promising, but demonstrate the challenge of overcoming tumor editing by T cells leading to ALVs. For vaccine strategies to be effective, they must also induce a strong immune response that persists indefinitely until the tumor is eliminated. Concerns about the induction of autoimmunity by cellular vaccines has generated interest in other tumor-specific approaches including passive treatment with tumor-specific antibodies or adoptive transfer of tumor-specific T cells.
Passive Immunotherapy

Passive immunotherapy involves the administration of molecules, typically antibodies, to elicit tumor cytotoxicity. The recipient does not have to be fully immune-competent, which bears importance for glioma patients who often have low peripheral numbers of B cells and helper CD4+ T cells. In addition, antibodies are not susceptible to soluble tumor-suppression mechanisms and provide an advantage over cell-based therapies.

Examples of passive immunotherapy involve injection of unlabeled antibodies into the resected tumor cavity to elicit antibody dependent cellular cytotoxicity (ADCC) by phagocytic immune cells bearing Fc receptors, or the use of radiolabeled or toxin-linked antibodies to localize their cytotoxic effects. Monoclonal antibodies against the extracellular matrix protein tenascin (131I labeled) and EGFR (wild type or mutant vIII), both overexpressed in gliomas, have been best characterized and show some modest increases in survival time when administered intratumorally. Passive immunotherapeutic approaches have potential because they avoid the need to overcome defects in priming the immune system. However, they face other challenges such as production of human anti-mouse antibodies (HAMA) which may prohibit a patient from receiving multiple rounds of treatment. Recombinant DNA technology or chimeric mouse/human antibodies can circumvent this problem. Other challenges include identification of truly tumor-specific antibodies to prevent damage to healthy tissue and delivery of sufficient doses to invasive brain tumor locations.

Adoptive T Cell Therapy

A third immunotherapeutic approach and the focus of our research involves the transfer of antigen-specific cellular immunity conferred by T cells. Tumor-specific T cells can be
isolated, activated and expanded *ex vivo*, and then re-infused back into a patient either intravenously or directly into the tumor cavity. The motility, sensitivity, specificity, and cytotoxic effector function of T cells make adoptive therapy an excellent candidate for targeting invasive, surgically inaccessible disease and micrometastases within the brain, while causing minimal collateral damage to healthy brain tissue.

Adoptive therapy has a number of significant advantages over other forms of immunotherapy. T cells can be expanded *ex vivo* away from tolerizing or inhibitory conditions and subsequently retain cytolytic ability *in vivo*\(^ {103}\). Anergic (unresponsive) T cells can be rescued by IL-15 exposure prior to adoptive transfer\(^ {104}\). Furthermore, effector T cells can be introduced into a patient or tumor-bearing animal in larger numbers (\(10^{10} - 10^{11}\)) than may be possible to generate by active vaccination strategies. As a result, robust T cell priming in a potentially immune-compromised patient is unnecessary. In addition, genes encoding T cell receptors recognizing glioma and melanoma tumor-associated antigens have been identified and transduced into human T cells\(^ {105-107}\). These engineered lymphocytes have been shown to persist in patients and mediate tumor regression. The ability to transduce peripheral blood T cells with antigen-specific TCRs eliminates the need to identify and expand tumor-specific T cells from patients, a slow and labor-intensive process. Recent identification of many additional glioma- and melanoma-associated antigens will undoubtedly aid the search for TCRs appropriate for treating brain tumors in this way.

The use of lymphocytes to treat brain tumors is not new. Observations 35 years ago that leukocytes were cytotoxic to glioblastoma cells in culture\(^ {108}\) led to an early clinical trial by Young and colleagues. Patients with GBM tumors with life expectancy under one month were enrolled and several dramatic clinical responses were observed\(^ {109}\). Patients were injected
intratumorally with autologous leukocytes from peripheral blood. Almost 50% of the patients (8/17) in this study had clinical improvement, although no survival benefit. In one remarkable case, a 49-year old man diagnosed with GBM underwent surgical resection, radiotherapy, and chemotherapy but deteriorated rapidly five months after treatment. He was readmitted to the hospital comatose, and a CT scan showed tumor growth. Following injection of $5 \times 10^7$ autologous leukocytes into the tumor, improvements were noted within 5 hours. In three days, he was walking and eating and remained well for 17 months. It is not known what roles the mixed immune cells in this preparation played in the observed responses, but this trial provided proof-of-concept for further adoptive immunotherapy studies.

Different sources of T cells have been used for preclinical and clinical adoptive therapy studies including peripheral blood, tumor or vaccine draining lymph nodes, lymphokine acitivated killer (LAK) cells, tumor-infiltrating lymphocytes (TILs), and most recently genetically engineered T cells transduced to express TCRs that recognize cancer antigens$^{105-107,110-116}$. Discovery of the T cell growth factor IL-2 led to the ability to expand and activate lymphocytes in culture. High concentrations of IL-2 produce LAK cells, which can be expanded and activated in vitro and subsequently re-infused back into brain tumor-bearing animals or patients in combination with IL-2 treatment$^{117-124}$. A few instances of improved survival with LAK cell treatment have been observed$^{123,124}$, especially with intracavitary brain injections, but concerns about overall efficacy$^{121}$, IL-2 toxicity$^{119}$, non-specific killing, and poor trafficking to the brain have generally shifted interest towards using tumor-specific T cells or TILs.

Tumor draining lymph nodes and lymph nodes receiving antigen from subcutaneous vaccinations with irradiated tumor cells or peptide-pulsed DCs both contain T cells that have been sensitized to tumor antigens. Although preparations of these T cells were unable to cure
tumor-bearing recipient mice after adoptive transfer, *ex vivo* activation with IL-2, anti-CD3, and superantigens such as staphylococcal enterotoxin was effective in eliciting anti-tumor effector function after adoptive transfer into mice with established brain tumors\textsuperscript{110,125-128}.

The combination of vaccination followed by adoptive immunotherapy with T cells from peripheral blood or tumor draining lymph nodes is in clinical development. Wood and colleagues have immunized patients with irradiated, autologous tumor cells and collected peripheral blood to activate and expand lymphocytes with anti-CD3 and IL-2 *ex vivo*\textsuperscript{115}. \(10^{10} - 10^{11}\) cells were reinfused back into patients resulting in regression of three of nine patients’ tumors.Remarkably, two patients were still alive four and five years later. Another trial using a similar protocol in combination with high dose chemotherapy for recurrent pediatric brain tumors resulted in tumor-specific immune responses in all patients and ongoing survival at two years\textsuperscript{129}. Similar trials by Sloan *et al.* saw 8/19 partial responses with activated T cells purified from blood after vaccination\textsuperscript{116} and Plautz *et al.* observed 4/12 partial responses using T cells from vaccine draining lymph nodes\textsuperscript{113}, but survival times were not increased in either study.

*Ex vivo* activation of T cells is an effective method to generate *in vivo* anti-tumor effector function, but to date, this approach has been generally insufficient to produce cures in patients. This may be due, in part, to the inclusion criteria of the clinical trials. All but the Plautz study treated patients that failed to respond to multiple previous forms of therapy. Patients also were not lymphodepleted prior to adoptive therapy, an important criterion for the success of adoptive therapy for metastatic melanoma (see below). Furthermore, no effort was made in these studies to isolate and expand TILs or highly reactive tumor-specific T cells.

According to the NIH clinical trials database, there are three active trials using adoptive T cell therapy for malignant glioma (www.clinicaltrials.gov, accessed 11/17/09). Two of these
trials employ T cells recognizing a viral antigen from cytomegalovirus (CMV). A high percentage of human gliomas have been reported to be infected with CMV\textsuperscript{130}, implying that viral epitopes could also serve as immunotherapeutic targets. It remains to be seen if highly reactive T cells recognizing any of the recently identified glioma-specific antigens (IL-13Rα2, gp100, MAGE-1, EGFRvIII, etc.) can be translated into clinical success.

Adoptive T cell therapy for brain tumors has a long history of sporadic responses, and lags behind more promising results for other solid peripheral tumors, particularly melanomas. Steven Rosenberg and colleagues at the National Cancer Institute were the first to show that adoptive transfer of TILs purified from resected melanomas, expanded \textit{in vitro}, and injected back into patients concurrently with high doses of IL-2 produced some responses in patients with metastatic melanomas refractory to other treatments\textsuperscript{131,132}. A major problem noted was the disappearance of adoptively transferred cells, presumably due to competition with endogenous T cells for cytokines required for survival (e.g., IL-7 and IL-15). Subsequent studies evaluated adoptive T cell therapy combined with lymphodepleting chemotherapy that allowed homeostatic repopulation with tumor-specific T cells, increased persistence of T cells in circulation, and depleted T\textsubscript{reg} cells. This protocol has resulted in 50\% objective clinical results in patients with metastatic melanoma, including responses to brain metastases, and some complete responses have apparently been achieved\textsuperscript{132,133}. Yee and colleagues have observed similar encouraging results after adoptive transfer of CD8\textsuperscript{+} T cells specific for two melanoma-associated antigens with systemic low dose IL-2, and reported 8/10 partial or stable responses in patients with metastatic disease\textsuperscript{134}. Most recently, a protocol that includes lymphodepletion with fractionated total body irradiation and non-myeloablative chemotherapy prior to T cell transfer with
concurrent IL-2 treatment has shown a 72% objective response rate and metastatic tumors are cleared from multiple tissues including brain\textsuperscript{135}.

Adoptive T cell therapy seems to be well-tolerated overall, with the major complications stemming from IL-2 toxicity, lymphodepletion-related viral and bacterial infections, and autoimmune reactivity to normal melanocytes. Recently, adoptive transfer of TCR-transduced, highly reactive T cells resulted in autoimmune complications involving the eye, inner ear, and skin producing hearing loss, anterior uveitis, and vitiligo\textsuperscript{107,132}. These patients required treatment with corticosteroids and most symptoms resolved. Further studies characterizing the ideal transduced T cell population for therapeutic use are underway\textsuperscript{136}.

T cell adoptive therapy is a promising approach for treatment of melanomas, gliomas and other cancers, but the current challenge remains to translate sporadic responses into frequent and widespread responses. Isolation and clonal expansion of tumor-specific T cells are time-intensive and complex processes. Patients may not have CTLs with sufficient avidity for tumor antigens, or their tumors may lack immunogenic antigens to generate CTLs. This can be overcome by searching for T cell clones from other individuals that demonstrate tumor reactivity, cloning the TCR genes, and expressing them in T cells isolated from a patient. Another approach involves generating CTL clones through immunization of transgenic mice expressing human HLA (MHC I) molecules with human tumor antigens or tumor cells. Mouse TCR genes can be isolated from tumor-reactive T cells and transduced into human T cells for adoptive therapy\textsuperscript{107}. Fortunately, TCR-transduced T cells do seem to persist \textit{in vivo} and retain both proliferative and cytotoxic abilities\textsuperscript{106,137}.

To date, reliable transduction of autologous T cells using viral vectors encoding tumor-specific TCRs has been accomplished in only a few laboratories. The use of retroviral and
lentiviral vectors to transduce genes for the alpha and beta chains of tumor antigen-specific TCRs must proceed cautiously to prevent chain mispairing with endogenous TCRs and potential autoimmunity. Chimeric antigen receptors (CARs) that fuse the antigen-binding regions of a monoclonal antibody with the CD3 signaling domain of the TCR may circumvent this problem. CARs targeting IL-13Rα, which is overexpressed on greater than 80% of human gliomas but absent from normal CNS tissue, have been used successfully to treat xenograft models of glioma\textsuperscript{138} and demonstrated antitumor effects in one case study of a GBM patient\textsuperscript{105}.

Clinical use of adoptive T cell therapy for brain tumors still faces several hurdles including sufficient trafficking to the tumor, persistence of transferred cells, overcoming T\textsubscript{reg}-mediated suppression, and heterogeneous tumor antigen expression. Activated T cells must also be able to resist tumor-suppressive mechanisms (TGFβ, IL-10, IDO, FasL, PD-L1, etc.). Adjunct therapies may be necessary to inhibit these mechanisms. For example, humanized anti-PD-1 antibodies are currently being evaluated in a Phase I clinical trial\textsuperscript{139}. CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{reg} cells may also need to be actively excluded from T cell preparations to promote tumor rejection.

Clearance of heterogeneous tumors like gliomas will likely require T cells specific for several tumor antigens to prevent the outgrowth of antigen loss variant cancer cells. CD4\textsuperscript{+} T cells that recognize MHC class II bound antigens on tumor stromal cells could expand the number of potential tumor targets significantly. The importance of tumor-specific CD4\textsuperscript{+} T helper cells to promote tumor clearance is gaining appreciation\textsuperscript{48,140-142}. CD4\textsuperscript{+} T cells may act as cytotoxic effector cells themselves\textsuperscript{48,143} or as support cells to CD8\textsuperscript{+} T cells via production of IFN\textgamma and APC activation.

In cases of a strong antitumor response, T cells can ‘edit’ the tumor by selectively killing cells that express antigen while ignoring ALVs\textsuperscript{134}. This is likely to become a major obstacle as
more T cell clones with high avidity for tumor-associated antigens are used for adoptive transfer studies in patients. Preclinical studies have characterized some factors that improve the anti-tumor effects of adoptive therapy and prevent tumor recurrence from ALVs. For example, work from our laboratory suggests that increasing cross-presentation of tumor antigen on tumor stromal cells improves the effectiveness of adoptive T cell therapy by eliminating the stromal support cells for ALVs (39 and Chapter 2). T cell-based therapies in combination with other strategies, including the approach of combining T cell therapy with oncolytic virotherapy described in Chapter 3, may also overcome these challenges.

**Oncolytic Virotherapies for Brain Tumors**

The potential use of viruses to treat cancer was recognized over 100 years ago when cancer regression was observed in patients that developed viral diseases or were inoculated with viral vaccines. Reports of clinical use of oncolytic viruses began in the 1950s, with some sporadic tumor regressions observed in patients injected with Hepatitis B virus, West Nile virus, and adenovirus (reviewed in 144 and 145). These observations, combined with the development of *in vitro* virus propagation methods and advances in molecular biology for characterizing viral genetics and pathogenesis, have led to the present-day investigation of dozens of viruses for their oncolytic potential.

Oncolytic viruses are replication-competent viruses that selectively infect and kill malignant cells by exploiting genetic mutations in cancer cells that permit viral entry and/or viral replication146,147. Productive replication leads to amplification of viral titer within a tumor and virus can spread between neighboring tumor cells. Preclinical models and clinical trials have evaluated many families of viruses including adenoviruses, herpesviruses, poxviruses,
parvoviruses, reoviruses, orthomyxoviruses, paramyxoviruses, rhabdoviruses, picornaviruses and others. For treatment of gliomas, four viruses have completed testing in Phase I clinical trials: two herpes simplex virus (HSV) mutants\textsuperscript{148,149}, a mutant adenovirus (ONYX-015)\textsuperscript{150}, a reovirus (REOLYSIN)\textsuperscript{151}, and two strains of Newcastle disease virus\textsuperscript{152,153}. Some of these viruses are normally pathogenic in humans and have been attenuated with various mutations to limit the infectivity of normal cells. All were shown to be safe in patients with occasional objective responses (some are summarized below). Clinical trials using vaccinia virus, poliovirus, and measles virus for treating malignant gliomas are also ongoing.

Martuza and colleagues developed the HSV virus mutant G207 with mutations in both copies of a neurovirulence gene that conditionally replicates in dividing cells but not in normal brain cells\textsuperscript{154,155}. G207 is also touted as safe because of its susceptibility to ganciclovir and acyclovir (anti-viral drugs) to treat HSV encephalitis, if it were to develop. Preclinical studies demonstrated the efficacy of G207 against intracranial human xenograft tumors in a mouse model\textsuperscript{155}. G207 was also well-tolerated by non-human primates\textsuperscript{156}. A phase I clinical trial published in 2000 showed patient tolerance of up to $3 \times 10^9$ pfu (plaque forming units) injected intratumorally into gliomas with no dose-limiting toxicity, and there was some evidence of anti-tumor activity and long term presence of viral DNA\textsuperscript{148}. One patient remains alive almost six years after treatment. A follow-up trial showed that multiple doses of virus can be safely delivered directly to brain tumor cavities via a catheter\textsuperscript{157}. A second similar HSV mutant, 1716, showed no rate-limiting toxicity in nine patients in a phase I clinical trial and tumor biopsies recovered virus at higher titers than the injected dose\textsuperscript{149}. A phase II trial showed responses in two of 12 patients\textsuperscript{158}. Phase III clinical trials are underway in Europe.
A conditionally replicative adenovirus, ONYX-015, was one of the first viruses to show good clinical success and was approved in China for the treatment of head and neck cancer\textsuperscript{150}. Disappointingly, ONYX-015 does not seem to be as effective for gliomas. It was originally thought that ONYX-015 could not replicate in cells with functional p53 tumor suppressor molecules, but tumor cells with non-functional p53 would allow viral replication and lysis\textsuperscript{159}. However, a subsequent study showed that the anti-tumor efficacy of ONYX-015 in a human xenograft glioma mouse model was independent of p53 status\textsuperscript{160}. Despite this preclinical study showing efficacy, a phase I clinical trial of intratumoral injection in gliomas showed no significant responses in 24 patients and 96\% of patients had disease progression. However, none of the patients had an adverse response to the virus and three patients that received the highest titers were alive at 19 months follow-up\textsuperscript{150}. The case of ONYX-015 illustrates that human xenograft brain tumors growing in immune-compromised mice do not necessarily predict the success of virotherapies in patients, nor does the success of an oncolytic virus for treating one type of cancer necessarily transfer to other types of cancer.

With dozens of candidate viruses being studied, it remains to be seen which will be most successful for treating malignant brain tumors. The most important feature of these viruses (other than efficacy) must be safety. Some candidate oncolytic viruses cause disease in non-human hosts, but have the benefit of being nonpathogenic in humans. Viruses such as Newcastle disease virus (NDV), vesicular stomatitis virus, and myxoma virus can infect and kill human cancer cells by exploiting their altered signaling pathways, and may be potentially safer and more potent than human viruses because attenuation is unnecessary. A Phase I /II clinical trial of patients with GBM evaluated the oncolytic effects of NDV, an avian virus, delivered intravenously. Three of 14 patients survived 15 months after treatment, but no long term
survival was attained\textsuperscript{153}. However, another study describes four patients that have survived long-term (five to nine years) following treatment with a different NDV strain\textsuperscript{152}.

