# Validating transgenic Farmington viruses for the treatment of glioblastoma multiforme

by

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**ABSTRACT**

Glioblastoma is the most common primary brain tumour in adults. Despite the aggressive standard of care currently used, median patient survival following treatment is only 14 months. Innovative treatment options are needed for these patients. Recently, oncolytic viruses have emerged as promising immunotherapies for the treatment of solid tumours. Preliminary work in our lab has demonstrated that Farmington virus, a novel brain-safe oncolytic rhabdovirus, can be engineered to encode a tumour-associated antigen (TAA) to prime and boost antigen-specific adaptive immune responses. Since other rhabdoviruses share this boosting capacity, a heterologous rhabdovirus prime/boost regimen can be designed to combine two powerful oncolytics and a robust anti-TAA adaptive immune response. We evaluated Farmington's ability to vaccinate against a self-glioblastoma antigen and two foreign glioblastoma-associated antigens. Farmington was able to vaccinate against the foreign antigens, leading to efficacy in prophylactic and therapeutic glioblastoma models. Additionally, treatment with heterologous rhabdoviruses demonstrated efficacy in an aggressive murine mammary carcinoma model. Herein, we demonstrate promising preliminary results for a novel glioblastoma therapeutic approach.

Le glioblastome est la tumeur primaire la plus fréquente chez l'adulte. La survie moyenne des patients n'excède pas 14 mois malgré une prise en charge thérapeutique agressive. Par conséquent, la mise au point de traitements innovants et efficaces est une nécessité pour ces patients. Des avancées récentes ont mise en évidence l'intérêt des virus oncolytiques dans le traitement des tumeurs solides. Des travaux préliminaires réalisés au sein de notre laboratoire ont, en effet, démontré que le virus Farmington pouvait être modifié afin d'exprimer un antigène associé aux tumeurs (AAT), pour initier et potentialiser une réponse immunitaire adaptative spécifique. D'autres rhabdovirus possèdent des capacités de potentialisation immunitaire similaires et peuvent être utilisés en association avec le virus Farmington modifié pour amorcer et amplifier la réponse immunitaire oncolytique de l'hôte. Le but de ce projet était d'évaluer le potentiel du virus Farmington comme vaccin contre des antigènes tumoraux d'origine endogène ou exogène associés au glioblastome. Nos résultats ont montré que le virus Farmington a la capacité d'induire une réponse immunitaire prophylactique et thérapeutique contre les antigènes tumoraux exogènes dans des modèles de glioblastome. De plus, l'utilisation de rhabdovirus hétérologues s'est aussi révélée efficace pour le traitement de carcinome mammaire agressif chez la souris. Cette étude préliminaire apporte des résultats prometteurs pour le développement de nouvelles approches thérapeutiques efficaces dans le traitement du glioblastome.

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**TABLE OF CONTENTS**

**ABSTRACT** ..... **ii**

**ACKNOWLEDGEMENTS** ..... **iii**

**LIST OF ABBREVIATIONS** ..... **vi**

**LIST OF FIGURES** ..... **x**

**LIST OF TABLES** ..... **xii**

**1 INTRODUCTION** ..... **1**

1.1 MALIGNANT BRAIN TUMOURS ..... 1

*1.1.1 Epidemiology of glioblastoma* ..... *1*

*1.1.2 Current treatment of glioblastoma* ..... *3*

1.2 EXPERIMENTAL TREATMENTS FOR GLIOBLASTOMA ..... 5

*1.2.1 Vaccination against GBM* ..... *7*

Epidermal growth factor receptor variant III (EGFRvIII)-targeted vaccines ..... 7

Tumour lysate-pulsed DC vaccines ..... 8

Tumour stem cell vaccines ..... 10

Human cytomegalovirus (hCMV)-targeted vaccines ..... 11

Survivin vaccines ..... 14

Concluding remarks ..... 16

*1.2.2 Traditional oncolytic virus treatment for glioblastoma* ..... *17*

Herpes Simplex Virus 1 (HSV1) ..... 18

Adenovirus (AdV) ..... 19

Newcastle Disease Virus (NDV) ..... 20

Reovirus ..... 20

Poliovirus ..... 21

Measles virus (MV) ..... 21

Parvovirus H-1 ..... 22

Concluding remarks ..... 23

1.3 A NOVEL COMBINATORIAL TREATMENT APPROACH FOR GLIOBLASTOMA ..... 23

*1.3.1 Oncolytic rhabdoviruses* ..... *23*

*1.3.2 Oncolytic rhabdoviruses as vaccine vectors* ..... *25*

*1.3.3 Rationale and hypothesis* ..... *27*

**2 MATERIALS AND METHODS** ..... **31**

2.1 CELL CULTURE ..... 31

2.2 MICE AND CELL LINES ..... 31

2.3 VIRUSES ..... 32

2.4 MOUSE VACCINATION ..... 33

2.5 PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) ISOLATION ..... 33

2.6 PEPTIDES ..... 34

2.7 ANTIBODIES ..... 34

2.8 DETECTION OF ANTIGEN-SPECIFIC T CELL RESPONSE ..... 34

2.9 SUBCUTANEOUS 4T1 CELL IMPLANTATION IN MICE ..... 35

2.10 TRANSGENIC CELL LINES ..... 36

2.11 SUBCUTANEOUS CT2A IMPLANTATION IN MICE ..... 36

2.12 INTRACRANIAL CT2A IMPLANTATION IN MICE ..... 36

2.13 MAGNETIC RESONANCE IMAGING (MRI) OF MICE ..... 37

2.14 VIRUS TREATMENT OF INTRACRANIAL TUMOUR BEARING MICE ..... 38

2.15 STATISTICAL ANALYSIS ..... 38

**3 RESULTS** ..... **39**

3.1 TESTING THE ABILITY OF TRANSGENIC FMT VIRUSES EXPRESSING SELF TUMOUR-ASSOCIATED ANTIGENS (TAAS) TO GENERATE TAA-SPECIFIC CD8+ T CELL RESPONSES IN NAÏVE MICE....39
*3.1.1 Vaccination against wild-type (wt) murine survivin in naïve mice....39*
*3.1.2 Vaccination against an inactive mutant of survivin in naïve mice....40*
3.2 EVALUATING THE THERAPEUTIC AND IMMUNOLOGICAL CAPABILITIES OF FMT VIRUS EXPRESSING A SELF-TUMOUR-ASSOCIATED ANTIGEN (TAAS) IN TUMOUR-BEARING MICE....48
*3.2.1 A murine mammary carcinoma model chosen to evaluate anti-survivin responses....49*
*3.2.2 Anti-survivin rhabdoviral prime/boost regimens control tumour progression and metastasis in the 4T1 model....52*
*3.2.3 Standalone heterologous rhabdovirus treatment demonstrates efficacy in the 4T1 model....62*
3.3 INVESTIGATING THE ABILITY OF FMT VIRUSES EXPRESSING FOREIGN TUMOUR-ASSOCIATED ANTIGENS (TAAS) TO GENERATE PROPHYLATIC OR THERAPEUTIC TAA-SPECIFIC CD8+ T CELL RESPONSES....67
*3.3.1 Vaccination against mCMV antigens in naïve mice....68*
*3.3.2 Prophylactic mCMV antigen-based vaccination against tumour challenge....72*
*3.3.3 Therapeutic mCMV antigen-based vaccination against GBM tumours....78*
*3.3.4 Evaluating the efficacy of a therapeutic prime/boost using m38 as a target TAA to treat GBM....85*
*3.3.5 Comparing the efficacy of FMT-m38 vs. FMT-eGFP and evaluating different viral delivery routes in the CT2A-m38 model....94*
**4 DISCUSSION....99**
4.1 SURVIVIN VACCINATION IN NAÏVE MICE....100
4.2 SURVIVIN VACCINATION IN A TUMOUR MODEL....109
4.3 TARGETING A FOREIGN GLIOBLASTOMA-ASSOCIATED ANTIGEN....118
4.4 POTENTIAL FUTURE DIRECTIONS....130
4.5 CONCLUDING REMARKS....131
**REFERENCES....133**
**CONTRIBUTION OF COLLABORATORS....148**
**APPENDIX....149**
SUPPLEMENTARY FIGURES....149

## LIST OF ABBREVIATIONS

| | |
|---|---|
| Ad | replication-deficient human serotype 5 Adenovirus vector |
| Ad-(h)DCT | Ad encoding human dopachrome tautomerase |
| Ad-IE3 | Ad encoding mCMV IE3 protein |
| Ad-m38 | Ad encoding mCMV m38 protein |
| Ad-OVA | Ad encoding chicken ovalbumin |
| Ad-SurvGFP | Ad encoding mouse survivin fused to green fluorescent protein |
| Ad-TAA | Ad encoding a tumour-associated antigen |
| AdV | replication competent Adenovirus |
| AICD | activation-induced cell death |
| APC | antigen presenting cell |
| BMDC | bone marrow-derived dendritic cells |
| CAR | chimeric antigen receptor |
| CBTRUS | Central Brain Tumour Registry of the United States |
| CEA | carcinoembryonic antigen |
| CMV | cytomegalovirus |
| CNS | central nervous system |
| cRPMI media | complete Roswell Park Memorial Institute media |
| CT | computed tomography |
| CTL | cytolytic T cell |
| CTLA-4 | cytotoxic T-lymphocyte-associated protein 4 |
| DAMP | danger associated molecular pattern |
| DC | dendritic cells |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DNA | deoxyribonucleic acid |
| EGFR | epidermal growth factor receptor |
| ELISPOT | enzyme-linked immunospot assay |
| EORTC/NCIC CTG | The European Organisation for Research and Treatment of Cancer and the NCIC Clinical Trials Group |
| FACS | flow cytometry analysis |
| FBS | fetal bovine serum |

| | |
|---|---|
| FCS | fetal calf serum |
| FMT | Farmington virus |
| FMT-eGFP | Farmington virus encoding enhanced green fluorescent protein |
| FMT-IE3 | Farmington virus encoding mCMV IE3 protein |
| FMT-m38 | Farmington virus encoding mCMV m38 protein |
| FMT-OVA | Farmington virus encoding chicken ovalbumin |
| FMT-Surv | Farmington virus encoding wt murine survivin protein |
| FMT-TAA | Farmingon virus encoding a tumour-associated antigen |
| FMT-T48A | Farmington virus encoding the mutant form of survivin T48A |
| GAA | glioma-associated antigen |
| GBM | glioblastoma multiforme |
| G-CSF | granulocyte-colony stimulating factor |
| HBSS | Hank's balanced salt solution |
| hCMV | human cytomegalovirus |
| HLA | human leukocyte antigen |
| HRV2 | human rhinovirus type 2 |
| HSV1 | Herpes Simplex virus 1 |
| H-1PV | Parvovirus H-1 |
| IAP | inhibitor of apoptosis family |
| IC | intracranial(ly) |
| ICS | intracellular cytokine staining |
| IM | intramuscular(ly) |
| IRES | internal ribosomal entry site |
| IV | intravenous(ly) |
| IFN(y/α/β) | interferon, gamma or alpha or beta |
| IL-# | interleukin-# |
| KC | chemokine (C-X-C motif) ligand 1 (CXCL1) |
| LCMV | lymphocytic choriomeningitis virus |
| mCMV | murine cytomegalovirus |
| MCP-1 | monocyte chemoattractant protein-1 |
| M-CSF | macrophage-colony stimulating factor |

| | |
|---|---|
| MDSC | myeloid derived suppressor cell |
| MG1 | Maraba MG1 virus |
| MG1-(h)DCT | Maraba MG1 virus encoding human dopachrome tautomerase |
| MG1-OVA | Maraba MG1 virus encoding chicken ovalbumin |
| MG1-Surv | Maraba MG1 virus encoding wt murine survivin protein |
| MG1-TAA | Maraba MG1 virus encoding a tumour-associated antigen |
| MHC (I/II) | major histocompatibility complex, class I or class II |
| MIP-1(α/β) | macrophage inflammatory protein I |
| MOI | multiplicity of infection |
| MRB | Maraba virus |
| MRB-MG1 | Maraba MG1 virus |
| MRI | magnetic resonance imaging |
| MTD | maximum tolerated dose |
| MV-(Edm) | Measles virus, Edmonston strain |
| NDV | New Castle Disease virus |
| NLS | nuclear localization signal |
| ns | not significant |
| OS | overall survival |
| OV | oncolytic virus |
| OVA | chicken ovalbumin protein |
| OVT | oncolytic virus therapy |
| PBMC | peripheral blood mononuclear cell |
| PBS | phosphate buffered saline |
| PD-1 | programmed cell death protein 1 |
| PDGFR | platelet-derived growth factor receptor |
| PFS | progression free survival |
| PFU | plaque forming units |
| PKR | protein kinase RNA-activated |
| PVS-RIPO | Poliovirus with HRV2 IRES |
| RNA | ribonucleic acid |
| Rb | retinoblastoma |

| | |
|---|---|
| SubQ | subcutaneous |
| Surv | wildtype survivin |
| Surv-T48A | mutant survivin with threonine at position 48 replaced by alanine |
| TAA | tumour-associated antigen |
| TCR | T cell receptor |
| TCID50 | tissue culture infectious dose 50 |
| Td | tetanus/diphtheria toxoid |
| TMZ | Temozolomide |
| TNF-α | tumour necrosis factor alpha |
| T48A | mutant survivin with threonine at position 48 replaced by alanine |
| qPCR | quantitative polymerase chain reaction |
| VSV | vesicular stomatitis virus |
| VSVΔ51 | VSV containing a deletion in methionine 51 in the viral genome |
| VSV-(h)DCT | VSV encoding human dopachrome tautomerase |
| WHO | World Health Organization |
| WT | wildtype |

## LIST OF FIGURES

Figure 1. FMT can prime robust CD8+ T cell responses against a foreign antigen, but not against a self-antigen.....41

Figure 2. FMT can prime robust CD8+ T cell responses against OVA, but not survivin T48A....44

Figure 3. Anti-survivin CD8+ T cell responses are not detected when using Ad-SurvGFP as a priming agent in a heterologous prime/boost vaccination regimen.....46

Figure 4. The 4T1 murine mammary carcinoma cell line expresses survivin and is susceptible to oncolysis by MG1, but not FMT virus.....50

Figure 5. Experimental timeline comparing the efficacy of different anti-survivin heterologous prime/boost vaccination regimens in a murine mammary carcinoma model.....53

Figure 6. Heterologous rhabdovirus prime/boost regimens control 4T1 tumour burden.....55

Figure 7. Tumours treated with MG1-T48A prime/ FMT-T48A boost appear necrotic.....57

Figure 8. Heterologous rhabdovirus prime/boost regimens significantly reduce 4T1 lung metastases.....60

Figure 9. Experimental timeline comparing the efficacy of non-TAA-encoding, heterologous dual oncolytic treatments in a murine mammary carcinoma model.....63

Figure 10. Non-TAA-encoding heterologous rhabdovirus treatments significantly control 4T1 tumour burden and lung metastases.....65

Figure 11. Ad-mCMV can prime anti-mCMV CD8+ T cell responses and FMT-mCMV can act as both a priming and a boosting agent against mCMV antigens.....70

Figure 12. Ad-mCMV prime/FMT-mCMV boost regimens generate robust CD8+ T cell responses against IE3 and m38 mCMV proteins.....73

Figure 13. Mice vaccinated against m38 control subcutaneous CT2A-m38 tumour progression, but IE3-vaccinated mice do not control CT2A-IE3 tumours.....75

Figure 14. Experimental timeline comparing wt CT2A vs. mCMV CT2A intracranial tumour progression.....79

Figure 15. MRI images of wt CT2A and CT2A-m38 intracranial tumours at day 10 post-tumour seed demonstrate slightly increased progression of CT2A-m38 tumours.....81

Figure 16. A single IV dose of FMT-m38 generates a greater anti-m38 CD8+ T cell response in CT2A-m38 tumour-bearing mice compared to wtCT2A tumour-bearing mice.....83

Figure 17. MRI imaging 7 days post-virus treatment reveals large, brightly defined tumours in wt CT2A mice and dark, undefined tumours in CT2A-m38 mice.....86

Figure 18. Ad-m38 prime significantly extends survival in an intracranial CT2A-m38 model.....89

Figure 19. Prime/boost vaccination strategies generate variable anti-m38 responses in intracranial CT2A-m38 tumour-bearing animals.....92

Figure 20. Treatment with a single dose of FMT-m38 delivered IC or IV significantly extends survival in an intracranial CT2A-m38 model ..... 95

Figure S1. Survivin is highly expressed in CT2A and GL261 GBM cells and TAA encoding rhabdoviruses.....149

Figure S2. Inactive survivin T48A mutant is highly expressed in TAA encoding rhabdoviruses.....149

Figure S3. Splenocytes isolated from 4T1 tumour-bearing mice treated with MG1-T48A prime/ FMT-T48A boost kill significantly more 4T1 cells *in vitro* compared to splenocytes from untreated tumour-bearing mice.....150

Figure S4. Frequencies of survivin-specific CD8+ T cells are similar between treatment groups in the 4T1 model.....151

Figure S5. CD11b+ myeloid cells isolated from 4T1 tumours treated with MG1-T48A prime/ FMT-T48A boost appear to express higher levels of co-stimulatory molecules compared to myeloid cells isolated from tumours of untreated mice.....151

Figure S6. 4T1 tumour-bearing mice treated with MG1-T48A prime/FMT-T48A boost and FMT-T48A prime/MG1-T48A boost appear to have more CD4+ and CD8+ T cells in their tumours when compared to untreated mice.....152

Figure S7. Replicating Farmington virus is detected in the brain and the spleen post-intracranial and post-intravenous injection.....153

Figure S8. Intracranial injection of FMT-eGFP causes the upregulation of important type I inflammatory chemokines and cytokine detectable 48 hours post injection in the tumour-bearing hemisphere of the brain.....154

## LIST OF TABLES

Table 1. Peptides used in detection of antigen-specific T cell responses.....34

# 1 INTRODUCTION

## 1.1 Malignant brain tumours

### *1.1.1 Epidemiology of glioblastoma*

Twenty seven Canadians are diagnosed with a brain tumour every day (1). The Brain Tumour Foundation of Canada estimates that 55,000 Canadians are currently living with a brain tumour. Common symptoms of brain cancer are headaches, seizures, neurological deficits, confusion, memory loss and personality changes (2). These symptoms can drastically affect a patient's cognitive abilities and quality of life. Brain tumours are diagnosed with magnetic resonance imaging (MRI) or computed tomography (CT). On average, patients with brain cancer will make 52 visits to their health care team in the first year after their diagnosis. Brain cancer patients require access to high quality special care, clinical trials, follow-up care and rehabilitative services. This is not only a burden on our health care system but is also a burden on patients and their families.

The Central Brain Tumour Registry of the United States (CBTRUS) indicates that the annual incidence rates for malignant primary brain and central nervous system (CNS) tumours in the United States is ~7/100,000 (3). This translates to ~14,000 new cases of malignant primary brain and central nervous system (CNS) tumours diagnosed in the US each year. Currently, the Canadian medical system does not have a standard nationwide registry to accurately record complete information on primary brain tumours. However, a plan to develop a Canadian Brain Tumour Registry with a standardized system to gather brain tumour statistics has recently been put into development.

Glioblastoma multiforme (GBM) is the most advanced stage of brain cancer with the worst prognosis and outcome for patients. It accounts for 45.2% of all malignant brain tumours, translating to an annual incidence rate of 3/100,000. GBM occurs twice as frequently in men than women, and the age range with peak incidence rates is 45 to 75 years. Glioblastomas are 2-3 times more likely to occur in Caucasians than African Americans and they ultimately recur in 90% of patients. GBM is classified by the World Health Organization (WHO) as a grade IV astrocytoma (2, 4). Invasive, heterogeneous GBM tumours are derived from glial cells, and are characterized by increased cellularity, abnormal nuclei, active division, microvascular proliferation and regions of necrosis. The progressive accumulation of genetic mutations and the dysregulation of growth factor signaling pathways leads to these malignant transformations (2).

GBMs can be classified into 2 subtypes of cancers arising directly in the brain: primary and secondary, based on biological and genetic differences (5, 6). Primary GBMs occur most commonly in patients over the age of 50 and are characterized by epidermal growth factor receptor (EGFR) amplification/mutation, deletion of the phosphatase and tensin homologue on chromosome 10 (PTEN), loss of heterozygosity of chromosome 10q and p16 deletion. Far less common than primary GBMs, secondary GBMs occur in younger patients and start off as lower-grade astrocytomas that progress over time into malignant glioblastomas. Loss of heterozygosity of chromosome 10q, mutations in the p53 tumor-suppressor gene, overexpression of platelet-derived growth factor receptor (PDGFR) and mutations in the p16 and retinoblastoma (Rb) pathways characterize secondary GBMs (2, 5, 6). Despite the apparent genetic difference between

subtypes, primary and secondary GBMs are morphologically indistinguishable and have similar responses to current standard therapy.

### *1.1.2 Current treatment of glioblastoma*

Current standard therapy for GBM is maximal surgical resection, followed by radiotherapy with concomitant and adjuvant temozolomide (TMZ) (7). With surgery alone, patient survival is typically 3 to 4 months, but when radiotherapy is added, survival is increased to 7 to 12 months. In 2005, Stupp et al. reported in a EORTC/NCIC CTG phase III trial that radiotherapy plus concomitant and adjuvant TMZ significantly increased the survival rate of GBM patients compared to radiotherapy alone (median survival= 14.6 months vs 12.1 months; 2 year survival rate= 26.5% vs. 10.4%), with acceptable side-effects (8). This has now become standard-of-care for GBM patients.

The current standard of care for GBM patients is extremely difficult to tolerate. It comprises 60 Gy of partial-field external-beam irradiation delivered 5 days per week in increments of 1.8 to 2.0 Gy for 30 days. This is accompanied by TMZ given at 75 $mg/m^2$/day, 7 days per week from the first day of radiotherapy until the last day of radiotherapy (7). After a 4-week break, patients receive up to six cycles of adjuvant TMZ according to the standard 5-day schedule every 28 days. The dose starts at 150 $mg/m^2$ for the first cycle and is increased to 200 $mg/m^2$ beginning with the second cycle if there are no toxic hematologic effects. Patients may fail to complete this standard of care for GBM treatment because of thrombocytopenia, extreme myelosuppression, profound lymphopenia, early progression of their tumour, deterioration in quality of life or transfer to palliative care (9).

In a retroactive study performed on information gathered by Cancer Care Ontario

between 2004-2008, it was found that despite the institution of TMZ in an improved standard of care, the overall survival of patients in the entire cohort was only 9.7 months (9). In this study, only 9% of the cohort survived beyond 2 years and less than half the patients even completed the radiotherapy with concomitant and adjuvant TMZ. According to this study, the implementation of radiotherapy+TMZ as the standard of care did not significantly improve overall survival at 28 months follow up compared to the previous regimen of radiation alone (9).

The patients that benefited in the EORTC/NCIC CTG phase III trial for radiotherapy+TMZ reported by Stupp et al. (2005), were those with the best prognosis at the time of diagnosis; therefore it is unclear if patients with worse prognoses gain any benefit from this new treatment regimen (9). Only a fraction of GBM patients actually received the recommended standard of care (radiotherapy+TMZ) and improved outcome was only observed in the subset of patients who completed the high dose radiotherapy and the grueling concomitant and adjuvant TMZ (9). Lwin *et al.* (2013) claim the EORTC/NCIC CTG phase III trial for radiotherapy+TMZ was biased because the patients that showed an improved response lived longer, had longer progression free survival and ultimately survived long enough to get more cycles of TMZ (9).

In general, it has been found that patients benefit the most when maximal surgical resection is achieved; however studies have shown that is only achievable for as little as 18.5% of patients (7, 10) Patients with poor prognosis are more likely to receive only palliative or best supportive care, therefore recent therapeutic advances have not benefitted this subset of patients. In the context of recurrent GBM, where a treated tumour ultimately regrows, the benefit of radiotherapy is questionable and the response

rate to TMZ is only 5.4%(2). Since nearly all malignant gliomas eventually recur, the outlook for these patients is grim.

Thus, it is clear that the current standard of care is not benefiting the majority of GBM patients. Fewer than 3% of GBM patients are still alive 5 years after diagnosis (11). Venur et al. (2014) go as far as recommending GBM patients enroll in clinical trials whenever possible because the overall survival with standard care is so dismal (7). Some patients even refuse the standard therapy because it will greatly decrease their quality of life in the short time they have left. Some GBM patients even choose to undergo medically assisted suicide in order to die with dignity. The need for better treatments for GBM patients is painfully apparent.

### 1.2 Experimental treatments for glioblastoma

Immunotherapy represents an attractive experimental approach for the treatment of cancer. This refers to harnessing one or more aspects of the patients' immune system to fight against the tumour. It provides a cellular level of specificity unlike radiation or chemotherapy and it has the potential to generate long-term protection against recurrence. The efficacy of this therapeutic approach has recently been highlighted by the U.S. FDA approval of sipuleucel-T, a dendritic cell vaccine for castration-resistant prostate cancer, as well as ipilimumab, a humanized monoclonal antibody that blocks cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) to improve antitumor T-cell responses (12, 13). Other immunotherapies for cancer have also seen success in early trials, such as monoclonal antibody blockade of programmed death 1 (PD-1) and its ligand PD-L1 as well as cancer-specific bioengineered chimeric antigen-receptor (CAR) T cells (14, 15). The encouraging results from these studies have led to immunotherapy advances in the

field of brain cancer treatment.

For many years it was thought that the brain was an immunoprivileged organ, however there is increasing evidence supporting the immunocompetence of the CNS that also suggest it has dynamic interactions with the systemic immune system (15, 16) . For example, the blood-brain barrier becomes disrupted during septic encephalopathy and inflammatory tissue damage that is associated with brain tumours (17). This allows immune cells to enter the CNS. Microglia, macrophages and dendritic cells (DCs) expressing MHC class II and T-cell costimulatory receptors and cytokines act as powerful antigen-presenting cells (APCs) in the CNS (18-21). In addition, CNS antigens drain to nasal and cervical lymph nodes via cerebrospinal fluid where they may be presented to naive T cells by APCs in the lymph nodes (15, 16, 21). It has also been shown that the brain microenvironment helps antigen-specific T cells mature and acquire effector function as well as express specific integrins that enhance retention in the CNS (22). Very recently, functional lymphatic vessels lining the dural sinuses of the brain were discovered (23). This CNS lymphatic system was found to transport immune cells from the cerebral spinal fluid, further demonstrating the dynamic relationship between the CNS and the systemic immune system.

There are two main avenues of development for immunotherapy in neuro-oncology: passive and active. Passive immunotherapy refers to the direct exploitation of patients' existing cancer-specific immune cells. For example, adoptive transfer of *ex vivo* stimulated tumour-specific T cells isolated from a patient, or checkpoint inhibitor blockade used to relieve immunosuppression of existing tumour-specific T cells in patients (24). Active immunotherapy aims to stimulate or activate a patients' immune

system to generate a *de novo* cancer-specific response. A common way to accomplish this is via vaccination. For the purpose of this thesis, active immunization will be further discussed.

The two main types of vaccine that are currently being explored for the treatment of GBM are cell- and peptide-based (25)**.** In cell-based vaccination, autologous APCs (usually DCs) are loaded with tumour antigens using tumour cell lysates, peptides, proteins or transfection with antigen-expression vectors *in vitro*. These DCs are then expanded and injected back in to patients to stimulate tumour-specific T cell responses. Peptide-based vaccines are considered to be simpler because they do not require the *ex vivo* culturing of APCs and simply comprise direct injection of cancer-specific peptides or proteins plus immune adjuvants. Numerous studies of GBM-specific vaccines have been completed, and many more are underway.

### *1.2.1 Vaccination against GBM*

***Epidermal growth factor receptor variant III (EGFRvIII)-targeted vaccines***

Epidermal growth factor receptor (EGFR) plays a role in normal cellular migration, differentiation and apoptosis (26). EGFRvIII is a variant of the EGFR that contains a deletion mutation resulting in constitutive receptor activation that contributes to uncontrolled proliferation and malignancy (27, 28). EGFRvIII is expressed in ~30% of GBMs and this variant is not found in normal brain tissue, making it a valid target for vaccination (29, 30).

A peptide vaccine with synthetic EGFRvIII (rindopepimut, CDX-110) has been tested in phase I and phase II trials for newly diagnosed GBM (31-33). The vaccine was well tolerated and ~60% of patients mounted an immune response to the EGFRvIII

peptide. The median progression-free-survival (PFS) for these trials was 12.3-15.3 months from diagnosis and the median overall-survival (OS) was 24 months from diagnosis (33). Standard of care for GBM has been shown to have a median PFS of ~8 months and a median OS of 16-19 months from diagnosis (9). The results of these studies suggest that EGFRvIII-specific immune responses correlate with modestly improved clinical outcome. It was also found that lymphopenia resulting from concomitant TMZ did not impair the activity of rindopepimut. It is interesting to note that recurrent tumors were found to be histologically negative for EGVRvIII staining, suggesting effective eradication of EGFRvIII+ tumor cells, but also indicating immunological escape via antigenic loss (34). A double-blind, randomized phase III trial comparing vaccine plus standard of care vs. standard of care alone is currently under way (NCT01480479).

The safety and validity of using a peptide vaccine targeting a single GBM-specific antigen have been demonstrated by the EGFRvIII trials to date. There does unfortunately seem to be selective deletion of EGFRvIII from recurrent tumours, indicating this treatment is incapable of preventing recurrence. This treatment is also only effective for patients with EGFRvIII+ tumour cells. It is worth considering the extreme heterogeneity that characterizes GBM when choosing to vaccinate against a single tumour antigen, as this may prove ineffective for long-term survival.

***Tumour lysate-pulsed DC vaccines***

There are two significant advantages when using whole tumour lysates as an antigen source for therapeutic vaccination (15, 26). First, the lysate provides a broad antigen pool. Second, the pool of potential immunologic targets is patient-specific. This treatment also has the potential to be used for any GBM patient with a resectable tumour.

Tumor lysate vaccines are generated by feeding autologous DCs surface proteins associated with major histocompatibility complexes (MHC) isolated from resected tumours cultured *ex vivo* (26).

In 2005, Liau *et al*. published a phase I clinical trial investigating the safety and feasibility of using a tumour lysate pulsed-autologous DC vaccine to treat GBM (NCT00068510) (35). The vaccination was not associated with any dose-limiting toxicity or any serious side effects. Fifty percent of the patients showed activated tumour-specific T cells in peripheral blood and a small increase in PFS and OS was observed. This group also reported that systemic anti-tumour CTL responses generated by their vaccine did not necessarily translate to clinical responses or increased survival.

Currently, there are multiple phase II trials and one phase III trial ongoing for validating tumour lysate vaccines. There is a phase II trial from the group described above using immune adjuvants in conjunction with tumour lysate-pulsed DCs (NCT01204684). This same group is also conducting a phase III trial using vaccine in addition to standard of care radiation and TMZ (NCT00045968). A phase II trial completed by the Dartmouth-Hitchcock Medical Center matured loaded DCs with prostaglandin E2 (PGE2) and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) prior to injection directly into cervical lymph nodes (NCT00323115) (36). Patients were vaccinated after radiation and TMZ therapy. Fadul *et al.* found that ~50% of their patients had a systemic immune response to the vaccine but there was no statistically significant increase in PFS or OS. Interestingly, the majority of the responding patients were still alive after 2 years, whereas the nonresponders did not survive to the 2-year mark. Taken together, these data

support the feasibility and safety of tumour lysate DC vaccines as well as suggest they are capable of inducing tumour-specific immune responses.

Some downfalls for this therapeutic approach are the requirement of surgery for vaccine generation as well as the lengthy time period taken for vaccine preparation (15). Additionally, there is the possibility that whole tumour lysates might trigger autoimmune responses against normal cells. Dr. Hideho Okada's group has overcome this caveat by identifying several glioma-associated antigen (GAA)-derived cytotoxic T lymphocyte epitopes (37-42). They generated multi-peptide-specific DC vaccines that target more than one antigen to avoid immune escape while also preventing an autoimmune reaction. Other factors such as tumour bulk and immunosuppressive tumour microenvironment need to be considered when evaluating this therapy.