**Poxviruses and Virotherapy**

Poxviruses are candidates for both oncolytic virotherapy and gene therapy due to their large, double-stranded DNA genomes that allow the insertion of eukaryotic genes\textsuperscript{161,162}. Gene delivery by poxviruses is considered safe because they replicate in cytoplasm and do not integrate their DNA into the host cell genome. Poxviruses can replicate in about 24 hours, with early gene expression starting within two to four hours after cell entry\textsuperscript{163}, making them ideally suited to deliver genes for immunomodulators or tumor antigens that get expressed before the infected cell dies. The short replication time also leads to rapid spread of virus *in vivo*.

Vaccinia, the most well-known member of the large poxvirus family, was most famously used in humans as a live vaccine to eradicate smallpox. Vaccinia virus infects multiple species including humans, but replicates more efficiently in rapidly dividing cells such as those found in tumors\textsuperscript{164}. Leaky tumor vasculature is important for the accumulation of systemically delivered, relatively large (~200nm) virus particles. In animal models, vaccinia accumulates in multiple tumor types after systemic delivery, with low yield in most other tissues. However, vaccinia does collect and replicate in highly vascularized tissues such as the ovaries, producing concern regarding its use in patients\textsuperscript{165,166}. Attenuated vaccinia viruses, such as thymidine kinase knockouts, have reduced ability to replicate in normal tissues\textsuperscript{167}. A recombinant vaccinia virus expressing p53, IL-2 and IL-12 was shown to inhibit rat C6 glioma growth *in vivo*, although some cytokine toxicity was observed\textsuperscript{168}. Recombinant vaccinia virus encoding GM-CSF has
been evaluated for the treatment of cutaneous and metastatic melanoma, with some clinical responses\textsuperscript{169,170}.

Other poxviruses considered for cancer therapy with tropism for species other than humans include yaba-like disease virus (primate), canarypox virus, fowlpox virus, and myxoma virus (rabbit). Myxoma virus has demonstrated oncolytic effects \textit{in vitro} in many permissive human cancer cell lines including melanoma and glioma, making myxoma virus an attractive candidate for treatment of brain tumors \textit{in vivo}\textsuperscript{171,172}. In contrast to vaccinia, humans lack pre-existing immunity against myxoma virus that could eliminate the virus before carrying out its oncolytic and gene delivery functions.

\textit{Myxoma Virus}

Myxoma virus is a rabbit poxvirus with a strict species tropism for European rabbits and a small number of other lagomorphs. Myxoma virus infection results in a fatal disease called myxomatosis for European rabbits, but is abortive in other species\textsuperscript{164,173-175}. The stringent host specificity of myxoma virus was the basis of efforts to eradicate feral rabbits in Australia in the 1950s and resulted in 99\% reduction in the rabbit population with no evidence of infection in other native species or in humans\textsuperscript{176}. In fact, historical data indicate that humans living in very close contact with infected rabbits and mosquitoes, the natural vectors of the virus, do not show evidence of infection or produce antibodies against myxoma virus\textsuperscript{177}. Virally encoded rabbit-specific immunomodulators enhance the ability of myxoma virus to replicate in rabbit cells, but its tropism for cancer cells seems to be related instead to defective antiviral defenses and signaling pathways in transformed cells (discussed below).
In the last five years, myxoma virus has shown oncolytic effects on many human and mouse cancer cell lines. Sypula and colleagues tested the oncolytic effects of myxoma virus on a National Cancer Institute collection of human cancer lines derived from various tissues and found that myxoma virus could kill 15/21 lines including melanoma\textsuperscript{171}. Subsequent studies have shown human glioma, pancreatic adenocarcinoma, medulloblastoma and rhabdoid tumor cells are permissive to infection and killing\textsuperscript{172,178-180}. Most recently it was discovered that myxoma virus can selectively target human leukemic stem cells while sparing normal stem cells, an exciting strategy that may allow purging of leukemic progenitors from autologous blood and marrow \textit{ex vivo} before grafting back into patients with acute myelogenous leukemia that were lymphodepleted with chemotherapy\textsuperscript{181}.

\textit{Mechanisms of Tumor-Selective Infection by Oncolytic Viruses}

Oncolytic viruses utilize two main mechanisms that determine tropism for tumor cells. First, most viruses enter cells by binding to specific cell surface viral receptors. Several of these proteins are overexpressed on many cancer cell types. For example, intracellular adhesion molecule (ICAM) and decay accelerating factor (DAF) serve as viral receptors for Coxsackievirus A21, and ICAM/DAF expression levels on melanoma cells correlate with infection and oncolysis by this virus\textsuperscript{182}.

In addition to receptor overexpression, tumor selectivity by oncolytic viruses is strongly related to aberrant signaling pathways found in cancer cells. For example, endogenous Type I interferon (IFN\textalpha{} and IFN\textbeta{}) anti-viral and anti-proliferative signaling pathways are disrupted in many cancers\textsuperscript{183,184}. Several oncolytic viruses have now been shown to exploit this defect and productively replicate in malignant cells\textsuperscript{185-187}, but not in normal cells of the same tissue, or in
permissible tumor cells treated with IFN-α\textsuperscript{185}. Other antiviral pathways are also mutated in cancer cells. dsRNA viruses such as reovirus can infect cells with a common cancer mutation involving Ras that lack an enzyme that suppresses translation of viral proteins\textsuperscript{188}.

In contrast to some oncolytic viruses, binding and entry by poxviruses into mammalian cells is generally not restricted at the cell surface level. Until recently, the mechanism of cellular entry by the well-characterized vaccinia virus was still unknown. Mercer et al. determined that vaccinia induces cellular uptake via endocytosis triggered by exposed phosphatidylserine residues on the viral membrane, mimicking absorption of apoptotic cells\textsuperscript{189}. The uptake mechanism of myxoma virus is still unknown, but the specificity of both vaccinia virus and myxoma virus for cancer cells is probably not at the level of cell entry. Instead, normal cells can abort the replication cycle of the virus, while signaling defects in cancer cells permit productive viral replication. These defects have been linked to impaired Type I interferon responses in mouse cancer cells\textsuperscript{186,190}, and dysregulated activation of Akt kinase in human cancers\textsuperscript{191,192}.

Cell culture experiments with mutant myxoma viruses lacking different host range genes revealed that one gene product significantly affects whether human cancer cells are permissive to infection\textsuperscript{171,191}. M-T5 protein is necessary for viral replication in CD4\textsuperscript{+} rabbit T cells and the development of myxomatosis in rabbits. M-T5 also forms complexes with two human intracellular proteins: Cullin-1 and Akt-1\textsuperscript{193}. The interaction of M-T5 with Cullin-1, an E3 ubiquitin ligase involved in cell cycling, prevents cell cycle arrest and apoptosis to allow viral replication. Upon binding Akt, M-T5 activates Akt, an important serine/threonine kinase involved in survival, proliferation and cell death. Permissiveness of myxoma infection is directly dependent on activation (phosphorylation) of Akt. Many human cancers exhibit hyperactivated Akt, and viral M-T5 interactions with Akt allows some non-permissive cancer
cells with lower levels of Akt phosphorylation to become permissive to myxoma infection\textsuperscript{192}. The molecular mechanism of Akt/MT-5 interaction is under active investigation\textsuperscript{164,194}.

McFadden and colleagues have also demonstrated that rapamycin, a potent inhibitor of mTOR signaling and a cancer chemotherapeutic, also increases the permissiveness of some cancer cells \textit{in vitro}\textsuperscript{179,195}. Using siRNA knockdown of signaling proteins, Stanford \textit{et al.} have shown that rapamycin binding to its target mTORC1 leads to increased replication of myxoma virus that correlates with Akt activation\textsuperscript{196}. Treatment of semi-permissible, but not fully permissive or non-permissible, human cancer cells with rapamycin increases viral replication and spread \textit{in vitro}\textsuperscript{195}. However, treatment of non-cancerous cells (e.g., human fibroblasts) with rapamycin does not lead to infection\textsuperscript{164}. Rapamycin may become an important enhancer of oncolytic virotherapy \textit{in vivo}\textsuperscript{179,197-200}.

\textbf{In Vivo Tumor Treatment with Myxoma Virus}

Myxoma virus was initially tested \textit{in vivo} using human glioblastoma cell line xenografts after it was shown to infect and kill several human glioma cell lines \textit{in vitro}\textsuperscript{172}. Myxoma virus demonstrated extremely potent antitumor activity in orthotopic mouse brain tumor models, as well as fresh human glioma explants. Intratumoral injection of myxoma virus into established U87 or U251 human gliomas growing in nude mice (lacking T cells) resulted in the selective and persistent infection of tumors that progressively shrank and eventually cured 92\% of mice\textsuperscript{172}. Injection of live virus into the brains of nude mice was safe and remained tumor-restricted, peaking in titer at two weeks in nude mice. Histologic examination revealed minimal inflammation but the authors noted that some lymphocytes and microglia/macrophages were present\textsuperscript{172}. 
These provocative findings were also extended to a murine model of metastatic melanoma using immune-competent mice. B16-F10 melanoma cells are highly permissive for myxoma virus infection and replication, and multiple intravenous injections of virus demonstrated therapeutic effects against B16-F10 lung metastases\textsuperscript{200}. Concurrent treatment with rapamycin reduced the size and number of metastases, and importantly, reduced the antiviral neutralizing antibody titers.

Myxoma virus oncolysis of human and mouse glioma and melanoma cancer cells holds promise as an effective virotherapy for malignant brain tumors. However, the immunogenicity of myxoma virus in the brains of immunocompetent mice or humans has not been characterized and may limit efficacy. However, combined with other conventional therapies or immunotherapy, myxoma virus may expand the available treatment options for malignant brain tumors.

\textbf{Statement of Problem and Research Significance}

Adoptive immunotherapy and oncolytic virotherapy are two strategies that have provided encouraging but infrequent successful outcomes for patients with malignant brain tumors. Current standard-of-care treatments require the combination of surgery, chemotherapy, radiotherapy, and temozolomide to extend patient survival approximately one year. Single modes of therapy are not effective. The complex pathology of brain tumors (and cancer in general) will likely continue to require multimodal treatments incorporating new strategies that target minimal residual disease. Both adoptive T cell therapy and oncolytic virotherapy complement standard treatments and have significant potential to improve the overall outcome.
Promising results from adoptive therapy clinical trials for metastatic melanoma provide momentum to further develop this strategy for treatment of brain tumors. Careful attention to the isolation and adoptive transfer of an appropriate subset of glioma antigen-specific T cells and lymphodepletion prior to transfer are likely to improve the effectiveness of adoptive therapy for GBM patients. Current challenges in the field relate to the identification of this subset and the factors that influence the success or failure of these cells to eliminate tumors in the brain. These issues can be readily examined in murine brain tumor models. For example, factors that prevent or allow selective immune editing of a brain tumor resulting in antigen loss variant cells need to be further characterized. These issues can be evaluated in tumor-bearing mice using tumor-specific T cells in our model systems.

A large body of work by Hans Schreiber and colleagues using peripheral tumor models indicates that tumor stromal cells play a critical role in T cell-mediated tumor rejection, including the elimination of ALV cancer cells\textsuperscript{201-205}. Tumor stroma includes the endothelial cells, fibroblasts, macrophages/microglia, and extracellular matrix proteins that support growth and tissue invasion by a tumor. Subcutaneous tumors in mice that express high levels of tumor antigen are rejected by antigen-specific T cells while the same T cells fail to eliminate low-expressing tumors due to ALV cells\textsuperscript{204}. The elimination of ALV cells from high antigen-expressing tumors was found to depend on the ability of CD11b\textsuperscript{+} macrophage stromal cells to cross-present antigen to T cells. T cells directly eliminated both cancer cells and cross-presenting stromal cells, indirectly preventing ALV cells from growing out, and resulted in complete cures in mice. Furthermore, low antigen-expressing tumors could be eliminated following treatment with chemotherapy or local irradiation. These treatments were found to induce loading of antigen onto stromal cells, and produced tumor rejection similar to that of
high-expressing tumors\textsuperscript{205}. Whether similar mechanisms relate to T cell rejection of tumors growing in the CNS with a different population of stromal cells were unknown. These questions were the basis of studies outlined in Chapter 2.

The second major focus of this research was to evaluate the combination of adoptive T cell therapy and oncolytic virotherapy with myxoma virus as a potential synergistic approach for treating malignant brain tumors. Myxoma virus is highly effective at eliminating orthotopic human glioma cells growing in immunocompromised nude mice\textsuperscript{172}. To our knowledge, there has been no characterization of the oncolytic effects of myxoma virus in immunocompetent, syngeneic mouse brain tumor models that may respond quite differently to viral challenge. This is an important issue considering that many therapies appear to be very efficient in xenograft tumor models but do not reliably translate to clinical use.

We evaluated the oncolytic effects of myxoma virus \textit{in vitro} and \textit{in vivo} using a syngeneic mouse melanoma brain tumor model. We also characterized the immune response to viral infection of murine brain tumors and evaluated a combination therapy of T cell adoptive transfer, injection of myxoma virus, and cytokine neutralization. Our results in the syngeneic brain tumor model using the same initial inoculum of myxoma virus differ significantly from the potent killing observed in xenograft models, and suggest that syngeneic tumor models may more accurately reflect the local environment that oncolytic viruses encounter in immune-competent patients.

\textbf{2C T Cell System and Brain Tumor Models}

\textit{2C T Cell System}
To study cytotoxic T cell responses to brain tumors in vivo, our laboratory works with a well-characterized CD8^+ T cell clone called 2C that was originally isolated 25 years ago\textsuperscript{206}. The 2C T cell receptor (TCR) recognizes and binds several defined peptide antigens bound to either allogeneic H-2L\textsuperscript{d} or syngeneic H-2K\textsuperscript{b} class I MHC molecules (Figure 1.1). There are many useful reagents related to this defined system. 1B2, a monoclonal antibody against the 2C TCR, can be used to identify 2C T cells by flow cytometry or immunohistochemistry. Biotinylated soluble single-chain high affinity mutant TCR monomers called 2C-m67 (Figure 1.2) can be used to detect the model peptide antigen SIYRYYGL bound to H-2K\textsuperscript{b} at very low concentrations with a K\textsubscript{D} of approximately 16 nM\textsuperscript{207}. 2C TCR transgenic mice on the C57BL/6 background and 2C TCR mice backcrossed with recombinase-activating gene-1 knockout (RAG1\textsuperscript{--/--}) mice that lack B and T cells express the 2C TCR on approximately 80% or 100% of their CD8^+ T cells respectively. Lymphocytes from these mice can be collected and used for tumor-specific adoptive immunotherapy in recipient mice with brain tumors.

Brain Tumor Models

The 2C TCR binds to the K\textsuperscript{b}-bound peptide ligand SIYRYYGL (referred to as SIY) that acts as the model tumor antigen in this system with a K\textsubscript{D} of approximately 32 μM\textsuperscript{207} (Figure 1.1). We used several mouse cancer lines that stably express SIY/K\textsuperscript{b} complexes to establish tumors by intracranial infusion into syngeneic C57BL/6 or C57BL/6 RAG1\textsuperscript{--/--} mice. MC57 is a fibrosarcoma cell line that expresses either high (MC57-SIY-Hi) or low (MC57-SIY-Lo) levels of SIY/K\textsuperscript{b} expressed as a fusion protein with enhanced green fluorescent protein (eGFP)\textsuperscript{205}. PRO41 is a fibrosarcoma/spindle cell line that expresses high levels of SIY and eGFP, but expresses MHC class I H-2\textsuperscript{k}. SIY peptide does not bind K\textsuperscript{k}, so 2C T cells do not directly

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30
recognize PRO4L-SIY cells\textsuperscript{201}. However, 2C T cells may recognize SIY peptide derived from PRO4L-SIY tumors when it is cross-presented on K\textsuperscript{b} molecules by brain tumor APCs\textsuperscript{39}.

T cell adoptive therapy studies using mice with these different SIY-expressing brain tumors are outlined in Chapter 2. In an effort to develop immunotherapies using realistic models that encompass many features of malignant brain tumors, a murine melanoma cell line was used for the studies in Chapter 3. The B16.SIY melanoma brain tumor model was chosen because it accurately reflects the clinical situation in many ways. Both tumors are highly aggressive and anti-tumor immune responses are insufficient due in part to low surface levels of tumor antigen and class I MHC expression.

The parental B16-F10 melanoma cell line was originally derived from a spontaneously arising melanoma in a C57BL/6 mouse and thus has an MHC class I H-2\textsuperscript{b} haplotype\textsuperscript{208}. Later, B16.SIY cells were engineered to stably express SIY peptide bound to K\textsuperscript{b}, making them recognizable to 2C transgenic T cells. Retroviral transduction was used to introduce cDNA encoding SIY peptide fused in frame with enhance green fluorescent protein (GFP)\textsuperscript{209} similar to the MC57-SIY cell lines. Fluorescence activated cell sorting for GFP-positive cells selected cells that expressed high levels of the fusion protein. Some downward drift in GFP fluorescence over time has been observed, so highly positive cells have been re-selected several times.

B16.SIY melanoma cells are poorly immunogenic and express low to absent levels of class I MHC without IFN\(\gamma\) treatment\textsuperscript{209}. SIY/K\textsuperscript{b} complexes on B16.SIY cells are detectable by 2C-m67 TCR monomers following IFN\(\gamma\) treatment (unpublished data).

B16-F10 and B16.SIY tumors grow very aggressively. C57BL/6 mice infused in the brain with 1x10\textsuperscript{3} B16.SIY cells (Figure 1.3) have a median survival of only 16 days\textsuperscript{210}. \textit{In vitro} killing assays and adoptive transfer of 2C T cells into minor histocompatibility mismatched
129S6 mice with B16.SIY brain tumors revealed poor cytotoxic effector function by 2C T cells\textsuperscript{209,210}. However, IFN\(\gamma\) upregulation of class I MHC expression on the cancer cells could improve the 2C T cell killing response \textit{in vitro}. Much improved effector function (cytolysis) by 2C T cells against B16.SIY cells was observed upon blocking PD-1, a cell surface inhibitory receptor of T cell activation that interacts with PD-L1 on B16.SIY cells\textsuperscript{209}. This suggests that given proper activation (e.g., IFN\(\gamma\)) and removal of tumor suppression (e.g., PD-L1) on B16.SIY cells, 2C T cell responses to melanoma brain tumors could be augmented \textit{in vivo}. We are the first to evaluate this model using wild-type C57BL/6 mice instead of 129S6 mice that are able to reject B16.SIY as a subcutaneous tumor but not in the brain\textsuperscript{210}. 
References

1. CBTRUS. Table 23 (Central Brain Tumor Registry of the United States, 2008).


Chapter 1 Figures

Figure 1.1

T cell/cancer cell interaction. Transgenic CD8\(^+\) 2C T cells express the heterodimeric \(\alpha\) and \(\beta\) subunits of the 2C T cell receptor (TCR) that recognizes SIYRYYGL peptide bound to the class I major histocompatibility (MHC) molecule H-2K\(^b\) on the surface of mouse cancer cells.
Figure 1.2

A soluble, single-chain monomer comprised of the variable regions of the 2C TCR high affinity mutant m67. 2C-m67 recognizes SIY peptide bound to MHC class I H-2K\(^b\) on cancer cells or APCs with low nanomolar affinity. Monomers were chemically biotinylated at multiple sites via free amine groups.
Figure 1.3


D3  
D10  
D15
CHAPTER 2. RECURRENCE OF INTRACRANIAL TUMORS FOLLOWING ADOPTIVE T CELL THERAPY CAN BE PREVENTED BY DIRECT AND INDIRECT KILLING AIDED BY HIGH LEVELS OF TUMOR ANTIGEN CROSS-PRESENTED ON STROMAL CELLS

Abstract

Elimination of peripheral tumors by adoptively transferred tumor-specific T cells may require killing of cancer cells and tumor stromal cells. Tumor antigens are cross-presented on stromal cells resulting in direct cytotoxic T cell (CTL) killing of both antigen-expressing cancer cells and stromal cells. Indirect killing of antigen loss variant cells (ALVs) also occurs. We show here that similar processes occur in a brain tumor stromal environment. We employed murine cancer cell lines that express high or low levels of a peptide antigen, SIYRYYGL (SIY) recognized by transgenic 2C CD8+ T cells. The two cell lines were killed with equivalent efficiency by 2C T cells in vitro. Following adoptive transfer of 2C T cells into mice with established SIY-Hi or SIY-Lo brain tumors, tumors of both types regressed but SIY-Lo tumors recurred. SIY-Hi tumors contained CD11b+ cells cross-presenting SIY peptide and mice were cured following adoptive transfer. To further test the role of cross-presentation, RAG1−/− H-2b mice were infused with H-2k tumor cells expressing high levels of SIY peptide. Adoptively transferred 2C T cells are able to kill cross-presenting H-2b stromal cells but not H-2k tumor cells. In peripheral models this paradigm led to a small static tumor. In the brain, activated 2C T cells were able to kill cross-presenting CD11b+ cells and completely eliminate the H-2k tumors in

most mice. Targeting brain tumor stroma or increasing antigen shedding from tumor cells to enhance cross-presentation may improve the clinical success of T cell adoptive therapies.

**Introduction**

T cell-mediated immunotherapies for cancer currently in development include vaccination strategies and adoptive transfer of tumor-specific T cells (reviewed in 1-3). The results of clinical trials evaluating adoptive T cell therapies have been mixed, with particular types of cancer showing some beneficial response. Treatment of melanoma in particular shows promise 4-6, but antigen loss variant (ALV) cancer cells remain a challenge for antigen-specific T cell therapies 5,7.

Adoptive T cell therapies for brain tumors have also been investigated for more than 25 years in animal models 8-15 and in clinical trials 16-26. Early trials used activated T cell populations that were not specific for tumor antigens, but more recent preclinical studies have outlined approaches to improve the effectiveness of adoptively transferred antigen-specific T cells 27-37. Clinical trials evaluating autologous, *ex vivo* activated T cells from peripheral blood or tumor- or vaccine-draining lymph nodes have demonstrated partial responses against gliomas, even in patients that previously failed to respond to multiple forms of therapy 20,21,26,38.

Tumors outside the central nervous system are easily accessed by primed CTLs, but even in cases of strong anti-tumor responses, it is clear that new approaches for targeting ALV cancer cells that lead to tumor recurrence are needed to improve the success of T cell-based therapies 5,7,39,40. Similarly, T cell responses can also be induced in the brain following peptide vaccination targeting EGFRvIII on malignant gliomas, but EGFRvIII negative tumors recur in patients 41.
Successful clearance of solid peripheral tumors and prevention of relapse from ALV outgrowth depend on elimination of malignant cancer cells as well as bone-marrow and non-bone-marrow-derived stromal cells that support the growing tumor \(^{42-47}\). Adoptively transferred CTLs can target both cancer cells and stromal cells that cross-present tumor peptides shed by cancer cells or internalized via phagocytosis. ALV cancer cells sheltered within the tumor stroma can be eliminated via a bystander effect by killing cross-presenting CD11b\(^+\) stromal cells that are sensitized with sufficient tumor antigen released by dying cancer cells, irradiation, or chemotherapy \(^{46}\).

The importance of targeting stroma has previously been established for a variety of peripheral tumors \(^{42-47}\), but brain tumor stroma differs from other tissues in several ways. For example, gliomas do not induce lymphangiogenesis \(^{48}\) and microglia associated with gliomas are particularly active at secreting TGF\(\beta\) that contributes to invasiveness \(^{49}\). Gliomas are also comprised of different stromal elements than peripheral tumors. Fibroblasts contribute to T cell-mediated peripheral tumor rejection \(^{44}\). However, fibroblasts are absent from gliomas and orthotopic murine brain tumors \(^{50}\).

Differences in brain and peripheral immune responses to tumors, and in the stroma of brain and peripheral tissues, led us to investigate whether cross-presentation of brain tumor antigens by stroma is also important for brain tumor rejection by adoptively transferred CTLs. Mice were intracranially infused with cancer cells expressing high or low levels of a defined model peptide antigen, SIYRYYGL (SIY). High- and low-antigen expressing cell lines have been shown to have differences in ALV outgrowth in subcutaneous models \(^{46}\). Tumors were allowed to establish for several days before treatment to allow tumor stromal cells to organize. Mice received an adoptive transfer of 2C T cells that recognize SIY peptide presented on MHC I
haplotype K^b and T cell responses to both tumors were characterized. Cross-presentation of
tumor antigens by CD11b^+ brain tumor stromal cells was directly assessed using high affinity
single-chain T cell receptor (TCR) monomers that bind tumor peptide/MHC complexes. We also
observed anti-tumor effects of adoptively transferred 2C T cells when stromal cross-presentation
was the only mechanism of peptide/MHC presentation in the tumors.

Methods

Mice, Cell Lines, and Reagents

C57BL/6 RAG1^-/- mice from The Jackson Laboratory are maintained as a colony at the
University of Illinois. 2C TCR transgenic mice are maintained as a heterozygous colony by
crossing with C57BL/6 mice and screened for expression of the 2C TCR on peripheral blood
leukocytes (PBLs) by flow analysis with 1B2 clonotypic antibody. Mice were 2–6 months of
age at the time of experiments. All procedures were approved by the Institutional Animal Care
and Use Committee at the University of Illinois at Urbana-Champaign.

MC57-SIY-Lo and MC57-SIY-Hi cells (derived from C57BL/6 mouse fibrosarcoma
MC57), and PRO4L-SIY and PRO4L-gp33 cells (derived from C3H/HeN mouse
undifferentiated spindle cell cancer 1591-PRO4L) were generated at the University of Chicago
and have been described previously^{45,51,52}. Cancer cells were grown in complete RPMI 1640
medium containing 5 mM HEPES, 10% FCS, 1.3 mM L-glutamine, 50 pM 2-ME, penicillin, and
streptomycin.

Anti-K^b and isotype control antibodies were purchased from eBiosciences and anti-CD31
(PECAM-1) from Pharmingen. 1B2 monoclonal antibody specific for the 2C TCR was purified
from the hybridoma and biotinylated in our laboratory. Expression and purification of soluble,
single-chain 2C-m67 TCR monomers that bind SIY/K\(^b\) with low nanomolar affinity were described previously \(^{46}\). Monomers were chemically biotinylated at multiple sites via free amine groups using EZ-Link NHS-PEO\(_4\) Biotinylation Kit (Pierce). Streptavidin-allophycocyanin (SA-APC, Invitrogen) was used as a fluorescently labeled secondary reagent. For flow cytometry experiments, cancer cells were stained and washed twice with PBS/BSA before analysis on a BD FACS Canto or BD LSR II.

**In Vitro Cytotoxicity Assay**

2C T cells from spleens of 2C TCR transgenic mice were prepared by mechanical dissociation and ammonium chloride buffer lysis of erythrocytes. Splenocytes were incubated for 48 hours with 3 \(\mu\)M SIY peptide (SIYRYYGL) and 10 ng/mL murine rIL-2 (R&D Systems) to activate effector 2C T cells. Target cancer cells were incubated with 100 \(\mu\)L of 2 mCi/mL \(^{51}\)Cr for two hours at 37°C and washed three times before incubating with 2C T cells at different effector-to-target ratios for four hours. Chromium release was measured using a Beckman gamma counter and specific release was calculated using the standard formula: \(((\text{sample counts}-\text{spontaneous counts})/(\text{maximum counts}-\text{spontaneous counts}))\times100.\)

**Cancer Cell Infusion and T Cell Adoptive Transfer**

Cancer cells were trypsinized and collected, washed twice with Hanks Balanced Salt Solution, and stereotaxically infused into the brain. Each mouse received an infusion of 5 \(\times\) 10\(^4\) cells in 300 nL in ventral striatum (AP 0.5mm, lat 2.5mm, DV -4mm). In all experiments unless specified otherwise, a small tumor challenge of approximately 1-2 \(\times\) 10\(^5\) cells was prepared the
same way and injected subcutaneously into the flank to ensure peripheral afferent immune
responses leading to activation of adoptively transferred 2C T cells.

2C T cells were adoptively transferred by preparing a single cell suspension of lymph
node and spleen cells from 2C TCR transgenic mice, lysing erythrocytes with ammonium
chloride buffer, and washing twice with HBSS. Mice were injected with 5 x 10⁷ naive 2C
lymphocytes into the tail vein one, five, six or ten days after tumor implantation.

**Histological Analysis of T cell Response**

Mice were sacrificed at one, three, or five days following 2C T cell transfer or followed
for survival. Criteria for euthanasia were 75% of baseline body weight or signs of neurological
impairment, in accordance with IACUC guidelines. To identify 2C T cells, unfixed frozen 10
micron tissue sections were stained with 1B2 antibody amplified with HRP/tyramide-biotin
(Perkin Elmer) and streptavidin-Alexa 594 (Invitrogen), and counter-stained with DAPI
(Invitrogen). Other sections were stained with anti-CD31 (PECAM), followed by biotinylated
rabbit anti-rat IgG (Vector) and streptavidin-Alexa 594 to detect endothelial cells. Tumor
volume was systematically reconstructed by measuring tumor area on hematoxylin stained
sections taken at regular intervals through the brain.

**Analysis of Cross-Presentation by CD11b⁺ Stromal Cells**

CD11b⁺ cells were purified from MC57, MC57-SIY-Lo, MC57-SIY-Hi brain tumors
(established for 10 days before adoptive transfer of 2C T cells) and PRO4L-SIY brain tumors
(established for seven days before adoptive transfer of 2C T cells) two or three days following
2C T cell adoptive transfer using anti-CD11b-labeled magnetic beads (Miltenyi). Brains tumors
were dissected, minced, and incubated with Dispase (Gibco, 5U/mL) at 33°C for 30 min in Hibernate A media (BrainBits). Cells were then washed with RPMI media containing 10% FCS to inactivate Dispase and pushed through 100 μm and 40 μm filters sequentially. Erythrocytes were lysed with ammonium chloride buffer and cells were washed with Hibernate A before following the manufacturer’s protocol for separation of CD11b+ cells using a magnetic column. Purified cells were then stained with anti-CD11b-Pacific Blue (Caltag Laboratories), 2C-m67 TCR detected with streptavidin-Alexa594, and/or propidium iodide (1 μg/mL, BD Pharmingen) and analyzed on a BD LSR II flow cytometer.

Statistical Analyses

GraphPad Prism software was used to analyze survival curves by Logrank test and area/volume data by unpaired T test. Significance was considered p < 0.05.

Results

Cancer cell lines expressing different surface levels of SIY peptide are killed equally well by 2C T cells in vitro

MHC I haplotype K\textsuperscript{b} expression on MC57 cancer cell lines was evaluated to rule out that differences in surface levels of MHC I result in differences in killing by CD8\textsuperscript{+} 2C T cells. All three MC57 lines (parental, SIY-Lo, SIY-Hi) express similar levels of K\textsuperscript{b} (Figure 2.1A). We also confirmed that PRO4L-SIY cancer cells (H-2\textsuperscript{k}) do not express K\textsuperscript{b}. Next we measured the relative surface levels of SIY peptide complexed with K\textsuperscript{b} on the cancer cells using biotinylated single-chain high-affinity 2C TCR monomers (2C-m67 TCR). 2C-m67 TCR binding was detected with fluorescent streptavidin-APC. The mean fluorescence intensity of MC57-SIY-Lo
cells was increased approximately 2.6-fold over untransduced MC57 parental cells. MC57-SIY-Hi cell levels were increased approximately 16-fold over MC57-SIY-Lo cells (Figure 2.1B). Enhanced GFP (eGFP) and SIY are expressed as a fusion protein in the construct used to generate these cell lines, therefore eGFP levels can also serve as an index of SIY antigen expression. The mean eGFP fluorescence of MC57-SIY-Lo and MC57-SIY-Hi cancer cells were 1.6 and 17-fold over untransduced parental MC57 respectively (Figure 2.1C). PRO4L-SIY cells express the highest level of eGFP, but SIY cannot be presented on H-2^k and thus is not recognized by the wild-type 2C TCR or single-chain high affinity 2C-m67 TCR monomers. eGFP expression serves as the only relative readout of SIY peptide present in PRO4L-SIY cancer cells.

Next we evaluated the cytotoxic effector function of 2C T cells against the MC57 cancer cell lines in vitro using a chromium release killing assay. Equivalent ^51^Cr release was measured from MC57-SIY-Hi and MC57-SIY-Lo cells incubated with preactivated 2C T cells at different effector to target cell ratios (Figure 2.1D). The parental MC57 line was poorly killed by 2C T cells, demonstrating specificity for the SIY antigen. In a separate assay, PRO4L-SIY showed low levels of chromium release similar to parental MC57 when incubated with activated 2C T cells, confirming the MHC restriction of the 2C TCR to K^b^ (data not shown).

**Adoptively transferred 2C CTLs initially cleared established brain tumors expressing high or low levels of antigen**

To establish an in vivo brain tumor model to assess the therapeutic effect of adoptively transferred tumor-specific 2C T cells, we infused MC57, MC57-SIY-Lo and MC57-SIY-Hi cancer cells into the brains of syngeneic C57BL/6 RAG1^-/-_ mice and waited several days for the
tumor and stroma to establish. Unactivated, naïve 2C T cells were adoptively transferred into the tumor-bearing mice on day 5. On the same day, a small number of the same cancer cells was injected subcutaneously to ensure a robust afferent immune response and activation of the transferred 2C CTLs.

One day following treatment, there were few 2C T cells infiltrating either SIY-Hi or SIY-Lo brain tumors (Figure 2.2A), and both tumors were well-established and growing (Figure 2.3A). Three days following transfer of 2C T cells, both MC57-SIY-Hi and MC57-SIY-Lo tumors were infiltrated by massive numbers of 2C T cells (Figure 2.2B). Five days following T cell transfer, both tumor types were almost completely eliminated based on histological examination (Figure 2.3B). 2C T cell accumulation was still observed at the sites of both SIY-Hi and SIY-Lo tumors (Figure 2.2C), but there was little evidence of residual tumor. In these brains, T cells were mostly restricted to the tumor hemisphere but not restricted to the tumor, raising some concern about autoimmunity. However, brain histopathology was unremarkable and long-term survivors showed no evidence of behavioral abnormalities at any point. Impressively, MC57-SIY-Hi tumors were almost completely eliminated within two days of the surge of T cell infiltration despite a faster growth rate than MC57-SIY-Lo (Figures 2.3C and 2.3D, note difference in Y-axis scale). This faster growth rate was also observed in cell culture (data not shown).

If the subcutaneous injection of cancer cells to activate the transferred 2C T cells was omitted, some 2C T cells infiltrated both SIY-Hi and SIY-Lo brain tumors by day 5, but the response was greatly diminished (Figure 2.2D). We also observed that 2C T cells activated by a subcutaneous injection of MC57-SIY-Hi cells were able to infiltrate parental (SIY-negative) MC57 tumors in the brain (Figure 2.2E), suggesting local inflammatory cues existed that
recruited the activated 2C T cells. However, the possibility that transgenic 2C T cells expressing an additional endogenous TCR that may have recognized other tumor antigens cannot be ruled out. Compared to MC57-SIY-Hi or SIY-Lo brain tumors, substantially fewer 2C T cells were retained in parental MC57 brain tumors at five days post-transfer (Figure 2.2E compared to Figure 2.2C) and the tumors grew out.

**In a unilateral brain tumor model, adoptively transferred 2C CTLs completely eliminated SIY-Hi brain tumors and cured mice while mice with SIY-Lo tumors usually relapsed**

Experiments following mice for survival outcome were done to determine if tumor-infiltrating 2C CTLs were able to completely cure mice with brain tumors expressing high or low levels of SIY peptide. Mice with five day-old unilateral MC57-SIY-Hi, MC57-SIY-Lo, or MC57 parental brain tumors received unactivated 2C T cells and a small subcutaneous injection of the same cancer cells to ensure activation of the transferred cells. Subcutaneous cancer cells did not grow out. 2C T cell treatment cured 100% of mice with MC57-SIY-Hi tumors and significantly extended survival of mice with MC57-SIY-Lo tumors (median survival 46 days vs. 17 days with no 2C transfer, Figure 2.4A). Mice with MC57-SIY-Lo brain tumors that reached criteria for euthanasia were confirmed to have recurring brain tumors post-mortem. Mice with either MC57-SIY-Hi or MC57-SIY-Lo brain tumors that did not receive 2C T cells survived similarly as mice with parental MC57 brain tumors treated with 2C T cells (median survival times of 16, 17, and 18 days, respectively, Figure 2.4A).