***Tumour stem cell vaccines***

Another attractive vaccination strategy is to target a specific malignant cell type; cancer stem cells (15, 25, 26). This cell subtype, identified in GBM, shows resistance to radiation and chemotherapy as well possessing the capacity for self-renewal and differentiation (43-45), (46, 47). Glioma stem cells have also been found to express higher levels of some tumor-associated antigens (TAAs) including HER2/neu, TRP-2, AIM-2, gp100, MAGE1 and IL13R$\alpha$2 (42, 48-50) .

In 2009, Xu *et al.* demonstrated that DCs loaded with cancer stem cell lysates, which originate from GBM neurospheres grown in neural stem cell permissive culture, produced greater T cell responses than whole tumour lysate loaded DCs (51). In 2012, Phuphanich *et al*. reported the results of a phase I clinical trial with ICT-107, a DC vaccine loaded with peptides from antigens known to be overexpressed on glioma stem

cells (HER2/neu, TRP-2, AIM-2, gp100, MAGE1 and IL13R$\alpha$2) (52). It was found that 33% of patients responded to the vaccine. Phuphanich *et al.* also found that PFS and OS was prolonged in newly diagnosed GBM patients whose pre-vaccine tumours expressed HER2/neu, AIM-2, MAGE1 and gp100. In patients who required a second tumour resection, they observed a decreased number of cancer stem cells in their tumour samples. This suggests that the vaccine successfully targeted this aggressive cell subtype within the tumour. Targeting cancer stem cells with vaccination could prove effective at increasing GBM sensitivity to radiation and TMZ by reducing this resistant population. A randomized, double blind phase II trial to evaluate the efficacy of ICT-107 is currently underway (NCT01280552).

***Human cytomegalovirus (hCMV)-targeted vaccines***

Human cytomegalovirus (hCMV, or human herpes virus-5) is a member of the subfamily *betaherpesviridae*. It has a ~230-kb double-stranded DNA genome that encodes approximately 200 proteins (53). In industrialized countries, 60-70% of the adult population is seropositive for hCMV, whereas the prevalence in emerging countries is almost 100% (54). Primary infection with hCMV is usually asymptomatic in otherwise healthy adults but a life-threatening active virus infection can occur in immunocompromised individuals, such as transplant and AIDS patients (55).

Human cytomegalovirus has been detected in a large proportion of GBMs in hCMV seropositive patients. In 2002, Cobbs *et al.* were the first to detect hCMV proteins in GBM patient samples by immunohistochemistry and *in situ*-hybridization. They also discovered that hCMV antigens were not detectable in normal brain tissue. Following this, several other groups surveyed GBM patient samples for hCMV proteins and hCMV

DNA and there was a discordance among results (56). In 2011, a consensus conference on the role of hCMV in GBM was held to decide the importance of hCMV in glioma patients. They came to the conclusion that there was enough positive evidence for detectable hCMV nucleic acids and proteins in most malignant gliomas to validate hCMV as an antigen present in some GBM patients. However, this is still quite controversial.

In terms of the biological significance of CMV antigens in GBM tumours, one study analyzed the effect of murine CMV (mCMV) infection in a mouse model that spontaneously develops high grade glioma (57). The authors found that mice infected with mCMV as pups to establish a latent infection demonstrated decreased survival and developed higher-grade gliomas compared to uninfected or mock-infected mice. This represents a useful animal model for examining the relationship between cytomegaloviruses and brain cancer.

In 2010, Lucas *et al*. also detected hCMV proteins in GBM patient samples: the pp65-tegument protein was detected in 51% of samples and the immediate-early protein IE1 was found in 16% (58). They also isolated pp65- and IE1-specific cytotoxic T cells from healthy seropositive donors and demonstrated that they were capable of lysing tumour cells infected with hCMV *in vitro*. Following this, two other groups demonstrated that pp65- and IE1-specific T cells could be isolated from hCMV seropositive GBM patients.

Ghazi *et al.* (2012) found that the precursor frequency of hCMV-specific CD8+ T cells was lower in GBM patients compared to healthy seropositive controls, but this could be rescued by expanding the T cells *in vitro* using APCs transduced with an adenoviral

vector encoding pp65 and IE1 (59). Crough *et al.* (2012) discovered that pp65- and IE1-specific T cells from GBM patients lacked polyfunctionality (impaired production of multiple cytokines and cytolytic function) in comparison to healthy seropositive donors (60). However, *ex vivo* stimulation with HLA-matched CMV peptides in combination with interleukin-2 rescued the polyfunctional response of patients' hCMV-specific T cells. They also injected one recurrent GBM patient with autologous, *ex vivo* expanded and activated hCMV-specific T cells with concomitant TMZ and this patient achieved 17 months progression-free survival (PFS). Importantly, this study showed that hCMV-specific T cell frequencies were not affected by previous or ongoing anti-cancer therapy. Together, these studies demonstrate the potential for targeting hCMV-positive GBM with hCMV-specific T cells.

A phaseI/II clinical trial performed by Duke University scientists evaluated an hCMV pp65 RNA loaded DC vaccine where randomized patients received preconditioning with tetanus/diphtheria (Td) toxoid or mature unpulsed DCs (NCT00639639) (61). Thirteen newly diagnosed GBM patients underwent standard radiation and concomitant TMZ therapy and then received preconditioning followed by vaccination after the first adjuvant TMZ cycle. Fifty percent of the patients from the Td cohort did not progress and survived over 40 months after diagnosis. This greatly exceeded the expected survival of GBM patients receiving standard therapy alone (16-19 months) (8). Mitchell *et al.* successfully demonstrated that using a tetanus vaccine booster to prime the immune system before DC vaccination significantly enhanced the lymph node migration and efficacy of tumour-antigen-specific DCs. Annias Immunotherapeutics, the company that owns the patents for this vaccination strategy, is

planning to launch a clinical trial in 2015 with a second-generation version of this vaccine, PEP-CMV. Additionally, a third phase I clinical is being conducted by the same group investigating the combination of basiliximab, a monoclonal antibody targeting CD25, with DC pp65 vaccine+GM-CSF (NCT00626483)(62). Finally, there is also an ongoing phase I clinical trial assessing the safety of pp65 RNA-loaded DC vaccination in combination with adoptive transfer of *ex vivo* expanded CMV-specific T cells (NCT00693095) (62). Although these therapies are still in the early phase of clinical trials, the results are inspiring for CMV-targeted vaccine treatment of GBM and suggest that this treatment path may offer significant clinical benefit.

***Survivin vaccines***

Survivin is a 16.5 kDa protein identified as a member of the inhibitor of apoptosis family (IAP) (63, 64). It is associated with cell division and inhibition of caspase 3 and 7. It has been characterized as a universal self-tumour-associated antigen because of its expression in almost all malignancies. While survivin is expressed at substantial levels during fetal development, it is otherwise low or undetectable in most normal adult differentiated tissue, apart from hematopoietic progenitor cells, lymphocytes undergoing development in the thymus and activated systemic lymphocytes (65, 66).

Survivin expression in cancer patients is correlated with poor prognosis. Xie et al. (2005) found that survivin expression was associated with accelerated GBM progression and a more aggressive phenotype (67). Survivin expression has also been associated with resistance to chemotherapy-induced apoptosis (63). The seemingly necessary expression of survivin for cancer cell division and resistance to apoptosis suggests that this antigen

may not be downregulated or lost for immune escape as is observed with other tumour antigens like EGFRvIII, making it an ideal target for anti-cancer vaccination (34).

Spontaneously occurring survivin-specific CTLs have been detected in humans. Schmitz *et al.* (2000) successfully stimulated T cells from healthy donors with rigorous rounds of stimulation with autologous DCs pulsed with soluble recombinant survivin protein (68). These activated survivin-specific CD8+ T cells were able to lyse autologous survivin-expressing B cell transfectants. Alternatively, Andersen *et al.* (2001) surveyed the survivin protein sequence for HLA-A2-binding motifs and used these epitopes to search for survivin-specific T cell responses in tumour patients (69). They discovered survivin-specific CD8+ T cells in leukemia, melanoma and breast cancer patients by ELISPOT assay using identified peptides for stimulation. In addition, Andersen and colleagues were able to detect survivin-specific CTLs *in situ* in the primary tumour of a melanoma patient and a breast cancer patient. In these studies, survivin-specific immune reactions were undetectable in any healthy donors, suggesting that anti-survivin responses in cancer patients were in fact tumour-specific (63). Rohayem *et al*. (2000) corroborated these findings by showing that only cancer patients have antibodies against survivin (70). These studies demonstrate that there is an anti-survivin memory T cell population in cancer patients with the potential to be boosted by vaccination.

There has been success in generating survivin-specific responses leading to anti-tumour efficacy in animal models of GBM (64, 71-73). Fenstermaker and Ciesielski (2014) report an experiment with the immunocompetent murine GBM model GL261, where mice were vaccinated with SurVaxM in incomplete Freund's adjuvant (non-metabolizable oils that stimulate Th2 biased responses) together with GM-CSF.

SurVaxM, as described by Fenstermaker and Ciesielski, "contains a synthetic peptide with an amino acid substitution at the MHC class I anchor position of one of its HLA-A*02 epitopes" to make it more immunogenic than the wild-type survivin peptide (64). They found that this treatment regimen achieved long-term cures in 25% of mice.

Studies such as these have led to the initiation of anti-survivin vaccine trials for the treatment of GBM. A Halifax-based company, Immunovaccine (IMV), has begun a randomized placebo-controlled phase II trial across four institutes in Italy. According to IMV, "the Phase II study will evaluate depovax (DPX)-Survivac therapy in combination with temozolomide, the standard of care therapy for newly diagnosed gliobastoma patients following surgery and radiation". DPX-Survivac contains survivin-based peptide antigens combined with the DepoVax™ adjuvanting platform, which is a patented technology used to envelop antigens and adjuvants in liposomes. The Roswell Park Cancer Institute is also conducting an ongoing phase I trial to verify the safety, tolerability and immunological effect of a survivin peptide vaccine (Montanide ISA-51/survivin peptide vaccine) plus sargramostim (recombinant human GM-CSF) in malignant glioma patients (NCT01250470). Results have yet to be reported from these studies.

***Concluding remarks***

Despite incredulous efforts being made in the field of immunotherapeutics for the treatment of GBM, clinical trials to date have only achieved modest clinical benefits (15). The extremely exciting data seen in preclinical models has yet to be effectively translated in patients. The immune responses generated are extremely inconsistent between patients and do not necessarily correlate with increased survival. On a positive note, the trials

have successfully demonstrated the safety of different vaccination techniques. To generate significant clinical benefit, vaccination therapies will need to produce robust, specific and long-lived anti-tumour immune responses that are able to beat systemic and local immunosuppression. It is unlikely that a vaccine alone will be able to achieve this, and thus a combinatorial approach will likely need to be taken. One novel idea may be to combine vaccination therapy with a cytotoxic therapy, such as oncolytic virus therapy (OVT).

### *1.2.2 Traditional oncolytic virus treatment for glioblastoma*

Over the past 100 years, viruses have attracted attention as potential anti-cancer agents (74). In the early 20$^{th}$ century, it was observed that some cancer patients had regression of their disease if they had a concurrent viral infection. Despite early observations of this phenomenon, important advances in this research area were not made until the modern era of oncolytic virus therapy (OVT) in the early 1990s. Oncolytic virus therapy is defined by selective, tumour-restricted viral replication with subsequent lysis of tumour cells. This is followed by spread of viral progeny to neighboring tumour cells to perpetuate tumour destruction. Viral tropism for tumour cells can be achieved based on naturally occurring or engineered mechanisms that take advantage of tumor-specific mutations for viral replication. Improvements in technology and our understanding of molecular biology and virology have allowed the generation of transgenic or engineered viruses with improved cancer-specific replication. The development and advancement of animal models has allowed most cancer types to be tested against OVT. In the last 20 years, the list of potential OVTs has grown to more than 20 viruses, all amenable to engineering improvements resulting in numerous successor generations (75).

GBM is an important target for oncolytic virus development, especially considering the poor survival rates under the current standard of care. GBM has several characteristics that make it particularly suitable to target with OVT (75). GBM is most often restricted to a single organ compartment (brain) and distant metastases are rare, potentially making local replication and intratumoral spread of the oncolytic virus (OV) easier and more efficient. The normal cells found in the brain that surround the tumour are non-dividing, increasing the tumour-specificity of viruses that require active cell cycles for their replication.

Over 250 articles have been published on OVT targeted to brain cancer (75). Out of almost 15 viruses studied and assessed for GBM targeting, 7 have advanced to clinical trials and are described below. As our understanding of GBM genetics and mechanisms of immunomodulation grows, so do the possibilities for generating more effective OVTs.

***Herpes Simplex Virus 1 (HSV1)***

HSV1 is a member of the *herpesviridae* family that consists of large double-stranded DNA viruses. It is a common human pathogen that establishes lifelong infection through latency. The wildtype (wt) HSV-1 contains two copies of the neurovirulence gene, ICP34.5. Both copies of the gene can be knocked out, while still allowing the virus to retain oncolytic capacity (76, 77). While HSV-1 requires the ribonucleotide reductase enzyme for replication, this gene can be deleted from the virus since actively dividing cells produce active ribonucleotide reductase for the virus to use *in trans*. This led to the generation of two clinical oncolytic HSV-1 strains: G207, which has both copies of the ICP34.5 gene deleted and an insertion interrupting the large subunit of the viral ribonucleotide reductase, and 1716, which lacks both copies of ICP34.5.

A phase I clinical trial reported by Markert *et al*. (2000) demonstrated that doses up to $3x10^9$ plaque forming units (pfu) of G207 virus are safe when injected in to human brain tumors (77). Mean survival from date of diagnosis for GBM patients in this study was 15.9 months, which is not a significant improvement compared to current standard of care. Another phase I clinical trial with oncolytic HSV1 strain 1716, reported by Rampling *et al.* (2000), demonstrated safety when virus was inoculated directly in to the tumours of patients that had failed all other radical treatment options.

***Adenovirus (AdV)***

AdV is a double-stranded, non-enveloped DNA virus and is a common human pathogen that causes mild respiratory infections. Some of the most studied OVs are genetically engineered recombinants based on AdV serotype 5 (Ad5) that demonstrate tumour-selective replication (75). AdV has several early proteins that manipulate host cell cycle to favour viral replication. Two genes of note are E1A and E1B, which trigger cells to enter S-phase and suppress apoptosis, respectively. AdVs with deficiencies in E1A or E1B proteins are replication-incompetent in normal cells, but are able to replicate in actively dividing tumours that inherently suppress apoptosis. A selectively replicating E1B-deleted mutant dl1520 Adenovirus 5 (also known as ONYX-015) has been tested in a glioblastoma clinical trial. A dose escalation was investigated, with high doses of ONYX-015 injected into a total of 10 sites within the resected glioma cavities of patients (78). The maximum tolerated dose was not reached at $10^{10}$ pfu and there were no serious side effects. Unfortunately, all but one patient had disease progression and no clear anti-tumor efficacy could be demonstrated.

***Newcastle Disease Virus (NDV)***

NDV is a negative-sense, single stranded RNA virus that is part of the avian *paramyxovirus* family. It can cause fatal disease in birds, but does not cause any serious disease in humans. Five strains, PV701, 73-T, MTH-68/H, NDV- HUJ, and V4UPM, have been tested for oncolytic potential (75). The mechanism behind the tumour-selective replication of this virus is not well understood. There have been 4 clinical studies on using NDV to treat GBM and some promising outcomes have been reported, such as 5-9 year survival in four GBM patients treated with MTH-68/H (79-81).

***Reovirus***

Reoviruses are non-enveloped viruses with segmented, double-stranded RNA genomes. The name Reovirus originated from the term "Respiratory Enteric Orphan" viruses, where "orphan" refers to the fact that these viruses are not associated with any apparent disease in humans. The double-stranded viral RNA of reovirus activates the host cell's protein kinase RNA-activated (PKR) pathways. PKR acts to block viral replication by inhibiting protein synthesis and promoting apoptosis. Tumours with activated Ras signaling pathways inhibit PKR function, allowing selective replication of wt reovirus (75).

In a phase I clinical trial reported by Forsyth *et al.* (2008), patients with recurring GBM received intratumoral injections with high doses of wt reovirus ($1x10^7$ – $1x10^9$ tissue culture infectious dose 50 (TCID50)) (82). A maximum tolerable dose was not reached and no adverse affects related to the virus injection were reported. One out of twelve patients was still alive 54 months after treatment with genetically unmodified reovirus.

***Poliovirus***

Poliovirus is a non-enveloped virus with a single-stranded positive-sense RNA genome. It is the causative agent for human poliomyelitis and belongs to the *picornaviridae* family There are two factors that determine the neurotoxicity of poliovirus. First, it selectively binds to the poliovirus receptor (PVR/CD155) that is expressed on motor neurons. Second, it has an internal ribosomal entry site (IRES) at the 5' end of its genome that allows for highly efficient cell type-specific translation of viral proteins in cells of neuronal origin (75, 83, 84).

Gromeier *et al.* (1996) discovered that the neurotoxicity of poliovirus could be eliminated by exchanging the entire poliovirus IRES with the IRES from human rhinovirus type 2 (HRV2) (83). The recombinant poliovirus (PVS-RIPO), derived from the attenuated Sabin polio vaccine strain, has demonstrated significant oncolytic capacity in glioma models (84). It is important to note that Merrill *et al.* (2004) examined high-grade glioma patient samples and the primary cell lines derived from these tumours and found that they expressed the PVR/CD155 receptor (85). In safety studies performed on CD155 transgenic mice and non-human primates, PVS-RIPO was shown to lack poliomyelitis-like neurotoxicity (86, 87). PVS-RIPO is currently in an ongoing clinical trial for high-grade glioma and initial reports have shown encouraging results (NCT01491893). However, the number of patients treated to date is still quite low.

***Measles virus (MV)***

Measles virus (MV), the causative agent of measles (an infection of the respiratory system), is an enveloped virus in the *paramyxoviridae* family and has a single-stranded negative-sense RNA genome (75). In 2003, Phuong *et al.* demonstrated

that the attenuated Measles strain used for vaccination (Edmonston, MV-Edm) had the potential to be used as an OV for the treatment of GBM (88). A mutation in the hemagglutinin glycoprotein of MV-Edm causes the virus to bind CD46 with high affinity as opposed to the wildtype H glycoprotein, which predominantly binds to SLAM on the surface of cells (89). CD46, a membrane-associated complement regulatory protein, is overexpressed in many different cancers, including glioma (90). Tumours use CD46 as a form of self-protection against complement-dependent lysis.

In order to monitor viral activity *in vivo* without invasive techniques, Peng et al. (2002) engineered a recombinant MV-Edm that expresses carcinoembryonic antigen (CEA) as a soluble reporter peptide (91). MV-CEA was shown to significantly increase survival in animal models of GBM and was also shown to lack neurotoxicity in CD46 transgenic mice and non-human primates(88, 92). Currently, there is an ongoing phase I clinical trial using MV-CEA for the treatment of glioblastoma (NCT00390299).

***Parvovirus H-1***

Autonomous parvoviruses are small, non-enveloped, single stranded DNA viruses that are non-pathogenic in humans. Parvovirus H-1 (H-1PV) is a wt rat virus that is capable of infecting and replicating in rodent and human glioma cells (93). This virus can be safely injected into adult rat brains without affecting normal tissues. H-1PV has been extensively studied for the treatment of glioblastoma *in vitro* and *in vivo* (94). A phase I clinical trial using H-1PV to treat glioblastoma was recently completed (NCT01301430). Initial reports suggest promising results: the H-1PV treatment was safe and a strong cellular immune response against the tumour was observed (94).

***Concluding remarks***

It is extremely encouraging that no major virus-related complications have been observed during OVT clinical trials and no maximum tolerated dose (MTD) has been reached in any trial. An important outcome of the trials described above is the proven safety of administering OVTs directly into the brains of patients. Unfortunately, the promising results seen in preclinical experiments using OVs to treat GBM have thus far only translated to marginal therapeutic efficacy in human clinical trials. The safety of the OVTs without significant therapeutic efficacy observed in human trials could partly be due to the decision to focus on the safest and most attenuated OVs and the fact that conservative dosing regimens were selected for most trials described herein.

Several other viruses are in preclinical studies for the treatment of GBM, such as Vaccinia virus and Vesicular Stomatitis Virus (VSV), that have yet to be tested in human clinical trials for direct intracranial injection treatment for brain cancer (75). Viruses with a more potent cytotoxic nature require more development to ensure no neurotoxicity will be associated with OVT. With our increasing knowledge and understanding of GBM and the molecular tools available to us today, there are tremendous possibilities for developing the ideal OVT for GBM. It is just a matter of narrowing down the candidates and completing the required preclinical studies to get novel OVs in to clinical trials for GBM.

## 1.3 A novel combinatorial treatment approach for glioblastoma

### *1.3.1 Oncolytic rhabdoviruses*

The field of experimental cancer therapy is ever evolving, constantly introducing new and novel therapeutic strategies. Dr. Stojdl has been a driving force in the

advancement of OVTs. In 2000, he and Dr. Lichty discovered a novel oncolytic virus that demonstrated impressive oncolytic capacity and was exquisitely sensitive to interferon (IFN), making it safe in normal tissues with an intact antiviral response (95). Vesicular stomatitis virus (VSV) is a member of the *rhabdoviridae* family and the *vesiculovirus* genus. It is characterized by a bullet-shaped virion and a negative sense, single stranded RNA genome. This virus showed impressive oncolytic capacity and inspired Dr. Stojdl to survey other rhabdoviruses for oncolytic potential. In 2009, Dr. Stojdl's group discovered a second oncolytic rhabdovirus in the *vesiculovirus* genus, even more potent than VSV (96). Maraba virus (MRB) was found to be highly lytic to 100% of a panel of 37 different cell lines originating from different types of cancer.

Mutations were made to both VSV and MRB to further attenuate them on normal cells and increase their selectivity for cancer cells with disrupted interferon pathways (VSVΔ51 and MRB MG1, respectively) (96, 97). These mutations rendered both viruses unable to completely block transport of host mRNAs from the nucleus to the cytoplasm, thereby allowing the host cells to mount an IFN response. Interestingly, MRB MG1 was hypervirulent and cytolytic on cancer cell lines to a greater degree than wt MRB (96). The maximum tolerable dose (MTD) of intravenous MRB MG1 in mice was two logs higher than that of wt MRB, indicating that the mutant is safer *in vivo*. However, lethal doses were achievable and resulted in central nervous system infection characterized by high infectious virus titers in the brain. Unfortunately, both VSVΔ51 and MRB MG1 are neurotoxic and are therefore unsuitable for the intracranial treatment of brain cancer. An attempt to further engineer these viruses to abrogate neurotoxicity, while maintaining

cytolytic capacity and the ability to replicate to high titers, is currently under investigation (98).

A third potent oncolytic rhabdovirus, Farmington virus (FMT), was identified in the screen performed by Brun *et al*. FMT was highly lytic against many different cancer cell lines and was particularly potent on those originating from the CNS (96). FMT is unique from VSV and MRB because it is most closely related to the *cytorhabdovirus* genus that infects plants (99). It is characterized by classic rhabdovirus features, including a bullet-shaped virion and a five gene, single-stranded, negative-sense RNA genome, but only its L protein shares any significant sequence homology with other rhabdoviruses. Stojdl's group found that FMT could be injected intracranially (IC) or intravenously (IV) in mice at high titers without any neurotoxicity (unpublished data). This indicates that FMT is novel brain-safe oncolytic rhabdovirus with the potential to treat brain cancer.

*1.3.2 Oncolytic rhabdoviruses as vaccine vectors*

Bridle and colleagues (2010) showed that a replication-deficient adenovirus (based on the human serotype 5) expressing a known self-melanoma-associated antigen, human dopachrome tautomerase (hDCT), could be administered prophylactically to prevent intracranial B16-F10 (murine melanoma) tumour formation (100). Administration of Ad-hDCT also prolonged survival of IC B16-F10 tumour-bearing mice when given therapeutically (100). Non-replicating Ad vectors are proven vaccine delivery vehicles that induce robust transgene-specific humoral and CD8+ T cell responses (101). Non-replicating Ad-vectors highly express their encoded transgene, quickly induce protective immune responses and can drive the maturation of DCs by

transducing them. Furthermore, because they are replication-deficient, they do not induce apoptosis in their host cells, which leads to long-lasting antigen presentation (101). The Ad-hDCT vaccination performed by Bridle *et al*. generated DCT-specific CD8+ T cells in mice that were necessary for anti-tumour activities (100). Importantly, Bridle *et al*. used the human version of the DCT protein because it is homologous to the murine version of DCT, but has sequence variation that provides more immunogenicity than the mouse self-protein. In the case of DCT, the higher immune response generated by vaccinating with the human version of the protein is due to a dominant CD4+ T cell epitope found only in hDCT (102).

The same group also tested if the adenovirus-primed CD8+ T cell response against DCT could be expanded to greater frequencies by subsequently treating with an OV expressing the same hDCT antigen (103). It has been shown that mice mount robust CD8+ T cell responses against VSV and they hypothesized that this heterologous prime/boost regimen would redirect the immune system against tumour-specific rather than viral-specific antigens (103). Bridle *et al*. found that VSV-hDCT alone was unable to prime a CD8+ T cell response against DCT and did not provide any therapeutic benefit to IC B16-F10 tumour-bearing mice. However, boosting a pre-established immune response (generated by Ad-DCT) with VSV-hDCT resulted in robust anti-DCT CD8+ T cell responses and prolonged survival in IC B16-F10 tumour-bearing mice compared to treatment with Ad-hDCT alone. This regimen cured 20% of mice compared to Ad-hDCT alone, which did not generate any cures. Bridle *et al*. also observed a marked decrease in anti-VSV CD8+ T cell responses in mice that received the heterologous prime/boost regimen.

In 2013, Pol *et al*. followed up on this work by engineering an alternative rhabdovirus, MRB MG1, to express hDCT (104). Similarly to the previous study, MRB MG1-hDCT alone was unable to prime a DCT-specific CD8+ T cell response. However, when used as a boosting vector to expand pre-existing DCT-specific immunity generated by an Ad-hDCT prime, MRB MG1-hDCT produced a robust anti-DCT CD8+ T cell response in mice. This immune response was superior to that generated by the Ad-hDCT prime/VSV-hDCT boost regimen in terms of frequency of anti-DCT IFNy+ CD8+ T cells generated, suggesting that MRB MG1 is better than VSV at boosting pre-established immune responses. This novel heterologous prime/boost regimen significantly increased survival in an IC B16-F10 model compared to Ad-hDCT alone. In addition, the Ad-hDCT prime/MG1-hDCT boost cured 20% of mice compared to treatment with an empty Ad vector alone. These studies demonstrate that oncolytic rhabdoviruses encoding known TAAs can boost pre-existing adaptive immunity against a cognate antigen. It is also worth noting that the Ad-TAA prime/MG1-TAA boost regimen is currently in phase I/II clinical trials targeting MAGE-A3 TAA in colorectal cancer, non-small cell lung cancer and melanoma (NCT02285816).

### *1.3.3 Rationale and hypothesis*

The current standard of care for GBM is difficult for patients to tolerate and does not greatly prolong their lives or prevent recurrence. It is clear that new treatment options are needed. The oncolytic virus and vaccination clinical trials for the treatment of GBM reviewed above have produced modest but promising results. It is likely that novel combinatorial approaches will provide more clinical benefit to patients.

Preliminary data in our lab suggests that FMT expressing the proof-of-principle

xenoantigen from chicken ovalbumin (OVA) can prime adaptive immune responses similarly to Ad in terms of IFNy+ OVA-specific CD8+ T cells. FMT is also capable of boosting pre-established anti-OVA responses produced by Ad-OVA. Engineering a heterologous prime/boost regimen, where both the priming agent and the boosting agent are OVs, has the potential to maximize the benefits of both cancer-specific lysis by the virus as well as generating a potent anti-tumour adaptive immune response that provides long term protection. Using dual OV prime/boost regimens will likely draw more immune cells to the tumour compared to replication-deficient Ad-prime/OV-boost vaccination because both OV vaccination vectors will replicate at the tumour site whereas a replication-deficient Ad-vector will not. OV replication at the tumour site also provides a greater chance for the tumour microenvironment to be polarized to a type I inflammatory response, favouring anti-tumour immune activities. We increase the likelihood of polarizing the tumour microenvironment by providing this potent type I inflammatory stimulus twice when using dual OV heterologous prime/boost vaccinations. Another advantage of using dual OV heterologous prime/boost vaccinations is that by increasing tumour cell lysis, we are also increasing the opportunities for tumour-epitope spreading and the generation of immune responses to a broad range of tumour antigens besides the one specifically targeted by our vaccination. Also, by using a heterologous vaccination regimen (ie: FMT followed by MG1), we can avoid anti-viral neutralizing antibodies that could interfere with virus delivery and effective transgene expression.

**We hypothesize that FMT virus encoding a known tumour-associated antigen (TAA) can be used in a heterologous dual oncolytic prime/boost vaccination regimen to generate TAA-specific CD8+ T cell responses that lead to better efficacy**

**in tumour-bearing animals.** Our lab set out to define TAAs that can be used to generate robust anti-tumour adaptive immune responses in a heterologous rhabdoviral prime/boost regimen. We chose to examine the self-TAA survivin, which as described above, is a widely recognized TAA expressed in GBM that is necessary for tumour survival and is therefore less likely to undergo immunogenic escape (63). Our first objective was to determine if an anti-survivin prime/boost regimen including FMT as a vector could generate robust anti-transgene CD8+ T cells responses in naïve mice. Our second objective was to determine if the potential anti-survivin immune response generated by our vaccination strategy provided any therapeutic benefits in a tumour model. It is possible that in using two immunogenic OVs, the foreign anti-viral response may dominate over the immune response to the self-TAA target, as there are peripheral and central mechanisms of tolerance controlling immune responses to self-antigens. For example, T cells that strongly bind to self-antigens in the thymus are deleted from the repertoire and only T cells with low avidity for self-antigens make it to the periphery; increasing the difficulty of generating responses against self-antigens (105). There is also some potential for autoimmunity to occur, as survivin is not exclusively expressed in malignant tissue (65, 66).

We also chose to examine foreign TAAs derived from CMV as vaccination targets for our heterologous prime/boost regimen for the treatment of GBM. CMV is a associated with GBM tumours, but not normal healthy brain tissue (58, 106). This target may overcome the potential of the anti-viral response to the OV platforms dominating the immune response and also avoid the risk of autoimmunity. As described above, it is more difficult to generate immune responses against self-antigens due to multiple tolerance

mechanisms in place to prevent autoimmunity; this can complicate targeting TAAs that are self-proteins. It is fortunate that the foreign-CMV antigens were found to be associated with GBM because it will likely be easier to generate CMV-specific responses since we do not need to overcome tolerance. Individuals that are seropositive for CMV already have moderate to high frequencies of circulating CMV-specific CD8+ T cells in their blood, representing a potent pre-existing memory pool to boost with our vaccination strategy. (59). A disadvantage to targeting the foreign CMV antigens is that they are likely not necessary for the tumour cells like the self-TAA survivin and therefore might undergo antigen loss for immunogenic escape.

We chose to investigate our ability to generate immune responses to two immunodominant mCMV proteins, m38 and IE3, using our heterologous prime/boost regimen. Our first objective was to determine if our vaccination strategy could generate anti-mCMV responses in naïve mice. Our second objective was to investigate the prophylactic and therapeutic efficacy of the vaccination against mCMV-positive GBM tumours. The limitation with this model is the species specificity. Unfortunately, we cannot make direct conclusions about clinical translation from studying mCMV antigen targets in murine GBM, since the precise antigen targets in human GBM are different. We can model hCMV antigens in murine GBM, but it is likely that these will be artificially immunodominant due to the fact that they are xenoantigens like OVA. Ultimately, the main aims of this project were to evaluate the ability of FMT virus to vaccinate against a self-TAA or foreign TAAs in the context of a heterologous prime/boost regimen and determine how this affects efficacy in tumour models.

# 2 MATERIALS AND METHODS

## 2.1 Cell culture

Cell lines were maintained in 1X Dulbecco's Modified Eagle's Medium (DMEM; HyClone™, Thermo Scientific) supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich®) at 37˚C and 5% $CO_2$. Adherent cell lines were maintained in 15 cm tissue culture plates (Corning™) until ~85% confluent. To passage these cells, media was aspirated and plates were washed in 10 mL 1X phosphate buffered saline (PBS). Three milliliters of 0.25% trypsin was then added to plates and incubated for 5 minutes at 37˚C. Trypsinized cells were collected by adding appropriate volumes of DMEM+10% FCS to the cell suspension to obtain the desired cell concentration. After mixing to ensure single cell suspension, an appropriate volume of the cell suspension was transferred to a new 15 cm plate containing ~25 mL of fresh DMEM+10% FCS.

Murine peripheral blood mononuclear cells (PBMCs) were maintained in complete Roswell Park Memorial Institute (cRPMI) media, which consisted of HyClone™ RPMI 1640 media (Thermo Scientific) supplemented with 10% FCS, 1% non-essential amino acids, 1% penicillin-streptomycin (HyClone™, Thermo Scientific), 1% HEPES (Stemcell™ technologies) and 55 nM beta-mercaptoethanol.

## 2.2 Mice and cell lines

BALB/c and C57BL/6 mice (6-8 weeks old) were purchased from Charles River Laboratories (ON, CA). Procedures and housing were carried out according to the rules and regulations of the University of Ottawa Animal Care and Veterinary Services.

Vero cells, kidney epithelial cell line isolated from African green monkeys

(ATCC®, CCL-81TM), were used to grow and titer virus. CT2A mouse astrocytoma cells were obtained as a generous gift from Dr. Forsyth at the University of Calgary. These adherent cells were used to generate a syngeneic mouse model of GBM and were used in *in vitro* cytotoxicity studies. 4T1 murine epithelial mammary carcinoma cells (ATCC® CRL-2539™) were used to generate a syngeneic mouse model of stage IV breast cancer and were used in *in vitro* cytotoxicity assays.

**2.3 Viruses**

All rhabdoviruses were grown and harvested from 15 cm tissue culture plates of confluent Vero cells infected at an MOI of 0.01 in 1X DMEM + 10% FCS. Briefly, infected plates of Veros were incubated at 37˚C for ~24 hours (MRB) or ~30 hours (FMT) before media was collected and centrifuged to remove whole cells. Supernatant was filtered using a sterile 0.2µm bottle top filter apparatus (Millipore, ON, CA; SCGPU05RE) to remove whole cell debris. Virus was pelleted by centrifugation (10,000 rpm, 2 hours, 4˚C), followed by resuspension of virus pellets in a maximum of 2 mL of Solution C (107) incubated overnight at 4˚C. The virus was then purified by OptiPrep™ (Sigma-Aldrich®) density gradient centrifugation as previously described (107). Purified virus collected was aliquoted in to 50 uL and stored at -80˚C.

Non-replicating Adenovirus (E1/E3-deletion), based on the human serotype 5, expressing murine survivin conjugated to green fluorescent protein was graciously given to our lab by Dr. Yonghong Wan at the McMaster Immunology Research Centre, Cancer Division (Hamilton, ON). Non-replicating adenoviruses expressing transgenic mCMV proteins were engineered and propagated by Charles Lefebvre of Dr. Stojdl's lab. Briefly, adenoviruses were generated using pAd/CMV/V5-Dest™ and the Gateway® Technology

with Clonase II (Invitrogen™, Life Technologies) and then propagated in 293A cells before purification with the Adeno-X™ Maxi Purification Kit (Clontech, CA, US).

### 2.4 Mouse vaccination

For adenovirus vectors, mice were injected intramuscularly (IM) with a dose of $2x10^8$ pfu (OVA, survivin) or $1x10^7$ pfu (mCMV IE3 or m38) in 100 uL (50 uL injected into each leg). For rhabdovirus delivery, mice were injected intravenously (IV) with a dose of $5x10^8$ pfu in 100 uL (tail vein injection). Prime injections were given on Day 0, followed by boost injections given on day 15 post-prime. In tumour models, boost injections were given on day 7 post-prime.

### 2.5 Peripheral blood mononuclear cell (PBMC) isolation

Approximately 100 uL of blood was collected from mice by puncturing the saphenous vein and collecting blood in a heparinized blood collection tube (Microvette® CB 300 LH, SARSTEDT). Samples were centrifuged to separate the serum from the plasma, after which the serum was discarded and the plasma was transferred to 15 mL conical tubes (Falcon, Corning™). ACK lysis buffer was used to lyse the red blood cells and following centrifugation, the PBMCs were resuspended in a desired amount of cRPMI to use for intracellular cytokine staining (ICS).

## 2.6 Peptides

**Table 1. Peptides used in detection of antigen-specific T cell responses**

| Target | Peptide sequence | MHC specificity | Working concentration (μg/ml) |
|---|---|---|---|
| $Ovalbumin_{257-264}$ | SIINFEKL | $H\text{-}2K^b$ | 0.5 |
| $Epha2_{682-689}$ | VVSKYKPM | $H\text{-}2K^b$ | 10 |
| $Survivin_{97-104}$ | TVSEFLKL | $H\text{-}2K^b$ | 10 |
| $Survivin_{57-64}$ | CFFCFKEL | $H\text{-}2K^b$ | 10 |
| $Survivin_{66-74}$ | GWEPDDNPI | $H\text{-}2K^d$ | 10 |
| $Survivin_{85-93}$ | AFLTVKKQM | $H\text{-}2K^d$ | 10 |
| mCMV $IE3_{416-423}$ | RALEYKNL | $H\text{-}2K^b$ | 5 |
| mCMV $m38_{316-323}$ | SSPPMFRV | $H\text{-}2K^b$ | 10 |
| Farmington | AVVLMFAQC | $H\text{-}2K^b$ | 1 |
| Maraba MG1 | RGYVYQGL | $H\text{-}2K^b$ | 1 |
| Adenovirus | FALSNAEDL | $H\text{-}2K^b$ | 20 |

Peptides were synthesized from Biomer Technologies (San Francisco, CA)

## 2.7 Antibodies

Monoclonal antibodies used in flow cytometry assays: anti-CD8α (53-6.7) and anti-TCRβ (H57-597) for detecting cell surface markers and anti-IFNy (XMG1.2) for intracellular staining. All flow cytometry antibodies were purchased from BD Biosciences (Missisauga, ON).

## 2.8 Detection of antigen-specific T cell response

Antigen-specific CD8+ T-cell responses were measured at specific time points post-prime and post-boost. For antigen-specific CD8+ T-cell re-stimulation, PBMCs were incubated in cRPMI with respective peptide concentrations listed in Table 1. Peptide stimulated cells were incubated for 1 hour at 37˚C before the addition of brefeldin A and another incubation at 37˚C for 4 more hours (1 μg/ml, GolgiPlug, BD Biosciences). Cells were spun down to remove brefeldin A and resuspended in cRPMI media before an overnight incubation at 4˚C. Cells were stained with fluorescent-conjugated antibodies

specific for T-cell surface markers. Cells were then permeabilized and fixed with Cytofix/Cytoperm (BD Biosciences) and stained for intracellular cytokines. Stained samples were run on a CyAn™ ADP Analyzer with HyperCyt Software Interface CyAn (Beckman Coulter), a FACSCanto™ flow cytometer with FACSDiva software (BD Biosciences) or a MoFlo™ XDP with Summit Software (Beckman Coulter). FCS files were analyzed with FlowJo Mac software (Treestar, Ashland, OR).

**2.9 Subcutaneous 4T1 cell implantation in mice**

For the 4T1 murine mammary carcinoma model, 4T1 cells were expanded in 15 cm tissue culture plates to reach 70% confluency on the day of implantation. Cells were collected by trypsinization and then washed 2x with sterile PBS (HyClone™, Thermo Scientific) (centrifuged 5 minutes at 900 rpm, then resuspended 10x). Cells were counted using a hemacytometer and made up to a final concentration of $1x10^7$ cells/mL in serum-free, HEPES buffered DMEM. Cells were kept on ice until the time of injection. For the injection, mice were maintained under anesthesia using isofluorane gas. Fifty microliters of the cell suspension was injected subcutaneously in the third mammary fat pads (right and left). Approximately one week post-injection, tumours were palpable and mice received the prime dose of virus. One week post-prime, the mice received the boost dose of virus. Tumour burden was monitored over time by caliper measurement. All mice in the experiments were sacrificed for tissue collection when the untreated group reached humane endpoint (approximately 22-27 days post tumour seed).

**2.10 Transgenic cell lines**

Briefly, CT2A cells were transfected with Lenti6/Ubc/V5-Dest vectors expressing either mCMV m38 or mCMV IE3 by Charles of the Stojdl Lab and were maintained under 2ug/ml blasticidin selection in DMEM+10% FCS.

**2.11 Subcutaneous CT2A implantation in mice**

For the subcutaneous CT2A mouse model, CT2A cells were expanded in 15 cm tissue culture plates to reach 70% confluency on the day of implantation. Cells were collected by trypsinization and then washed 2x with sterile PBS (centrifuged 5 minutes at 900 rpm, then resuspended 10x). Cells were counted using a hemacytometer and made up to a final concentration of $1x10^7$ cells/mL in serum-free, HEPES buffered DMEM. Cells were kept on ice until the time of injection. For the injection, mice were maintained under anesthesia using isofluorane gas. Fifty microliters of the cell suspension was injected subcutaneously in to the right flank (mCMV CT2A cells) and the left flank (wt CT2A cells) of the mouse. Approximately 20 days post-injection, tumours were palpable and tumour progression was monitored over time by caliper measurement. Mice were sacrificed when tumours reached a size of 15mm by 15mm or if the tumour ulcerated.

**2.12 Intracranial CT2A implantation in mice**

For the intracranial CT2A syngeneic mouse model of GBM, CT2A cells were expanded in 15 cm tissue culture plates to reach 70% confluency on the day of implantation. Cells were collected by trypsinization and then washed 2x with sterile PBS (centrifuged 5 minutes at 900 rpm, then resuspended). Cells were counted using a hemacytometer and made up to a final concentration of $1.67x10^7$ cells/mL in serum-free, HEPES buffered DMEM. Cells were kept on ice until the time of injection. For the

procedure, mice were maintained under anesthesia using isofluorane gas. In preparation for surgery, mice received a 1 mL subcutaneous bolus of 0.9% sodium chloride, 30 uL of buprenorphine (0.03mg/mL) subcutaneously and their heads were shaved and sterilized with a chlorahexidine solution (provided by University of Ottawa Animal Care and Veterinary Service (ACVS)). A small incision was made to expose the mouse's skull and a stereotaxic unit was used to find the appropriate injection coordinates (0.5 mm forward and 2.0mm to the right of bregma). A 25G needle was used to puncture the skull at the appropriate coordinates and a 26G 10uL gastight Hamilton® syringe loaded with the cell suspension was lowered in to the brain. Three microliters of cells were injected in to the brain at a rate of 1uL/min using the stereotaxic pump. Thirty seconds after the injection, the Hamilton® syringe was slowly withdrawn from the brain and VetBond tissue adhesive (3M, MN, USA) was used to close the incision. Topical 2% bupivacaine hydrocholoride was applied to the incision and mice were placed in an infant incubator to recover at 37˚C. Mice received two more doses of buprenorphine post surgery (administered by ACVS technicians).

**2.13 Magnetic resonance imaging (MRI) of mice**

MRI imaging was performed with a General Electric / Agilent MR901 7T small-animal scanner, in combination with a SA Instruments Inc. (SAII) physiological monitoring system. Each mouse was anaesthetized with isoflurane throughout the imaging procedure. The SAII system monitored the mouse's vital signs and maintained its body temperature via warm air delivery into the MRI bore. After the positioning of the anaesthetized mouse within the MRI bore, routine adjustments and localizer scans were performed. Images of the mouse brain were then obtained with a T2-weighted fast spin

echo pulse sequence with the following parameters: 15 axial (transverse) slices; slice thickness = 700 microns; in-plane resolution = 78 microns; echo train length = 8; echo time = 27 ms; scan time = 5 minutes.

**2.14 Virus treatment of intracranial tumour bearing mice**

One to two weeks post tumour seed, mice were treated with either an intravenous or an intracranial dose of virus. Briefly, for the intracranial virus treatment, mice underwent the surgical procedure described above in section 2.12 with some exceptions: $1x10^8$ pfu of virus suspended in 10 uL of PBS was injected at a rate of 2uL/min (unless otherwise stated). For intravenous virus treatment, mice were tail vein injected with $1x10^8$ pfu of virus suspended in 100 uL of PBS (unless otherwise stated).

**2.15 Statistical analysis**

Statistical analyses were conducted using Prism, version 5.0f (GraphPad Software, Inc., CA, USA). Statistical tests included: Student's t-test, one-way ANOVA with Dunnett's test and two-way ANOVA with Bonferonni test. Mean and standard error of the mean (SEM) are shown (unless otherwise stated). P-values are indicated as *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$ and ****=$p<0.0001$.

# 3 Results

## 3.1 Testing the ability of transgenic FMT viruses expressing self tumour-associated antigens (TAAs) to generate TAA-specific CD8+ T cell responses in naïve mice

### *3.1.1 Vaccination against wild-type (wt) murine survivin in naïve mice*

A previous study in the lab surveyed CT2A and GL261 syngeneic mouse GBM cell lines for expression of known TAAs by quantitative-polymerase chain reaction (q-PCR). The TAAs examined were survivin (surv), ephrin type-A receptor 2 (Epha2), dopachrome tautomerase (DCT), baculoviral IAP repeat-containing protein 5 isoform 3 (Birc5-iso3), GARC1, melanoma antigen recognized by T cells (Mart-1), melanoma antigen family D1 variant 1 (MAGED1-var1), MAGED1 variant 3, tyrosinase (Tyr), Gli family zinc finger 1 (Gli1), complement receptor 1 (CR1) and activating transcription factor 5 (ATF5). Survivin and Epha2 mRNAs were highly expressed in the CT2A cell line whereas the other TAAs were below the limit of detection or poorly expressed (Appendix, Figure S1. A). Due to the high expression of survivin mRNA in the GL261 cell line as well, survivin was chosen as the lead clinical candidate for our TAA vaccination study. Survivin is widely recognized as an immunogenic self-TAA target in the clinic (63, 66, 68, 69) and vaccination strategies against survivin are currently under investigation (39, 64, 108-110). To model self-TAA targeting in a therapeutic rhabdovirus vaccination setting, survivin was engineered into both Maraba MG1 (MG1) and Farmington (FMT) rhabdovirus expression vectors. The TAA expression from each virus was verified by Western blot analysis (Appendix, Figure S1. B, C).

An experimental timeline was designed to test the ability of these newly engineered viruses to generate anti-surv CD8+ T cell responses in a heterologous dual

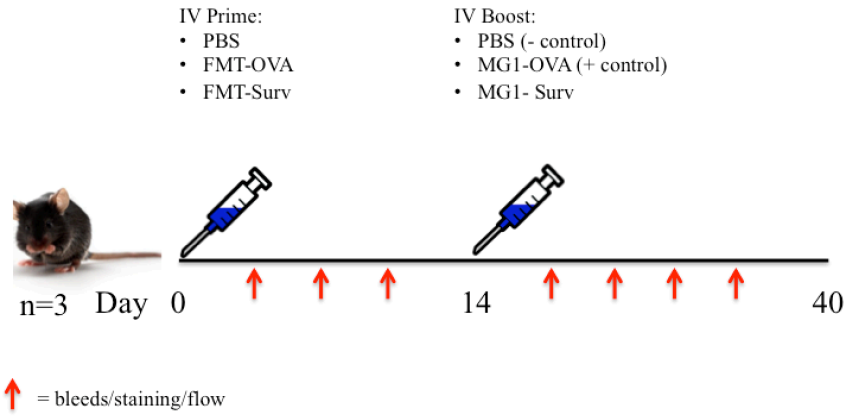
oncolytic prime/boost vaccination regimen (Figure 1. A). It has been previously shown that FMT vectors expressing a foreign TAA based on chicken egg ovalbumin (OVA) can prime OVA-specific CD8+ T cell responses; therefore the FMT-OVA prime/MG1-OVA boost regimen was used as a positive control for these experiments. Antigen-specific CD8+ T cell responses were evaluated by *ex vivo* peptide re-stimulation followed by intracellular cytokine staining (ICS) for interferon-gamma (IFNy). A robust anti-OVA CD8+ T cell response was detected following vaccination with FMT-OVA prime/MG1-OVA boost (Figure 1. B). No detectable anti-surv responses were generated in mice vaccinated with FMT-Surv prime/MG1-Surv boost (Figure 1. C). Successful inoculation of the mice with our viral vectors was confirmed through detection of an anti-FMT CD8+ T cell response; therefore failure to deliver the vector was not the reason for our inability to detect self TAA-specific CD8+ T cell responses (Figure 1. D).

*3.1.2 Vaccination against an inactive mutant of survivin in naïve mice*

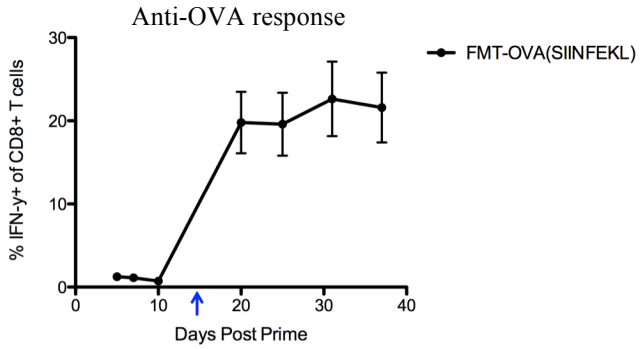
Survivin normally acts as an inhibitor of apoptosis and supports mitosis. Thus, while survivin may represent a rational cancer immunotherapy target, overexpressing the wild-type protein in a vector specifically targeting and replicating in cancer cells may increase their resistance to cell death. New rhabdoviruses were therefore engineered to express an inactive, mutant form of survivin, replacing the threonine at position 48 with an alanine (Surv-T48A or T48A). The mutant Surv-T48A still localizes normally within cells, but is incapable of supporting cell division and incapable of protecting the cells from TRAIL-mediated apoptosis when endogenous survivin is knocked down by siRNA (111). Overexpression of Surv-T48A from both MG1 and FMT vectors was verified by Western blot (MG1-T48A and FMT-T48A, respectively) (Appendix Figure S2. A, B).

**Figure 1. FMT can prime robust CD8+ T cell responses against a foreign antigen, but not against a self-antigen.** Experimental timeline for heterologous dual oncolytic prime/boost vaccination regimen (A). Naïve female C57BL/6 mice (8-12weeks) were injected intravenously with PBS, 5E8 pfu FMT-OVA or 5E8 pfu FMT-Surv on Day 0 (prime). On day 14 post-prime, mice were injected IV with the MG1 platform expressing the corresponding TAA (boost). Peripheral blood CD8+ T cells were analyzed at times indicated for IFNy expression by intracellular cytokine staining (ICS) following stimulation with immunodominant CD8+ peptides from OVA (B) or survivin (C). Anti-viral responses were assessed in a similar manner to verify inoculation (D). Arrow in B, C and D indicates time of boost. Data is plotted as mean of 3 animals ± SD.

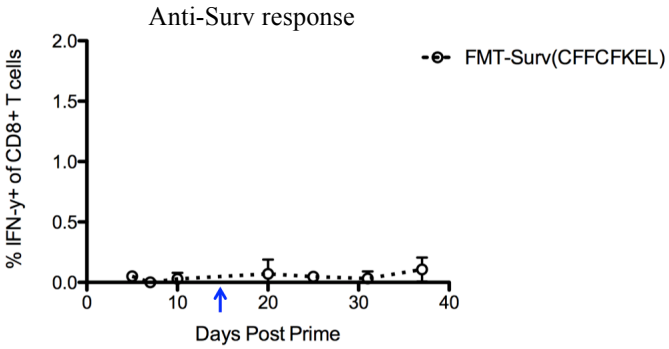
A.



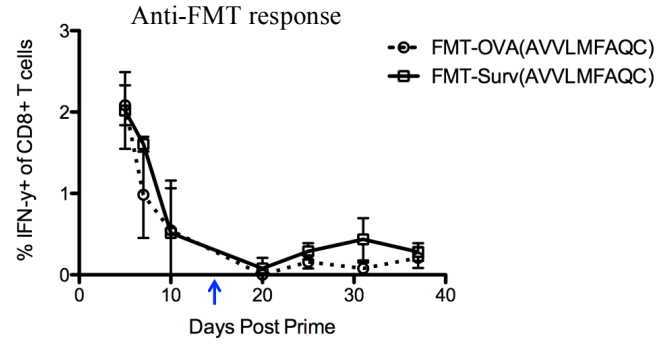

B.




C.




D.




= boost with MG1 expressing corresponding antigen

An experimental timeline was designed to test the ability of FMT-T48A prime/MG1-T48A boost to generate anti-survivin CD8+ T cell responses (Figure 2. A). FMT-OVA prime/MG1-OVA boost was used as positive control for the experiment. Antigen-specific CD8+ T cell responses were evaluated by *ex vivo* peptide re-stimulation followed by ICS for IFNy. A robust anti-OVA CD8+ T cell response was detected following vaccination with FMT-OVA prime/ MG1-OVA boost (Figure 2. B). No detectable anti-survivin responses were generated in mice vaccinated with FMT-T48A prime/MG1-T48A boost (Figure 2. C). Successful inoculation of the mice with our viral vectors was confirmed by assessing the anti-FMT and anti-MG1 CD8+ T cell responses, so our inability to detect self-TAA specific CD8+ T cell responses was not due to failure to deliver vector (Figure 2. D and E, respectively). It is interesting to note that the peak anti-viral responses in T48A-vaccinated mice were significantly higher than the peak anti-viral responses in OVA-vaccinated mice, suggesting that the immune system may be more focused on viral antigens instead of the self-TAA encoded in the rhabdovirus.

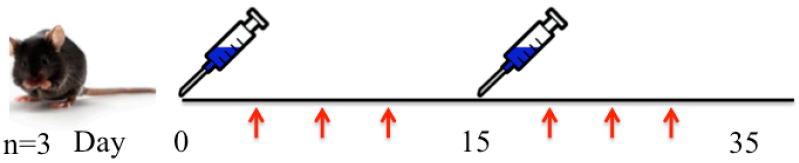
Due to our inability to detect anti-survivin responses when priming with FMT, a non-replicating adenoviral vector expressing survivin-GFP was generated (Ad-SurvGFP) (provided courtesy of Dr. Wan, McMaster University). The generation of a robust CD8+ T cell response against a self-TAA using an adenovirus-TAA prime/MG1-TAA boost vaccination regimen has been previously shown (104, 112). An experimental timeline was designed to assess the ability of Ad-SurvGFP to prime an anti-survivin response and to test the capacity of FMT-T48A or MG1-T48A to boost the pre-established adaptive response (Figure 3. A). FMT-OVA prime/MG1-OVA boost was used as a positive control and successfully generated a robust anti-OVA CD8+ T cell response (Figure 3.

**Figure 2. FMT can prime robust CD8+ T cell responses against OVA, but not survivin T48A.** Experimental timeline for heterologous prime/boost vaccination regimen using survivin-T48A mutant-encoding rhabdoviruses (A). Naïve female C57BL/6 (8-12weeks) were injected with PBS (IV), 5E8 pfu FMT-OVA (IV) or 5E8 pfu FMT-T48A (IV) (prime). The mice were injected IV with 5E8 pfu of the MG1 platform expressing the corresponding TAA 15 days post-prime (boost). Peripheral blood CD8+ T cells were analyzed at times indicated for intracellular IFNy staining in response to dominant epitopes for OVA (B), survivin (C), FMT (D) or MG1 (E) via flow cytometry. Arrow indicates time of boost in B, C, D and E. Data is plotted as mean of 3 animals ± SEM. Asterisks indicate significant difference as determined by Student's T-test. *$p<0.05$.

A.

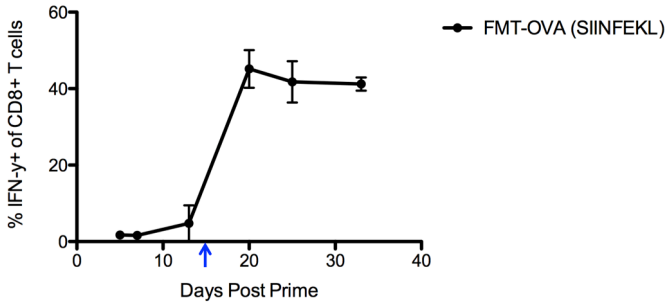
IV Prime:
- PBS
- FMT-OVA
- FMT-T48A

IV Boost:
- PBS (- control)
- MG1-OVA (+ control)
- MG1-T48A

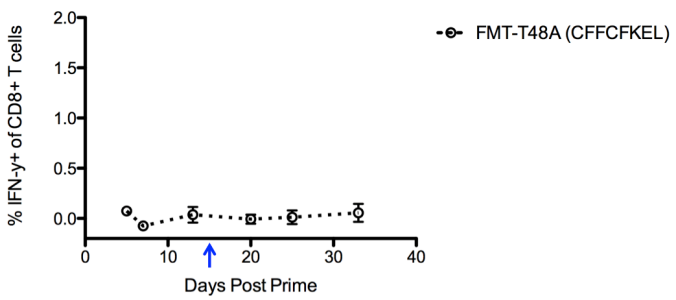



↑ = bleeds/staining/flow

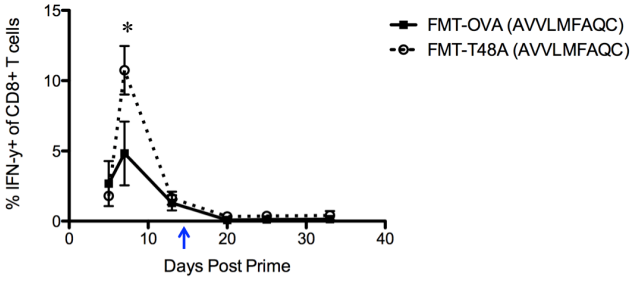
B.



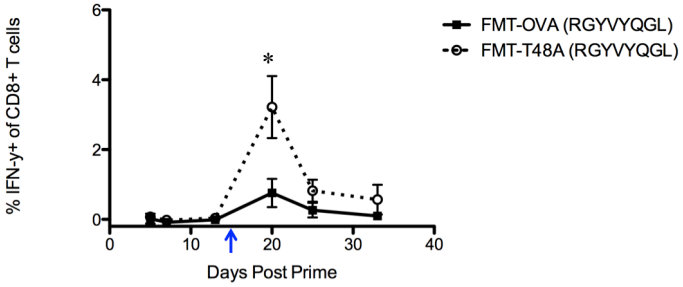

C.




D.

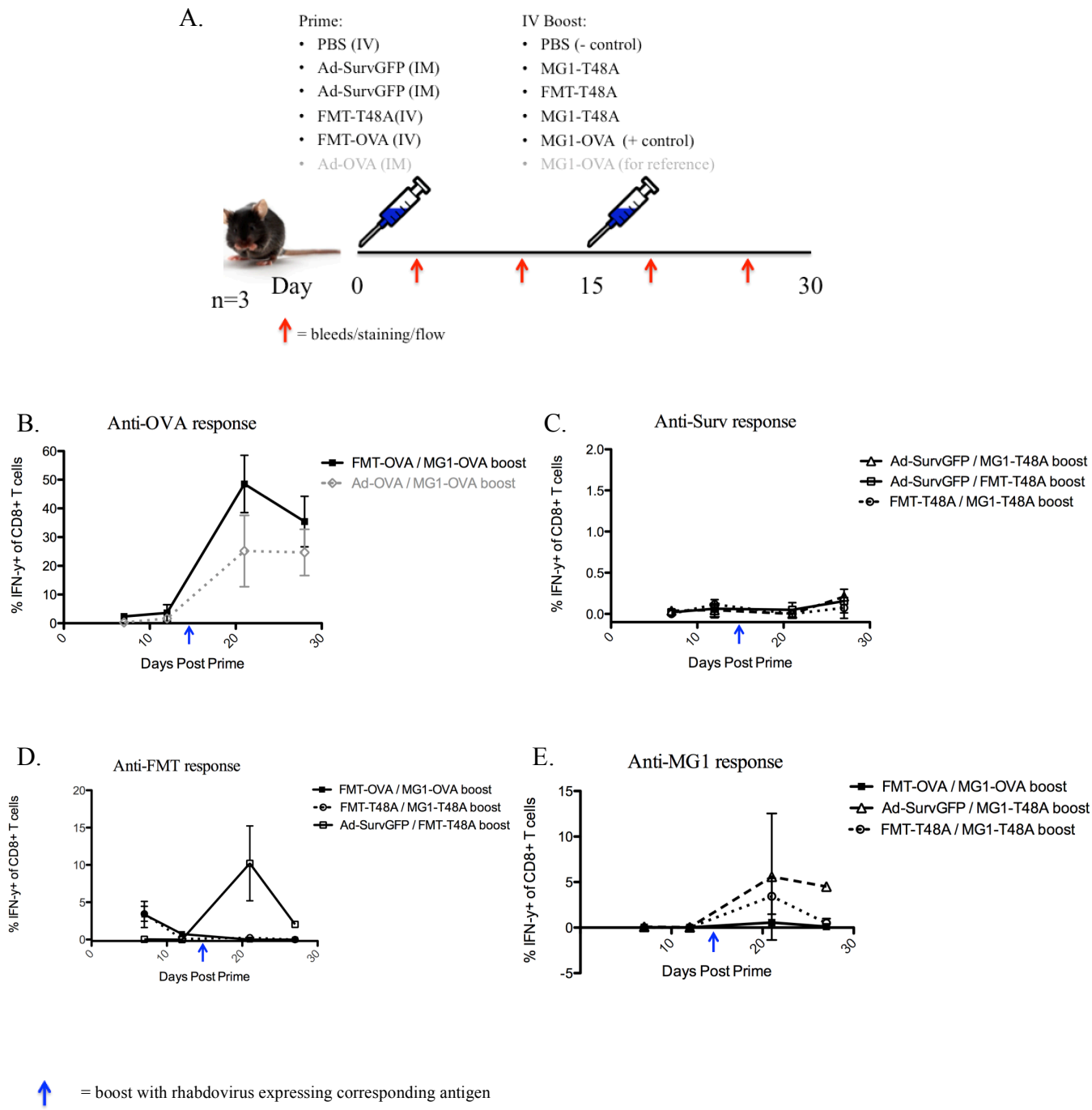



E.




↑ = boost with MG1 expressing corresponding antigen

**Figure 3. Anti-survivin CD8+ T cell responses are not detected when using Ad-SurvGFP as a priming agent in a heterologous prime/boost vaccination regimen.** Experimental timeline for heterologous prime/boost vaccination regimen comparing the priming ability of survivin-GFP encoding adenovirus vs. survivin-T48A encoding FMT (A). Naïve female C57BL/6 (8-12weeks) were injected with PBS (IV), 2E8 pfu Ad-SurvGFP (IM) (2 groups), 5E8 pfu FMT-T48A (IV) or 5E8 pfu FMT-OVA (IV) (prime). The mice were injected IV with 5E8 pfu of the MG1 platform expressing the corresponding TAA 15 days post-prime (boost). One Ad-SurvGFP primed group was boosted with FMT SurvT48A. Peripheral blood CD8+ T cells were analyzed at times indicated for intracellular IFNy staining in response to dominant epitopes for OVA (B), survivin (C), FMT (D) or MG1 (E) via flow cytometry. Grey plot in B is an example of Ad-OVA prime/ MG1 OVA boost from separate experiment. Arrow indicates time of boost. Data is plotted as mean of 3 animals ± SEM.