**In a bilateral brain tumor model, adoptively transferred 2C CTLs eliminated SIY-Hi tumors but failed to eliminate SIY-Lo tumors**
To evaluate whether ineffective priming or cytotoxic effector function of adoptively transferred 2C T cells were responsible for the observed relapses of mice with MC57-SIY-Lo brain tumors, additional survival experiments were done to determine if 2C CTLs could clear both SIY-Hi and SIY-Lo brain tumors present in the same mouse. MC57-SIY-Hi and MC57-SIY-Lo cells were infused bilaterally into the brains of mice. Mice received 2C T cell transfers and subcutaneous injections of both SIY-Hi and SIY-Lo cancer cells bilaterally to activate the CTLs five, six or ten days later to determine any effect of timing. In Figure 2.4B-D, each mouse that received 2C T cells is represented by two curves, showing survival time dependent on which brain tumor recurred. Brains of mice that reached criteria for euthanasia were histologically examined post-mortem to determine which tumor had recurred. When 2C T cell adoptive transfers were given on day 5, MC57-SIY-Hi tumors were eliminated in all mice, indicating that the 2C CTLs had been sufficiently activated and were able to kill (Figure 2.4B). However, two of five mice died from recurring MC57-SIY-Lo tumors even though MC57-SIY-Hi tumors were rejected in the same mice.

The timing of adoptive transfer with respect to tumor growth was critical. Control mice with bilateral tumors that did not receive 2C T cell treatment survived only 14 days (Figure 2.4B-D). Mice receiving 2C T cells on day 5 following cancer cell infusion included three long term survivors (out of five), and all mice rejected MC57-SIY-Hi tumors (Figure 2.4B). Mice receiving 2C T cells on day 6 did not survive, but death was significantly delayed by 2C T cell treatment (Figure 2.4C). In four of five cases, recurring MC57-SIY-Lo tumors were responsible for the relapse and MC57-SIY-Hi tumors were eliminated. If 2C T cell treatment was given on day 10 (i.e. only a few days before mice normally succumb to the tumors) both MC57-SIY-Hi and MC57-SIY-Lo tumors continued to grow and were rapidly fatal (Figure 2.4D). Presumably
CTL killing cannot keep up with the rapidly dividing cancer cells once the tumor reaches a critical mass. In this regard, it is important to note that by day 12 following cancer cell infusion, tumor masses occupied more than 50% of the brain hemisphere in some coronal planes assessed by MRI (data not shown).

*Adoptively transferred 2C T cells persist in vivo but no longer recognize MC57-SIY-Lo brain tumors that recur due to antigen loss*

To address the possibility that adoptively transferred 2C T cells did not persist in mice with MC57-SIY-Lo brain tumors that recurred, we examined tumors, draining cervical lymph nodes and spleen tissue collected from mice when they reached criteria for euthanasia. 1B2 antibody staining revealed substantial numbers of 2C T cells in recurring brain tumors and lymphoid tissue of all mice evaluated 34 or 35 days following adoptive transfer of 2C T cells (Figure 2.5, n=3).

To determine whether the recurring MC57-SIY-Lo brain tumors were SIY-antigen loss variant cells no longer recognized by 2C T cells, tumors were excised from mice (n=5) when they reached criteria for euthanasia following relapse (31 to 51 days following 2C T cell transfer). All mice had received 2C T cell transfers and subcutaneous cancer cell injections on day 5. Tumor explants were dissociated and then cultured for several days to enrich for live cancer cells. Explanted tumor cells were then stained with anti-K^b^ monoclonal antibody and analyzed by flow cytometry. All recurring tumors expressed normal levels of MHC I-K^b^ (Figure 2.6A). To detect surface levels of SIY peptide bound to K^b^, explanted cancer cells were also stained with biotinylated single-chain 2C-m67 TCR monomer (Figure 2.6B). Flow analysis revealed that levels of 2C-m67 TCR binding to explanted tumor cells were similar to SIY-
negative parental MC57 tumor cells, indicating that recurring MC57-SIY-Lo tumors developed from SIY-loss variants not recognized by 2C T cells.

**CD11b⁺ stromal cells in brain tumors expressing high levels of antigen cross-present tumor peptide on MHC I**

We hypothesized that one possible mechanism by which adoptively transferred 2C T cells can eliminate SIY-Hi but not SIY-Lo brain tumors may be related to stromal cell cross-presentation of available tumor antigen to CTLs in the tumors. To evaluate the role of CD11b⁺ stromal cells, MC57-SIY-Hi, SIY-Lo or parental cells were infused into the brains of mice. Ten days later, all mice received an adoptive transfer of naïve 2C T cells and a subcutaneous injection of MC57-SIY-Hi cells to activate the transferred cells. Two days following 2C T cell treatment, when T cells are extravasating into the brain tumors in large numbers and presumably interacting with stromal cells, mice were sacrificed and brains were excised. CD11b⁺ cells purified from dissociated brain tumors were incubated with biotinylated 2C-m67 TCR monomers. Flow cytometry analysis showed that CD11b⁺ cells purified from MC57-SIY-Hi brain tumors presented SIY peptide bound to Kᵇ on their surface (**Figure 2.7A, right panel**). However, no increase in surface levels of SIY/Kᵇ was detectable on CD11b⁺ cells purified from SIY-Lo brain tumors (**Figure 2.7A, middle panel**). CD11b⁺ cells from parental (SIY-negative) brain tumors served as a negative staining control (**Figure 2.7A, left panel**). Tumor peptide was therefore cross-presented by CD11b⁺ tumor stromal cells to 2C T cells infiltrating MC57-SIY-Hi brain tumors two days post-transfer, but not on CD11b⁺ cells from MC57-SIY-Lo brain tumors. **Figure 2.7B** shows 2C-m67 TCR staining of two independent CD11b⁺ cell isolations from MC57-SIY-Lo brain tumors which stain similarly to CD11b⁺ cells from MC57 parental tumors.
(12.7, 15.6, and 12.1 MFU respectively), while two independent CD11b+ cell isolations from MC57-SIY-Hi brain tumors express significantly higher levels of SIY/Kb on the cell surface (64.9 and 84.3 MFU). The percentage of dead CD11b+ stromal cells in SIY-Hi and SIY-Lo tumors two days following injection of 2C T cells were also compared and no differences were observed (data not shown). Whether bystander elimination of antigen loss variant cells in MC57-SIY-Hi tumors occurred as a result of increased 2C T cell killing of stromal cells cross-presenting SIY peptide could not be confirmed, perhaps because stromal cell killing occurs later than the point at which CD11b+ cells were collected for this experiment (two days after T cell transfer). 2C T cells only begin to appear in the brain between one to three days following adoptive transfer. However, direct assessment of the ability of brain tumor APCs to cross-present tumor-derived peptides demonstrated that cross-presentation can and does occur in the brain when sufficient tumor antigen is available.

Activated 2C CTLs can also suppress the growth of SIY-expressing tumors without direct cancer cell recognition, possibly by targeting CD11b+ stromal cells cross-presenting SIY peptide

To further examine whether stromal cell cross-presentation of tumor antigen enhances tumor elimination, we characterized the 2C T cell response to allogeneic H-2k PRO4L-SIY tumors in the brain. Despite the inability of 2C T cells to directly recognize SIY peptide on the cancer cell surface due to MHC mismatch, activated 2C T cells had potent anti-tumor effector function in these mice with high antigen-expressing tumors. PRO4L-SIY cells were infused into the brains of H-2b C57BL/6 RAG1-/- mice. Seven days later, naïve 2C T cells were adoptively transferred into the mice. Half of the mice were also received a small subcutaneous inoculation
of MC57-SIY-Hi cells to activate the T cells. In all cases, the subcutaneous MC57-SIY-Hi cells did not grow out in the mice. In the absence of the subcutaneous injection of MC57-SIY-Hi cells, no survival benefit was conferred by the adoptively transferred 2C T cells (Figure 2.8A). However, with the subcutaneous challenge of MC57-SIY-Hi cells to activate the adoptively transferred 2C cells, mice lived significantly longer than saline injected controls and 80% were cured, despite the lack of direct recognition of PRO4L-SIY cancer cells by 2C T cells (Figure 2.8B). The response was antigen specific, because mice that were injected bilaterally with PRO4L-SIY cells and PRO4L-gp33 cells, which express an irrelevant peptide, showed delayed growth of PRO4L-SIY tumors only five days after adoptive transfer (Figure 2.8C, D).

PRO4L-SIY and PRO4L-gp33 brain tumor tissue from untreated mice and mice that received 2C T cell adoptive transfers were immunostained using 4D11 antibody (anti-Ly-49G). Similar numbers of NK cells were observed in tumors of untreated mice and mice that received 2C T cells one to three days following T cell transfer, and the number of NK cells was proportional to the size of the tumor, suggesting that NK cells may also participate in the clearance of PRO4L-SIY brain tumors following 2C T cell transfer. However, the recruitment of NK cells to the brain was independent of the effect of T cell adoptive transfer (data not shown).

To determine if tumor peptides were cross-presented on CD11b+ stromal cells in this model of indirect T cell killing, we isolated CD11b+ cells from PRO4L-SIY brain tumors in mice that received either 2C T cell adoptive transfers or control lymphocytes from a C57BL/6 wild-type mouse three days earlier. Following incubation with biotinylated 2C-m67 TCR monomers, we were able to detect increased surface levels of SIY/Kb on CD11b+ cells from 2C T cell-treated mice compared to controls (Figure 2.9A). To address whether 2C T cells were targeting CD11b+ stromal cells cross-presenting SIY peptide and killing the tumor stroma directly (and
PRO4L-SIY cells indirectly), we assessed the viability of CD11b+ stromal cells purified from the tumors. Propidium iodide staining revealed proportionally higher percentages of dead CD11b+ cells in tumors pooled from mice that received 2C CTL adoptive transfers compared to C57 wildtype lymphocyte transferred controls (Figure 2.9B, 40% vs. 25% respectively, calculated by % PI+/CD11b+/all CD11b+).

Endothelial cells, another stromal cell type with the potential to cross-present tumor peptides, were analyzed by staining for expression of CD31/PECAM. The smaller tumors in 2C T cell-treated mice had substantially reduced staining (Figure 2.10), but no attempt was made to determine whether this was a cause or an effect of the smaller tumor size in the treated mice.

**Discussion**

Our finding of a differential effect of adoptive T cell therapy on brain tumors expressing high or low levels of the target antigen supports the idea that stroma plays a role in T cell-mediated rejection of brain tumors. In the present experiments, activated CD8+ T cells were able to kill cancer cells expressing high or low levels of antigen efficiently both in vitro and in vivo, yet brain tumors expressing low levels of antigen recurred as a result of antigen loss variant cancer cells.

In these studies, the tumor stroma was allowed to establish before initiation of treatment to best model the stromal environment T cells would encounter in an endogenously arising brain tumor. Stromal cells with macrophage/microglia surface markers represent approximately one-third of all cells in glioma biopsies 53,54 and between 5-35% of the total tumor burden in experimental rodent glioma models 55,56. We were able to purify and characterize a population of
CD11b⁺ macrophage/microglia stromal cells representing approximately 10% of total tumor burden in the present experiments.

From studies evaluating adoptive T cell therapy in peripheral tumor models, we know that cross-presentation of tumor antigens by stroma allows activated T cells to kill stromal cells. Using single-chain, high affinity TCR monomers, we have directly demonstrated that brain tumor CD11b⁺ stromal cells can cross-present tumor antigens at detectable levels in tumors expressing high levels of tumor antigen but not in tumors with low levels of antigen, and that these cross-presenting stromal cells are likely targeted and eliminated by antigen-specific, adoptively transferred T cells.

Stromal cell cytolysis may enhance the therapeutic effect conferred by adoptively transferred T cells in three ways. First, trophic support provided by stromal cells is eliminated. Stromal cells can enhance tumor growth by providing growth factors and stimulating angiogenesis. Destruction of tumor stroma results in a less hospitable environment for residual cancer cells including ALVs that escape direct T cell killing and continue to proliferate. Thus, cross-presentation of antigen by stromal cells could play an important role in preventing relapses. Second, elimination of stromal cells may remove a source of T cell suppression. Stromal cells can serve as a physical barrier for tumor-infiltrating T cells and have multiple mechanisms for suppressing CTLs. We did not examine the phenotype of the CD11b⁺ stromal cells in the present experiments, but in other tumor models we have found that cancer cells infused into the brain resulted in an influx of Gr1⁺ CD11b⁺ suppressor phenotype cells. Elimination of these myeloid derived suppressor cells removes one form of suppression on tumor-infiltrating CTLs. Third, brain tumor stroma may provide a niche that supports chemoresistant and radiation-
resistant cancer stem cells that express low or no MHC I or NK cell activating ligands on their surface. Targeting tumor stroma may be an ideal way to eliminate these cells.

In the present experiments, the extent to which tumors had progressed before T cell therapy was initiated determined whether a differential effect of T cells on the growth of high and low antigen-expressing tumors was observed. Following adoptive T cell treatment on day 5 after tumor cell infusion, both MC57-SIY-Hi and MC57-SIY-Lo tumors were established but treatable, and they initially regressed within 5 days. If mice had been monitored for only 35 days when all mice still appeared healthy, it would have seemed that all mice were long-term survivors. Instead, two mice relapsed from MC57-SIY-Lo tumors and were euthanized at 42 and 54 days. When the transfer of 2C T cells occurred on day 6 after tumor infusion, all of the MC57-SIY-Lo tumors recurred, with a median survival time of 65 days. However, the MC57-SIY-Hi tumors were completely cleared in nine of ten mice when the adoptive transfer occurred on day five or six after tumor infusion. Finally, if T cell adoptive therapy was delayed until day 10 following cancer cell infusion, it was too late for a therapeutic effect for either tumor, and mice died within the next four days with no prolongation of survival compared to untreated controls. Thus, at intermediate time points when tumors were well-established but treatable, the abundance of antigen available for cross-presentation may have prevented recurrences of the MC57-SIY-Hi tumors (9 of 10 long term survivors), and the scarcity of antigen made it more likely that recurrences would occur in the MC57-SIY-Lo tumors (only 3 of 10 long term survivors).

Two lines of evidence, histological evaluation and calculations of tumor growth patterns, suggest that T cells initially cleared nearly all of the cancer cells from the brain. Following T cell therapy at the most effective time point, there was no clear histological evidence of tumor on day
ten, five days after T cell transfer. Calculated estimations of tumor growth are consistent with
the idea that very few cancer cells remained at that time point. The median survival time for
untreated bilateral tumor mice was 14 days, and 17 days for unilateral tumors, in the absence of
T cell therapy. If there were only a single surviving cancer cell after the T cell treatment, with a
doubling time of 24 hrs it would take two weeks to re-establish the original tumor inoculation
and another two to three weeks to grow to a size of approximately one cm$^3$, the point of
producing neurological symptoms. In cases of recurring MC57-SIY-Lo tumors, we were able to
demonstrate that the cancer cells were antigen loss variants. It may be that the residual tumor
stroma nurtured a few antigen loss variant cells and allowed them to repopulate the tumor.

Our experiments with allogeneic PRO4L-SIY tumors suggest that activated T cells can
clear an established brain tumor by targeting the tumor stroma, without any direct recognition of
the cancer cells. PRO4L-SIY tumors cannot be directly killed by 2C T cells but do provide SIY
peptide for cross-presentation on stromal cells. Cross-presentation of brain tumor peptides to
CD4 T cells $^{59}$ and CD8 T cells $^{60}$ has been previously reported, inferred from experiments of
MHC mismatch between T cells and tumor cells. In the present experiments, we were able to
directly detect cross-presentation of tumor antigen on CD11b$^+$ stromal cells using single-chain,
high affinity TCRs, as well as demonstrate the apparent targeting and killing of these cross-
presenting stromal cells.

PRO4L-SIY brain tumor growth was controlled following an adoptive transfer of 2C T
cells, and the response was tumor specific because the growth of PRO4L-gp33 tumors was not
affected by 2C T cells. Indeed, activated 2C T cells were able to completely eliminate PRO4L-
SIY tumors from the brain. This is in contrast to peripheral tumor models where an equilibrium
was established between the transferred T cells and residual PRO4L-SIY cells $^{47}$ and tumors
grew out in mice that were subsequently depleted of CD8\(^+\) T cells. Plautz et al.\(^{59}\) observed a similar phenomenon with CD4 T cells adoptively transferred into mice bearing MCA tumors in the brain, which do not express MHC II, but were nevertheless eliminated by T cell treatment.

In addition to killing stromal cells, it is unknown whether other effector mechanisms are involved in 2C T cell rejection of PRO4L-SIY brain tumors. We did not examine the possibility that the degree of NK cell cytotoxicity may be changed in the presence of activated CD8\(^+\) T cells, but we did observe similar numbers of NK cells in PRO4L-SIY tumors with or without adoptive T cell therapy. However, it is possible that NK cells may have been more cytotoxic in the presence of 2C T cells interacting with cross-presenting stromal cells. Shanker et al.\(^{61}\) observed that CD8\(^+\) T cell transfer resulted in the rejection of antigen-negative cancer cell variants by a mechanism dependent on NK cells, and Wu et al.\(^{58}\) demonstrated that IFN\(\gamma\) produced by T cells causes glioma stem cells to become susceptible to NK cell killing. It is thus possible that the interaction of 2C T cells with cross-presenting stromal cells also enhances NK cell function, allowing rejection of allogeneic PRO4L-SIY cells but not PRO4L-gp33 cells. This possibility warrants further investigation.

In addition to stromal microglia/macrophages, other elements of tumor stroma may be targets of T cells. Liver endothelial cells are able to cross-present antigens from apoptotic tumor cells\(^{62,63}\). We observed a reduction of in the number endothelial cells in PRO4L-SIY brain tumors of mice treated with 2C T cells. This could be a consequence of the reduced tumor size, but requires further study.