A.
Prime:
• PBS (IV)
• Ad-SurvGFP (IM)
• Ad-SurvGFP (IM)
• FMT-T48A(IV)
• FMT-OVA (IV)
• Ad-OVA (IM)
IV Boost:
• PBS (- control)
• MG1-T48A
• FMT-T48A
• MG1-T48A
• MG1-OVA (+ control)
• MG1-OVA (for reference)
n=3
Day 0 15 30
= bleeds/staining/flow
B.
Anti-OVA response
% IFN-γ+ of CD8+ T cells
Days Post Prime
FMT-OVA / MG1-OVA boost
Ad-OVA / MG1-OVA boost
C.
Anti-Surv response
% IFN-γ+ of CD8+ T cells
Days Post Prime
Ad-SurvGFP / MG1-T48A boost
Ad-SurvGFP / FMT-T48A boost
FMT-T48A / MG1-T48A boost
D.
Anti-FMT response
% IFN-γ+ of CD8+ T cells
Days Post Prime
FMT-OVA / MG1-OVA boost
FMT-T48A / MG1-T48A boost
Ad-SurvGFP / FMT-T48A boost
E.
Anti-MG1 response
% IFN-γ+ of CD8+ T cells
Days Post Prime
FMT-OVA / MG1-OVA boost
Ad-SurvGFP / MG1-T48A boost
FMT-T48A / MG1-T48A boost
= boost with rhabdovirus expressing corresponding antigen

B). The anti-OVA CD8+ T cell responses generated by Ad-OVA prime/MG1-OVA boost from a separate experiment is provided as a reference to compare Ad vs. FMT priming against a foreign antigen. There were no detectable anti-survivin responses generated by any of the different survivin-targeting heterologous prime/boost regimens tested (Figure 3. C). Successful inoculation of the mice with our viral vectors was confirmed by assessing the anti-FMT and anti-MG1 CD8+ T cell response (Figure 3. D and E, respectively). The data described above suggests that FMT virus is able to prime robust responses, comparable to the well-established Ad priming vector, against a foreign xenoantigen (OVA), but not against the self-antigen survivin. The Ad vector used in this study also appeared to be unable to prime a response against survivin, but it has been previously shown to prime a robust response against a different self-TAA, DCT (104). This suggests that it may be properties of our target antigen (survivin) and not the priming vectors that are the issue here.

### 3.2 Evaluating the therapeutic and immunological capabilities of FMT virus expressing a self-tumour-associated antigen (TAAs) in tumour-bearing mice

While we failed to detect anti-self TAA immune responses in vaccinated naïve mice, it is notoriously difficult to overcome the natural tolerogenic mechanisms that suppress reactivity against self-antigens. Furthermore, self-specific T cells are present at very low frequencies in the periphery of naïve animals because strongly self-reactive T cells are deleted in the thymus; however some T cells evade this negative selection because they have low avidity for self-proteins (113). Thus it seemed reasonable to explore if it was possible to generate an anti-self TAA immune response in a tumour-bearing animal model, where the tumour expresses the self antigen against which we are

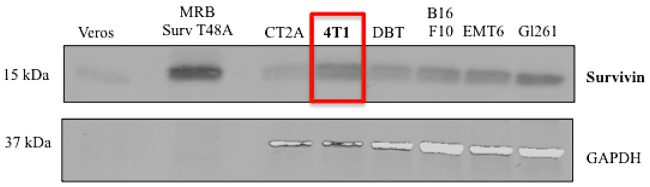
vaccinating. Initially, we examined which tumour model would be suitable for the purpose of testing our survivin-targeting heterologous prime/boost regimen by analyzing expression of survivin protein (as opposed to mRNA level which was previously examined in the lab).

*3.2.1 A murine mammary carcinoma model chosen to evaluate anti-survivin responses*

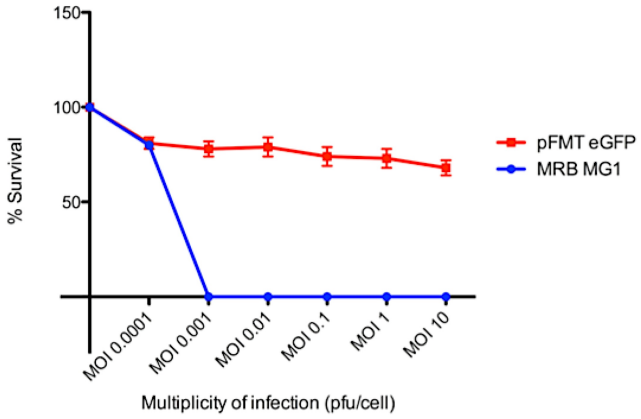
A panel of murine cancer cell lines was surveyed for survivin expression by Western blot to confirm the results of a qPCR analysis of survivin mRNA levels previously completed in the lab (Figure 4. A). All of the cell lines expressed varying levels of survivin, however the CT2A cell line that was previously found to express high levels of survivin mRNA did not appear to have robust survivin protein expression. It would likely be more difficult to examine the immune response against an intracranial (IC) tumour due to the complex relationship between the systemic immune system and the CNS. Furthermore, there is no way to monitor tumour progression in the IC CT2A model except for Magnetic Resonance Imaging (MRI), which is costly and time consuming. Thus the 4T1 murine mammary carcinoma model was chosen to evaluate the survivin-targeting rhabdoviral prime/boost regimen on the basis that the 4T1 cells appear to express higher levels of survivin compared to most other cell lines surveyed, tumour progression in this orthotopic model can be easily monitored and 4T1 cells are susceptible to killing by MG1, but not FMT virus *in vitro* (Figure 4. B). We hypothesized that an observation of FMT efficacy against 4T1 tumours *in vivo* would likely be attributable to the immune response generated by the virus and not its direct oncolytic capacity. Furthermore, the 4T1 model spontaneously metastasizes to the lungs, providing a secondary read-out for efficacy besides primary tumour progression (114).

**Figure 4. The 4T1 murine mammary carcinoma cell line expresses survivin and is susceptible to oncolysis by MG1, but not FMT virus.** Lysates of murine carcinoma cell lines were collected from confluent 15 cm tissue culture plates and analyzed for survivin protein expression by Western blot (A). 4T1 cells were plated in triplicate in a 96 well plate and infected with increasing multiplicities of infection (MOIs) (pfu/cell) of FMT-eGFP, MG1 or were mock treated. Cell survival was assessed 96 hours post-infection via AlamarBlue assay where % survival represents the mean absorbance of 3 infected wells per MOI divided by the mean absorbance of 6 uninfected wells multiplied by 100 (B).

A.


Veros
MRB Surv T48A
CT2A
4T1
DBT
B16 F10
EMT6
Gl261
15 kDa
Survivin
37 kDa
GAPDH

B.


150
100
50
% Survival
pFMT eGFP
MRB MG1
MOI 0.0001
MOI 0.001
MOI 0.01
MOI 0.1
MOI 1
MOI 10
Multiplicity of infection (pfu/cell)

An experimental design was generated to assess the effect of survivin-targeting, heterologous, dual oncolytic rhabdoviral prime/boost regimens on 4T1 tumours (Figure 5). These regimens were compared to the heterologous Ad-TAA prime/MG1-TAA boost regimen, which was previously proven to be efficacious in a murine model of metastatic melanoma (104). MG1-T48A and FMT-T48A were compared as priming agents in these experiments to determine if the order of the survivin-targeting rhabdoviral prime/boost regimens had an effect on tumour progression.

### *3.2.2 Anti-survivin rhabdoviral prime/boost regimens control tumour progression and metastasis in the 4T1 model*

Once tumours became palpable after implantation, their progression was monitored over time by caliper measurement until the untreated animals reached humane endpoint. Heterologous rhabdovirus prime/boost regimens significantly controlled 4T1 tumour burden compared to untreated tumour-bearing mice (Figure 6). Additionally, the heterologous rhabdovirus prime/boost regimens demonstrated significantly better control over tumour progression compared to Ad-SurvGFP prime/MG1-T48A boost, despite the fact that this treatment regimen showed modestly significant tumour control compared to PBS. The tumours of mice treated with MG1-T48A prime/FMT-T48A boost appeared to be necrotic (black/burgundy) when compared to healthy, untreated 4T1 tumours, which are typically a light pink-flesh colour (Figure 7). At endpoint, lungs were dissected from the mice and visible lung metastases were counted.

**Figure 5. Experimental timeline comparing the efficacy of different anti-survivin heterologous prime/boost vaccination regimens in a murine mammary carcinoma model.** Naïve female BALB/C mice (8-12weeks) were injected subcutaneously with 5E5 4T1 cells in left and right mammary fat pads. Once tumours were palpable, mice were treated with PBS (IV), 2E8 pfu Ad-SurvGFP (IM), 5E8 pfu FMT-T48A (IV) or 5E8 pfu MG1-T48A (IV) (prime). 7 days post-prime, mice were injected IV with PBS, 5E8 pfu MG1-T48A, 5E8 pfu MG1-T48A or 5E8 pfu FMT-T48A, respectively (boost). Peripheral blood CD8+ T cells were analyzed at times indicated for intracellular IFNy staining in response to dominant epitopes for survivin via flow cytometry. When control mice reached humane endpoint, all mice were sacrificed and tissues were collected for further analysis. The experiment was performed twice with n=3/treatment group.

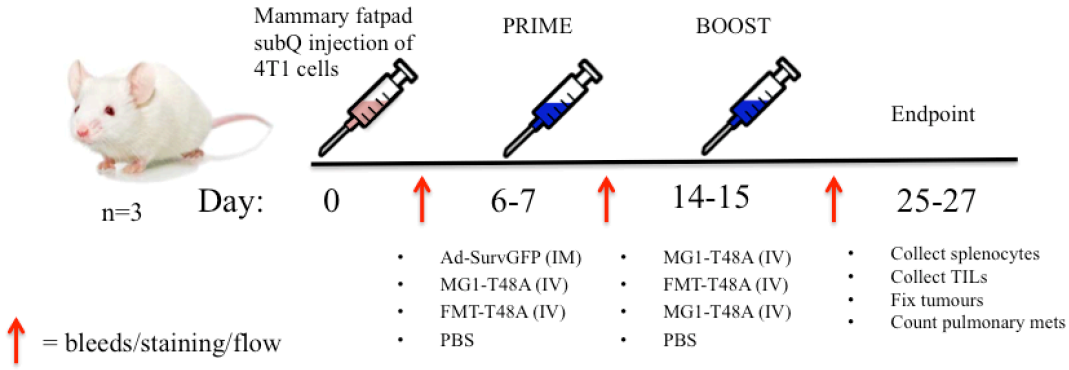

Mammary fatpad subQ injection of 4T1 cells
PRIME
BOOST
Endpoint
n=3
Day:
0
6-7
14-15
25-27
Ad-SurvGFP (IM)
MG1-T48A (IV)
FMT-T48A (IV)
PBS
MG1-T48A (IV)
FMT-T48A (IV)
MG1-T48A (IV)
PBS
Collect splenocytes
Collect TILs
Fix tumours
Count pulmonary mets
= bleeds/staining/flow

**Figure 6. Heterologous rhabdovirus prime/boost regimens control 4T1 tumour burden.** Groups of 4T1 tumour-bearing mice were treated with MG1-T48A prime/FMT-T48A boost, Ad-SurvGFP prime/MG1-T48A, FMT-T48A prime/MG1-T48A or PBS. Tumour progression was monitored over time (left tumour burden+right tumour=total tumour burden). Data is plotted as mean total tumour burden of 3 animals ± SD, but is representative of two experiments (total of n=6 for each treatment group). Asterisks (*) indicate significant values compared to PBS calculated by two-way ANOVA with Bonferroni test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, **** $p<0.0001$. Plus symbols (+) indicate significant values compared to Ad-SurvGFP prime/MG1-T48A boost calculated by two-way ANOVA with Bonferroni test. +$p<0.05$, ++$p<0.01$, +++$p<0.001$, ++++ $p<0.0001$. Pound symbol (#) indicates significant difference between MG1-T48A prime/ FMT-T48A boost and FMT-T48A prime/ MG1-T48A calculated by two-way ANOVA with Bonferroni test. #$p<0.05$.

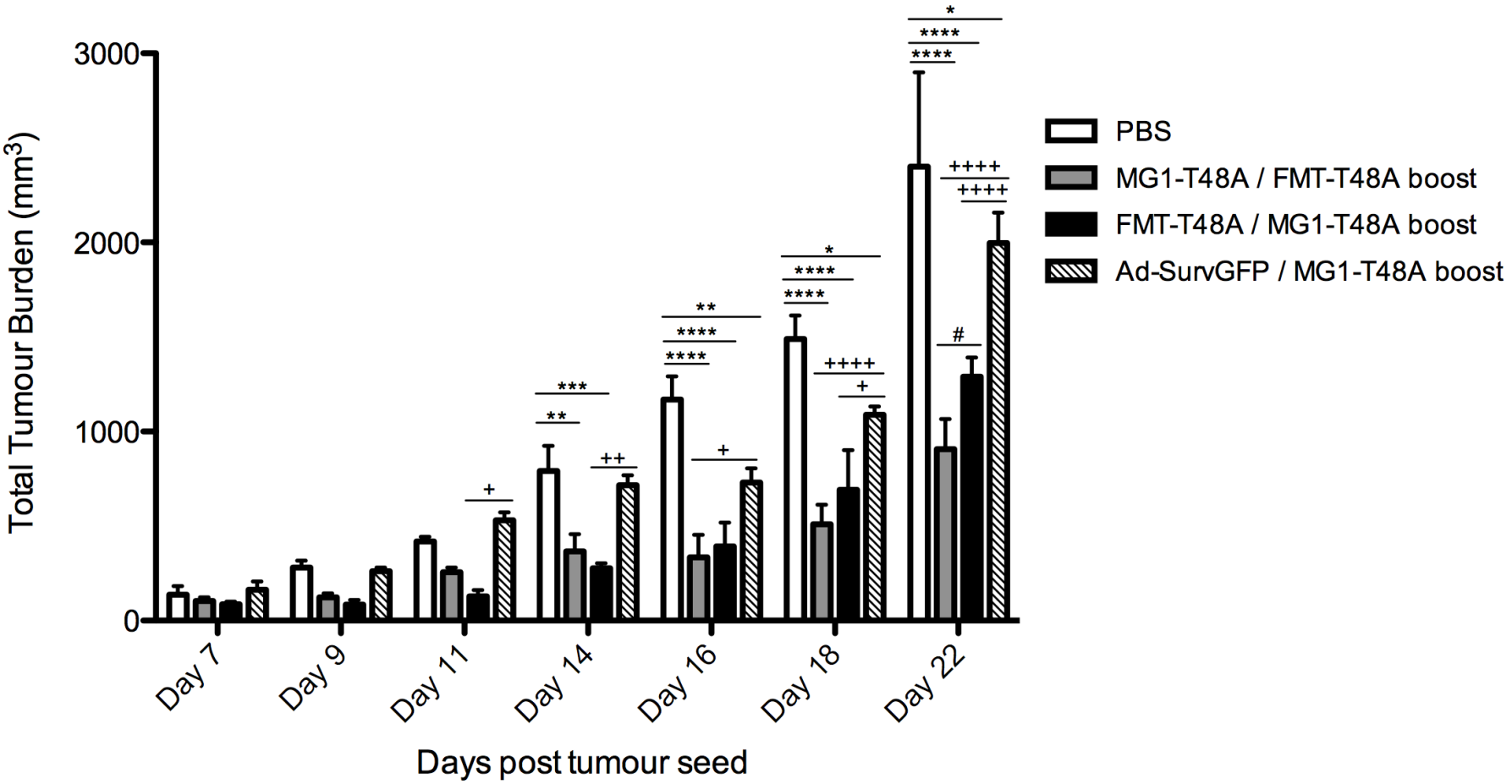
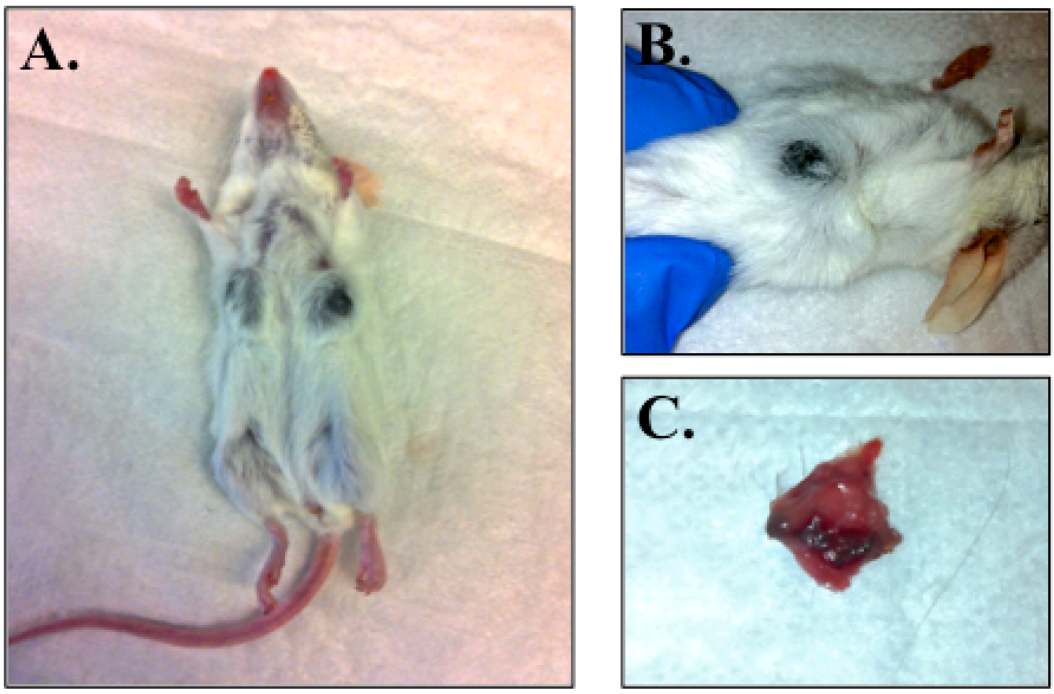

Total Tumour Burden (mm3)
3000
2000
1000
0
Day 7
Day 9
Day 11
Day 14
Day 16
Day 18
Day 22
Days post tumour seed
PBS
MG1-T48A / FMT-T48A boost
FMT-T48A / MG1-T48A boost
Ad-SurvGFP / MG1-T48A boost

**Figure 7. Tumours treated with MG1-T48A prime/ FMT-T48A boost appear necrotic.** Mouse treated with MG1-T48A prime/FMT-T48A boost (A). Close up view of mouse A's left tumour (B). Left tumour from B following dissection (C).

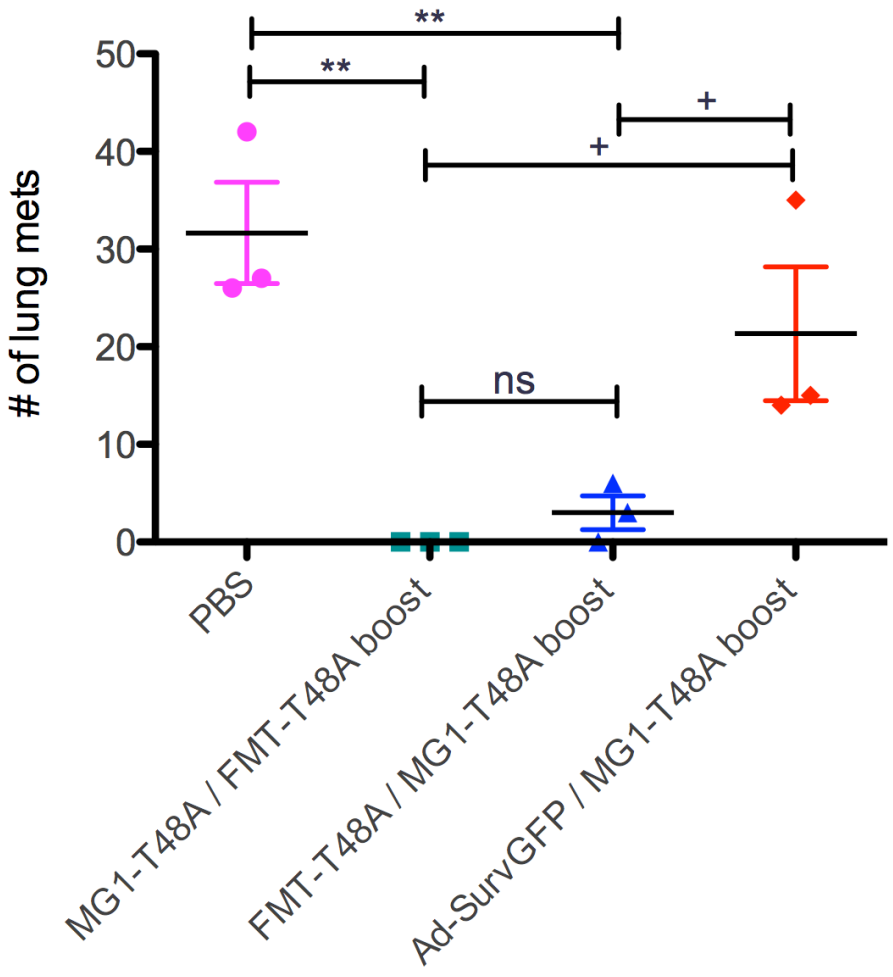

A.
B.
C.

The heterologous rhabdovirus prime/boost regimens also significantly controlled the formation of 4T1 lung metastases compared to PBS and to the Ad-SurvGFP prime/MG1-T48A boost vaccination regimen (Figure 8). This suggests that both oncolytic rhabdoviruses appear to do a better job in terms of controlling tumour burden and lung metastases compared to the Ad vector.

At endpoint, spleens were collected from the mice, and immune cells were isolated by Ficoll gradient centrifugation to use in a splenocyte killing assay of 4T1 cells *in vitro*. Splenocytes isolated from 4T1 tumour-bearing mice treated with MG1-T48A prime/FMT-T48A boost killed significantly more 4T1 cells *in vitro* compared to splenocytes from untreated tumour-bearing mice (Appendix, Figure S3.). The splenocytes isolated from other treatment groups (FMT-T48A prime/ MG1-T48A boost, Ad-SurvGFP prime/ MG1-T48A boost) did not demonstrate significant ability to kill 4T1 cells *in vitro* (Appendix, Figure S3).

Tumours were also excised from sacrificed animals, processed to a single cell suspension, and immune cells were isolated by Ficoll gradient centrifugation. The immune cells isolated from tumours were pooled within treatment groups due to low yield. These tumour-isolated immune cells were phenotyped for activated myeloid cell populations ($CD11b^+$, $CD40^+$, $CD80^+$) and T cell populations (CD4+, CD8+) by flow cytometry. The tumours of mice treated with MG1-T48A prime/FMT-T48A boost appeared to contain a large population of activated myeloid cell that were $CD40^+CD80^+$ whereas the tumours of mice from all other treatment groups contained a large population of non-activated $CD40^-CD80^-$ myeloid cells (Appendix, Figure S5). The tumours of mice treated with MG1-T48A prime/FMT-T48A boost and FMT-T48A prime/MG1-

**Figure 8. Heterologous rhabdovirus prime/boost regimens significantly reduce 4T1 lung metastases.** Groups of 4T1 tumour-bearing mice were treated with MG1-T48A prime/FMT-T48A boost, Ad-SurvGFP prime/MG1-T48A, FMT-T48A prime/MG1-T48A or PBS. At endpoint, lungs were collected from all the animals and inspected for visible lung metastases. (n=3 for each treatment group). Asterisks indicate significant values compared to PBS as calculated by one-way ANOVA with Dunnett's test. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Plus symbols (+) indicate significant values compared to Ad-SurvGFP prime/MG1-T48A boost calculated by one-way ANOVA with Dunnett's test. +$p<0.05$. ns = no significant difference between groups indicated, $p>0.05$.


**
**
+
+
ns
50
40
30
20
10
0
# of lung mets
PBS
MG1-T48A / FMT-T48A boost
FMT-T48A / MG1-T48A boost
Ad-SurvGFP / MG1-T48A boost

T48A boost seemed to contain more T cells when compared to the tumours of untreated mice and mice treated with Ad-SurvGFP prime/MG1-T48A boost (Appendix, Figure S6). Although there does appear to be considerably more activated myeloid cells in the tumours of MG1-T48A prime/FMT-T48A boost treated mice, the data described above suggests that survivin-targeting prime/boost regimens consisting of dual oncolytic rhabdoviruses generate immune responses in the 4T1 model, which may slow tumour progression.

### *3.2.3 Standalone heterologous rhabdovirus treatment demonstrates efficacy in the 4T1 model*

Since minimal anti-survivin CD8+ T cell responses were detected in the experiments described above (except for a single Ad-SurvGFP/MG1-T48A-vaccinated mouse in each repeat of the experiment) (Figure S4.), the experiment was repeated using non-TAA encoding FMT and MG1 to evaluate the contribution of encoding the self-TAA (Figure 9). Tumour progression over time was significantly slower in mice treated with the non-antigen-encoding heterologous rhabdovirus treatments compared to PBS (Figure 10. A). It was found that the non-TAA heterologous rhabdovirus treatments also significantly controlled 4T1 lung metastases compared to untreated mice (Figure 10. B). Taken together, these data suggest that the efficacy observed in the survivin-targeting rhabdoviral prime/boost regimens may not be dependent on anti-survivin T cell responses, but may be a result of *de-novo* anti-tumour responses generated by the oncolytic viruses releasing tumour-antigens and the ability of the rhabdoviruses to modulate the tumour microenvironment (Appendix, Figure S8). Since the immunological data generated during these experiments was only successfully performed once, further

**Figure 9. Experimental timeline comparing the efficacy of non-TAA-encoding, heterologous dual oncolytic treatments in a murine mammary carcinoma model.** Naïve female BALB/C mice (8-12weeks) were injected subcutaneously with 5E5 4T1 cells in left and right mammary fatpads. Once tumours were palpable, mice were treated with PBS (IV), 5E8 pfu FMT-eGFP (IV) or 5E8 pfu MG1-eGFP (IV) (prime). 7 days post-prime, mice were injected IV with PBS (n=3), 5E8 pfu MG1-eGFP (n=5) or 5E8 pfu FMT-eGFP (n=5), respectively (boost).

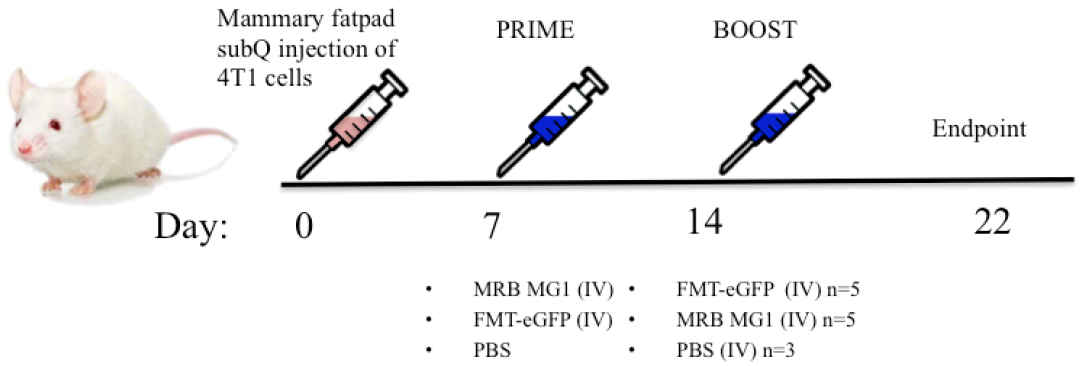
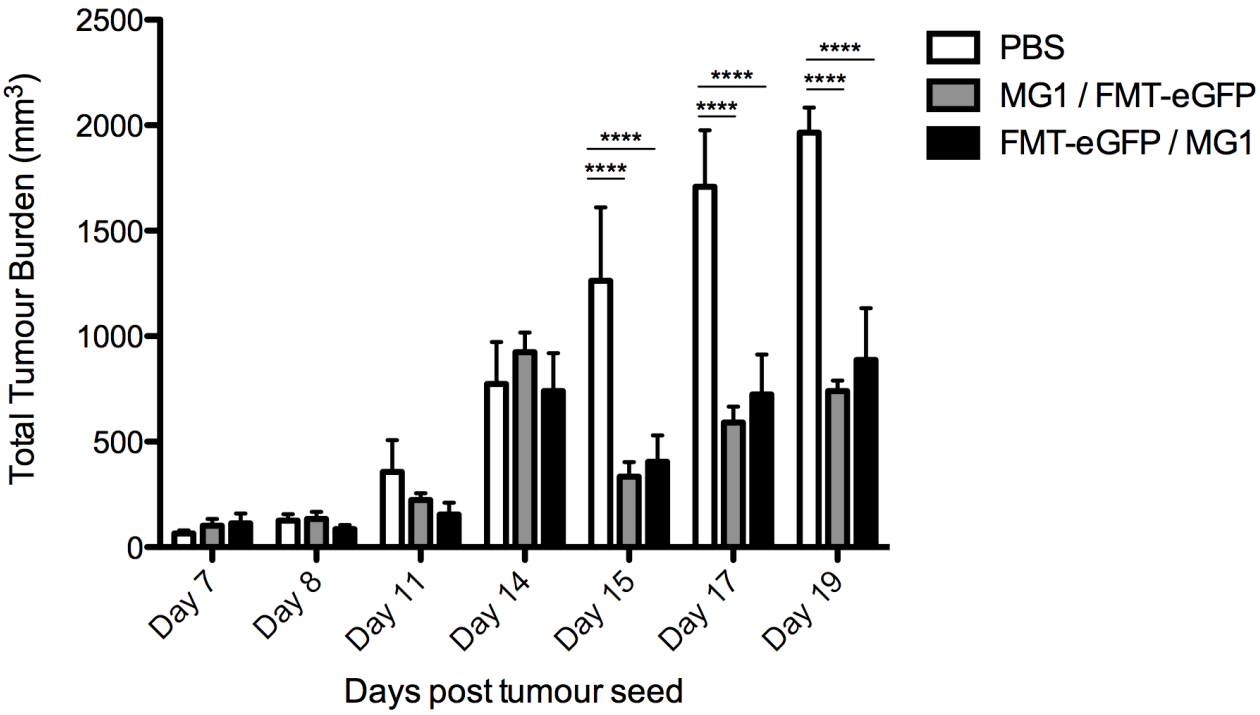
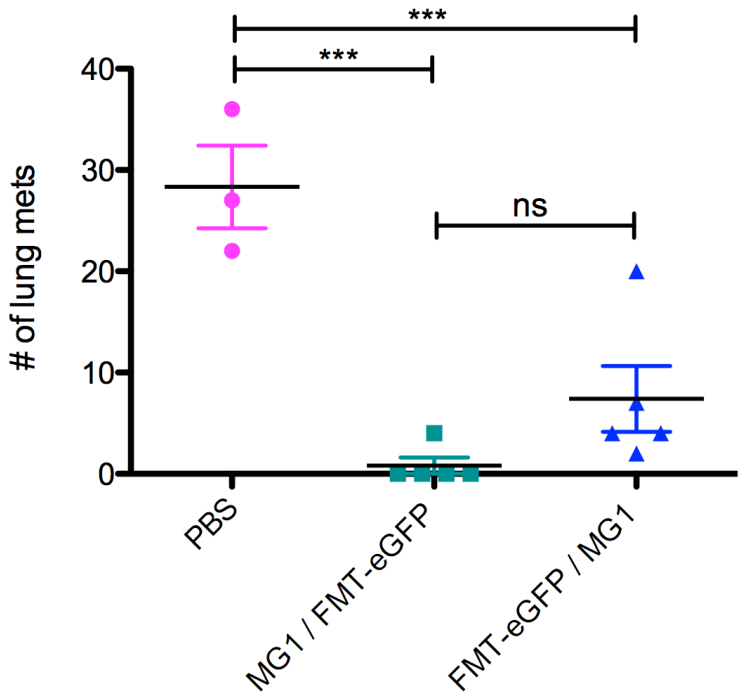

Mammary fatpad
subQ injection of
4T1 cells
PRIME
BOOST
Endpoint
Day:
0
7
14
22
• MRB MG1 (IV)
• FMT-eGFP (IV)
• PBS
• FMT-eGFP (IV) n=5
• MRB MG1 (IV) n=5
• PBS (IV) n=3

**Figure 10. Non-TAA-encoding heterologous rhabdovirus treatments significantly control 4T1 tumour burden and lung metastases.** Groups of 4T1 tumour-bearing mice were treated with MG1 /FMT-eGFP, FMT-eGFP/ MG1 or PBS. Tumour progression was monitored over time ((left tumour burden+right tumour burden=total tumour burden) (A). Data is plotted as mean ± SD. When untreated mice reached humane endpoint, lungs were collected from all the animals and counted for visible lung metastases (B). (n=3 for PBS group and n=5 for virus-treated groups). Asterisks indicate significant values compared to PBS as calculated by two-way ANOVA with Bonferroni test (A) or one-way ANOVA with Dunnett's test (B). *$p<0.05$, **$p<0.01$, ***$p<0.001$, **** $p<0.0001$. ns = no significant difference between groups indicated, $p>0.05$.

A.


Total Tumour Burden (mm3)
2500
2000
1500
1000
500
0
Day 7
Day 8
Day 11
Day 14
Day 15
Day 17
Day 19
Days post tumour seed
****
****
****
****
****
****
PBS
MG1 / FMT-eGFP
FMT-eGFP / MG1

B.


# of lung mets
40
30
20
10
0
***
***
ns
PBS
MG1 / FMT-eGFP
FMT-eGFP / MG1

repeats of the immunological experiments and the addition of other assays, such as immunohistochemistry to look at immune cell populations inside the tumours, would need to be executed to further support this hypothesis.

### 3.3 Investigating the ability of FMT viruses expressing foreign tumour-associated antigens (TAAs) to generate prophylatic or therapeutic TAA-specific CD8+ T cell responses

GBM tumours present a unique opportunity for oncolytic vaccination regimens since they express foreign, viral antigens derived from human cytomegalovirus (hCMV) (58, 60, 115, 116). Several immunodominant CMV antigens have been detected in the majority of GBM tumours, and are not found in surrounding healthy tissue. CMV antigens represent promising targets for our TAA-encoding FMT platform. Vaccinating against a foreign antigen also minimizes the potential for autoimmune consequences, which can arise following vaccination against self-antigen targets. For example, it has been previously shown that heterologous prime/boost vaccination against the self-melanoma antigen DCT leads to the targeted deletion of normal melanocytes resulting in vitiligo in long-term surviving mice (104). We hypothesized that FMT vectors would be better able to prime an immune response against a foreign antigen (as seen with OVA) compared to a self-antigen. To explore this hypothesis, two mouse CMV (mCMV) antigens were investigated as potential *in vivo* immunotherapeutic targets: IE3 and m38. FMT and Ad vectors were engineered to express either full-length IE3 or m38, and the corresponding CT2A GBM tumour cell lines were generated to express IE3 or m38 proteins.

*3.3.1 Vaccination against mCMV antigens in naïve mice*

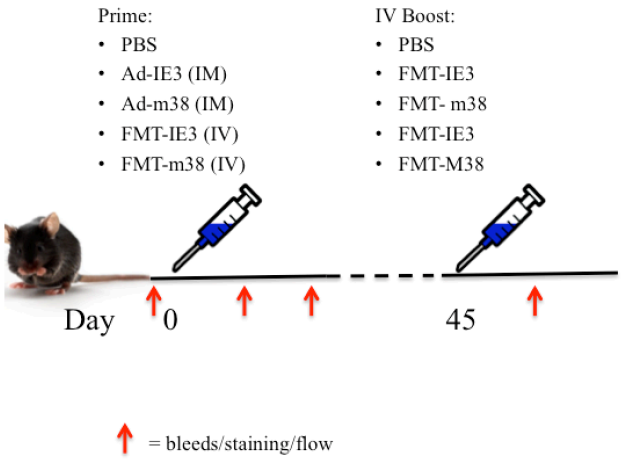
Initially, we wished to determine if FMT virus could prime a response against the mCMV antigens comparable to Ad, as it did with the OVA antigen. Thus the ability of the Ad-mCMV and FMT-mCMV viruses to prime a response against the target antigens in naïve animals was evaluated (Figure 11. A). Mice vaccinated with Ad-IE3 or FMT-IE3 both generated small, but detectable anti-IE3 CD8+ T cell responses (Figure 11. B). Mice primed with FMT-m38 generated slightly greater anti-m38 responses compared to Ad-m38 primed mice, but this was not statistically significant (Figure 11. C).

We subsequently investigated whether these responses could be boosted in a secondary recall response 45 days post prime. The purpose of this was to investigate if we can still boost responses at late time points post-prime to model the situation in patients who have been historically infected with hCMV. Unfortunately, the Maraba MG1 counterpart to the heterologous rhabdovirus prime/boost schedule was not generated, so the primed responses were all boosted by FMT mCMV. Robust anti-mCMV responses were observed in mice that were primed with Ad-mCMV and boosted at a late time point with FMT-mCMV (Figure 11. B, C). The homologous prime/boost regimens, where mice were primed and boosted with FMT mCMV, did not generate remarkable anti-mCMV responses. This is likely due to the fact that the second dose of FMT was rapidly deleted by virus-specific neutralizing antibody generated from the first dose of FMT (99). Pol *et al.* (2013) also found that robust anti-DCT responses could not be generated by homologous Ad-DCT prime/Ad-DCT boost or MG1-DCT/MG1-DCT boost regimens (104). These data suggest that Ad-mCMV can prime responses against

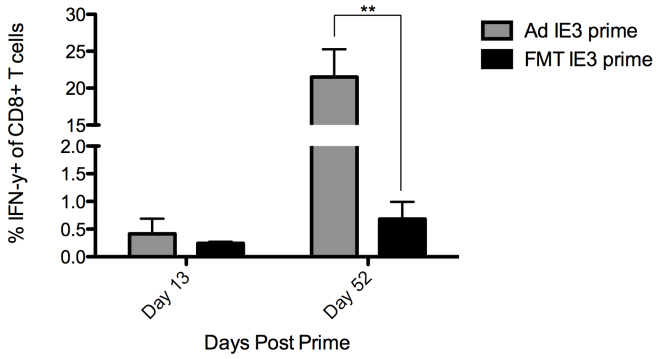
mCMV antigens and that FMT-mCMV vectors can acts as both priming and boosting agents against mCMV antigens.

**Figure 11. Ad-mCMV can prime anti-mCMV CD8+ T cell responses and FMT-mCMV can act as both a priming and a boosting agent against mCMV antigens.** Experimental timeline for comparing Ad-mCMV prime against FMT-mCMV prime (A): Naïve female C57BL/6 mice (8-12weeks) were injected with PBS (IV), 4E7 pfu Ad-IE3 (IM), 4E7 pfu Ad-m38 (IM), 5E8 pfu FMT-IE3 (IV) or 5E8 pfu FMT-m38 (IV) (prime). Peripheral blood CD8+ T cells were analyzed at times indicated for intracellular IFNy staining in response to dominant epitopes for IE3 (B), m38 (C) and antiviral (not shown) responses via flow cytometry. The mice were injected IV with the FMT platform expressing the corresponding mCMV antigen 45 days post-prime (boost) and were bled again for PBMC analysis a week later. n=3 for each group. Asterisks indicate significant values as calculated by a Student's T-test. *$p<0.05$, **$p<0.01$.

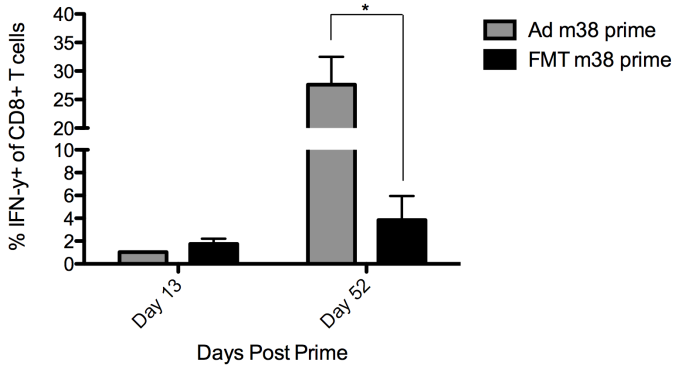
A.




B. Anti-IE3 response




C. Anti-m38 response



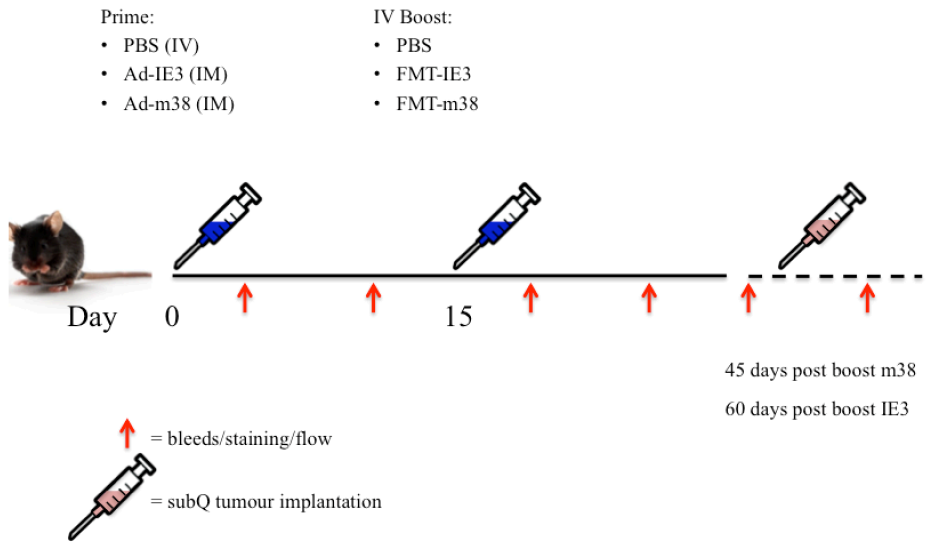
*3.3.2 Prophylactic mCMV antigen-based vaccination against tumour challenge*

Next, the anti-mCMV responses generated in a traditional heterologous prime/boost regimen (boost 15 days post prime) were investigated (Figure 12. A). The Ad-mCMV prime / FMT-mCMV boost generated robust CD8+ T cell responses against IE3 and m38 target antigens (Figure 12. B and C, respectively). Approximately 2 months post-prime, mice were challenged with subcutaneous wtCT2A tumours (left flank) and mCMV-CT2A tumours (right flank). Mice were bled pre-tumour implantation, when the anti-mCMV CD8+ T cell responses appeared to have contracted to lower frequencies (Figure 12. B and C). Mice were bled again post-tumour implantation, but there were no significant changes in anti-mCMV responses (Figure 12. B and C).

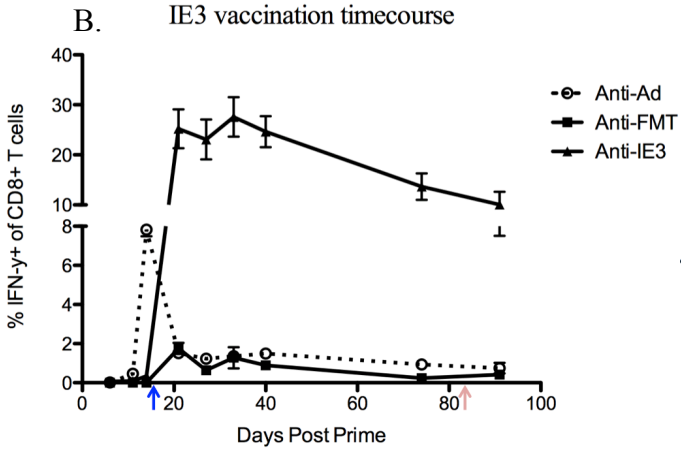
Tumour burdens were monitored over time. In unvaccinated animals, both wtCT2A and CT2A-mCMV tumours on either flank progressed at similar rates (Figure 13. A, D, E). In IE3-vaccinated animals, both wtCT2A and CT2A-IE3 tumours on either flank progressed at similar rates (Figure 13. B, D). In m38-vaccinated animals, wtCT2A tumours were quickly established on the left flank, while the growth of CT2A-m38 tumours was suppressed in the majority of animals on the right flank (Figure 13. C, E). Importantly, wtCT2A tumours grew at similar rates in unvaccinated and prime/boost-vaccinated animals, supporting the idea that the control of the m38 tumour burden in vaccinated animals was due to the presence of the m38-antigen and the pre-established CD8+ T cell response specific to this antigen. Taken together, these data suggest that m38, but not IE3, acts as a tumour-rejection antigen in this prime/boost mCMV vaccination model.

**Figure 12. Ad-mCMV prime/FMT-mCMV boost regimens generate robust CD8+ T cell responses against IE3 and m38 mCMV proteins.** Experimental timeline for heterologous prime/boost vaccination regimen using mCMV antigen-encoding Ad (prime) and FMT (boost) (A): Naïve female C57BL/6 mice (8-12weeks) were injected with PBS (IV), 4E7 pfu Ad-IE3 (IM) or 4E7 pfu Ad-m38 (IM) (prime). The mice were injected IV with 5E8 pfu of the FMT platform expressing the corresponding mCMV antigen 15 days post-prime (boost). Peripheral blood CD8+ T cells were analyzed at times indicated for intracellular IFNy staining in response to *ex vivo* stimulation with immunodominant CD8+ peptides for IE3 (B) or m38 (C) and antiviral responses (B,C) via flow cytometry. Approximately 2 months post-prime, vaccinated and unvaccinated mice were challenged with subcutaneous CT2A-wt tumours (left flank) and CT2A-mCMV (right flank) tumours. Data is plotted as the mean of 5 mice ± SEM.

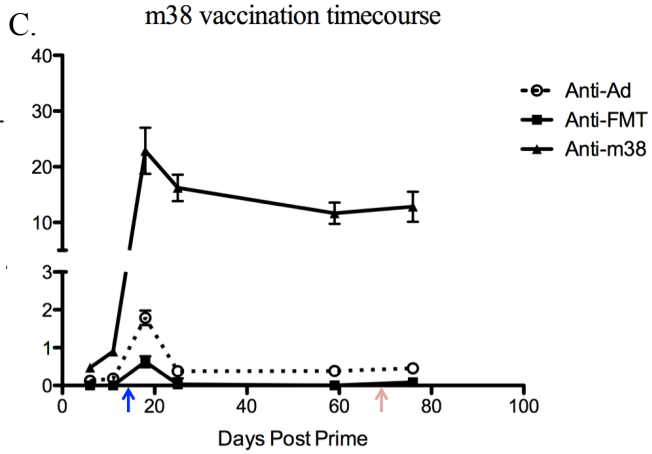
A.




B.




C.

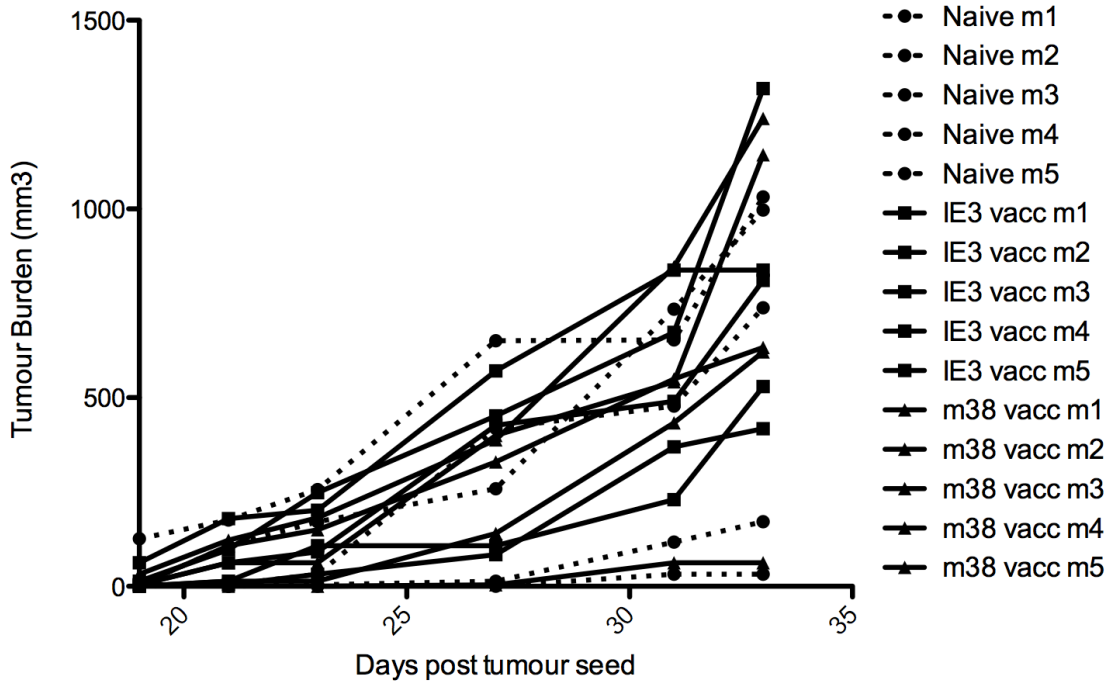



= boost with mCMV FMT vector

= subQ tumour implantation

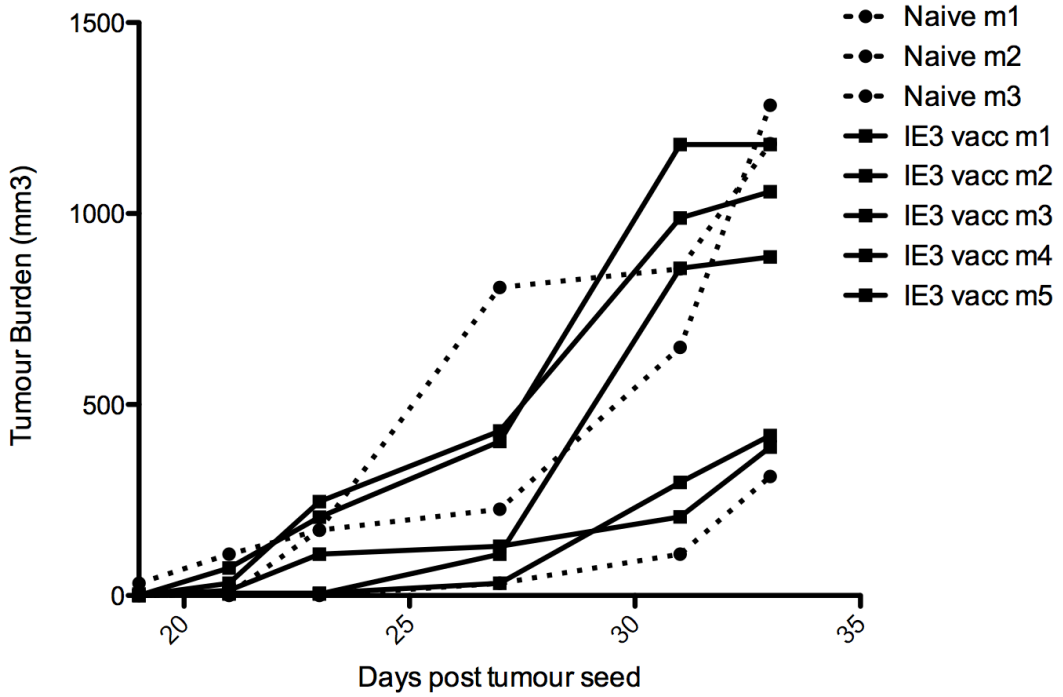
**Figure 13. Mice vaccinated against m38 control subcutaneous CT2A-m38 tumour progression, but IE3-vaccinated mice do not control CT2A-IE3 tumours.** Naïve female C57BL/6 mice (8-12weeks) were injected with PBS (IV), Ad-IE3 (IM) or Ad-m38 (IM) (prime). The mice were injected IV with the FMT platform expressing the corresponding mCMV antigen 15 days post-prime (boost). IE3- and m38-vaccinated mice generated robust anti-mCMV antigen CD8+ T cell responses. Approximately 2 months post-prime, vaccinated and unvaccinated mice were challenged with subcutaneous CT2A-wt tumours (left flank) and CT2A-mCMV (right flank) tumours. Wildtype CT2A tumours progressed similarly between vaccinated and unvaccinated animals (A). CT2A-IE3 tumours progressed similarly between IE3 vaccinated (n=5) and unvaccinated animals (n=3) (B). CT2A-m38 tumours were controlled in m38-vaccinated mice (n=5) compared to unvaccinated mice (n=2) (C). Day 31 post tumour seed, IE3-vaccinated and unvaccinated mice show similar CT2A-wt and CT2A-IE3 tumour burden (D). Day 31 post tumour seed, m38-vaccinated and unvaccinated mice show similar CT2A-wt tumour burden, however the CT2A-m38 tumour burden is significantly controlled in vaccinated mice compared to unvaccinated mice (as determined by a student's T-test, *$p<0.05$, **$p<0.01$) (E).

A. Wildtype CT2A tumour progression

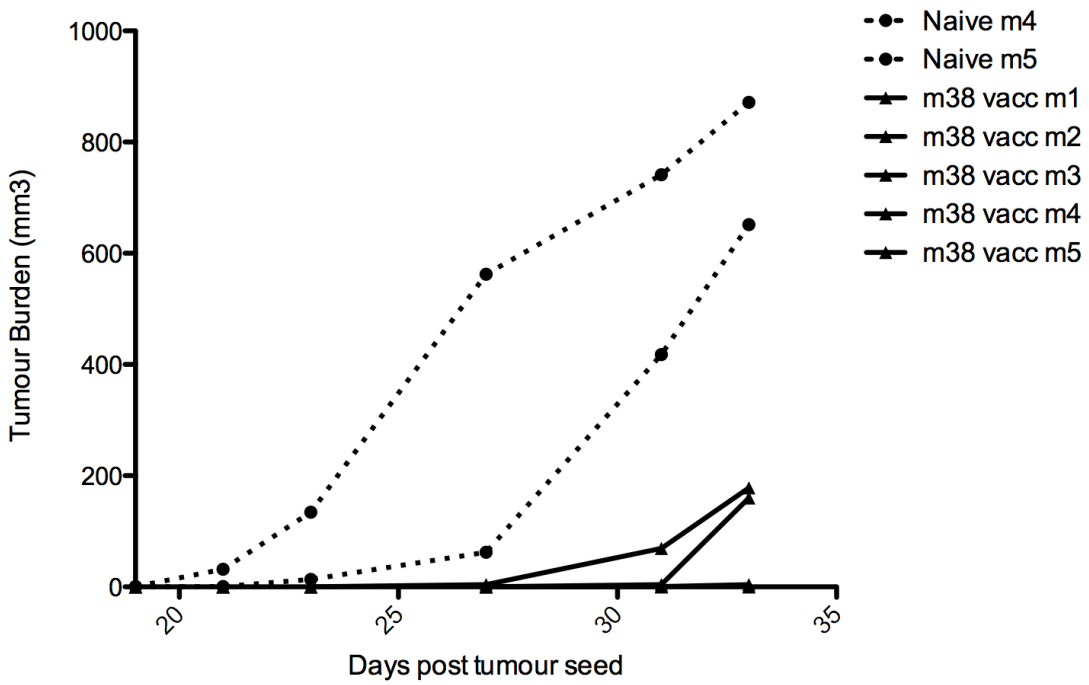



B.

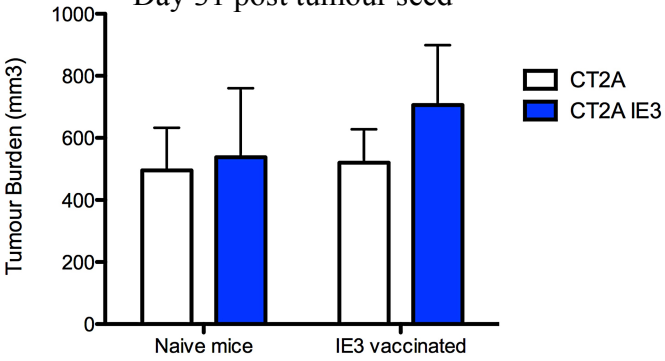
CT2A-IE3 tumour progression



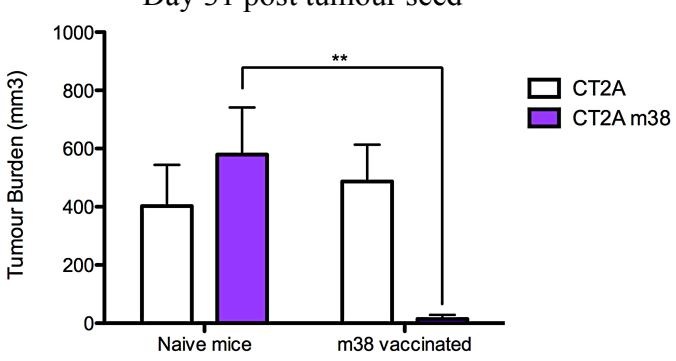
C. CT2A-m38 tumour progression




D. Day 31 post tumour seed




E. Day 31 post tumour seed



*3.3.3 Therapeutic mCMV antigen-based vaccination against GBM tumours*

It has previously been shown that a large percentage of hCMV seropositive GBM patients express immunogenic hCMV proteins in their tumours; making these a target for immunotherapies (58, 60, 115, 116). Murine CMV has also been shown to associate with mouse gliomas (57). We chose to model the disease in a species-specific manor and have demonstrated that we can successfully vaccinate naïve mice with a heterologous prime/boost regimen against mCMV antigens. We also demonstrated that prophylactic vaccination against the m38 antigen protects against subcutaneous CT2A-m38 tumour implantation. We had yet to investigate our vaccination system in a brain tumour model, thus we evaluated intracranial tumour progression and the effect of FMT-m38 treatment in the orthotopic model system of wt CT2A and CT2A-m38 as an initial pilot study (Figure 14).

Mice were injected intracranially with wtCT2A or CT2A-m38 tumour cells. Ten days post-tumour seed, MRI imaging was used to evaluate tumour progression (Figure 15). The CT2A-m38 tumours appeared slightly larger than the wtCT2A tumours, suggesting that the CT2A-m38 model might progress more rapidly than the wtCT2A model (Figure 15. A and B). Next, we evaluated if detectable anti-m38 responses were generated by the act of implanting a m38-expressing tumour. However, at 10 days post-tumour seed, no anti-m38 responses were detected (Figure 16. A), suggesting that either the tumour did not supply a sufficient prime, or that the responses were below the threshold of detection. Fourteen days post-tumour seed, wtCT2A and CT2A-m38 mice were treated with FMT-m38. At day 7 post-virus treatment, there were detectable anti-m38 CD8+ T cell responses in mice treated with FMT-m38. CT2A-m38 tumour-bearing

**Figure 14. Experimental timeline comparing wt CT2A vs. mCMV CT2A intracranial tumour progression.** Naïve female C57BL/6 mice (8-12weeks) were injected intracranially with either 5E4 wtCT2A cells or CT2A-m38 cells. Peripheral blood CD8+ T cells were analyzed at times indicated for intracellular IFNy staining in response to *ex vivo* stimulation with immunodominant CD8+ peptides from m38 via flow cytometry. Tumour progression was monitored by MRI imaging performed 10 days and 21 days post-tumour seed. At day 14 post-tumour seed, wtCT2A and CT2A-m38 tumour-bearing mice were treated with 5E8 pfu FMT-m38 (IV)). n=3 for each group.

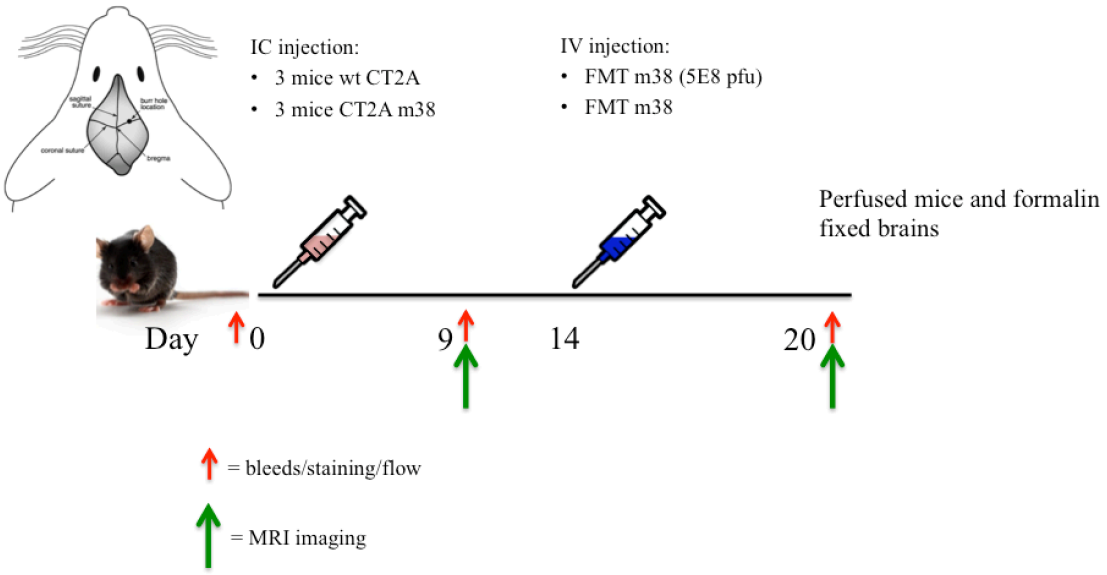
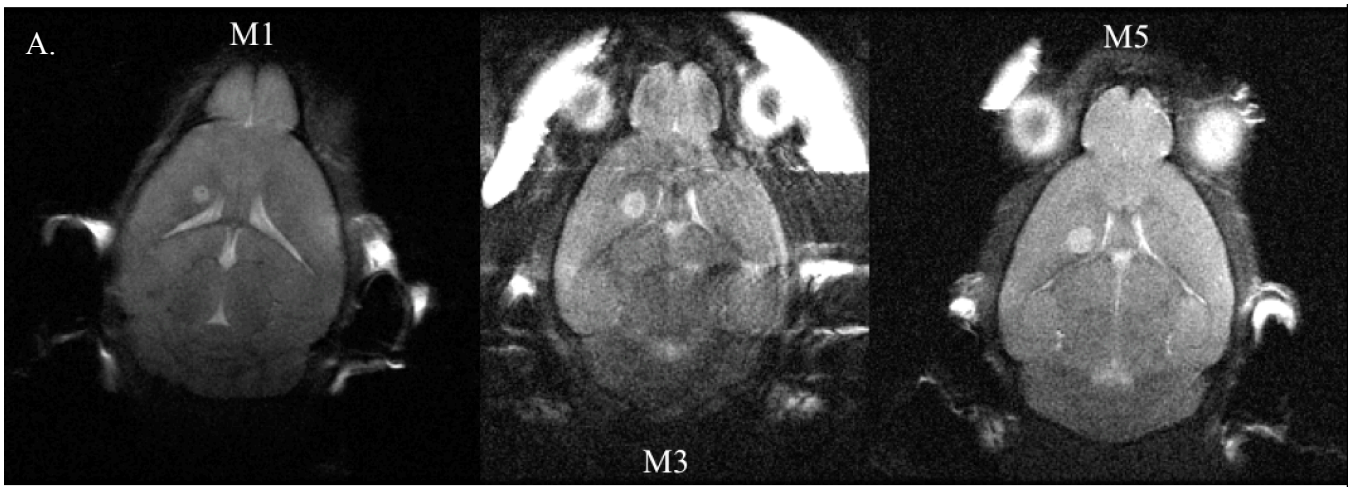

IC injection:
• 3 mice wt CT2A
• 3 mice CT2A m38
IV injection:
• FMT m38 (5E8 pfu)
• FMT m38
Perfused mice and formalin fixed brains
Day
0
9
14
20
= bleeds/staining/flow
= MRI imaging

**Figure 15. MRI images of wt CT2A and CT2A-m38 intracranial tumours at day 10 post-tumour seed demonstrate slightly increased progression of CT2A-m38 tumours.** Naïve female C57BL/6 mice (8-12weeks) were injected intracranially with wtCT2A (A) or CT2A-m38 (B) cells. MRI imaging was performed 10 days post tumour seed. n=3 for each group.