With respect to therapeutic implications of these results, targeting tumor stroma in addition to malignant cancer cells may help prevent tumor recurrence. In peripheral tumor models, low antigen-expressing tumors can be damaged by radiation or chemotherapy to produce
a surge of antigen available for cross-presentation, and an appropriately timed adoptive T cell treatment can become more effective. The present results suggest such a strategy may be effective for brain tumors expressing endogenous levels of antigen as well. Other strategies for delivering tumor antigen, for example via viral vectors, could also enhance T cell targeting of stroma and help prevent tumor recurrence.
References


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Figure 2.1
MC57 cancer cell lines that express equivalent levels of class I MHC K\(^b\) but differ in expression of SIY peptide are killed equally well by 2C T cells \textit{in vitro}.

A. MHC I levels detected by anti-K\(^b\) staining. PRO4L-SIY cells are H-2\(^k\) and therefore negative for anti-K\(^b\) staining.

B. Cell surface levels of SIY peptide bound to K\(^b\) detected by 2C-m67 TCR monomer staining.

C. SIY peptide expression levels correlate with eGFP expression.

D. 2C T cells kill MC57-SIY-Hi and MC57-SIY-Lo similarly \textit{in vitro}. Chromium release was measured from cancer cells incubated with preactivated 2C T cells at different effector to target ratios in triplicate. Parental MC57 cells were used as a control. All experiments were repeated at least three times and representative data are shown.
Figure 2.2

Following adoptive transfer and activation \textit{in vivo}, 2C T cells infiltrate all MC57 brain tumors and rapidly clear both MC57-SIY-Hi and SIY-Lo tumors. Equivalent numbers of 2C T cells and tumor destruction were observed in all SIY-Hi and SIY-Lo tumors at each timepoint so representative images were chosen. Red = 2C T cells stained with 1B2 clonotypic antibody. Blue = DAPI counterstain. Scale bar = 200 μm for all images. \(n = 2\) mice per group.

A. One day following 2C T cell transfer (6 days following infusion of SIY-Lo cancer cells into the brain).

B. Three days following 2C T cell transfer (8 days following infusion of SIY-Lo cancer cells into the brain).

C. Five days following 2C T cell transfer (10 days following infusion of SIY-Hi cancer cells into the brain).

D. \textit{In vivo} activation of 2C T cells is required. Five days following 2C T cell transfer (10 days following infusion of SIY-Hi cancer cells into the brain) but omitting the s.c. infusion of SIY-Hi cancer cells on day 5.

E. Tumor infiltration by 2C T cells is SIY peptide-independent. Five days following 2C T cell transfer (10 days following infusion of parental MC57 cancer cells into the brain). Mice also received a s.c. infusion of SIY-Hi cancer cells on day 5 to activate 2C T cells.
Figure 2.3
Regression of both MC57-SIY-Hi and SIY-Lo brain tumors after 2C T cell transfer.
A. One day after 2C T cell transfer (6 days after bilateral infusion of SIY-Hi and SIY-Lo cancer cells into striatum) the tumors were similarly well-established.
B. Five days after 2C T cell transfer (10 days after bilateral infusion of cancer cells into striatum) both tumors appear to have been eliminated. Arrows indicate former location of tumors.
C. and D. Quantitation of the size regression of both SIY-Hi and SIY-Lo brain tumors after 2C T cell transfer. Mice with bilateral tumors received 2C T cells and a small s.c. infusion of SIY-Hi cells on day 5 and were euthanized on day 6 (1 day after 2C transfer), day 8 (3 days after 2C transfer), or day 10 (5 days after 2C transfer). Both SIY-Hi and SIY-Lo tumors regressed within 5 days of receiving 2C T cells. \( n = 4 \) to 8 mice per group at each timepoint. Two way ANOVA of treatment and time, significant interaction, \( p < 0.001 \); Bonferroni comparison of 2C vs. control on day 5, \( p < 0.001 \).
Effect of adoptively transferred 2C T cells on survival of mice bearing unilateral or bilateral MC57-SIY-Hi and MC57-SIY-Lo brain tumors.

A. Median survival of untreated mice with unilateral brain tumors was 16 days for SIY-Hi and 17 days for SIY-Lo. Median survival was 18 days for parental MC57 mice given 2C T cell transfers. Mice with SIY-Hi or SIY-Lo tumors lived significantly longer when treated with 2C T cells five days following infusion of cancer cells, compared to no treatment (p < 0.01). Approximately 80% of mice with SIY-Lo brain tumors relapsed and died; median survival was 46 days. 100% of SIY-Hi tumor mice were cured by 2C T cell treatment. Survival of SIY-Hi mice that received 2C T cells differed significantly from SIY-Lo mice that received 2C T cells (p < 0.05).

B-D. Effect of adoptively transferred 2C T cells on survival of mice bearing bilateral brain tumors. 2C T cells eliminated SIY-Hi tumors, but some SIY-Lo tumors recurred in the same mice. Each mouse treated with 2C T cells is represented by two curves. When mice reached criteria for euthanasia, brains were examined histologically to determine which tumor proved lethal. The therapeutic effect of 2C T cells decreased with a delay in treatment as tumors became larger. In mice with bilateral tumors that did not receive 2C T cells, both tumors progressed rapidly and mice had a median survival of 14 days. The “no 2C” mice (n=2) in B-D are the same.

B. When 2C T cells were injected on day five, SIY-Hi tumors were eliminated in all mice, but two mice eventually succumbed to SIY-Lo tumor recurrences. No tumor was evident histologically in the SIY-Hi hemisphere. p < 0.01 for SIY-Hi vs. SIY-Lo.

C. When 2C T cells were injected on day six, one mouse died due to the SIY-Hi tumor, whereas four of five mice succumbed to SIY-Lo recurring tumors. p < 0.01 for SIY-Hi vs. SIY-Lo.

D. When 2C T cells were injected on day ten, a therapeutic effect was not observed for either tumor.
Figure 2.5

Adoptively transferred 2C T cells persist in mice that relapse from recurring MC57-SIY-Lo brain tumors. Mice infused in the brain with MC57-SIY-Lo cancer cells received 2C T cell transfers and s.c. MC57-SIY-Lo cell infusions to activate 2C T cells on day 5. Tissues were collected when mice (n=3) reached criteria for euthanasia following relapse 34 to 35 days later. Immunostaining with 1B2 antibody demonstrated persistence of 2C T cells in all mice. Representative images are shown. Red = 2C T cells stained with 1B2 clonotypic antibody. Blue = DAPI counterstain. A. Brain tumor, scale bar = 1 mm. B. Spleen, scale bar = 200 μm. C. Draining cervical lymph node, scale bar = 200 μm.
Figure 2.6
Recurring SIY-Lo brain tumors arise from variant cells that do not express SIY peptide. Explanted tumor cells from mice that relapsed from MC57-SIY-Lo brain tumors following adoptive transfer of 2C T cells (n=5) were evaluated for MHC I expression with anti-K<sup>b</sup> antibody and for SIY/K<sup>b</sup> expression with 2C-m67 TCR monomer staining.

A. Tumors that recurred expressed normal levels of K<sup>b</sup>.

B. Tumor cells no longer expressed measurable surface levels of SIY/K<sup>b</sup> recognized by 2C T cells. Cultured parental MC57 cells and MC57-SIY-Lo cells were used as negative and positive controls respectively.
Figure 2.7
CD11b+ stromal cells in MC57-SIY-Hi brain tumors but not SIY-Lo brain tumors cross-present tumor antigens on MHC I. CD11b+ cells were purified from established MC57, SIY-Lo and SIY-Hi brain tumors two days after adoptive transfer of 2C T cells and stained with 2C-m67 TCR monomers to detect SIY/Kb cross-presentation.
A. Representative plots of purified, dual-stained (with anti-CD11b Abs and biotinylated 2C-m67 TCR monomers) brain tumor APCs from MC57, SIY-Lo and SIY-Hi brain tumors.
B. 2C-m67 TCR staining of independent samples of purified live CD11b+ cells collected from different mice demonstrated that detectable levels of cross-presentation of SIY peptide on CD11b+ stromal cells occurs only in tumors with high levels of available tumor antigen (MC57-SIY-Hi). Staining levels of CD11b+ cells from MC57-SIY-Lo brain tumors were indistinguishable from those of CD11b+ cells from parental MC57 tumors. Two brain tumors were pooled per sample, two samples per group were evaluated.
Figure 2.8

Activated 2C CTLs cannot directly recognize H-2k expressing PRO4L-SIY brain tumors but are able to significantly prolong survival and achieve cures in tumor-bearing mice.

A. In the absence of a s.c. inoculation of MC57-SIY-Hi cancer cells, 2C T cell transfer had no effect on survival of mice bearing PRO4L-SIY brain tumors (n= 5 per group).

B. With a concurrent s.c. inoculation of MC57-SIY-Hi cancer cells, 2C T cell transfer significantly prolonged survival of mice with PRO4L-SIY brain tumors (n = 5 per group, p < 0.01). Brains were examined for residual tumor, but none was found in the long-term survivors.

C and D. Growth inhibition by 2C T cell adoptive transfer was specific to PRO4L tumors expressing SIY peptide. Mice received bilateral cancer cell infusions of PRO4L-SIY and PRO4L-gp33 in the brain, followed by 2C T cell transfer on day five. Mice were euthanized on day 10 (five days after 2C transfer). Brain tumor volumes were reconstructed by quantifying tumor areas of randomly selected sections of PRO4L-SIY tumors in (C.) and PRO4L-gp33 tumors in (D.) n=8 for 2C and n=5 for control, p < 0.05 for PRO4L-SIY 2C vs. control.
Figure 2.9

CD11b+ stromal cells from PRO4L-SIY brain tumors cross-present tumor peptide following 2C T cell adoptive transfer. Mice with established PRO4L-SIY brain tumors were adoptively transferred unactivated 2C T cells or control wildtype lymphocytes and given a s.c. infusion of MC57-SIY-Hi cells to activate T cells on day seven.

A. 2C-m67 TCR monomer staining detected higher levels of cross-presentation of SIY peptide by CD11b+ cells in tumors of mice treated with 2C T cells than in mice that received control lymphocyte transfers.

B. Proportionally more CD11b+ stromal cells were dead (based on propidium iodide uptake), possibly due to T cell killing in PRO4L-SIY brain tumors of mice that received 2C T cell transfers compared to controls (40% vs. 25% respectively, % PI−CD11b+/all CD11b+).
Figure 2.10

**Endothelial cell staining (CD31\(^+\)/PECAM\(^+\)) is reduced in PRO4L-SIY brain tumors of mice treated with 2C T cells.** Mice bearing PRO4L-SIY brain tumors were treated with 2C T cells and injected s.c. with a small burden of PRO4L-SIY cells on day five to activate 2C T cells, and euthanized on day ten. Brain sections were stained with a biotinylated antibody against CD31/PECAM, and visualized with SA-594. The area of endothelial cell staining was reduced in the 2C T cell treated mice (A) compared to control mice that did not receive 2C T cell adoptive transfers (B). Scale bars = 200 µm. C. The mean area of stained vessels was quantified. n=8 for 2C T cell treatment, n=5 for controls. One to two random brain tumor sections per mouse were analyzed. p < 0.01 for 2C T cell treatment.
CHAPTER 3. EVALUATION OF TUMOR-SPECIFIC ADOPTIVE T CELL THERAPY AND ONCOLYTIC VIROTHERAPY FOR THE TREATMENT OF METASTATIC MELANOMA BRAIN TUMORS

Abstract

Brain metastases commonly arise in patients diagnosed with melanoma and significantly shorten survival. Adoptive transfer of T cells recognizing melanoma-associated antigens has shown clinical success, but immune editing of tumors by T cells can result in antigen loss variant (ALV) cell outgrowth and tumor escape. We evaluated a novel strategy to overcome tumor escape using a combination therapy of adoptive T cell transfer and administration of oncolytic myxoma virus (MYXV) in a syngeneic murine B16.SIY melanoma brain tumor model. MYXV, a poxvirus with strict species tropism for European rabbits, is nonpathogenic in mice and humans but can infect and kill many mouse and human cancer cell lines including melanoma. We evaluated the ability of recombinant MYXV expressing Tomato red fluorescent protein to infect, spread, and persist in B16.SIY brain tumors. We also characterized the brain cytokine and immune cell responses to the virus. B16.SIY cells were infected and killed by MYXV in vitro, but several lines of evidence indicate that the brain immune response to the virus limited its oncolytic ability in vivo. Intratumoral injection of MYXV led to the robust expression of Tomato red protein that remained restricted to the tumor but waned after several days, recruited large numbers of CD11b\(^+\) macrophages/microglia and GR-1\(^+\) neutrophils to the tumor, and led to the production of IFN\(\beta\) and TNF\(\alpha\) by surrounding normal brain tissue. Adoptive transfer of activated CD8\(^+\) 2C T cells that recognize SIY peptide presented on B16.SIY cells resulted in a doubling in survival time, but tumors recurred and no longer expressed SIY. We tested the safety and efficacy of co-administering 2C T cells, MYXV, and neutralizing antibodies against
IFNβ to mice with established brain tumors. Mice that received the combination therapy lived significantly longer than those receiving any single treatment, with no apparent spreading of the virus outside the tumor or other safety concerns, but mice eventually relapsed due to ALV outgrowth. A small amount of infectious virus was recovered from the relapsed tumors, suggesting that strategies to improve viral replication and cell-to-cell spread in vivo via inhibition of additional cytokines or immune cells could further improve the survival outcome.

Introduction

Metastatic brain tumors have been identified as the most common cause of death in patients with cutaneous melanoma\(^1\). Cancer cells metastasize to the brain in greater than 50% of patients, leading to a median survival of three to four months. Melanoma brain tumors are generally resistant to chemotherapy\(^2\) and typically recur following surgery, chemotherapy and whole or partial brain irradiation\(^3\,^4\). In combination, these traditional therapies extend the post-diagnosis median survival to approximately eight months.

Adoptive T cell immunotherapy is currently the most successful treatment option for patients with metastatic melanoma\(^5\). Almost 50% of patients with metastatic melanoma show an objective response (partial or complete) after lymphodepletion with non-myeloablative chemotherapy and subsequent infusion of autologous, activated tumor-infiltrating T cells. This response rate can be improved to 52-72% upon addition and dose escalation of total body irradiation prior to T cell infusion. Almost 13% of treated patients (n=12 of 93) in recent clinical trials conducted by Rosenberg, Dudley and colleagues at the National Cancer Institute had complete responses and survived at least 18 months with ongoing responses as long as 75
months. Importantly, responses were observed in patients with metastases at various sites, including the brain.

Successful T cell therapy for metastatic melanoma relies on the expansion of autologous tumor-infiltrating T cells \textit{ex vivo} away from suppressive cells and cytokines before re-infusion into the patient. T cells are typically isolated from tumors following surgical resection, expanded and activated \textit{ex vivo} to $10^{10}$ or $10^{11}$ total cells, and infused back into the patient. Prior to adoptive transfer, patients undergo lymphodepletion to eliminate competition with endogenous T cells for cytokines and space, and to eliminate Tregs. In some cases, the transferred cytotoxic T cells initially control tumor growth, but selective pressure edits the tumors until only cancer cells that lack the antigen or MHC molecule recognized by the T cells remain. Recurrent tumors are comprised of antigen loss variant (ALV) cancer cells and escape T cell killing. Many clinical and preclinical studies evaluating both adoptive T cell therapy and tumor peptide vaccine approaches have documented tumor escape due to immune editing of the tumor, including in brain tumors, and highlight the importance of targeting ALV cancer cells. Data from our own laboratory suggest that an appropriate strategy would be to enhance cross-presentation of tumor antigen on brain tumor stromal cells, leading to the elimination of these support cells for ALVs by cytotoxic T cells.

Our previous work showed that adoptive transfer of tumor-specific T cells as a single therapy was effective, but not sufficient to cure established murine brain tumors. Concurrent delivery of an oncolytic virus to a tumor could complement adoptive T cell therapy. Oncolytic viruses have the potential to enhance anti-tumor responses in several ways: tumor-specific viral infection may lead to oncolysis, the production of pro-inflammatory cytokines, release of tumor antigen to stimulate an immune response, recruitment of different immune cells, and in the case
of recombinant viruses, tumor-specific delivery and expression of therapeutic genes. We chose to characterize the oncolytic potential of myxoma virus (MYXV), a replication-competent poxvirus with strict species tropism for European rabbits\textsuperscript{17,18}. MYXV can infect many human and mouse cancer cells and has a large dsDNA genome with the capability to deliver therapeutic eukaryotic genes (at least 25 kb)\textsuperscript{19-25}. The species barrier to MYXV replication depends on intact Type I interferon responses\textsuperscript{26}, but mouse cancer cells with mutations that disrupt production of these cytokines become permissive to infection by MYXV. MXYV tropism for mouse and human cancer cells also depends on hyperphosphorylation of the serine/threonine kinase Akt\textsuperscript{22,27}. Mouse and human cancer cells can be categorized as permissive, semi-permissive, or non-permissive according to their relative Akt activation. Rapamycin can activate Akt through an mTOR-dependent pathway, and rapamycin treatment can increase the replication of MYXV in cancer cells \textit{in vitro} and \textit{in vivo}, but does not convert normal cells or non-permissive cancer cells to replication-permissive cells\textsuperscript{21,22,28}.

Mouse and human melanoma and glioma cells are both permissive to infection by MYXV\textsuperscript{20,22,29,30}. Stanford and colleagues demonstrated improved survival of C57BL/6 mice with B16-F10 melanoma lung tumors by concurrent administration of MYXV and rapamycin\textsuperscript{22}. MYXV treatment has also been shown to significantly improve survival of CD-1 nude mice implanted in the brain with several human glioma cell lines\textsuperscript{20}. While this xenograft model demonstrated proof-of-concept, characterization of MYXV in a syngeneic, immune-competent mouse brain tumor model has not been reported. Due to the inherent immunogenicity of viruses, this is an essential test relevant to the translational potential of any oncolytic virotherapy.

In the present studies, we characterized the \textit{in vitro} and \textit{in vivo} ability of MYXV to infect, replicate, express a virally-delivered gene (tdTomato red fluorescent protein), and kill mouse
B16-F10 melanoma cancer cells expressing the model tumor antigen SIYRYYGL (B16.SIY) recognized by the 2C T cell receptor. We also evaluated the combination therapy of adoptive CD8⁺ 2C T cell transfer and MYXV treatment in mice with established B16.SIY brain tumors. Lymphopenic C57BL/6 RAG1⁻/⁻ mice lacking endogenous T and B cells were used to imitate the lymphodepletion protocol used in clinical trials evaluating adoptive T cell therapy. All innate immune cells are normal in these mice. We show that injection of MYXV into mice with established B16.SIY brain tumors is safe and leads to infection and virally-delivered gene expression. We also characterized the cytokine and immune cell response to brain tumor injection of MYXV to evaluate its oncolytic capabilities in vivo in a syngeneic mouse melanoma model. Our experimental findings differed significantly from published reports using xenograft models of brain tumors²⁰, highlighting the importance of choice of model. We also tested the safety and efficacy of intratumoral administration of neutralizing antibodies against interferon-β (IFNβ) and found modest survival benefits when mice received MYXV, 2C T cells, and anti-IFNβ.

**Methods**

**Cells**

B16-F10 mouse melanoma cells were purchased from ATCC. B16.SIY cancer cells were a gift from Dr. Thomas Gajewski (University of Chicago). Parental B16-F10 murine melanoma cells were transduced to express the model peptide antigen SIYRYYGL (SIY) fused in frame with GFP and expressed as a fusion protein as previously described³¹. 2C T cells collected from 2C TCR transgenic mice recognize SIY peptide in the context of MHC I Kᵇ. Cancer cells were grown in DMEM containing 5 mM HEPES, 10% FCS, 1.3 mM L-glutamine, 50 pM 2-ME,
penicillin, and streptomycin. RK-13 rabbit kidney cells used to propagate and titer MYXV were a gift from Dr. Richard Moyer (University of Florida, Gainesville). RK-13 cells were maintained in minimum essential medium (MEM) with Earle’s salts supplemented with 2 mM glutamine, 50 U/mL penicillin G, 50 µg/mL streptomycin, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids (MEM-C; Mediatech, Manassas, VA).

**Virus**

The Lausanne strain of myxoma virus (MYXV) was a gift from Dr. Richard Moyer. Recombinant MYXV-Red was provided by Dr. Grant McFadden (University of Florida, Gainesville). Both viruses were propagated using RK-13 cells. MYXV-red expresses tdTomato red fluorescent protein under the control of a synthetic vaccinia virus early/late promoter and was originally designated vMyx-tdTr²⁴. Virus replicated in RK-13 cells was sucrose pad purified prior to use in mouse experiments. UV-inactivated MYXV was produced by exposing sucrose pad purified MYXV-Red to a standard tissue culture hood UV light overnight. The inability of the UV inactivated virus to infect RK-13 cells was confirmed prior to use. Uninfected RK-13 cells also were sucrose pad purified and used as a control in vivo cytokine release experiments.

**In vitro infection of cancer cells by MYXV**

Adherent cancer cells at ~75% confluency were incubated with virus suspended in MEM-C without serum at different multiplicities of infection (MOI, ratio of infectious virions:cells) at 37°C in 5% CO₂ for 1 hr, with rocking at 15 min intervals. Similarly, mock-infected cells were incubated in MEM-C without serum. In some experiments, the inoculum was removed and cells were washed with PBS three times before adding fresh DMEM with 10% serum. For other
experiments, fresh DMEM with 10% serum was added after 1 hr and virus remained. For the pre-incubation survival experiment, cells were washed, trypsinized and prepared for brain injection after 1 hr incubation with virus at MOI=1.

*Virus recovery from mouse tissues*

For titration of infectious virions within tissue samples, samples were frozen then thawed three times, sonicated for 1 to 5 min, and then a series of 10-fold dilutions were made. Each dilution (400 μL) was added to confluent RK-13 cells in 35 mm diameter plates. After 1 hr of incubation at 37°C in 5% CO₂ with rocking at 15 min intervals, 3 mL of a 1:1 mixture of 1% SeaKem high gelling temperature agarose solution and 2× MEM-C with 20% serum was added to the infected cells. Plates were incubated at 37°C in 5% CO₂ for 5 to 7 days until viral foci (plaques) developed. A minimum of three separate series of 10-fold dilutions were processed to calculate the average number of viral plaque forming units per mL (pfu/mL; virus titer) in each sample.

*Cell Viability Assay*

B16.SIY cells growing in 96 well plates were infected at a MOI=1 or 10 with wild-type MYXV or mock-infected. The CellTiter-Blue Viability Assay (Promega) was used according to the kit instructions. Viable cells convert a redox dye (resazurin) into a fluorescent end product (resorufin) that can be detected at 590nm. Blue reagent was added 1.5 hrs prior to fluorescence readings. All measurements were done in at least triplicate and the assay repeated twice. Brightfield images of cytopathic effect were taken immediately prior to adding blue reagent to
wells and were acquired using a Leica DMI 4000 B microscope (magnification ×100) and ImagePro Express software (Media Cybernetics).

*Mice*

C57BL/6 and C57BL/6 RAG1−/− mice originally from The Jackson Laboratory are maintained as colonies at the University of Illinois. 2C TCR transgenic mice are maintained as a heterozygous colony by crossing with C57BL/6 mice and screened for expression of the 2C TCR on PBLs by flow analysis with 1B2 clonotypic antibody. Mice were 2–6 months of age at the time of experiments. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign.

*Intracranial cancer cell infusions*

Cancer cells were trypsinized and collected, washed twice with Hanks Balanced Salt Solution (HBSS), and stereotaxically infused into the brain while the mice were anesthetized using Isoflurane gas. Mice received an infusion of 2 × 10^3 B16.SIY cells in 300 nL HBSS into ventral striatum (AP 0.5mm, lat 2.5mm, DV -4mm). In some experiments, mice received bilateral infusions of cancer cells. Mice were sacrificed according to standard criteria for euthanasia: 75% of baseline body weight or signs of neurological impairment, in accordance with IACUC guidelines.

*Preparation of activated 2C T cells for adoptive transfer*

2C T cells from spleens and lymph nodes of 2C TCR transgenic mice were prepared by mechanical dissociation of the tissues and ammonium chloride buffer lysis of erythrocytes.
Lymphocytes were incubated for 48 hrs with 3 μM SIY peptide (SIYRYYGL) and 5% RCAS to activate effector 2C T cells. Cells were then collected using PBS/EDTA buffer and washed with HBSS. Mice were briefly restrained under a heat lamp and 1-2 x 10^6 cells in 200μl HBSS were injected into the tail vein.

**Histological analysis of 2C T cell response in brain**

Mice were sacrificed at two or seven days following 2C T cell transfer or followed for survival. Brains and cervical lymph nodes were collected and snap-frozen. 1B2 monoclonal antibody specific for the 2C TCR was purified from the hybridoma and biotinylated in our laboratory. Unfixed 10 μm tissue sections were stained with 1B2 antibody amplified with HRP/tyramide-biotin (Perkin Elmer) and detected with streptavidin-Alexa 594 (Invitrogen), and counterstained with DAPI (Invitrogen).

**Flow cytometry experiments**

Cancer cells were trypsinized and washed twice with PBS/0.5% BSA before analysis of GFP expression on a BD FACS Canto instrument. For analysis of cross-presentation, CD11b^+ cells were isolated from ten day-old B16-F10 or B16.SIY brain tumors injected with 5x10^6 pfu MYXV-Red or PBS three days earlier. Tumor hemispheres from two mice were pooled per sample. Tumors were minced and dissociated by tituration following a 30 min incubation in Hibernate A media (BrainBits) containing Dispase (5U/mL). Cells were then pushed through 100 μM and 40 μM filters sequentially. Erythrocytes were lysed with ACK buffer. CD11b^+ cells were isolated via positive selection using magnetic microbeads labeled with anti-CD11b antibodies (Miltenyi Biotec) and passing the cell mixture over LS columns in a VarioMACS
magnet. Cells were washed with PBS/1% BSA and stained with anti-CD11b antibody directly labeled with Pacific Blue (Caltag Laboratories) and 2C-m67 TCR multibiotinylated monomers detected with streptavidin-allophycocyanin (Invitrogen). Expression and purification of soluble, single-chain 2C-m67 TCR monomers that bind SIY/K\textsuperscript{b} with low nanomolar affinity were described previously\textsuperscript{16}. Monomers were chemically biotinylated at multiple sites via free amine groups using EZ-Link NHS-PEO\textsubscript{4} Biotinylation Kit (Pierce).

**Intratumoral injection of MYXV and characterization of infection in vivo**

Mice with four- to eight-day established B16.SIY brain tumors (depending on experiment) were anesthetized and injected intratumorally with approximately 5x10\textsuperscript{6} pfu of sucrose pad-purified MYXV in 0.7 \(\mu\)L total volume. For some experiments, brains were collected and snap-frozen in OCT media, immersion fixed in 10% formalin, or homogenized and used for cytokine ELISA experiments or to recover infectious virus. Brains required formalin fixation for preservation of Tomato red protein expression. Sections were counterstained with DAPI and analyzed with an Olympus fluorescence microscope.

**Immunostaining and qualitative analysis of brain tumor-infiltrating immune cells**

RAG1\textsuperscript{-/-} mice with 5 day-old B16.SIY brain tumors were injected intratumorally with MYXV, UV-MYXV or PBS. Mice were sacrificed 48 hrs later and brains were snap-frozen for cryosectioning. 10 \(\mu\)m sections were fixed in cold 95% ethanol for 20 min, rinsed with PBS, and blocked with Superblock (Pierce) for 4 hrs prior to incubation with primary antibodies. Slides were stained overnight with purified anti-mouse CD11b (BD Pharmingen), anti-mouse 4D11 (BD Pharmingen) or anti-mouse GR-1 (Becton Dickenson). Slides were then washed with PBS
+ 0.1% Tween and incubated with rabbit anti-rat-biotin (Vector) with 5% normal rabbit serum for 2 hrs, washed, and incubated with streptavidin-Alexa594 (Invitrogen) for 20 min. Sections were also co-labeled with DAPI (Invitrogen). Images were taken using Texas red and blue filter sets. Control slides omitting each primary antibody were negative for Alexa594.

**Cytokine ELISAs**

Mouse TNFα Ready-Set-Go ELISA kits (eBiosciences) and mouse IFNα and IFNβ ELISA kits (PBL Biomedical Labs) were used according to manufacturer instructions. Brain tumors from mice were homogenized in 2 ml ice cold PBS + 0.1% Igepal detergent and pooled, two brains per sample. Supernatants were concentrated and tested in triplicate.

**Interferon β neutralizing antibodies**

Rabbit polyclonal antibody against mouse IFNβ (PBL Interferon Source) was diluted in sterile saline and injected into mice intratumorally as a single dose (12.5 ng in 0.5 μL) or intraperitoneally at 100 μg/kg body weight diluted in 100 μL sterile PBS once daily for four days.

**Data Analysis**

GraphPad Prism software was used for all statistical analyses: cell viability data by two-way ANOVA, survival curves by Log-rank test, cytokine ELISAs by one-way ANOVA and unpaired t test. Significance was considered p < 0.05.
Results

**B16.SIY melanoma cancer cells are permissive to infection by MYXV**

The murine melanoma cell line B16-F10 is known to be permissive to MYXV infection and replication\(^2^2\). In collaboration with Dr. Amy MacNeill and Dr. Rosalinda Doty (University of Illinois at Urbana-Champaign), we extended this finding by testing the permissiveness of B16.SIY cancer cells derived from the parental B16-F10 line that express the model antigenic peptide SIYRYYGL. Two methods were used to confirm that MYXV can productively infect and replicate in B16.SIY cancer cells *in vitro*. First, both mature and immature virion stages were observed in infected cells by electron microscopy and confirmed intracellular viral trafficking and replication (data not shown, unpublished data from AM & RD). Second, multi-step growth curves demonstrated productive viral replication after inoculation of cells at a low multiplicity of infection (MOI), implying cell-to-cell spread over the first 48 hrs post-infection (p.i.), and an exponential increase in infectious virions. Similar kinetics of virus replication were observed in permissive RK-13 cells, B16-F10 cells, and B16.SIY cells (data not shown, unpublished data from AM & RD).

**MYXV infection of B16.SIY melanoma cancer cells results in oncolysis**

To determine whether MYXV has oncolytic effects on B16.SIY cancer cells *in vitro*, we infected confluent monolayers of cells at a MOI=1 or 10 to observe the cytopathic effect and measure cell viability. By 24 hrs, most cells rounded up or detached from the plates and by 48 hrs, significant numbers of cells detached from the wells at both MOIs compared to mock-infected cells (Figure 3.1). By 72 hrs post-infection, only a few viable cells were evident at the highest MOI. Mock-infected cells remained adherent and overgrew the wells by 72 hrs.
To quantitatively assess the decrease in cell viability following infection with MYXV, we measured the metabolic activity of infected cells over time using a commercially available fluorescence based assay (Figure 3.2). Mock-infected cells continued to multiply over 72 hrs with a corresponding increase in metabolic activity. At MOI=1, viability was significantly decreased by 48 hrs p.i. At MOI=10, viability was significantly decreased by 24 hrs p.i. Over 72 hrs, the viability of melanoma cells infected at both MOIs remained low, but some cancer cells did survive.

**Survival duration of mice with B16.SIY brain tumors increases significantly following adoptive transfer of 2C T cells**

B16.SIY cancer cells express low to no class I MHC on their surface and are not efficiently killed by 2C T cells *in vitro*\(^3\)\(^\text{1}\). However, the *in vivo* antitumor efficacy was not known. We tested the effect of transferring activated CD\(^8\)\(^+\) 2C T cells that recognize SIY peptide presented on the MHC class I molecule K\(^b\) into mice with established B16.SIY brain tumors. We hypothesized a potential effect due to homeostatic proliferation of the transferred cells in lymphopenic animals, so both RAG1\(^{-/-}\) and wild-type C57BL/6 mice were infused with B16.SIY cells in the brain. On day seven when the tumors were well-established, mice received intravenous injections of preactivated 2C T cells and were followed for survival (Figure 3.3). Control mice that were injected with saline died within two weeks (median survival of RAG1\(^{-/-}\) mice was 14 days, C57BL/6 mice 13 days) while mice that received 2C T cells survived over twice as long (median survival of RAG1\(^{-/-}\) mice was 35 days, C57 mice 30 days). However, no mice were cured by T cell adoptive therapy alone.
2C T cells infiltrate and initially destroy brain tumors, but tumors recur and no longer express SIY tumor antigen

The ability of transferred 2C T cells to control tumor growth and increase survival duration, but not successfully eliminate the tumors, led us to further characterize the timecourse of response. Mice with seven day-established B16.SIY brain tumors received intravenous injections of activated 2C T cells and were euthanized two or seven days after transfer. Immunostaining with 1B2 antibody specific for the 2C TCR revealed low numbers of 2C T cells in the tumors on day two, but a massive infiltration of 2C T cells in the tumors on day seven (Figure 3.4). The T cells were restricted to the tumor bed and were not found in surrounding normal brain tissue. Tumors were mostly necrotic and had lost clear margins by day seven. Lymph nodes also contained large numbers of T cells at both timepoints, while untreated RAG1−/− control mice had none (data not shown). Immunostaining was also done on brains collected when mice reached criteria for euthanasia. 2C T cells were absent from the large tumors comprising almost the entire hemisphere of the brain. However, 2C T cells were present in the draining cervical lymph nodes, but in much smaller numbers than those observed on day seven (data not shown).

The presence of an early anti-tumor T cell response that disappears in late-stage tumors led us to hypothesize that the tumors may have recurred from outgrowth of antigen loss variant (ALV) cancer cells that are not recognized by 2C T cells. When mice reached criteria for euthanasia, brain tumors were collected and mechanically dissociated. Cells were then cultured for four to seven days to enrich for live cancer cells and analyzed by flow cytometry (Figure 3.5). GFP expression was used as a surrogate marker for expression of SIY peptide as these genes are fused in frame and expressed as a fusion protein. Tumor explant cells from four mice
were compared to fresh B16.SIY cells and parental B16-F10 cells that do not express GFP. All cancer cells recovered from the brain tumors had lost expression of GFP indicating that ALV outgrowth led to tumor recurrence in the mice.

**Intratumoral injection of MYXV into established brain tumors results in tumor-specific infection and viral gene expression**

To evaluate whether concurrent delivery of MYXV could complement adoptive T cell therapy, we characterized the oncolytic effects of MYXV *in vivo*. MYXV-Red was injected directly into established B16.SIY brain tumors to assess safety and characterize the timecourse and spread of viral infection. Intratumoral injection of virus resulted in robust expression of Tomato red fluorescent protein in the tumor but not in surrounding normal brain tissue 24 hrs following injection (*Figure 3.6A*). Some cancer cells expressing Tomato red protein could be found up to eight days post-injection, but expression peaked at 48-72 hrs post-injection (data not shown). UV-inactivated MYXV was used as a control and expression of Tomato red protein was not observed in tumors at any time (*Figure 3.6B*). We also tested the ability of live MYXV to spread to contralateral brain following intratumoral injection in a bilateral tumor model. We did not observe any spread of virus to contralateral brain tumors (*Figure 3.6B*). Peripheral tissues were collected and homogenized one to five days post-injection of virus and titered to check for spread of virus outside the brain. Live virus was not detected in blood, spleen, liver or lymph nodes in any mice (n=9). In all experiments, mice did not exhibit any behavioral abnormalities or weight loss following injection of live or UV-inactivated virus into the brain. Intratumoral injection of MYXV in the brains of semi-immunocompetent mice was safe and resulted in transient, tumor-specific infection and gene expression.
Pre-infection of B16.SIY cancer cells prior to infusion leads to modest increases in survival

To further characterize the oncolytic effects of MYXV in vivo, we evaluated whether virus treatment could improve the survival of mice infused in the brain with B16.SIY cells that were pre-incubated with a low MOI (=1) of MYXV-Red. 2 x 10^3 pre-infected or mock-infected cells, or a 1:1 mixture of both (2 x 10^3 total), were injected into the brains of RAG1^-/- mice (Figure 3.7). Mice injected with either 50% or 100% pre-infected cells lived significantly longer than mice that received mock-infected cells (18 and 20 days vs. 15 days, respectively, p < 0.05), but there were no long-term survivors. Mice injected with only 1 x 10^3 mock-infected cancer cells were included for comparison to mice injected with 2 x 10^3 50% pre-infected cells to determine if all infected cells were killed, and to test the ability of the virus to spread from infected cells to non-infected neighboring tumor cells leading to additional oncolysis. There was a small increase in survival of the 50% pre-infection group compared to the 1 x 10^3 mock group, but the difference was not statistically significant (18 vs. 16 days, respectively, p = 0.096). Despite initial infection of the cancer cells with a low MOI, our results demonstrated modest oncolytic effects by MYXV and suggest potent anti-viral mechanisms exist in vivo that hinder the replication and spread of virus.