Wildtype CT2A IC tumours




CT2A-m38 IC tumours

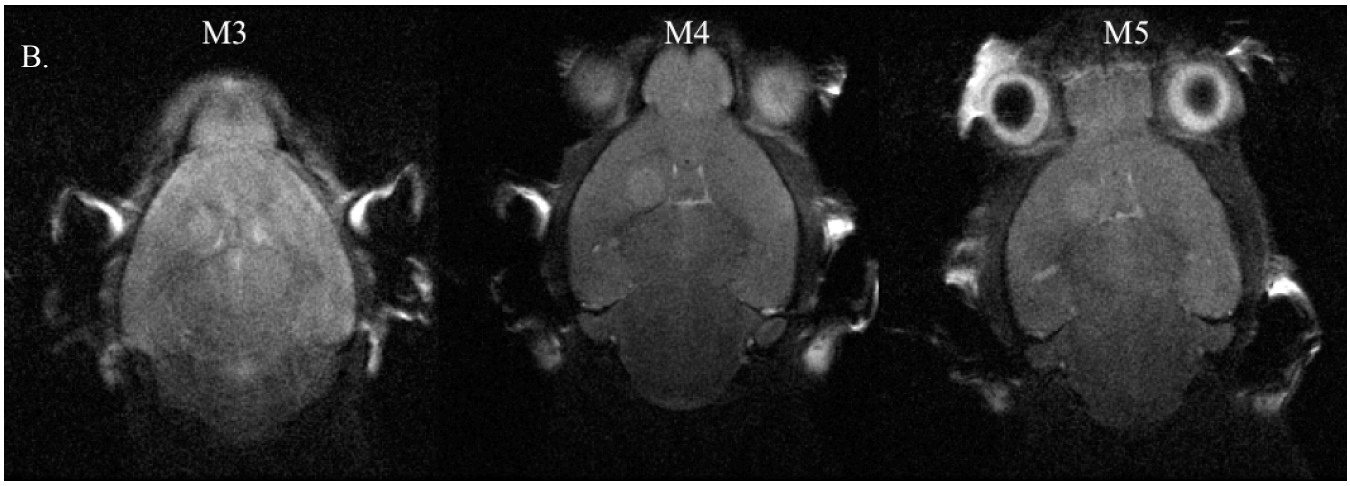
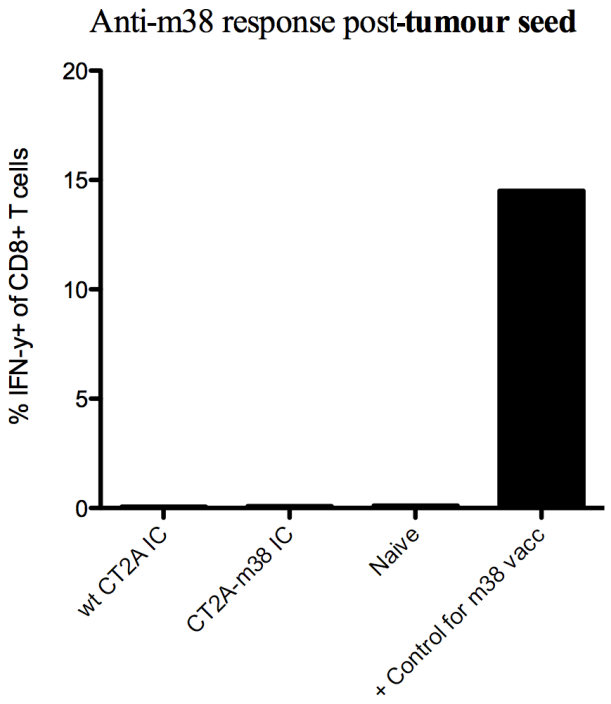
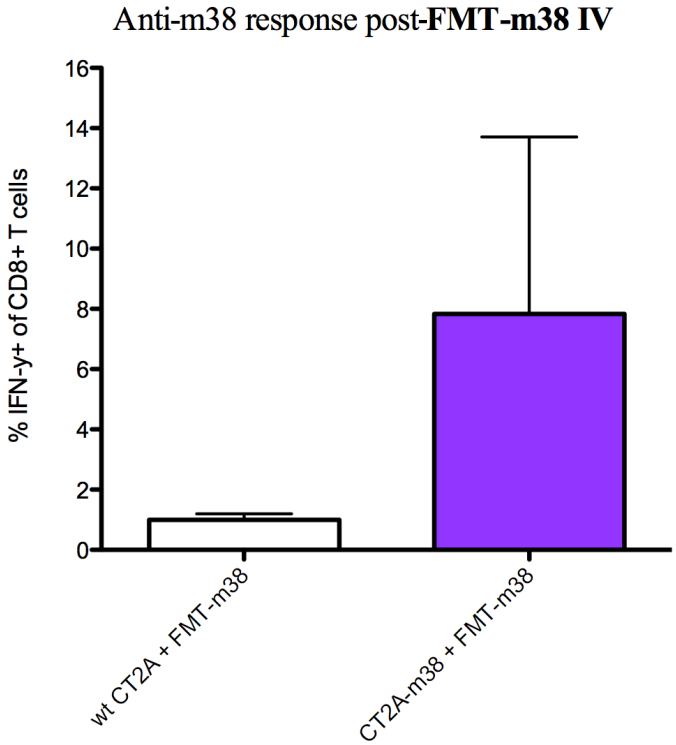


**Figure 16. A single IV dose of FMT-m38 generates a greater anti-m38 CD8+ T cell response in CT2A-m38 tumour-bearing mice compared to wtCT2A tumour-bearing mice.** Naïve female C57BL/6 mice (8-12weeks) were injected intracranially with either 5E4 wtCT2A cells or CT2A-m38 cells. At day 14 post-tumour seed, wtCT2A and CT2A-m38 tumour-bearing mice were treated with 5E8 pfu FMT-m38 (IV). Peripheral blood CD8+ T cells were analyzed at 10 days post tumour seed and again at 7 days post virus treatment for intracellular IFNy staining in response to dominant epitopes for m38 via flow cytometry. At day 10 post tumour seed, there were no detectable anti-m38 (A). A naïve mouse previously vaccinated by Ad-m38 prime/FMT-m38 boost was used as a positive control for m38 response in A. At 7 days post virus treatment, there were detectable anti-m38 responses in mice treated with FMT-m38. CT2A-m38 tumour-bearing mice had higher anti-m38 responses compared to wtCT2A tumour-bearing mice (B). Data is plotted as mean of 3 animals (A) ± SEM (B).

A.


Anti-m38 response post-tumour seed
% IFN-y+ of CD8+ T cells
20
15
10
5
0
wt CT2A IC
CT2A-m38 IC
Naive
+ Control for m38 vacc

B.


Anti-m38 response post-FMT-m38 IV
% IFN-y+ of CD8+ T cells
16
14
12
10
8
6
4
2
0
wt CT2A + FMT-m38
CT2A-m38 + FMT-m38

mice had higher anti-m38 responses compared to wtCT2A tumour-bearing mice (Figure 16. B).

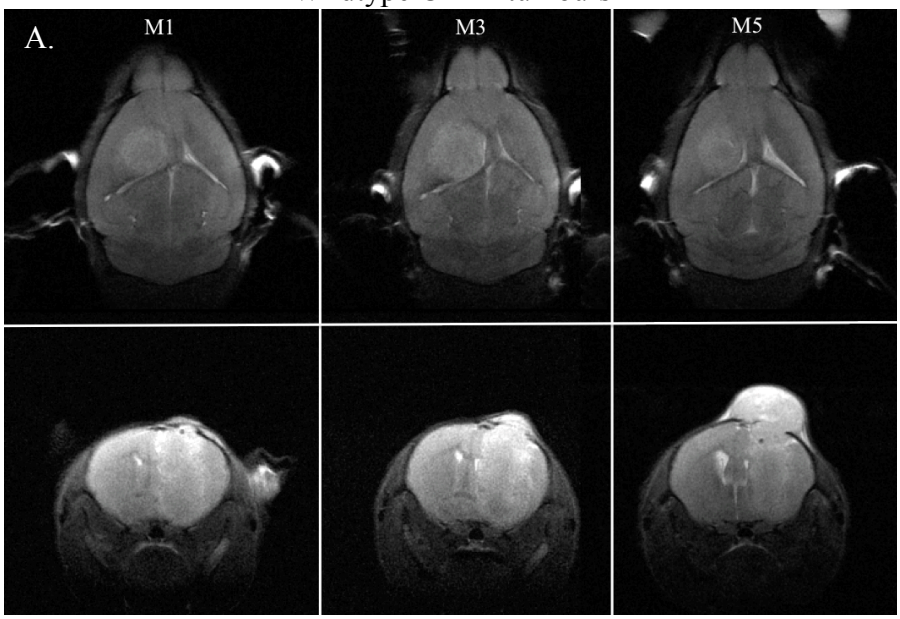
Mice were also MRI imaged 7 days post-virus treatment. Wild-type CT2A tumours were large, with brightly defined borders whereas the CT2A-m38 tumours appeared dark and undefined (Figure 17. A and B). It was interesting to note that CT2A-m38 tumour-bearing mouse #3 had the highest anti-m38 CD8+ T cell response out of the FMT-m38 treated mice (15%), and its axial MRI scan also showed an unusually large ventricle (Figure 17. B). In the brain, antigen-presenting cells (APCs) are found in the ventricles, and the data suggests that the ventricles in mouse #3 may appear so large due to proliferation of APCs in response to treatment with FMT-m38 (21, 117, 118). The data described above also suggest that the m38 model engineered in our lab provides a promising mouse CMV system to investigate oncolytic vaccination, and other immunotherapies designed to target a foreign tumour-associated antigen to treat GBM.

### *3.3.4 Evaluating the efficacy of a therapeutic prime/boost using m38 as a target TAA to treat GBM*

In section 3.3.2, we demonstrated that an Ad-m38 prime followed by a FMT-m38 boost activated a robust CD8+ T cell response that was able to prophylactically protect against subcutaneous CT2A-m38 tumours. To test this approach in a more challenging therapeutic model against established tumours, mice were inoculated intracranially with CT2A-m38 cells and then primed with Ad-m38 on day 7 post-tumour seed. The prime

**Figure 17. MRI imaging 7 days post-virus treatment reveals large, brightly defined tumours in wt CT2A mice and dark, undefined tumours in CT2A-m38 mice.** Naïve female C57BL/6 mice (8-12weeks) were injected intracranially with 5E4 wtCT2A cells (A) or CT2A-m38 cells (B). At day 14 post-tumour seed, wtCT2A and CT2A-m38 tumour-bearing mice were treated with 5E8 pfu FMT-m38 (IV). MRI imaging was performed 7 days post virus treatment. Mouse 3 (M3) had a robust anti-m38 CD8+T cell response (15%) and the axial image shows dramatically increased ventricle size (indicated by arrow). Mouse 4 (M4) and M5 demonstrated smaller anti-m38 responses and do not have large ventricles. Top row represents axial scans. Bottom row represents coronal scans. n=3 mice per group.

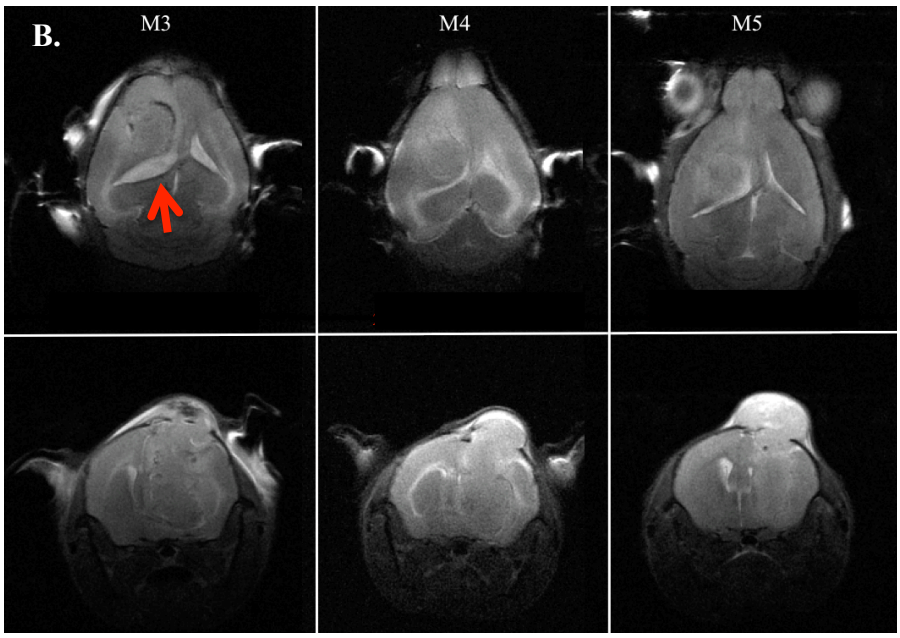
Wildtype CT2A tumours




axial

coronal

CT2A-m38 tumours




axial

coronal

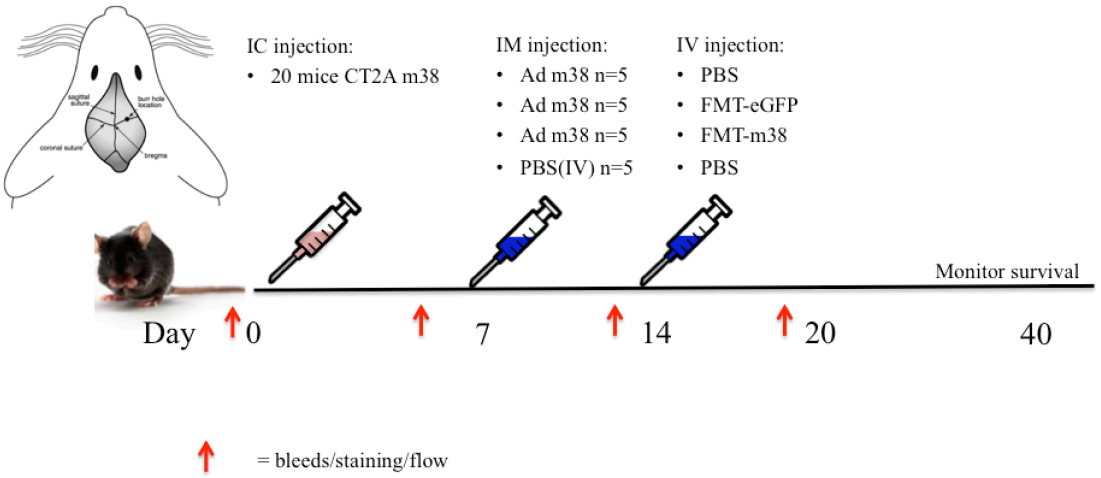
was followed by a boost 14 days post-tumour seed (7 days post-prime) with PBS as a negative control (n=5), FMT expressing the irrelevant antigen, eGFP, to control for the contribution of the TAA (n=5), or FMT-m38 (n=5). Mice were bled at indicated time points for *ex vivo* T cell stimulation and intracellular cytokine staining (Figure 18. A).

We found that all of the treatments significantly prolonged the survival of tumour-bearing animals compared to PBS treated mice, however none of the mice were ultimately cured (Figure 18. B). Unfortunately, 2 out of 5 mice in the Ad-m38 prime/FMT-m38 boost were found dead 4 days post boost and were therefore excluded from the survival curve because their cause of death was unknown. Upon necropsy, it was observed that these mice had large, bloody masses in their brains in the place of tumours, suggesting that maybe a successful anti-tumour immune response was mounted, but the tumour mass was too large to clear. This is supported by the fact that PBS treated mice reached endpoint from large tumour burden only 5-6 days post boost (~20 days post tumour seed), indicating that the tumours may have been a substantial size at the time the boost was given. Without MRI imaging to confirm tumour progression and the inability to measure immune responses due to the mice being found dead, no concrete conclusions can be made. It is interesting to note that there were no statistically significant differences between any of the vaccination groups (Ad-m38 prime/PBS boost, Ad-m38 prime/FMT-eGFP boost, Ad-m38 prime/FMT-m38 boost).

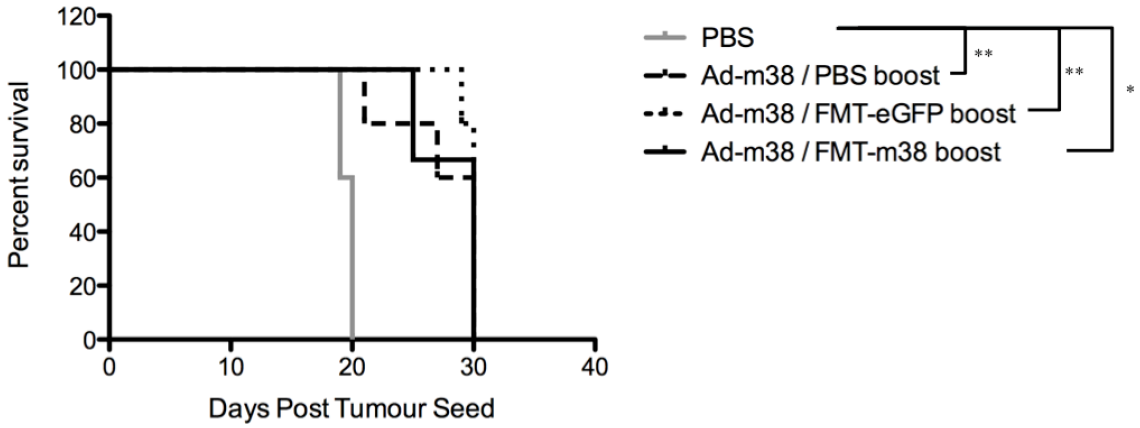
The anti-m38 CD8+ T cell responses were monitored throughout the experiment by ICS for IFNy following PBMC stimulation *ex vivo* with the immunodominant epitope of m38 (Figure 19). Some mice appeared to mount a small but detectable anti-m38 response 6 days post-tumour seed, indicating that the inflammation resulting from the

**Figure 18. Ad-m38 prime significantly extends survival in an intracranial CT2A-m38 model.** Experimental timeline for Ad-m38 prime/FMT-m38 boost intracranial efficacy experiment (A). Naïve female C57BL/6 mice (8-12 weeks) were injected intracranially with 5E4 CT2A-m38 cells. Seven days post tumour seed, mice were primed with 1E7 pfu Ad-m38. Seven days following the prime, mice were boosted with 5E8 pfu FMT-eGFP, 5E8 pfu FMT-m38 or PBS. Peripheral blood CD8+ T cells were analyzed at times indicated for intracellular IFNy staining in response to stimulation with the immunodominant CD8+ epitopes from m38 via flow cytometry. Efficacy was scored by overall mouse survival illustrated by Kaplan-Meier curve (B). n=5 for each group. Asterisks indicate statistically significant difference in survival compared to PBS as determine by Mantel-Cox log-rank test. *$p<0.05$, **$p<0.01$.

A.


IC injection:
• 20 mice CT2A m38
IM injection:
• Ad m38 n=5
• Ad m38 n=5
• Ad m38 n=5
• PBS(IV) n=5
IV injection:
• PBS
• FMT-eGFP
• FMT-m38
• PBS
Monitor survival
Day 0
7
14
20
40
= bleeds/staining/flow

B.


Percent survival
120
100
80
60
40
20
0
0
10
20
30
40
Days Post Tumour Seed
PBS
Ad-m38 / PBS boost
Ad-m38 / FMT-eGFP boost
Ad-m38 / FMT-m38 boost
**
**
*

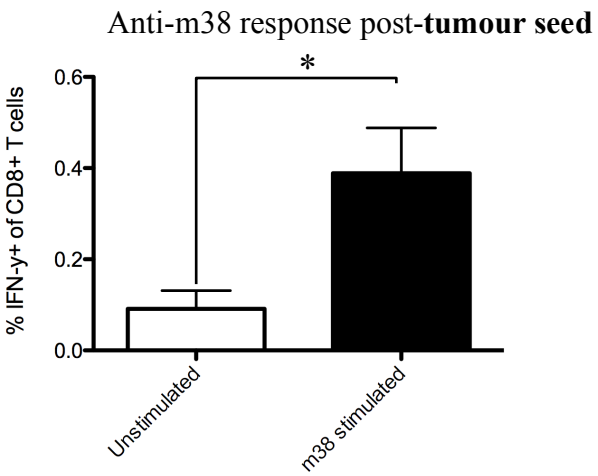
intracranial cell implantation may trigger priming against the TAA expressed by the CT2A cells in some mice (see significant difference between unstimulated vs. m38 stimulated IFNy+ CD8 T cells in Figure 19.A).

Anti-m38 responses were analyzed again 6 days post-Ad-m38 prime and 20% of the mice generated a strong anti-m38 response exceeding the responses observed following Ad-m38 prime in naïve animals (~10% vs. ~1%) (Figure 19. B vs. 11. C). The variability observed between mice could be due to the restriction presented by a low titer stock Adenovirus; meaning a lower dose of Ad-m38 was used to prime compared to other studies (1E7 vs. 2E8). This supports the hypothesis that the implantation of the tumour cells may drive priming against the TAA in some mice. Six days post boost, we found that the Ad-m38 prime/PBS boost treated mice had anti-m38 responses between 1-8%, the Ad-m38 prime/FMT-eGFP boost treated mice had anti-m38 responses between 2-4% and the Ad-m38 prime/FMT-m38 boost treated mice had variable responses to m38 (Figure 19. C). One mouse vaccinated with Ad-m38 prime/FMT-m38 boost had an extremely robust anti-m38 CD8+ T cell response (61%) whereas the other two mice in the group had responses below 10%. Interestingly, the mouse with the large anti-m38 response did survive the longest within its treatment group, but it was not cured.

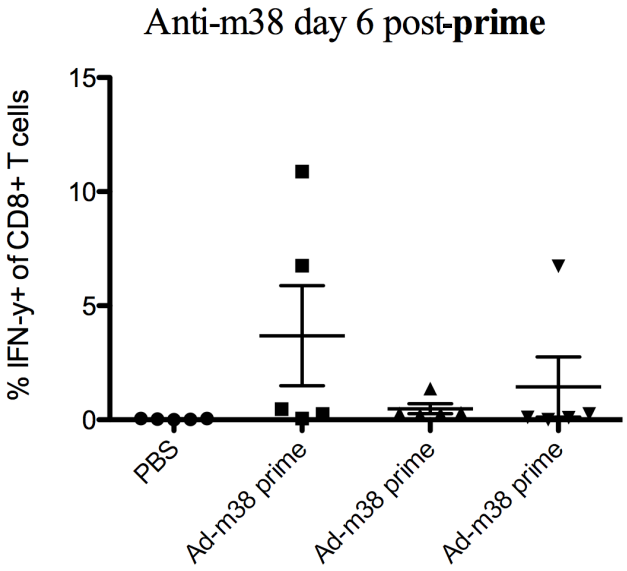
It is clear from these data that the anti-m38 response generated from vaccination in a tumour-bearing animal is different from what is observed in naïve animals and that prophylactic protection in a subcutaneous tumour model does not necessarily translate to a therapeutic intracranial tumour model. Factors such as large, well-established tumours at the time of vaccination could have affected the immune response via immunosuppressive mechanisms. Repeating the experiment using fewer cells for tumour

**Figure 19. Prime/boost vaccination strategies generate variable anti-m38 responses in intracranial CT2A-m38 tumour-bearing animals.** Naïve female C57BL/6 mice (8-12 weeks) were injected intracranially with 5E4 CT2A-m38 cells. Seven days post tumour seed, mice were primed with 1E7 pfu Ad-m38. Seven days following the prime, mice were boosted with 5E8 pfu FMT-eGFP, 5E8 pfu FMT-m38 or PBS. Peripheral blood CD8+ T cells were analyzed 6 days post-tumour seed (A), 6 days post-prime (B), and 6 days post-boost (C) for intracellular IFNy staining in response to stimulation with the immunodominant CD8+ epitopes from m38 via flow cytometry. For A, asterisks indicate statistically significant difference as determined by a Student's T-test. *$p<0.05$.

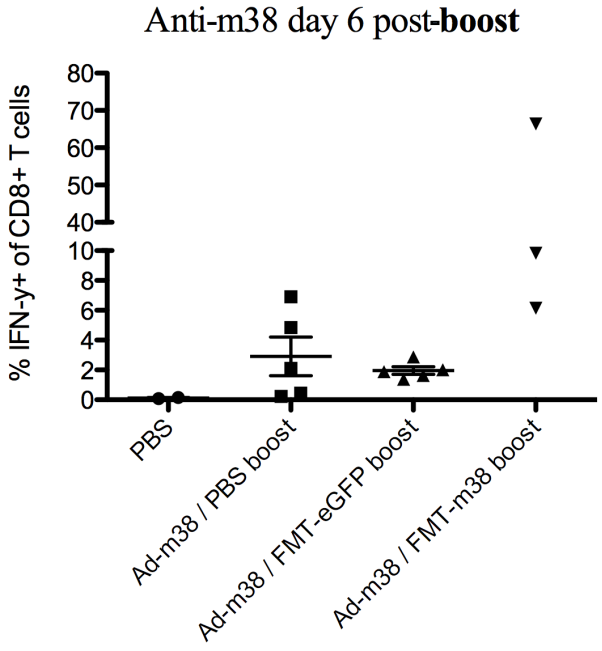
A.


Anti-m38 response post-**tumour seed**
*
% IFN-y+ of CD8+ T cells
0.6
0.4
0.2
0.0
Unstimulated
m38 stimulated

B.


Anti-m38 day 6 post-**prime**
% IFN-y+ of CD8+ T cells
15
10
5
0
PBS
Ad-m38 prime
Ad-m38 prime
Ad-m38 prime

C.


Anti-m38 day 6 post-**boost**
% IFN-y+ of CD8+ T cells
80
70
60
50
40
10
8
6
4
2
0
PBS
Ad-m38 / PBS boost
Ad-m38 / FMT-eGFP boost
Ad-m38 / FMT-m38 boost

implantation may provide a better window for generating TAA-specific immune responses that may lead to cures. One conclusion that can be made from this experiment is that Ad-m38 significantly prolongs survival in an intracranial CT2A-m38 model.

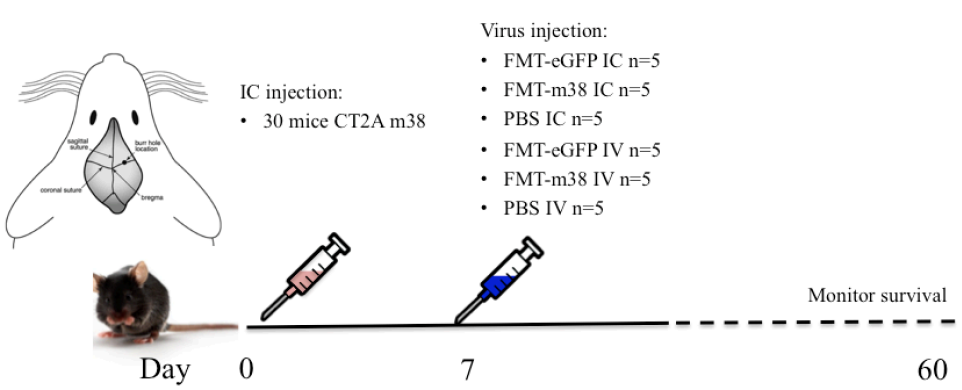
*3.3.5 Comparing the efficacy of FMT-m38 vs. FMT-eGFP and evaluating different viral delivery routes in the CT2A-m38 model*

In section 3.3.3, we found evidence that a single intravenous dose of FMT encoding the mCMV antigen m38 generates decent anti-m38 CD8+ T cell responses in mice bearing IC CT2A-m38 tumours. We also observed, via MRI imaging, a notably different appearance between wtCT2A tumours treated with FMT-m38 compared to CT2A-m38 tumours treated with FMT-m38. This led us to further investigate the therapeutic benefit of single dose FMT-m38 in an IC CT2A-m38 tumour model. We wished to compare two different treatment routes that are relevant to the clinical application of this virus: direct intracranial injection vs. systemic intravenous injection. Farmington virus expressing enhanced green fluorescent protein (FMT-eGFP) has shown efficacy in the wt CT2A IC model, significantly extending survival and curing ~25% of mice (data not shown). Thus we wanted to evaluate the importance of encoding a relevant TAA in our OV vector by comparing the efficacy of FMT-eGFP vs FMT-m38 in an IC CT2A-m38 model.

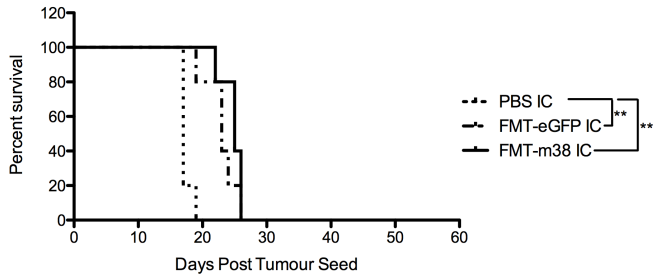
Mice were treated with virus a week after IC CT2A-m38 tumour implantation and were monitored for survival (Figure 20. A). There were 6 different treatment groups tested: PBS IC, FMT-eGFP IC, FMT-m38 IC, PBS IV, FMT-eGFP IV, and FMT-m38

**Figure 20. Treatment with a single dose of FMT-m38 delivered IC or IV significantly extends survival in an intracranial CT2A-m38 model.** Experimental timeline for therapeutic efficacy of FMT-eGFP vs. FMT-m38 delivered IV or IC in a murine GBM model (A). Naïve female C57BL/6 mice (8-12 weeks) were injected intracranially with 5E4 CT2A-m38 cells. Seven days post-tumour seed, mice were treated with PBS IC, 1E7 pfu FMT-eGFP IC, 1E7 pfu FMT-m38 IC, PBS IV, 1E7 pfu FMT-eGFP IV or 1E7 pfu FMT-m38 IV. Treatments with different viruses were compared within IC or IV delivery routes (B, C, respectively) and treatment with the same virus, but by different route, were also compared (D, E). Efficacy was scored by overall mouse survival illustrated by Kaplan-Meier curves (B, C, D, E). n=5 for each group. Asterisks indicate statistically significant difference in survival as determine by Mantel-Cox log-rank test. *$p<0.05$, **$p<0.01$. ns = not-significant, $p>0.05$.

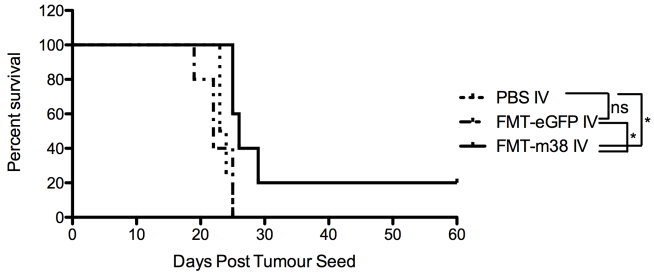
A.


IC injection:
• 30 mice CT2A m38
Virus injection:
• FMT-eGFP IC n=5
• FMT-m38 IC n=5
• PBS IC n=5
• FMT-eGFP IV n=5
• FMT-m38 IV n=5
• PBS IV n=5
Monitor survival
Day 0
7
60

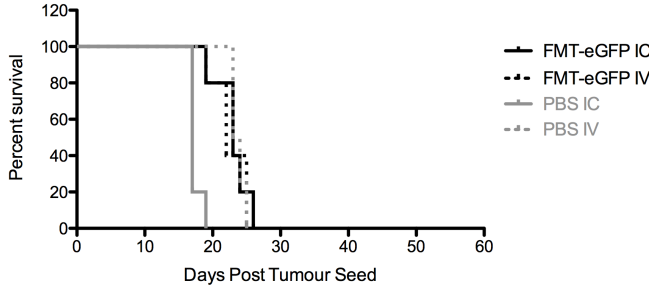
B.