Strong cellular immune response to MYXV-Red may promote clearance of virus and prevent oncolysis

The striking difference we observed regarding the oncolytic ability of MYXV in vitro and the modest survival advantage conferred in vivo may be attributed to immune clearance or inactivation of the virus. Expression of Tomato red protein was observed out to eight days post-
injection, but only in a few cancer cells, suggesting that MYXV can readily infect tumor cells, but does not spread due to physical barriers or anti-viral immune mechanisms triggered in the brain that abort viral replication.

To characterize the cellular immune response to the virus, RAG1^-/- mice with five day-old B16.SIY brain tumors (n=12) were injected intratumorally with live MYXV-Red, UV-inactivated MYXV-Red or PBS (n=4 per group). Brains were collected after 48 hrs and sections were stained for the immune markers CD11b (on macrophages and microglia), GR-1 (on neutrophils) and Ly49G2 (on NK cells). In a parallel experiment, brains were formalin fixed and prepared for H&E staining. The immunostained tissues revealed a large infiltration of CD11b^+ and GR-1^+ cells in tumors injected with live or UV-inactivated virus, especially adjacent to the virus injection site (Figure 3.8). We do not believe these cells are dual-labeled myeloid-derived suppressor cells that express these phenotypic markers because equivalent H&E stained sections showed local microabscesses created by an influx of inflammatory cells, typically macrophages and neutrophils, in the same location (data not shown). The pathology was limited to the virus injection site in all cases. CD11b^+ cells were also found around the rim of all tumors including those injected with PBS. GR-1^+ cells and NK cells were only found in tumors injected with live or UV-inactivated virus. NK cells were found very rarely.

**Brain cytokine response to MYXV may also restrict spread of virus**

In addition to immune cell clearance of the virus, production of cytokines by immune cells and normal non-tumor brain cells may abort viral replication and spread and prohibit the robust infection of a tumor. To characterize the cytokine response in vivo in response to intratumoral injection of virus, eight day-old B16.SIY brain tumors growing in RAG1^-/- mice
were injected with MYXV-Red, a mock preparation from uninfected RK-13 cells, or PBS. 24 hrs later, tumors were excised and homogenized and the supernatants were tested for IFNβ, IFNα, and TNFα by ELISA (Figure 3.9). We observed significant increases in brain levels of both IFNβ and TNFα in response to virus. There was no detectable change in IFNα. Data are normalized to PBS-injected brain samples. The cytokine response was virus-specific because the mock preparation of virus (a sucrose pad- purified extract of RK-13 cells used to propagate the virus) did not elicit a response that was different from that of PBS. In addition, the cytokine response to MYXV injection in mouse brain was similar whether or not a tumor was present (data not shown). We did not attempt to characterize the cell types responsible for the production of these cytokines.

*Treatment with neutralizing antibodies against IFNβ is safe and modestly improves survival of mice treated with a combination of MYXV and adoptive 2C T cell transfer*

Based on the cytokine profile characterizing the pro-inflammatory response to MYXV in the brain, we considered whether cytokine neutralization could be an appropriate strategy to pursue *in vivo*. Due to the known anti-tumor effects of TNFα, we selected a strategy of treating tumor-bearing mice with MYXV-Red and neutralizing antibodies against IFNβ, rather than suppressing the TNFα response. We tested the safety of this approach in short-term experiments followed by survival experiments to determine the efficacy.

RAG1−/− mice with seven day-established B16.SIY brain tumors were injected intratumorally with MYXV-Red. At the same time mice received intratumoral (i.t.) or intraperitoneal (i.p.) injections of polyclonal neutralizing antibodies, or both. Intraperitoneal injection of neutralizing antibodies against IFNα has been shown to be effective for decreasing
brain cytokine levels. Brains were collected, fixed and sectioned two or four days after treatment. In all mice, the expression of Tomato red protein remained limited to the tumor and did not spread to surrounding normal brain cells after any combination of treatment (Figure 3.10). At four days post-injection of virus, strong expression of Tomato Red protein was still observed in tumor. No differences were noted in the expression of Tomato Red protein in mice that received i.p. or i.t. delivery of neutralizing antibody.

After determining that co-administration of neutralizing antibodies against IFNβ and MYXV did not result in infection of normal brain, we evaluated the effect of a combination therapy on survival of mice with established B16.SIY brain tumors. Mice received an intratumoral co-injection of MYXV and anti-IFNβ Abs on day six, intravenous injections of activated 2C T cells on day seven, and intraperitoneal injections of anti-IFNβ Abs for four days following virus administration (Figure 3.11). Mice that received MYXV, anti-IFNβ Abs and 2C T cells lived three times longer than untreated mice (39 vs. 13 days, respectively), and significantly longer than mice receiving only 2C T cells (39 vs. 32 days, p<0.05 Log-rank test). Injection of anti-IFNβ alone increased survival time modestly (15 vs. 13 days) but the difference was not statistically significant. Data are pooled from two separate experiments.

It was unknown whether MYXV was cleared over time from the brain tumors, or if the virus was inactivated but still present in the tumors. When mice in the survival experiments reached criteria for euthanasia, brain tumors were excised and tumor homogenates were tested to determine whether MYXV-Red could be recovered from the tumors several weeks after injection. Tumor homogenates were titered on permissive RK-13 cells using a plaque forming assay (Figure 3.12). A small number of infectious MYXV-Red virions were recovered from 11 of 14 total tumors tested. This effect was time-dependent and more virus was isolated from
A subset of CD11b+ stromal cells purified from B16.SIY brain tumors cross-present SIY peptide but MYXV-infection does not increase cross-presentation

We tested the hypothesis that MYXV infection of B16.SIY brain tumors would lead to oncolysis and release of tumor peptides for cross-presentation. We purified CD11b+ cells from B16.SIY tumors 72 hrs after intratumoral injection of MYXV-Red and analyzed surface levels of SIY/Kb complexes using single-chain 2C-m67 TCR multibiotinylated monomers (Figure 3.13). MYXV-injected B16-F10 tumors and PBS-injected B16.SIY tumors were used as controls. A subset of cross-presenting CD11b+ cells were detected in B16.SIY brain tumors compared to B16-F10 tumors, but there was no difference between MYXV and PBS injected tumors at 72 hrs post-injection.

Discussion

T cells are exceedingly proficient at antigen-specific killing, but successful elimination of any tumor requires concurrent destruction of both antigen-expressing and ALV cancer cells. Our experiments confirmed that immune editing by T cells occurs in the brain, and allows ALV cells to repopulate a tumor. 2C T cell killing of B16.SIY melanoma cells in vitro is inefficient\textsuperscript{31}, presumably due to low MHC I expression, thus it was unknown if adoptive transfer of activated 2C T cells would have any beneficial effect on controlling aggressive B16.SIY tumors in vivo. Impressively, 2C T cells almost completely eliminated established brain tumors and significantly improved survival time, but the primary cause of death in all mice was outgrowth of ALV cancer
cells. These studies have identified a realistic model of metastatic melanoma ideal for testing strategies to eliminate ALV cancer cells from brain tumors.

To better understand the low levels of 2C T cell killing previously observed \textit{in vitro} \textsuperscript{31} compared to our results \textit{in vivo}, we treated B16.SIY cells with IFN\(\gamma\) for 24 hrs to test for upregulation of MHC I. Cell staining and flow cytometry analysis revealed strong upregulation of both MHC I \(K^b\) and SIY/\(K^b\) complexes (data not shown). We assume IFN\(\gamma\) release from activated T cells in the brain leads to upregulation of MHC I by tumor cells resulting in efficient killing, though this remains to be tested.

Lymphodepletion prior to adoptive T cell therapy has proved critical to the successful persistence and effector function of transferred T cells in preclinical and clinical studies\textsuperscript{33-35}. We observed a modest increase in survival of RAG1\textsuperscript{-/-} mice lacking endogenous T cells and B cells compared to wild-type mice following T cell transfer (35 vs. 30 days respectively), but the sample sizes were small (\(n=3\)). All subsequent experiments were done using only RAG1\textsuperscript{-/-} mice. It is interesting to note that it took more than two days following adoptive transfer for activated T cells to arrive in the brain in large numbers, but in less than five days the fast-growing tumors were mostly dissolved and only a small necrotic core was evident.

Many potential approaches could be used to target ALV cancer cells. We tested whether a relatively novel oncolytic virus, MYXV, could augment the efficacy of adoptive T cell therapy and prevent outgrowth of ALV cells. We hypothesized that MYXV could enhance T cell therapy for melanoma brain tumors by four potential mechanisms. First, MYXV can directly kill melanoma cancer cells (including ALV cells) and has the potential to replicate and spread within the tumor, but not to normal brain tissue, making MYXV very safe. Second, the large dsDNA genome of MYXV can accommodate the insertion of large eukaryotic genes. The present
experiments demonstrated viral gene delivery and robust expression of Tomato red fluorescent protein. Our laboratory is currently engineering MYXV to express therapeutic genes such as mIL-15/mIL-15Rα, a cytokine and its receptor subunit important for T cell activation, persistence, and anti-tumor effector function of CD8+ T cells and NK cells. Third, MYXV tumor infection can lead to local production of immunostimulatory cytokines and recruit innate and adaptive immune cells to aid in tumor clearance. Fourth, dying cancer cells killed by MYXV may be a source of tumor peptides that could be cross-presented on brain APCs to further enhance a T cell response.

Previous work in our laboratory using a different SIY-expressing brain tumor showed that T cell elimination of cross-presenting CD11b+ stromal cells prevented the outgrowth of ALV cells and cured mice with established brain tumors. We hypothesized that oncolysis by MYXV could lead to antigen release and cross-presentation. In the present studies, we were unsuccessful in our attempts to demonstrate increased levels of cross-presentation of SIY peptide on CD11b+ stromal cells following intratumoral injection of MYXV. This may be due to choice of timing (3 days after injection of virus when Tomato red expression peaks) or the mechanism of cell death. MYXV probably kills cancer cells via apoptosis, but this has not yet been confirmed. It is unknown if apoptotic removal of cancer cells would generate enough peptide to produce detectable cross-presentation.

MYXV can productively infect and replicate in B16.SIY melanoma cells in vitro, resulting in cell death. In the brain, MYXV can safely and selectivity infect tumor but not normal brain. In one experiment when the virus injection missed the tumor by several millimeters and was instead infused adjacent to the lateral ventricle, we observed Tomato red expression in ependymal cells (which has been previously reported for other oncolytic viruses).
but it disappeared by four days post-injection. Peripheral organs (lung, heart, intestine, spleen, kidney, reproductive tract, and liver) were also tested for infectious virus at one, two, three and five days post-injection by incubating tissue homogenates with RK-13 cells and analyzing for expression of Tomato red protein. No virus was detected in any sample (n=2 or 3 at each timepoint).

MYXV had potent and persistent oncolytic effects leading to long-term survival in several xenograft mouse tumor models including brain tumors\textsuperscript{20,21,25}. In contrast to these findings, the oncolytic effects of MYXV in our syngeneic brain tumor model were substantially diminished. Viral expression of Tomato red protein in the tumor was present 24 hrs post-injection and peaked by 48-72 hrs, but then rapidly disappeared and only a few infected cells were observed at one week. Proximity to the virus injection site was important. Fewer infected cells were observed at distant sites within the tumor, but infection of cultured cells at a low MOI allowed viral spread, suggesting impaired spread of the infection \textit{in vivo}. We characterized the oncolytic ability of MYXV in both immunocompetent and lymphopenic RAG1\textsuperscript{-/-} mice. We observed similar levels of Tomato red expression, innate immune cell recruitment, and survival in both strains of mice. We attribute, at least in part, the modest oncolytic effect of MYXV \textit{in vivo} to the presence of anti-viral cytokines (IFNβ and TNFα) produced by surrounding brain or immune cells in response to the virus. Macrophages/microglia and neutrophils are also recruited to the tumors and may have a role in viral clearance or inhibition of replication. Our survival experiment using pre-infected cancer cells suggests that these mechanisms are very potent.

We did observe a small but significant survival benefit from our combination therapy of T cell transfer and administration of MYXV and anti-IFNβ antibodies. Infusion of neutralizing antibodies against IFNβ was safe and did not lead to the productive infection of normal cells.
outside the tumor. Infectious virions were recovered from most brain tumors, even several weeks following injection, supporting the hypothesis that overcoming local inactivation of the virus would improve the spread of infection.

We are exploring several strategies that could potentially enhance the oncolytic effects of MYXV in vivo. TNFα in combination with IFNβ are known to synergize to produce an anti-viral state\textsuperscript{37-39}. Transient, local neutralization of TNFα within the brain by antibodies or other agents has been shown to be safe\textsuperscript{40,41} and is a possible strategy in addition to IFNβ neutralization to improve viral replication for at least a few days. The pre-infection survival experiment in the present studies used a very low MOI and the survival benefit was small, but an initial infusion of more viral particles should improve the extent of infection. Practical limitations on the volume of virus that can be injected intracranially could be skirted if MYXV was delivered intravenously and sufficient amounts reached the brain. The blood brain barrier is likely to be leaky enough to allow virus to enter a tumor\textsuperscript{42} and we plan to purify large amounts of virus to test intravenous administration.

Several recent studies have revealed significant synergistic effects of treating tumor-bearing animals with an oncolytic virus, including MYXV, and rapamycin or rapamycin derivatives\textsuperscript{21,22,43,44}. Rapamycin is under active investigation for treatment of many types of cancer\textsuperscript{45} and is FDA approved for treatment of renal cell carcinoma. A recent report documented beneficial responses in patients with melanoma co-treated with rapamycin and chemotherapy\textsuperscript{46}. Rapamycin is best known for its immunosuppressive effects and can suppress T cell rejection of organ transplants. We plan to test if co-treatment of mice with low doses of rapamycin and T cell adoptive transfer would improve viral spread but spare T cell effector functions. Finally, MYXV-mediated delivery and expression of therapeutic genes would be an ideal way to
transiently express tumor antigens or other immune modulators in the brain that could enhance
cross-presentation and promote tumor clearance by innate immune cells such as NK cells,
potentially resulting in the elimination of ALV cancer cells.

We evaluated a novel strategy using MYXV to target syngeneic brain tumors and
complement adoptive T cell therapy. The safety, tumor-selectivity, and robust expression of
virally delivered genes by MYXV support its further investigation as a cancer therapeutic.
References

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Figure 3.1

Cytopathic effects of MYXV infection on B16.SIY cells *in vitro*. Cells were mock-infected or infected at a MOI=1 or 10. Images were taken over 72 hrs post-infection.

**Left column, mock infected:** The adherent monolayer of cells becomes more confluent over 24 to 72 hrs. Cells are overgrown by 72 hrs and multiple cell layers are evident.

**Middle column, infected at MOI=1:** Cells begin to round up and detach by 24 hrs. Some cells do remain adherent at 72 HPI.

**Right column, infected at MOI=10:** Clear cytopathic effect and very few cells remain adherent at 72 hrs. All images taken using 10x objective, 100x total magnification.
**Figure 3.2**

**B16.SIY cell viability decreases following infection with MYXV *in vitro***. Viable cells convert a redox dye (resazurin) into a fluorescent product (resorufin) that can be detected at 590nm. Cells were infected at a MOI=1 or 10, or mock-infected. Measurements were taken over 72 hours post-infection (p.i.). Mock-infected cells continued to multiply resulting in an increase in fluorescence over time. At MOI=1, viability was significantly decreased at 48 and 72 hours p.i. At MOI=10, viability was significantly decreased at 24, 48 and 72 hrs p.i. p<0.05 for two-way ANOVA with Bonferroni post-test. Data are representative of two independent experiments.
Adoptive transfer of activated, tumor-specific 2C T cells significantly increases survival duration of both wild-type C57BL/6 and lymphopenic RAG1−/− mice with established B16.SIY brain tumors. Mice were infused with 2x10^3 B16.SIY cells in the brain and tumors were allowed to establish for seven days prior to treatment. Mice then received intravenous injections of approximately 1x10^7 activated 2C T cells or saline and were followed for survival. Both C57BL/6 and RAG1−/− mice treated with 2C T cells lived significantly longer than untreated controls (p < 0.05 Log-rank test), but no cures were achieved. Survival of C57BL/6 and RAG1−/− mice treated with 2C cells (30 vs. 35 days respectively) were not significantly different (p = 0.1) so a small effect due to homeostatic proliferation of 2C T cells in lymphopenic RAG1−/− mice was not conclusive. Numbers on graph indicate median survival.
A. Two days post-transfer of 2C T cells

B. Seven days post-transfer of 2C T cells

Figure 3.4
Activated 2C T cells infiltrate and destroy established B16.SIY brain tumors within one week after transfer. B16.SIY cells were infused into the brains of RAG1−/− mice. On day 7, mice were injected with activated 2C T cells. Mice were sacrificed on day two or seven after transfer (n=3 per group). Brains were sectioned and stained with a clonotypic antibody against the 2C TCR. A few 2C T cells can be observed in the tumors on day two (A), but a massive response is present on day seven (B) and the tumors are almost completely destroyed. Red = 2C T cells stained with biotinylated 1B2 antibody and detected with streptavidin-Alexa 594. Blue = DAPI counterstained cell nuclei. Scale bar = 200µm. Images are from two different mice per group.
Figure 3.5
Cancer cells recovered from recurring B16.SIY brain tumors in mice treated with 2C T cells no longer express SIY peptide. When mice reached criteria for euthanasia (all >30 days out), brain tumors were excised and dissociated. Tumor cells were cultured *in vitro* for approximately one week to enrich for live cancer cells. B16.SIY cells express enhanced green fluorescent protein (GFP) as a fusion protein with SIY peptide. Loss of GFP expression was used as a marker for loss of SIY expression. Cancer cells from four tumor explants were analyzed by flow cytometry. All cancer cells were similar to parental B16-F10 melanoma cells that do not express GFP, indicating antigen loss (or antigen low) variant outgrowth in these mice. Fresh B16.SIY cancer cells (not from a mouse tumor) were included as a positive control and expressed GFP at much higher levels. All cancer cells retained expression of melanin confirming that the expanded tumor explant cells were cancer cells.
Figure 3.6
Injection of MYXV-Red into established B16.SIY mouse brain tumors results in tumor-selective infection and viral gene expression.  