Intracranial Treatment
Percent survival
Days Post Tumour Seed
PBS IC
FMT-eGFP IC
FMT-m38 IC
**
**

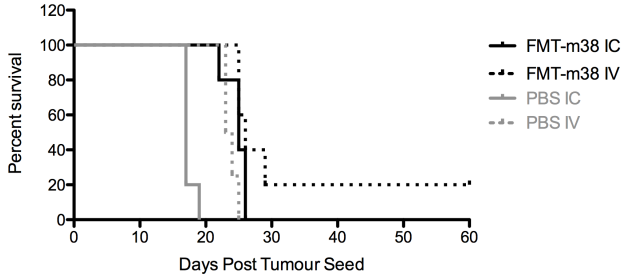
C.


Intravenous Treatment
Percent survival
Days Post Tumour Seed
PBS IV
FMT-eGFP IV
FMT-m38 IV
ns
*

D.


FMT-eGFP IC vs FMT-eGFP IV
Percent survival
Days Post Tumour Seed
FMT-eGFP IC
FMT-eGFP IV
PBS IC
PBS IV

E.


FMT-m38 IC vs. FMT-m38 IV
Percent survival
Days Post Tumour Seed
FMT-m38 IC
FMT-m38 IV
PBS IC
PBS IV

IV. In the IC treatment groups, both FMT-eGFP and FMT-m38 extended survival significantly compared to PBS, but no cures were generated (Figure 20. B). There was no difference in survival between the IC virus treatment groups. In the IV treatment groups, FMT-m38 significantly prolonged survival compared to PBS and FMT-eGFP (Figure 20.C). A twenty percent cure rate was achieved with FMT-m38 IV treatment, with 1 out of 5 mice becoming a long-term survivor.

When comparing the survival curves of mice treated by the same virus but by different delivery routes, there does not appear to be an advantage for either route (Figure 20. D, E). It is interesting to note that mice treated with PBS IV had significantly longer survival compared to mice treated with PBS IC. This is not entirely surprising as the mice treated with PBS IC undergo a much more traumatic and invasive procedure compared to IV treated mice. Furthermore, it has been previously shown that surgical stress in untreated tumour-bearing mice is immunosuppressive and leads to decreased survival rates (119).

In the experiments described herein, the IC CT2A-m38 model appears to be more aggressive than the IC wtCT2A model. Untreated wtCT2A tumour-bearing mice usually reach endpoint at ~ 30-35 days post-tumour seed (data not shown) whereas untreated CT2A-m38 tumour-bearing mice reached endpoint ~17-25 days post-tumour seed. We need to determine what concentration of CT2A-m38 cells for implantation generates similar tumour progression to the wtCT2A in order to have a larger window for treatment. In conclusion, the results of this experiment suggest that there may be a trend favouring IV delivery of TAA-encoding FMT over IC delivery, as only IV treatment

generated a cure. However, this experiment needs to be repeated several more times to increase the n value to prove whether or not this trend is real.

# 4 DISCUSSION

Glioblastoma multiforme (GBM) is the most common primary malignant brain tumour that occurs in adults (2). This cancer is highly invasive, rapidly develops resistance to treatment and ultimately recurs in 90% of patients. The current standard of care involves maximal surgical resection followed by radiation therapy with concomitant and adjuvant Temozolomide (TMZ) (8). This therapy is extremely difficult for patients to endure and only a fraction of GBM patients actually complete the entire treatment course (9). Despite the aggressive treatments in place, median patient survival with standard of care is only ~14 months. It is apparent that novel treatment options are needed for these patients.

When generating novel therapies for the treatment of GBM, factors such as heterogeneity of GBM tumours, systemic and local immunosuppression and potential antigenic immune escape need to be considered. An approach combining vaccination with a cytolytic agent may prove to be more effective than vaccine or OV treatment alone. In 2013, Pol *et al.* demonstrated that a potent oncolytic rhabdovirus discovered by our lab, Maraba MG1, encoding a tumour-associated antigen (TAA), DCT, could successfully boost pre-existing TAA-specific memory pools produced by a non-replicating Adenovirus vector (Ad) prime (104). This generated efficacy against an intracranial model of metastatic murine melanoma.

Our lab recently discovered a novel oncolytic rhabdovirus that was particularly cytotoxic on cell lines originating from the CNS (96). Unlike the Maraba MG1 virus described above, we found that Farmington virus (FMT) could be safely injected into the brain, and was capable of priming TAA-specific immune responses, as well as boosting

pre-existing TAA-specific memory T cell pools (unpublished data). Engineering a heterologous prime/boost regimen, where both the priming agent and the boosting agent are oncolytic viruses, has the potential to maximize the benefits of both cancer-specific lysis by the virus as well as generating a potent anti-tumour adaptive immune response that provides long term protection. **We hypothesized that FMT virus encoding a known tumour-associated antigen (TAA) can be used in a heterologous prime/boost vaccination regimen to generate TAA-specific CD8+ T cell responses that lead to better efficacy in brain tumour-bearing animals.** The project described herein set out to investigate self- and foreign-tumour-associated antigens that can be used to generate robust anti-tumour adaptive immune responses in a heterologous prime/boost regimen.

### 4.1 Survivin vaccination in naïve mice

Survivin is considered a universal tumour antigen (63). Its expression has been identified in numerous malignancies, but is not found in normal adult differentiated tissue. It is a member of the inhibitor of apoptosis family (IAP) and plays a role in cell division and inhibition of caspase 3 and 7. Its expression in tumours is associated with worse prognosis, aggressive phenotypes and resistance to chemotherapy-induced apoptosis (63, 67). Tumours appear to be addicted to survivin expression and are therefore less likely to lose this antigen for immune escape, making it an ideal target for vaccination. This is particularly true since survivin-specific CD8+ T cells have been detected in cancer patients (68, 69). Peripheral blood T cells from patients were able to lyse target cancer cells after stimulation with survivin peptide-pulsed DCs and survivin-specific CD8 + T cells were detectable by ELISPOT assay. These studies demonstrate

that there is an anti-survivin memory cell population in cancer patients, with the potential to be boosted by vaccination.

Based on this rationale, we decided to target survivin with our heterologous rhabdovirus prime/boost regimen. We first needed to prove the validity of this vaccination strategy in naïve mice. Initially, we vaccinated animals with rhabdovirus vectors expressing wt murine survivin. We were able to detect robust anti-OVA CD8+ T cell responses in our positive control mice; however mice primed with FMT-surv and boosted with MG1-surv did not generate a detectable IFNy+ CD8+ T cell response. There are several possible reasons as to why there was no detectable response to survivin.

Mice were vaccinated with full-length wt murine survivin and stimulated *ex vivo* with two different H-2K$^{b}$ restricted immunodominant peptide epitopes, respectively. These two peptides, Msur57–64 (CFFCFKEL) and Msur97–104 (TVSEFLKL), have previously demonstrated the ability to stimulate survivin-specific CD8+ T cells from mice vaccinated with survivin peptide-pulsed DCs (71, 72). Similarly to Ciesielski *et al*. (2008), we stimulated our PBMCs with the Msur57 peptide because this sequence is 100% homologous to the human survivin protein. The peptide was tested at several different concentrations (2 ug/ml, 5 ug/ml, 10 ug/ml), but none of these concentrations appeared to elicit a detectable IFNy+ CD8+ T cell response in our vaccinated mice. It is possible that due to expression from the rhabdoviral vector, the survivin protein was not processed in an appropriate way to generate the Msur57 peptide. However, the Msur57 peptide has previously been shown to be the best survivin immunodominant peptide for binding MHC class I molecules in C57BL/6 mice (71, 72). Therefore it is unlikely that the peptide chosen for *ex vivo* stimulation was the issue, but without an appropriate

positive control for survivin vaccination, no definite conclusions can be drawn.

Our failure to detect a response could also be due to the fact that thymic selection and central immune tolerance deletes T cells with high avidity for self-antigens, thereby leaving only T cells with low avidity for self-antigens (105). Low avidity T cells are notoriously difficult to expand *in vivo*, even with potent vaccination strategies (120). Upon successful stimulation with large amounts of peptide *in vitro*, low avidity T cells have impaired IFNy production, but are capable of lysing cells pulsed with target peptides or cells that endogenously process and present the target. Many of the studies that claim to have generated successful survivin vaccines use splenocyte cytotoxicity assays against target cells to measure specific lysis rather than measuring intracellular IFNy expression in PBMCs following *ex vivo* peptide stimulation (71, 72, 110, 121). Since low avidity T cells can still effectively mediate target-specific lysis, it is likely that the survivin-specific CD8+ T cells observed in previous studies were low avidity T cells. This possibility is also supported by the fact that in intracranial murine models, time to progression was only modestly increased compared to controls after survivin vaccination (71, 121). Low avidity T cells fail to traffic into tumours effectively, cannot overcome the immunosuppression in the tumour microenvironment and ultimately fail in eradicating tumour under optimal vaccination conditions; providing further evidence suggesting that low avidity T cells are the product of survivin vaccines (122). If this is in fact the case, it is possible that looking for intracellular IFNy positivity in PBMCs is not an optimal read-out for successful vaccination against survivin.

As mentioned above, our method for detecting survivin-specific T cells involved *ex vivo* peptide re-stimulation of PBMCs whereas the read-out in other studies using

survivin vaccines in mice used splenocytes (71, 72, 110, 121). If the survivin-specific T cells generated are indeed low avidity T cells, then they may require a very high density of peptide/MHC complexes to become activated (123). The spleen contains many mature, professional APCs, whereas the peripheral blood is more likely to contain monocytes and immature DCs that are not as efficient at presenting peptide/MHC complexes. It is possible that using splenocytes and high concentrations of survivin peptides (100 ug/ml) for an extended period of stimulation (72 hours) is why the other studies successfully stimulated the survivin-specific low avidity T cells (71, 72, 110, 121). In our assay, PBMCs were incubated with a maximum of 10 ug/ml of peptide for 4 hours, potentially with less efficient APCs presenting the peptide/MHC complexes to the T cells. The advantage of using PBMCs over splenocytes is that immune responses can be monitored over a period of time without having to sacrifice the animals. It is also more reflective of the methods that would be used in the clinic to evaluate a patient's immune response to a vaccine. In the future, it would likely be better to culture autologous bone-marrow-derived DCs (BMDCs), pulse them with survivin peptide after maturing them and then use these cells to stimulate our PBMC samples, similarly to the studies performed by Schmidtz (2000) and Andersen (2001) (68, 69).

Our inability to detect an anti-survivin response could also be due to the fact that self-antigens have poor immunogenicity. Ciesielski *et al.* (2006) used the human survivin protein to vaccinate mice because the proteins are homologous, yet there is sufficient variations in protein sequence to provide the immunogenicity needed to generate a better immune response in mice to a self-protein (121). They found that vaccination with DCs transfected with a full-length human survivin xenoantigen protected mice significantly

more against intracranial GL261 tumours compared to mice vaccinated with DCs transfected with full-length mouse survivin. Furthermore, Pol et al. (2013) used the human DCT protein encoded in vaccine vectors to generate robust anti-DCT responses in mice (104). Previous to the study by Pol et al., the vaccination capabilities of vectors expressing hDCT or murine DCT were directly compared and it was found that hDCT had a large advantage vaccinating mice against DCT due to a dominant CD4+ T cell epitope found only in the hDCT protein (102). It is possible that re-engineering the rhabdovirus vectors to express human survivin might generate a detectable survivin-specific IFNy+ CD8+ T cell response in mice due to cross species sequence variation increasing the immunogenicity of the transgene.

When survivin expression from the rhabdoviral vectors was examined by Western blot, we found that the level of the wildtype protein expressed from the viruses was not much greater than the background survivin expression in the cell line alone control sample (Appendix, Figure S1. B, C). The higher the transgene expression, the more likely it is for this protein to be processed and presented to immune cells as well as compete against the massive amounts of virus proteins being produced, processed and presented. We also considered that *in vivo,* it is possible that expression of the active full-length survivin could make rhabdovirus-infected cells resistant to apoptosis, thereby decreasing the chance for phagocytosis and presentation of survivin peptides by APCs. In efficacy experiments, it may be counterproductive to have viruses that target and replicate in tumour cells, but also express a transgene that may provide the tumour cells with a mechanism to increase their resistance to infection. Thus, the vaccination vectors were re-engineered to express an inactive mutant version of murine survivin (survT48A). By

replacing the threonine at position 48 in the survivin protein with alanine, survivin's ability to inhibit apoptosis and aid in cell division is abrogated by preventing phosphorylation at this site (111).

The rhabdovirus vectors expressing survT48A were used to vaccinate naïve C57BL/6 mice. In our Western blot verification of transgene expression, it appeared that the vectors expressing the T48A mutants had more robust transgene expression than the vectors encoding the wt survivin. This may be due to the fact that we mutated one of the most important activation/phosphorylation sites on the protein, and therefore changed the protein degradation dynamics. FMT-T48A was tested as a priming agent, with MG1-T48A being used to boost. In this second vaccination experiment, the proven priming ability of FMT-OVA was included as positive control. Again, we were unable to detect anti-survivin responses by IFNy+ CD8+ T cell ICS. We did, however, observe robust anti-OVA responses in the group primed by FMT-OVA. Despite the fact that there appeared to be greater transgene expression, thereby increasing the chance of survivin presentation on the surface of infected cells, we were still unable to measure survivin-specific responses.

In our third attempt to demonstrate vaccination against survivin, we received the appropriate reagent to reproduce the heterologous prime/boost regimen similarly to Pol *et al.* (2013) (courtesy of Dr. Wan's group at McMaster University). In using a non-replicating Ad-survivin vector to prime the immune system, we hoped that we would be able to detect an anti-survivin response post-boost. Pol *et al.* previously demonstrated that a non-replicating Ad vector-TAA prime/Maraba MG1-TAA boost was capable of generating a robust T-cell response to the self-tumour antigen DCT (104). We

hypothesized that perhaps FMT was not an effective priming agent against self-antigens because it was only previously shown to prime well against an immunodominant xenoantigen (OVA). Farmington virus itself generates a strong CD8+ T cell response and it is possible that this response dominates over a response to the self-antigen survivin. Thus we set up several new vaccination groups, where two groups were primed with Ad-survGFP and then boosted with either MG1 T48A or FMT T48A. We also tested FMT T48A prime/MG1 T48A boost for a second time. Again, we detected no measurable anti-survivin response by IFNy+ CD8+ T cells; however, interestingly we found that 2 out of 3 mice receiving the Ad-surv prime/MG1 T48A boost regimen died approximately one-week post-boost.

It has previously been shown that survivin-specific T cells are capable of killing activated immune cells (66, 124). In Lesiegang *et al*.'s study in 2010, survivin expression was highly upregulated in activated T cells and resultant presentation of survivin-specific MHC ligands led to death by survivin-specific T cells in co-culture, irrespective of the TCR specificity of the activated cells (124). In another study, Schmidt *et al*. (2003) produced survivin-specific CD8+ T cells from the peripheral blood cells of healthy donors by stimulating them *ex vivo* with DCs pulsed with survivin peptides (66). They found that these survivin-specific T cells lysed activated T cells, B cells and mature DCs when they were pulsed with target peptides or transfected with survivin cDNA isolated from survivin+ tumours. Furthermore, Black et al. (2014) found that low avidity T cells, which are likely generated by our survivin vaccine, had elevated expression of pro-apoptotic proteins such as DR5, FasL, and CD24, which caused their own cell death and also that of other CD8+ T cells in the surrounding environment (122). It may be possible

that the mice in our experiment treated with Ad-surv prime/MG1 T48A boost might have suffered a severe lymphopenia if we successfully generated a low avidity anti-survivin CD8+ T cell response. Maraba MG1 activates a strong anti-viral immune response with high avidity T cells, so it is likely that survivin would be upregulated in many different immune cell types, making them targets for lysis by anti-survivin CD8+ T cells. However, without repeating the experiment and carefully tracking/quantifiying the immune cell populations in the mice, no conclusions can be made.

Another phenomenon that may have contributed to the death of the Ad-surv prime/MG1-T48A boost vaccinated mice is the marked lymphopenia observed in mice following treatment with a rhabdovirus. This lymphopenia following administration of a rhabdovirus lasts for a few days post-virus treatment. It has been shown that during infection with the rhabdovirus VSV, non-virus-specific bystander CD8+ T cells undergo apoptosis as a result of partial activation by IFNα/β without appropriate TCR/MHC+peptide stimulation and costimulatory signals (125-127). It has also been observed in previous studies that during high dose virus infections, CD8+ T cells and other immune cells can be lysed by virus-specific T cells if they are passively loaded with relevant viral peptides released (123, 128). In the case of acute lymphocytic choriomeningitis virus (LCMV) infection, macrophages are the first cells infected in lymphoid organs, and their lysis by anti-viral CD8+ T cells causes the release of viral peptides, making lymphocytes in the vicinity susceptible targets for anti-viral CD8+ T cells (128). Additionally, it has been shown that high avidity CD8+ T cells can become over-stimulated and then deleted by interacting with APCs that have a very high density of peptide/MHC complexes (activation induced cell death – AICD), which could happen

when treating mice with a large dose of virus (123)**.** Furthermore, it has been shown that VSV, another oncolytic rhabdovirus, infects and kills dendritic cells in mice (129). VSV and Maraba are closely related; therefore it may be possible that MRB MG1 also infects DCs *in vivo*, resulting in a phenomenon similar to that occurring in acute LCMV infection, described above. Furthermore, Zhang *et al*. (2014) have demonstrated that MG1 can infect DCs *in vitro*, but it is unclear whether the infection leads to DC death *in vivo* (119).

If our Ad-SurvGFP prime/MG1-T48A boost treated mice generated an anti-survivin CD8+ T cell response that targeted other activated immune cells and there was additional virus-induced lymphopenia, then the mice may have suffered disruption of their cellular homeostasis. In addition, it has recently emerged that multiple normal adult tissues express survivin. If survivin-specific T cells were generated, it is possible that they could attack the thymus, centers of hematopoiesis, neutrophils, arterial muscle, the liver, the brain and/or the spleen (130). With immune cell homeostasis disrupted, it may have been possible that regulatory mechanisms in place to protect mice against autoimmune reactions were weakened. As a result, damage may have been caused to important organs and this may have lead to the mice being found dead post-boost. This hypothesis is purely speculative and many further studies would need to be performed to to support it.

Several different approaches could be used to improve our anti-survivin vaccine for future studies. Xiang *et al.* (2005) demonstrated improved vaccination with murine survivin in mice by replacing a single amino acid in the wild-type self-peptide that made it more immunogenic and better at expanding low avidity T cells (131). They also

encoded a polyubiquitination signal along with the full-length survivin to ensure its efficient delivery to the proteasome for optimized antigen processing and presentation by MHC I. In addition, they included CCL21 in their vector, which is a murine chemokine that attracts APCs and naïve T cells. Another option may be to conservatively mutate the dominant FMT epitope to make it less immunogenic, so that the immune response could focus on survivin epitopes. Another important factor would be to include a proven positive control for survivin vaccination, such as a peptide-pulsed DC vaccine, to determine if our assay is capable of measuring the anti-survivin CD8+ T cell response. It is likely that we might have to switch to PBMC stimulation with peptide-pulsed autologous BMDCs to generate a response that is detectable by IFNy ICS.

**4.2 Survivin vaccination in a tumour model**

Since self-specific T cells are present at very low frequency in naïve animals (113), we decided to test the anti-survivin vaccination in a survivin-positive tumour model. We hypothesized that anti-survivin CD8+ T cells may be spontaneously generated against the tumour, increasing the frequency of self-specific T cells available to boost with our vaccination strategy. We also analyzed if a dual heterologous rhabdovirus prime/boost regimen in a tumour model could demonstrate an advantage over a regimen that uses one non-replicating vector followed by a single OV.

After surveying several tumour cell lines for their survivin expression, the 4T1 murine mammary carcinoma cell model was chosen to explore our anti-survivin heterologous prime/boost vaccination strategy. The 4T1 cell line was derived from a spontaneously occurring mammary carcinoma in a BALB/C mouse and was selected for its resistance to the anti-cancer drug 6-thioguanine (114, 132). In the 4T1 murine

mammary carcinoma model, the tumour implantation is rapid and caliper measurements can easily monitor the progression of the orthotopic tumour. It is also simpler to evaluate an anti-tumour immune response in a subcutaneous model as opposed to in an intracranial model. The brain has a complex and dynamic relationship with systemic immunity.

Maraba MG1, but not FMT, was found to be highly cytotoxic on 4T1 cells *in vitro*. We hypothesized that if efficacy was observed *in vivo* using FMT in the 4T1 model, it would most likely be because of immune activation caused by the virus and not due to its oncolytic capacity. The 4T1 model rapidly progresses and spontaneously metastasizes to the lungs of mice, providing a second read-out for efficacy besides primary tumour progression (114). In some models, primary 4T1 tumours are surgically removed after establishing metastases; however, since the purpose of our tumours was to provide a survivin-positive target, the primary tumours were not removed from the mice. The 4T1 model is also known for being poorly immunogenic, which presented a challenge for our vaccination strategy (133-135).

Established 4T1 tumours in BALB/C mice were treated with FMT-T48A prime/MG1-T48A boost, MG1-T48A prime/FMT-T48A boost, Ad-SurvGFP prime/MG1-T48A boost or PBS. Tumour burden was significantly controlled in mice treated with either dual heterologous rhabdovirus regimen when compared to the PBS and the Ad-SurvGFP prime/MG1-T48A boost treated groups. There was also a trend towards mice receiving the MG1 T48A prime/FMT T48A boost having slightly better tumour control than those receiving FMT T48A prime/MG1 T48A boost. There was modest control of tumour progression in mice treated with Ad-SurvGFP/MG1-T48A after

the boost, but the heterologous rhabdovirus vaccinations were significantly better. We also observed that the tumours of mice treated with MG1 T48A prime/FMT T48A boost had a notably black appearance suggestive of tumour necrosis.

We also found that mice treated with FMT-T48A prime/MG1-T48A boost or MG1-T48A prime/FMT-T48A boost had a significantly lower number of visible lung metastases when compared to PBS-treated and Ad-SurvGFP/MG1-T48A-treated mice. Mice treated with Ad-SurvGFP prime/MG1-T48A boost did not significantly control lung metastases compared to PBS. However, counting the lung metastases by eye only gives an approximation of metastatic control because we cannot observe micrometastases or metastases imbedded within the lung tissue. The most quantitative way to analyze lung metastases would be to dissociate the lung tissue and perform a clonogenic assay for 6-thioguanine-resistant cells (114).

Interestingly, mice with 4T1 tumours typically suffer from extreme granulocytosis and massive splenomegaly (136-139). Gr-$1^+$ myeloid cells with granulocyte morphology have been shown to increase from 15% of total $CD45^+$ leukocytes in the peripheral blood to >80% in 4T1 tumour-bearing mice (137). The majority of these cells were found to be Gr-$1^{dim}CD11b^+$, suggesting they are immature myeloid cells/myeloid derived suppressor cells (MDSCs) capable of suppressing T cell function. The absolute numbers of B cells and T cells in peripheral blood did not change. DuPre *et al.* (2007) also found that primary 4T1 tumours had massive myeloid cell infiltrates, where 36% of total cells from homogenized tumour were myeloid cells. When examining the spleens of 4T1 tumour-bearing mice, they found an expanded red pulp area with signs of extramedullary hematopoiesis. There was also a decrease in Gr-$1^+$ staining intensity on Gr-$1^+CD11b^+$

cells and a lower percentage of T cells and B cells found in the spleen. Studies by DuPre *et al.* reported that 4T1 tumours produce a variety of myeloid chemokines and growth factors such as GM-CSF, G-CSF, MCP-1, RANTES, KC, MIP-1a and MIP-1b (137, 138). Their data suggests that 4T1 tumours themselves are a major contributor to the large expansion of myeloid cells in this model.

Another study by DuPret *et al*. (2008) further analyzed the immune cell populations found in 4T1 tumours (139). They found that $CD11b^+$ myeloid cells made up 90% of the $CD45^+$ leukocytes found in day 22 4T1 tumours. Almost no CD8+ T cells or natural killer (NK) cells were found in the tumours, but a small number of CD4+ T cells were observed. However, DuPre *et al*. did not look for regulatory T cell (Treg) markers so it is possible that the CD4+ T cells found in the tumours were Tregs. The massive amount of immature myeloid cells found in 4T1 tumour-bearing mice act as MDSCs that contribute to tumour immune-escape and block adaptive and innate immunity (133, 140). Danna *et al.* (2004) found that primary 4T1 tumour-bearing BALB/C mice were incapable of rejecting allogeneic B16-F10 (MHC mismatched H2-$K^b$) melanoma cells (133). This illustrates the severity of the immunocompromised state of primary 4T1 tumour-bearing mice.

When examining the lymphocyte populations isolated from the 4T1 tumours in our experiments, we found that MG1-T48A primed/FMT-T48A boosted mice had 7-fold more CD4+ T cells and 18-fold more CD8+ T cells in their tumours compared to PBS-treated mice (Appendix, Figure S6). Mice treated with FMT-T48A prime/MG1-T48A boost had 13-fold more CD8+ T cells and 4-fold more CD4+ T cells in their tumours compared to PBS-treated mice. These cells likely contributed to controlling tumour

progression as almost no T cells were found in the rapidly progressing tumours of PBS- or Ad-SurvGFP prime/MG1-T48A boost-treated mice. The MG1-T48A prime/FMT-T48A boost-treated mice also had a remarkable $CD40^{+}CD80^{+}$ $CD11b^{+}$ population that was absent from the tumours of all other treatment groups (Appendix, Figure S5). This suggests that treatment with heterologous rhabdovirus vaccination regimens may increase T cell trafficking to the 4T1 tumours or expansion of tumour-specific T cells and that the MG1-T48A prime/FMT-T48A boost regimen may activate APCs in the tumour to express co-stimulatory molecules needed for effective T cell activation. Immunohistochemical staining of fixed 4T1 tumour cells would be a good way to verify the immune cell infiltrates described above.

We also performed a splenocyte cytotoxicity assay on 4T1 cells *in vitro* with whole splenocytes isolated from the treated, tumour-bearing mice (Appendix, Figure S3). We observed that at an effector:target ratio of 50:1 and 200:1, the splenocytes isolated from mice treated with MG1-T48A prime/FMT-T48A boost killed significantly more 4T1 cells *in vitro* compared to splenocytes isolated from PBS-treated mice. No other treatment groups demonstrated significant cytotoxicity on 4T1 cells *in vitro*. This suggests that treatment with MG1-T48A prime/FMT-T48A boost may generate a successful anti-tumour immune response. The splenocytes were not pre-activated by survivin-pulsed DCs and the 4T1 tumours were not pulsed with survivin peptide, therefore we cannot determine whether survivin-specific T cells played a role in this cytotoxicity assay. It is more likely that a wide repertoire of tumour epitopes were recognized on the surface of the 4T1 cells by CD8+ T cells with different specificities. Additionally, an AlamarBlue assay was used to determine 4T1 cell survival in our

experiment, which does not have the most precise read-out for cytotoxicity as it is based on actively metabolizing cells. A chromium$^{51}$ release assay may have given a more precise readout for specific lysis.

The survivin-specific CD8+ T cell response was monitored throughout these experiments by stimulating PBMCs *ex vivo* with H-2K$^{d}$-restricted survivin peptides previously shown to elicit CD8+ T cell responses in BALB/C mice (141). In both repeats of the experiment, a total of 2 mice had a detectable anti-survivin response by IFNy ICS above background. These mice were both in the Ad-SurvGFP prime/MG1-T48A boost treatment group, but in separate experiments. This suggests that we may have successfully generated a survivin-specific CD8+ T cell response in a small number of mice, but its contribution to efficacy could not be established due to the inconsistency of the response. Due to the massive MDSC population in the peripheral blood of 4T1 tumour-bearing mice, it is possible that sufficient peptide stimulation was lacking during the *ex vivo* activation of the CD8+ T cells and we may have missed some low avidity survivin-specific T cell responses. One way to overcome this may have been to isolate T cells from the peripheral blood samples to remove the suppressive myeloid populations and then stimulate the T cell population with survivin peptide-pulsed autologous BMDCs. This technique is more likely to remove immunosuppressive factors as well as deliver better peptide/MHC density to stimulate low avidity T cells to allow us to analyze anti-survivin responses more accurately. However, this would not reflect the immunological state in the tumour-bearing animals.

A small number of studies have demonstrated successful vaccination against survivin in the BALB/C animal model. Siegel *et al.* (2003) achieved successful anti-

survivin vaccination in BALB/C mice with peptide-pulsed DCs, demonstrating lysis of survivin-positive tumour cells with splenocytes *ex vivo* (141). Prophylactic vaccination improved survival in an A20 lymphoma model, but therapeutic vaccination did not show the same efficacy. Yang et al. (2008) generated a mimovirus vaccine composed of the cell penetrating HIV Tat protein sequence fused to an H-2K$^d$ survivin peptide and a plasmid encoding murine IL-15 (142). Their vaccine induced long-lasting survivin-specific CD8+ T cell immunity, controlled tumour progression and increased survival rates in a CT26 tumour model. Yang et al. found that IL-15 was necessary for the success of their vaccine. NoeDominguez-Romero *et al.* (2015) chose a different vaccination approach where they introduced many different mutations into the immunodominant H-2K$^d$ survivin peptide, GWEPDDNPI, to generate a variable epitope library (VEL) that was delivered in a phage library or a synthetic peptide library (143). They demonstrated modest control of 4T1 tumour progression with a prophylactic vaccination and a therapeutic vaccination with 4 treatments; however a single treatment did not slow 4T1 tumour progression. NoeDominguez-Romero *et al.* did not detect any survivin-specific CD8+ T cell responses in their VEL-vaccinated mice above the spontaneously generated anti-survivin responses detected in untreated 4T1 mice.

Chandra *et al.* (2014) observed a remarkable phenomenon while treating 4T1 tumours with a TAA-targeting vaccine (144). Initially, they used a *Listeria monocytogenes* vector expressing the self-TAA Mage-b (LM-Mb) and cyclic diguanylate (c-di-GMP) as an adjuvant. This regimen significantly slowed tumour burden, cleared almost 100% of metastases and generated a Mage-b-specific CD8+ T cell response. Although Chandra *et al.*'s target antigen was not survivin, they did demonstrate

successful vaccination against a self-TAA in the poorly immunogenic 4T1 model. However, they also found that a single high dose of c-di-GMP followed by multiple low doses of c-di-GMP controlled tumour burden and metastases similarly to the LM-Mb vaccination. During further investigation, Chandra *et al.* found that high dose c-di-GMP killed 4T1 cells *in vitro* and low doses of c-di-GMP decreased the % MDSC in peripheral blood of 4T1 tumour-bearing animals and also increased IL-12 secretion from MDSCs. Chandra *et al.* also found that the high dose followed by low dose c-di-GMP treatment generated Mage-b-specific as well as survivin-specific CD8+ T cells and led to a higher number of IFNy+ cells in the peripheral blood of 4T1 tumour-bearing mice. They also found that this regimen increased the expression of CD80/CD86 on splenic DCs, which is important for effective T cell activation. Due to the observed efficacy of this regimen and the detectable response to more than one TAA, Chandra *et al.* hypothesized that the treatment with high dose c-di-GMP caused immunogenic 4T1 tumour cell death leading to tumour epitope spreading and cross-presentation of TAAs and that the subsequent low doses of c-di-GMP decreased the immunosuppression in the mice, allowing them to mount a stronger anti-tumour adaptive immune response.

To determine if the survivin antigen encoded in our heterologous rhabdovirus regimen played a role in the efficacy we observed in the 4T1 model, we repeated the experiment with FMT and MRB MG1 vectors that did not encode a TAA. We observed that the heterologous rhabdovirus treatments significantly slowed 4T1 tumour progression and controlled 4T1 lung metastases compared to PBS treated mice. Although not statistically significant, there was a trend favouring MRB MG1 followed by FMT as the more effective order for the treatment. A group treated with a non-TAA encoding,

non-replicating Ad followed by MRB MG1 should have been included as another control in this experiment. A previous pilot experiment, where 4T1 tumour-bearing mice received multiple doses of FMT or MRB MG1, demonstrated that either of these viruses on their own only modestly control tumour progression in the left tumour and do not control tumour progression in the right tumour. For this reason, FMT or MRB MG1 alone treatments were not included as controls for this experiment. Although not directly comparable, we observed similar outcomes in separate experiments with wt or survivin-targeted rhabdovirus treatments, suggesting that an anti-survivin response was not necessary for tumour and metastases control.