A. Tomato Red fluorescent protein is expressed in B16.SIY cancer cells (GFP+ cells) but not in normal surrounding brain tissue 24 hours after intratumoral injection of live virus into a four-day old tumor.  

B. Tomato Red protein is observed 24 hours after injection of live virus (left panel) but not after injection of UV-inactivated dead virus (right panel) into bilateral B16.SIY brain tumors in the same mouse. Top = brightfield, bottom = Texas Red filter. All images taken using 10x objective, 100x total magnification.
Figure 3.7

Pre-incubation of B16.SIY cancer cells with MYXV-Red prior to infusion into the brain results in a modest increase in survival. Cells were incubated with virus at a MOI=1 for 1 hr, washed, and prepared for injection. 2 x 10^3 pre-infected or mock-infected cells, or a 1:1 mixture of both (2 x 10^3 total), were infused into the brains of RAG1^-/- mice. Three mice per group were followed for survival. Mice that were injected with 2 x 10^3 cells that were either 50% or 100% pre-infected with MYX-Red lived significantly longer than mice injected with 2 x 10^3 mock-infected cells (18 and 20 vs. 15 days respectively, p<0.05 for both by Log-rank test). Mice that received only 1 x 10^3 mock-infected cells were included for comparison to mice that received 2 x 10^3 cells that were 50% pre-infected to assess the ability of the virus to spread from infected cells to non-infected neighboring tumor cells. There was a small increase in survival of the 50% pre-infection group compared to the 1 x 10^3 mock group, but the difference was not statistically significant (18 vs. 16 days, respectively, p = 0.096). Numbers indicate median survival.
Figure 3.8

**Strong cellular immune response to intratumoral injection of live or UV-inactivated MYXV-Red.** Five day-old B16.SIY brain tumors growing in RAG1<sup>−/−</sup> mice were injected with live virus, UV-inactivated virus or PBS. 48 hours later, brains were collected, sectioned and stained for immune markers (CD11b on macrophages/microglia, GR-1 on neutrophils, Ly49G2 on NK cells). All tumor sections from mice injected with UV-inactivated virus stained similarly to those that received live virus. Only images with live virus are shown (A., C., and E.). Scale bar = 200 μm. Red= 1° Abs detected by Alexa594, Blue= DAPI. **A-B.** CD11b+ cell infiltration in B16.SIY brain tumors injected with **(A.)** MYX-Red and **(B.)** PBS. In all tumors, CD11b+ cells were found around the rim and scattered through the core, but were also found in massive numbers at the site of live or inactivated virus injection. (see right side of A.) **C-D.** Large numbers of GR-1+ neutrophils in tumors injected with **(C.)** MXYV-Red but not **(D.)** PBS. **E.** NK cells were present in low numbers in tumors injected with MYXV-Red but not PBS.
Figure 3.9

Intratumoral injection of MYXV-Red increases local concentrations of IFNβ and TNFα but not IFNα. Mice with eight-day-old B16.SIY brain tumors received intratumoral injections of MYX-Red, a mock preparation of the virus propagation cell line, or PBS. At 24 hours, tumors and surrounding brain tissue were excised, homogenized, and the supernatants were assessed using cytokine ELISAs. Horizontal lines indicate baseline response defined as cytokine release following intratumoral injection of PBS. P<0.05 by unpaired t test. Data from two separate experiments were pooled. 3 samples per group (MYXV, mock, PBS), two brains pooled per sample and normalized by tissue weight.
Figure 3.10
MYXV infection remains restricted to the tumor and does not spread to normal brain following injection of neutralizing antibodies against IFNβ. Mice with seven day-established B16.SIY brain tumors were injected intratumorally with MYXV-Red. Mice received intraperitoneal (i.p.) injections of neutralizing antibodies at the same time for two consecutive days. Some mice were also injected intratumorally (i.t.) with anti-IFNβ on day seven. Two or four days following treatment, brains were collected, fixed, and sectioned. A. Two days following i.p. injection of anti-IFNβ. Normal brain at periphery of image where DAPI-stained nuclei are less dense does not express Tomato Red protein. B. Four days following i.p. injection of anti-IFNβ. Normal brain is not infected and approximately 20% of tumor is infected (expressing Tomato red protein). Scale bar = 200 μm. Three mice were analyzed per group. Intratumoral administration of neutralizing antibodies had similar results (data not shown).
Figure 3.11

Combination treatment of RAG1^−/− mice with B16.SIY brain tumors leads to a small but significant improvement in survival time. Mice with seven day-established brain tumors received activated 2C T cells i.v., intratumoral injection of MYXV-Red, and neutralizing antibodies (Abs) against IFNβ (single intratumoral and 4 days intraperitoneal injections). Mice receiving MYXV with or without anti-IFNβ Abs did not survive longer than untreated historical controls. Mice treated with MYXV, 2C T cells, and anti-IFNβ Abs lived significantly longer than mice that received only MYXV and 2C T cells (39 vs. 32 days respectively, p < 0.05 by Log-rank test). Data from two separate experiments was pooled. Numbers indicate median survival.
Infectious MYXV-Red virions can be recovered from B16.SIY brain tumors weeks after intratumoral injection. Brain tumors were excised when mice reached criteria for euthanasia. Tumor homogenates were titered on permissive RK-13 cells using a plaque forming assay. Each dot represents virus recovered from one mouse. The range of recovered virus is approximately 50-5000 times less than number of infectious particles originally injected. Data collected with Dr. Amy MacNeill.
Figure 3.13

**MYXV infection did not increase cross-presentation of SIY peptide on CD11b+ cells at 72 hrs post-injection.** Mice were injected in the brain with 1x10^4 B16-F10 or B16.SIY cancer cells. On day 7, 5x10^6 pfu MYX-Red or PBS were injected intratumorally. Three days later CD11b^+ cells were isolated from dissociated tumor tissue using magnetic beads labeled with anti-CD11b antibodies. CD11b^+ cells were stained with anti-CD11b antibody and multibirotinylated 2C-m67 TCR monomers that recognize SIY/K^b^. Monomers were detected with streptavidin-allophycocyanin. Events are gated on live cells on the basis of propidium iodide exclusion. Dead cells were not characterized. Two samples per treatment, two brains pooled per sample.

(A.) Infection of B16.SIY brain tumors with MYXV-Red did not increase SIY peptide cross-presentation on tumor CD11b^+ stromal cells at 72 hrs compared to PBS injected controls (C.). B16-F10 tumors were included as a control for non-specific binding (B.).
CHAPTER 4. SUMMARY AND CONCLUSIONS

Adoptive T cell therapy following lymphodepletion is currently the most successful treatment for patients with metastatic melanoma and can mediate tumor regression in over 50% of patients, including those with brain metastases\(^1\). Adoptive therapy for patients with glioblastoma has been less successful and future clinical trials should be designed to include some of the important criteria learned from (1) results of adoptive therapy trials for metastatic melanoma and (2) preclinical animal studies that demonstrate effective ways to prevent immune editing of tumors by antigen-specific T cells.

For example, patients with gliomas may require pre-conditioning with non-myeloablative chemotherapy or total body irradiation prior to adoptive therapy. Transferred T cells should be highly reactive and CD4\(^+\) regulatory T cells (T\(_{\text{regs}}\)) should be actively excluded. Furthermore, successful therapies will likely need to target multiple tumor antigens as well as tumor stromal cells that shelter ALV cells. Inclusion of CD4\(^+\) T cells that can target MHC class II restricted tumor peptides on tumor stromal cells may be important in addition to CD8\(^+\) effector T cells. Information on glioma-associated antigens was previously lacking but these peptides can now be used to generate highly reactive antigen-specific T cells. The field is moving towards identifying TCR genes specific for glioma antigens and viral transduction of autologous cells for adoptive transfer may be a possibility in the near future. Finally, multimodal therapies will likely be critical to prevent tumor escape.

In the present studies, we evaluated the effects of 2C T cell adoptive therapy for MC57-SIY-Hi and SIY-Lo mouse brain tumors growing in lymphopenic RAG1\(^{-/-}\) mice to mimic the condition of lymphodepleted patients. Activated 2C T cells could kill SIY-Hi and SIY-Lo cancer cells equally well \textit{in vitro} and adoptively transferred 2C T cells were able to rapidly...
eliminate both SIY-Hi and SIY-Lo established brain tumors. The transferred T cells were naïve and required *in vivo* activation with a subcutaneous cell vaccine to gain effector function.

Similar to the clinical situation, tumor recurrences were frequent and resulted from immune editing of tumors and not the lack of persistence by the transferred T cells. T cell therapy led to the elimination of antigen-expressing tumor cells, but was only effective for eliminating antigen loss variant (ALV) cancer cells in certain cases.

Our experiments demonstrated that the level of tumor antigen present affects the success of adoptive T cell therapy with regard to ALV cells. With high levels of antigen, tumor stromal cells such as microglia/macrophages present tumor peptide on their surface. As a result, T cells directly eliminate cancer cells and probably kill cross-presenting stromal cells as well. This was observed even in tumors in which there was no direct recognition of the tumor cells, yet adoptive T cell therapy led to the rejection of antigen-negative tumors and cured the mice. While the exact mechanism by which stromal cells are mediating this effect in the brain is still unknown, T cell therapy for tumors expressing high levels of antigen did lead to the indirect elimination of ALV cells and cured mice with established tumors. Much debate has surrounded the issue of whether brain tumor associated macrophages and microglia are capable of enhancing CD8⁺ T cell responses against brain tumors by cross-presenting MHC I restricted tumor peptides. We were able to show the first direct evidence of tumor antigen cross-presentation on CD11b⁺ stromal cells in brain tumors using soluble high affinity single-chain T cell receptor monomers.

While patients are certainly unable to choose to have high expression of a tumor antigen, these findings suggest that strategies that target brain tumor stroma or increase antigen shedding from tumor cells to enhance cross-presentation may improve the clinical success of T cell adoptive therapies. Both chemotherapy and irradiation are proven ways to induce local necrosis
and antigen shedding leading to enhanced cross-presentation and are already approved treatments for brain tumors. An appropriately timed transfer of tumor-specific T cells could potentially be much more successful in combination with these therapies. In support of this, treatment of mice with B16-F10 melanoma tumors with cyclophosphamide can lead to the release of tumor peptide and improve CD8+ cell cytotoxicity while also depleting Tregs from the tumor. In terms of combining multiple treatment modalities, cyclophosphamide could also eliminate innate immune cells responsible for clearing oncolytic viruses, making all of these therapies complementary.

We also characterized the effect of 2C T cell adoptive therapy on aggressive B16.SIY melanoma brain tumors. Like many cancer cells, B16.SIY cells express very low levels of MHC I and 2C T cells have limited cytotoxic effects in vitro. It was unknown if adoptive therapy would have any effect in an in vivo tumor model. Impressively, strong antigen-specific responses by 2C T cells were observed suggesting that activated T cells produce sufficient amounts of IFNγ to upregulate surface levels of MHC I. Alternatively, RAG1−/− mice do have NK cells that can produce IFNγ, and we have not ruled out this possibility or a synergistic effect of NK and 2C T cells. Furthermore, the preparation of cells for adoptive transfer did not exclude CD4+ T cells, another possible source of IFNγ (discussed more below).

This fast-growing tumor model provides a very brief therapeutic window of opportunity. Adoptive therapy led to more than a two-fold increase in survival time and similar to the MC57 model, ALV cells repopulated the tumors leading to death. 2C T cells persisted in the mice, but could no longer recognize the tumors. We did detect a small percentage of CD11b+ cells in the tumors that were cross-presenting SIY peptide on day 10, but this was in mice that did not
receive 2C T cells. 2C T cell killing may release additional peptide available for cross-presentation.

We have not determined why MC57-SIY-Hi brain tumors are cured by 2C T cell adoptive transfer while B16.SIY brain tumors are not. Based on levels of GFP expression that should be directly equivalent to levels of SIY expression, B16.SIY cells express similar if not higher levels of SIY than MC57-SIY-Hi cells. However, we have not directly compared surface levels of SIY/K\textsuperscript{b} on these cell lines following IFN\textgreek{y} treatment. There are several possible reasons for the survival difference, including the observation that activated 2C T cells express the inhibitory programmed death receptor PD-1 and upon stimulation with IFN\textgreek{y}, B16.SIY cells upregulate its ligand PD-L1. It would be valuable to determine if PD-1 inhibition improved the antitumor response by 2C T cells. We have also not characterized whether MC57 cancer cells express PD-L1. Another potential reason that MC57-SIY-Hi tumors are easier to cure following adoptive 2C T cell transfer may be related to minor histocompatibility antigens. MC57 cell lines are rejected by wild-type C57BL/6 mice without any intervention, but they do grow out in lymphopenic RAG1\textsuperscript{-/-} mice. We adoptively transfer a mixed population of cells including some CD8\textsuperscript{+} T cells that do not express the 2C TCR and instead express an endogenous TCR. These cells may be able to eliminate MC57-SIY-Hi cells but not B16.SIY cells based on these antigens.

It would be simple to conclude from these experiments that activated CD8\textsuperscript{+} tumor-specific T cells are solely responsible for the antitumor responses we observed in both models. However, careful consideration must be given to the population of T cells adoptively transferred into these mice. Transferred cells were isolated from the lymph nodes of 2C TCR transgenic mice and activated \textit{in vitro} with SIY peptide and cytokines for 48 hours prior to injection. We characterized the activated transfer population and identified approximately 50% of the cells as
expressing the 2C TCR and CD8. The remaining cells were approximately 5-10% CD4+ T cells (half of which are CD25+), 25% B cells, 4% NK cells and 6% CD11b+ cells (unpublished data). Furthermore, approximately 50% of the 2C T cells expressed the inhibitory receptor PD-1. Additional characterization of the critical components of the antitumor responses we observed is important.

We explored one potential mechanism to enhance T cell elimination of brain tumors by characterizing the oncolytic effects of myxoma virus in combination with adoptive therapy. Oncolytic virotherapy, while tumor-specific, is not antigen-restricted. For example, productive infection by myxoma virus should not discriminate between antigen-positive or antigen-negative cancer cells, or even cancer stem cells, although this needs to be explored further.

Myxoma virus (MYXV) is a rabbit poxvirus with strict species tropism for European rabbits. MYXV can also infect mouse and human cancer cell lines due to signaling defects in innate antiviral mechanisms and hyperphosphorylation of Akt5,6. MYXV demonstrated impressive killing of human glioma cell lines in mouse xenograft brain tumors7. Following injection of the same number of plaque forming units of MYXV into syngeneic B16.SIY brain tumors, we observed tumor-specific but abortive infection and evidence of immune clearance of the virus. The combination of T cell adoptive transfer and myxoma virus injection resulted in a small but significant increase in survival time, but neutralization of the cytokine IFNα was required. In the xenograft models, viral titer peaked two weeks after injection in the tumors and was detectable for more than 40 days. We were also able to detect infectious virus particles in the brain several weeks after injection, but at very low titers. Viral gene expression peaked at 48 to 72 hours post-injection and then decreased rapidly. We hypothesize that the difference in xenograft versus syngeneic graft tumor models may relate to the unresponsiveness of human
cancer cells to mouse Type I interferons produced in the mouse brain. We are planning experiments to test this hypothesis.

Interestingly, many oncolytic viruses approved for clinical trials are not tested in syngeneic tumors growing in immune-competent animals prior to use in patients. Given the sporadic objective responses in clinical trials of patients with malignant brain tumors thus far, and the allocation of millions of dollars for conducting these trials, this could perhaps be an important checkpoint for evaluating immunogenicity and improving efficacy preclinically.

There are many potential ways to enhance the oncolytic efficacy of MYXV and other oncolytic viruses in brain and peripheral tumors. Based on the results of our experiments, two obvious strategies would be to enhance viral replication in infected tumor cells and limit the initial clearance of the virus by transiently depleting neutrophils and macrophages. Rapamycin has been shown to enhance viral replication in cancer cells\textsuperscript{6,8-11} and could help prevent abortive infections \textit{in vivo}. For example, we have injected MYXV-Red into mice with syngeneic GL261 glioma brain tumors and expression of Tomato red protein is detectable in these tumors only after mice have been pretreated with rapamycin (unpublished data). Rapamycin (also known as sirolimus) and rapamycin-derivatives are currently being evaluated in 159 different clinical trials for the treatment of many cancers (www.clinicaltrials.gov, accessed 11/22/09), and if approved, would potentially complement oncolytic virus strategies. Neutrophils are likely responsible for phagocytosing extracellular virus and clearing it before it can be internalized by tumor cells. Transient host neutrophil depletion using anti-GR-1 antibodies has been shown to improve viral spreading in tumors\textsuperscript{12} and should be characterized in our tumor models prior to administration of MYXV-Red.
Multi-modal combination therapies for brain tumors will likely require innate and adaptive immune participation. Due to their impressive degree of tumor-specificity, oncolytic viruses such as myxoma virus may be ideal vectors for delivering therapeutic genes encoding additional tumor antigens for cross-presentation on tumor stromal cells or NK cell activating ligands (UL-16BP, MICA, Rae-1b/H-60 ligands). Our laboratory is currently engineering a recombinant MYXV encoding mouse IL-15 and its receptor subunit IL-15Ra. In addition to the effects of IL-15 on improving antitumor responses by CD8+ T cells, IL-15 can recruit and activate NK cells that may be able to help eliminate ALV cancer cells. It would also be interesting to see if MYXV delivery of additional SIY peptide to a B16.SIY brain tumor would produce similar effects (cures) as seen in the MC57-SIY-Hi model due to cross-presentation.

Tumor-specificity is an important requirement of an ideal brain tumor therapy due to low cell turn-over in the brain. Myxoma virus has demonstrated robust, tumor-specific gene delivery in established brain tumors. Combined with the antigen-specificity of adoptively transferred T cells, this combination treatment should be further explored for treatment of malignant brain tumors.
References

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