It is possible that what we observed in our heterologous rhabdovirus treatments is similar to what Chandra *et al.* (2014) found with combined high and low dose c-di-GMP described above. Maraba MG1 is capable of lysing 4T1 cells *in vitro*, whereas FMT is not. Both rhabdoviruses have been found to cause marked transient lymphopenias following injection in to mice, potentially decreasing the massive MDSC population in the 4T1 mice allowing expansion of activated T cells. There is preliminary evidence suggesting that FMT replicates to high titers in the spleen and is capable of stimulating the secretion of type I inflammatory chemokines/cytokines (Appendix, Figure S7 and S8). Initial immunological tests performed during the 4T1 experiments described above imply that FMT virus might increase co-stimulatory molecule expression on APCs, similarly to low dose c-di-GMP observed by Chandra *et al*. Further investigations by methods such as immunohistochemical staining of 4T1 tumours and more complex flow cytometry panels to better define observed immune populations are needed before any conclusions can be made from these 4T1 experiments.

Nevertheless, based on these preliminary tests, it may be hypothesized that the initial injection with MRB MG1 leads to immunogenic 4T1 tumour cell death resulting in epitope spreading and cross-presentation of TAAs, and that the resulting lymphopenia decreases the MDSC population allowing for efficient activation of naïve T cells. Since rhabdovirus-induced lymphopenia is temporary, it is likely that these *de novo* tumour-specific T cells ultimately become suppressed by MDSCs. The follow-up dose with FMT may help to polarize the immunosuppressive environment to a type I inflammatory response by transiently decreasing the MDSC population and replicating at the tumour site and in the spleen, allowing more efficient TAA presentation by APCs and better anti-tumour responses by TAA-specific CD8+ T cells. Many more experiments are required to prove or disprove this hypothesis, but the data described above suggest that heterologous rhabdovirus treatments have the potential to benefit mice with metastatic mammary carcinoma.

### 4.3 Targeting a foreign glioblastoma-associated antigen

GBM presents a unique opportunity for oncolytic vaccination regimens since most tumours express foreign viral immunodominant antigens derived from human cytomegalovirus (hCMV) (55, 56, 58, 59, 115). These hCMV antigens are not found in surrounding healthy tissue. Indeed, latent mCMV infection in mice leads to more aggressive glioma phenotypes and progression in a spontaneous GBM mouse model (57). As such, CMV antigens represented promising targets for our GBM-targeted TAA-encoding FMT platform. While FMT was unable to prime or boost a response against the self-antigen survivin, it has been previously shown to prime and boost adaptive immune responses against a foreign antigen (OVA). Because of the species specificity of CMV

viruses, murine CMV (mCMV) antigens in mice were used to model hCMV-positive GBM patients.

Two mCMV model antigens were investigated as potential targets in a syngeneic animal model (IE3 and m38). Both IE3 and m38 have been previously shown to generate strong CD8+ T cell specific responses in C57BL/6 mice in the context of a chronic mCMV infection (145). This property is similar to that of the two hCMV antigens identified in the tumours of GBM patients (IE1 and pp65). Pools of IE1-specific and pp65-specific T cells are found in seropositive patients and are generated as a result of chronic hCMV infection (60). The chosen mCMV antigens are highly immunodominant when compared to other mCMV antigens, as are the hCMV antigens expressed in human GBM tumours (58, 60, 115, 145).

Importantly, the hCMV IE2 protein is homologous to the mCMV IE3 protein and hCMV IE1 and IE2 proteins have similar functions (146, 147). The hCMV IE1 protein localizes to the nucleus and inhibits apoptosis, type I IFN signaling, and other host-cell antiviral genes. It is important for viral genome replication and promoting transcriptional activation of the early viral genes by interacting with many different host-cell and viral proteins (146). HCMV IE1 protein has been found to interact with histone modifying proteins. The mCMV IE3 protein has many similar functions to hCMV IE1 and also localizes to the nucleus. MCMV IE3 is also important for the initiation of viral replication by interactions with viral and host-cell proteins and has been shown to interact with histone deacetylase 2 (HDAC2) to eliminate its suppression of early viral gene transcription (148). The hCMV pp65-tegument protein is the most abundant CMV protein during infection and is the major constituent of virions and non-infectious virus

particles (115). It localizes mainly to the nucleus after virus entry, but also accumulates in the cytoplasm over the course of infection. It has been shown to dampen host-antiviral responses and modulate antigen presentation (149). It is interesting to note that in the context of GBM cells, reports have shown that pp65 expression is primarily in the nucleus and that IE1 is found in the cytoplasm (59). The localization of IE3 and m38 in our model CT2A cells is unknown due to lack of antibodies specific to these proteins, however IE3 is predicted to have a nuclear localization signal (NLS). The mCMV m38 protein does not have a defined function, but it does not encode a NLS.

Non-replicating adenovirus vectors, FMT vectors and syngeneic CT2A mouse GBM cells were engineered to express mCMV proteins, IE3 or m38. These tools were used to assess the capacity of FMT virus to prime and boost adaptive immune responses against foreign GBM-associated antigens in naïve C57BL/6 mice. First, we compared the priming capabilities of the mCMV FMT vectors with the mCMV Ad vectors and found that both viruses primed adaptive immune responses equally in terms of % mCMV-specific IFNy+ CD8+ T cells. Unfortunately, the Maraba MG1 platform for these antigens was unavailable, so these primed responses could only be boosted with FMT vectors and heterologous rhabdovirus regimens could not be tested. The Ad prime followed by late FMT boost 45 days post prime generated robust anti-IE3 and anti-m38 CD8 + T cell responses (~22% and ~27%, respectively). Pol *et al.* (2013) previously demonstrated that homologous prime/boost regimens with Ad-DCT/Ad-DCT or MG1-DCT/MG1-DCT could not generate robust anti-DCT CD8+ T cell responses (104). In agreement with their findings, we found that FMT IE3 could not boost a FMT IE3 prime (<1%); however the anti-m38 CD8+ T cell response primed by FMT m38 appeared to

modestly expand following the second dose of FMT m38 (~5%). Since mice generate robust anti-viral humoral responses against FMT (unpublished data), it may be likely that the mild expansion we observed was due to modest memory inflation as opposed to boosted expansion from the second FMT dose. The presence of chronic low doses of m38 antigen have been shown to lead to inflation of m38-specific memory CD8 + T cells (145).

Next, we examined the mCMV-specific responses generated in a traditional prime/boost regimen schedule (boost administered at 15 days post-prime). We found that Ad-IE3 prime/FMT IE3 boost and Ad-m38 prime/FMT m38 boost both generated robust specific-CD8+ T cell responses. The anti-IE3 response contracted significantly over time whereas the anti-m38 response displayed a prolonged period of CD8+ T cell maintenance with only mild contraction. Interestingly, a robust anti-Ad CD8+ T cell response was only detected in IE3-vaccinated mice compared to m38-vaccinated mice (8% vs <2%). Similarly, there was also a larger anti-FMT response in IE3-vaccinated mice compared to m38-vaccinated mice. This suggested that the immune system focused on anti-TAA over anti-viral responses for m38 model, but maybe not for IE3. Vaccinated mice were prophylatically challenged with subcutaneous wt CT2A and mCMV CT2A cell lines over 2 months after prime. Wildtype CT2A, CT2A-IE3 and CT2A-m38 established well subcutaneously in unvaccinated mice. Mice vaccinated against IE3 were unable to reject wt CT2A or CT2A-IE3 tumours. Mice vaccinated against m38 were unable to reject wt CT2A tumours, but they did have significantly slowed CT2A-m38 tumour progression, with 2/5 mice completely blocking this tumour from establishing. This demonstrated that m38-vaccinated mice likely rejected CT2A-m38 tumours in an m38-dependent fashion.

The m38-specific CD8+ T cells were likely the effectors of this protection, but this would have to be verified by blocking CD8+ T cells during the tumour challenge in m38-vaccinated mice. Alternatively, T cells from m38-vaccinated mice could be adoptively transferred to naïve, irradiated mice followed by a challenge with CT2A-m38 tumours.

We only observed tumour rejection with one of our two mCMV antigens. If we look back on the survivin vaccination experiments, we observed greater anti-viral CD8+ T cell responses in mice vaccinated against survivin compared to the positive controls vaccinated against OVA. Similarly, we observed greater anti-viral CD8+ T cell responses in IE3-vaccinated mice compared to mice vaccinated against m38. This may suggest that m38 is a more potent antigen than IE3 and does a better job swaying the immune system towards a transgene-specific response as opposed to a vector-dominated response. Munks *et al.* (2006) have also stated that m38 is higher in immunodominance hierarchy than IE3 (145).

Munks *et al*. (2006) have also made remarkable observations about the pattern of CD8+ T cell responses to m38 and IE3 in a natural chronic mCMV infection (145). m38 responses did not peak until 14 days post-infection, underwent a small contraction at 35 days post-infection and subsequently reached a long-term plateau. They found low IE3 responses detectable above background post-infection, but robust responses were not observed until 4 months post-infection (145). In our experiments, the anti-IE3 CD8+ T cell response in IE3-vaccinated mice slowly contracted over time and actually continued to decrease after CT2A-IE3 tumour implantation. The anti-m38 CD8+ T cell response in m38-vaccinated mice remained steady after a small contraction, and a slight increase (but not statistically significant) in % IFNy+ CD8+ T cells was observed after CT2A-m38

tumour implantation. While we are vaccinating our mice with vectors expressing mCMV antigens rather than exposing them to chronic mCMV infection, there nevertheless appears to be parallels between the pattern of CD8+ T cell responses observed between our work and work done by Munks *et al*. Of note, Munks *et al*. (2006) also observed that IE3- and m38-specific CD8+ T cells had a permanent effector memory phenotype ($CD62L^{low}$, $CD127^{-}$ and $CD122^{-}$). It would be interesting to phenotype the CD8 T cells generated in our vaccine for comparison.

Another reason why the IE3 vaccination could not reject CT2A-IE3 tumours may be because IE3 expressed in the CT2A cell line likely traffics to the nucleus and is not efficiently processed and presented on MHC I (148). When examining the protein sequences with a nuclear localization sequence (NLS) mapper (Kosugi et al., (2009) PNAS 106, 10171-10176), the IE3 sequence was found to have a predicted NLS, but the m38 protein was not. It is therefore possible that nuclear localization of IE3 reduces its presentation on the surface of CT2A cells for the IE3-specific T cells to see. Due to lack of IE3- or m38-specific antibodies, surface expression of these proteins from the CT2A cells could not be verified. Therefore, it is also possible that the CT2A-IE3 cells simply did not express their mCMV protein as well as the CT2A-m38 cells. To indirectly test this, the tumours were excised from mice, dissociated and plated back under the blasticidin selection conditions originally used to select for clones expressing the mCMV antigen construct. We found that wt CT2A cells died under selection conditions, but the CT2A-IE3 and CT2A-m38 cells survived. This suggested that the mCMV cell lines still contained their expression vectors, but formally did not prove IE3 protein expression.

A pilot intracranial (IC) injection study showed that CT2A cells expressing m38

established tumours similarly to wt CT2A tumours. However, it does appear that the CT2A-m38 tumours might progress slightly faster, generating slightly larger masses than the wt CT2A tumours at 10 days post tumour-seed. This is supported by the results from the therapeutic m38 IC studies showing that untreated mice bearing CT2A-m38 tumours succumb to tumour burden 17-25 days post cell injection, compared to untreated wtCT2A tumour-bearing mice that reach endpoint around day 30-35 post tumour seed (data not shown). Preliminary MRI imaging suggested that a single dose of intravenous (IV) FMT-m38 altered the structure of IC CT2A-m38 tumours. These tumours appeared dark and did not have the bright, defined borders characteristic of the wtCT2A IC tumours, suggesting perhaps tumour necrosis or destruction of tumour vasculature. Brains from these mice were perfused and fixed; future immunohistochemical staining may better indicate what effect the single IV dose of FMT-m38 had on the IC CT2A-m38 tumours. Additionally, IFNy ICS of PBMCs in the pilot study suggested that a single dose of IV FMT-m38 generated a significant anti-m38 CD8+ T cell response in CT2A-m38 tumour bearing mice. An anti-m38 response was not detected following tumour implantation, but the responses observed at one-week post-virus treatment were greater than the responses observed in naïve mice primed with FMT-m38. This suggests that a m38 response below detection thresholds may have been established by the tumour following implantation, or that epitope spreading via oncolysis of CT2A-m38 tumours in addition to m38-expression from FMT itself contributed to the observed m38-specific response.

The efficacy of FMT-m38 was tested in two different therapeutic GBM models. First, mice were inoculated with CT2A-m38 cells IC, primed with Ad-m38 7 days post-tumour seed and then boosted with PBS, FMT-eGFP or FMT-m38 7 days post-prime.

Second, mice were inoculated with CT2A-m38 cells IC and then treated either IC or IV with PBS, FMT-eGFP or FMT-m38 7 days post-tumour seed. Survival was monitored in both experiments, but immune responses were only followed in the prime/boost efficacy experiment.

In the prime/boost efficacy experiment, all vaccination regimens significantly prolonged survival compared to PBS treatment; however no mice were cured. There were detectable anti-m38 CD8+ T cell responses in all vaccination groups that were much more variable than the responses observed in previous experiments vaccinating naïve mice. There was also a small detectable prime against m38 in 3 out of the 9 mice evaluated post-tumour implantation (~0.5% IFNy+ CD8+ T cells). The fact that some mice in the Ad-m38 prime alone group mounted robust anti-m38 responses (~12%) also supports the idea that the tumour implantation may represent a priming event against the target antigen in some animals. Similar to our observations, Pol et al. (2013) found that treating B16-F10 IC tumour-bearing mice with Ad-DCT alone generated strong anti-DCT CD8+ T cell responses in some mice (~10%); Ad-DCT vaccination in naive animals typically generates frequencies approximating 0.5-3% (104). This supports the idea that the tumour itself can prime TAA-specific CD8+ T cell responses. An interesting trend we observed was that the group treated with Ad-m38 prime/FMT-eGFP boost had the lowest frequency of m38-specific CD8+ T cell responses, but also had the most prolonged survival compared to untreated mice (although this difference did not reach statistical significance).

Unfortunately, 2 out of 5 mice treated with Ad-m38 prime/FMT-m38 boost were found dead several days after receiving the boosting dose of FMT-m38. During

dissection, large bloody masses were found in the brains of these mice in place of the tumours. This suggests that a robust anti-m38 CD8+ T cell response may have been generated in these mice, but because the tumour was too large to be cleared, the mice died from massive inflammation and clotting in the brain. The untimely death of these animals made quantification of m38-specific CD8+ T cell responses impossible. Since PBS mice reached endpoint ~20 days post tumour seed and the boost was given 14 days post tumour seed, the tumour masses were likely quite large at the time of boost, but this would need to be confirmed by MRI. Out of the remaining three mice, we observed that one mouse had an extremely large anti-m38 CD8+ T cell response (~61% of total CD8+ T cells) and the other two mice had relatively low anti-m38 responses compared to naïve mice that had received the Ad-m38 prime/FMT m38 boost (~8% vs ~28%). Despite demonstrating detectable responses against the m38 target antigen, none of these mice were cured. Again, this can likely be attributed to the size of the tumour at the time of treatment.

A larger tumour would impose a much greater systemic immunosuppression and also present a more challenging tumour microenvironment for immune cells to penetrate and retain their activity. This could be confirmed by fixing the brains of the mice and then performing immunohistochemical staining for immunosuppressive cell populations (Tregs, MDSCs) or immunosuppressive molecules on the surface of tumour cells (PD-L1, CTLA-4, Fas-L). Alternatively, isolating immune cell populations from the tumour and analyzing them by flow cytometry could also verify this. Finally, serum samples and protein homogenates from the brain could be analyzed for the presence of immunosuppressive chemokines/cytokines (TGF-B, IL-10).

In future repeats of this experiment, the number of implanted CT2A-m38 tumour cells could be titrated down to a dose that would allow for slower tumour progression in order to give more time for vaccination before the tumours grow too large. Another option would be to prime mice earlier with Ad-m38, at day 4 or 5 post-tumour seed as opposed to 7. In the extremely aggressive IC B16-F10 model, Pol *et al.* (2013) inject $10^3$ cells IC, prime with Ad day 5 post tumour seed and boost 9 days post prime (104). Adenovirus vectors are known to generate substantial numbers of antigen-specific effector cells approximately 14-25 days post injection, therefore maybe giving the prime earlier, but waiting longer before boosting, would have achieved greater efficacy (101, 150).

Ciesielski *et al*. (2006) observed that vaccinating against tumour antigens in a prophylactic regimen, where mice were challenged subcutaneously with a mouse GBM cell line, demonstrated significant efficacy (121). However, when translating this to a therapeutic model where the GBM cells were implanted IC, survival was only modestly extended and there were no cures. In addition, Pol *et al*. (2013) found that heterologous prime/boost vaccination against an immunogenic TAA had profound effects on survival in a metastatic lung cancer model, but when evaluating the regimen in an intracranial tumour model, they observed much more modest cure rates (104).

Our second IC CT2A-m38 efficacy experiment assessed both the value of encoding a TAA (comparing FMT-eGFP vs. FMT-m38) and changing the route of virus delivery (IC vs IV) during OV therapy. We observed significantly longer survival in animals treated with FMT-eGFP IC and in animals treated with FMT-m38 IC or IV when compared to PBS treated animals. Statistically, there was no difference between the

treatment routes, but there was a 20% cure rate in the group treated with FMT-m38 IV. When comparing FMT-eGFP IC to FMT-m38 IC there was no significant difference between these treatments. However, FMT-m38 IV significantly prolongs survival compared to FMT-eGFP IV. The mice treated with PBS IV had significantly extended survival compared to mice treated with PBS IC (Median survival 23.5 days vs 17 days). Zhang *et al.* (2014) observed a similar phenomenon where surgical stress in tumour-bearing untreated animals increased metastases, decreased immune cell activity and shortened survival compared to untreated tumour-bearing animals that did not receive surgical insult (119). This preliminary data suggests that the tumour-associated antigen did not improve the outcome when treatment was delivered directly into the brain, but it did provide an advantage over the virus alone when the treatment was delivered systemically. It should be noted that the survival rates were not different between FMT-m38 IC vs FMT-m38 IV, but because there was a cure in the IV treatment group, this might indicate a trend towards an advantage in delivering the FMT-m38 intravenously.

We have demonstrated that IV delivery of FMT achieves a very high titer of replicating virus in the spleen 48 hours after injection in brain tumour-bearing animals (~$10^8$ pfu) (Appendix, Figure S7). Only a small amount of replicating FMT virus was observed in the brain following IV delivery (~$10^4$ pfu). When FMT was delivered IC, there was a high titer of replicating virus detected in the brain, but a much smaller titer was detected in the spleen. The observed trend that FMT-m38 delivered IV might have an advantage over FMT-m38 delivered IC may indicate that it is important to have high titers of replicating, antigen-encoding virus in the spleen to generate protective antigen-specific CD8+ T cell responses. The spleen contains large populations of mature DCs that

are extremely important for the cross-presentation of antigens (150). Dendritic cells infected with viruses have been shown to present antigens and prime CD8+ T cell responses *in vivo* via cross-presentation (151). Both VSV and MRB MG1 can infect DCs, and it is highly likely that FMT also infects DCs (119, 129). It is possible that in the brain, there is a greater population of immature APCs (MDSCs) compared to mature APCs due to the immunosuppressive nature of the GBM microenvironment. When antigens are cross-presented to CD8+ T cells by immature DCs, it leads to abortive proliferation or expansion of cells without effector function (152). Perhaps the advantage of having more replicating, antigen-encoding virus in the spleen simply increases the chances of successful cross-presentation of the target antigen by mature DCs that expands greater frequencies of cytotoxic T cells. In the context of the clinic, it is certainly more appealing to deliver treatments using the less invasive intravenous route as opposed to intracranially.

The intracranial efficacy experiments described above need to be repeated several more times to confirm the hypotheses made above. The m38 model system currently in use is a somewhat an artificial imitation of the situation in hCMV-infected GBM patients. Ideally, tumours could be implanted in mice chronically infected with mCMV to test our m38-targeted oncolytic virotherapy; however, since mice would already be primed against m38 in this context, it may be difficult to get the CT2A-m38 cells to take in these animals (145). Another useful model would be the one described by Price *et al,* where mice spontaneously develop high grade glioma following the establishment of chronic mCMV infection (57). These models obviously only evaluate species-specific consequences; therefore it is also useful to model the hCMV antigens found in GBM

patients in our mouse model. Heterologous vaccination platforms against pp65 and IE1 have also been generated in the lab and are currently being evaluated for immunogenicity and efficacy against CT2A cells engineered to express the same hCMV antigens. By combining data from the species-specific experiments that model the timeline in GBM patients chronically infected with hCMV, and the preliminary mouse experiments evaluating our potential clinical candidates targeting hCMV, we can demonstrate a powerful proof-of-principle to validate the hCMV targeting of rhabdoviruses to GBM for clinical studies.

### 4.4 Potential future directions

An oncolytic virus typically polarizes the immune system to a type I inflammatory response, but because the infection is transient and virus is usually rapidly cleared, it might be advantageous to deliver adjuvants at later time points to keep the immune system polarized after the virus is gone. Dr. Okada's group has demonstrated that DC vaccinations targeting glioma-associated antigens (GAAs), where the DCs are encoded to express high levels of IFN-α, are more effective vaccine vectors against gliomas because they generate a robust type I inflammatory cytokine/chemokine response (40). Dr. Okada's group has also demonstrated that using a simple adjuvant that is known to generate a type I inflammatory response, such as polyinosinic-polycytidylic acid (poly[I:C]) stabilized by lysine and carboxymethylcellulose (poly-ICLC), drastically improves the effectiveness of vaccines targeting GAAs (39, 153). The strategies employed by Dr. Okada's group improve T cell homing to the tumour site in the CNS and the type I inflammatory cytokine and chemokines produced help these GAA-specific T cells retain their function.

Despite our ability to generate robust TAA-specific CD8+ T cells, our data may indicate that the immunosuppressive tumour microenvironment presents an enormous challenge. For potent immune responses to be effective at the tumour site, strategies need to be applied to polarize the tumour microenvironment in a way that allows activated immune cells to traffic to the tumour in high frequencies and to allow these activated immune cells to retain their function. One possibility is to combine immune checkpoint inhibitors such and CTLA-4 or PD-1 antibodies with our vaccination regimens, to block T cell exhaustion and anergy at the tumour site (16). Another option is to use adjuvants to polarize the immune system to a type I inflammatory phenotype. These are just a few examples of strategies that could be used in combination with our heterologous prime/boost vaccination to improve the effectiveness against intracranial tumours.

### 4.5 Concluding remarks

This project set out to investigate if FMT virus encoding a known tumour-associated antigen (TAA) could be used in a heterologous prime/boost vaccination regimen to generate TAA-specific CD8+ T cell responses that lead to better efficacy in tumour-bearing animals. We found that the self-antigen survivin does not appear to be a good candidate for oncolytic vaccination using FMT virus. However, FMT virus does appear to be effective in controlling tumour progression and metastasis in an aggressive survivin-positive murine mammary carcinoma model when combined in a heterologous dual rhabdoviral treatment regimen along with MRB MG1. Farmington virus was able to both prime and boost adaptive responses against foreign GBM-associated CMV antigens. Prophylactic vaccination against these antigens demonstrated efficacy in a subcutaneous GBM tumour model, but this efficacy did not translate as well in therapeutic approaches

against intracranial GBM.

Many more experiments are required to validate FMT viruses encoding TAAs for the treatment of GBM before a clinical candidate is chosen. However, the data described herein provide a glimpse into the potential utility that FMT virus has for GBM patients. Identifying the most protective TAAs, uncovering how FMT polarizes the tumour microenvironment, assessing how FMT primes adaptive immune responses, determining which delivery route is the most effective and determining which combination treatments can provide the greatest advantage are some very important questions that need to be investigated to validate this treatment. There is currently a global effort to generate novel, potent immunotherapeutics for the treatment of GBM, and FMT virus has many strengths that recommend its use in this effort.

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## CONTRIBUTION OF COLLABORATORS

Dr. Kyle Stephenson, who at the time was in Dr. Wan's Lab at McMaster University, provided me with the Ad-SurvGFP virus used in the survivin-targeted prime/boost vaccinations. Dr. Vera Tang performed the initial experiments validating the FMT-OVA vaccination vector and the qPCR experiment surveying GBM lines for TAAs. Christiano Souza, senior research technician from Dr. Auer's lab at OHRI, skillfully executed the IM and IV injections necessary for the first two survivin-targeted vaccination experiments as well as taught me how to implant 4T1 tumours.

## APPENDIX

### Supplementary Figures

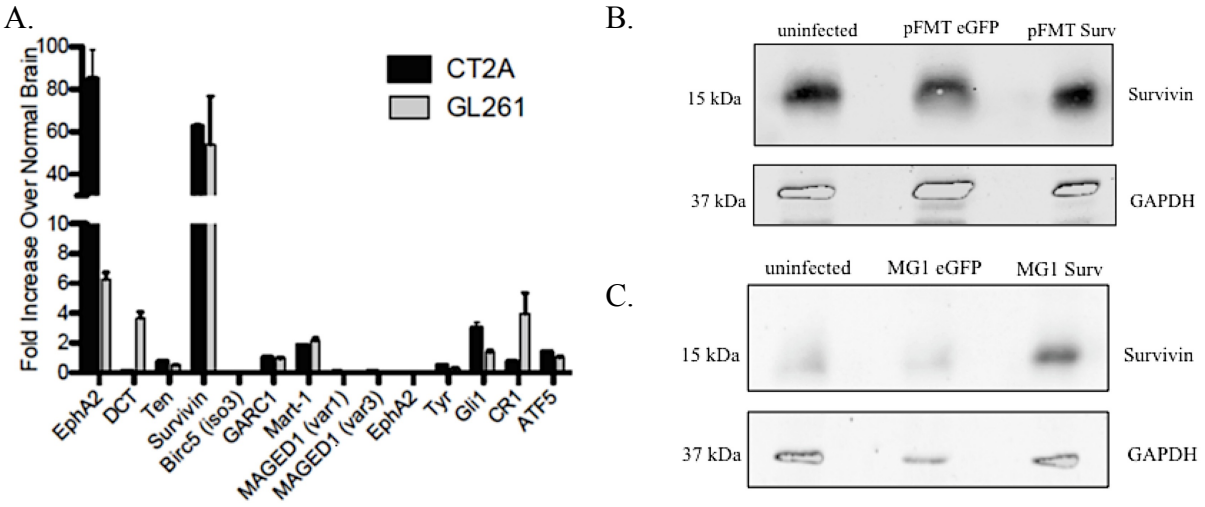



**Figure S1. Survivin is highly expressed in CT2A and GL261 GBM cells and TAA encoding rhabdoviruses**. RT-qPCR was used to quantify the mRNA expression of a panel of known TAA genes in CT2A and GL261 murine glioma cells (compared to normal whole brain mRNA) (A). Vero cells were infected with mock treatment, pFMT eGFP, pFMT Surv (B), MRB MG1 eGFP, and MRB MG1 Surv (C) and lysates were collected after 24hrs. Western blot was used to verify the TAA expression in engineered FMT and MRB variants (B,C,).

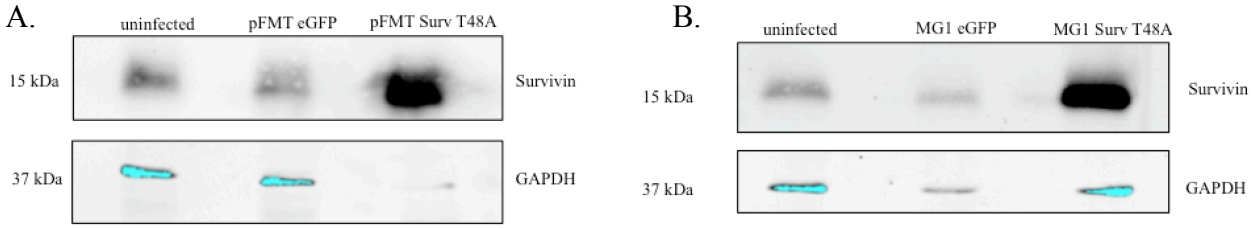



**Figure S2. Inactive survivin T48A mutant is highly expressed in TAA encoding rhabdoviruses.** Vero cells were infected with pFMT Surv T48A (A) or MRB MG1 Surv T48A (B) and lysates were collected after 24 hrs. Western blot was used to verify the TAA expression in engineered FMT and MRB variants (A,B). Uninfected and eGFP viruses used as controls.

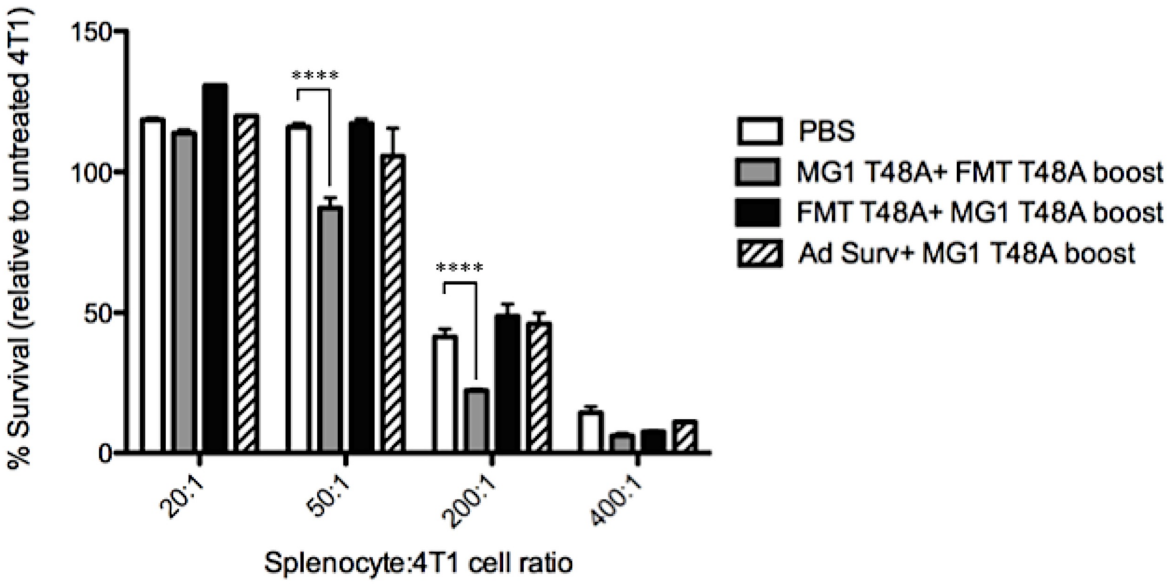



**Figure S3. Splenocytes isolated from 4T1 tumour-bearing mice treated with MG1-T48A prime/ FMT-T48A boost kill significantly more 4T1 cells *in vitro* compared to splenocytes from untreated tumour-bearing mice.** Groups of 4T1 tumour-bearing mice were treated with either MG1-T48A prime/ FMT-T48A boost, Ad-SurvGFP prime/ MG1-T48A, FMT-T48A prime/ MG1-T48A or PBS. At endpoint, spleens were collected from all the animals and processed in to single cell suspensions. Splenocytes from each animal (n=3/group) were then plated in triplicate on 4T1 cells in a 96 well-plate (ratios 20:1, 50:1, 200:1, 400:1). Eighteen hours post co-culturing, splenocytes were washed from the wells and AlamarBlue assay was used to assess 4T1 survival. Data is plotted as mean of 3 animals ± SEM (n=3 for each treatment group). Asterisks indicate significant values as calculated by two-way ANOVA with Bonferroni test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, **** $p<0.0001$.

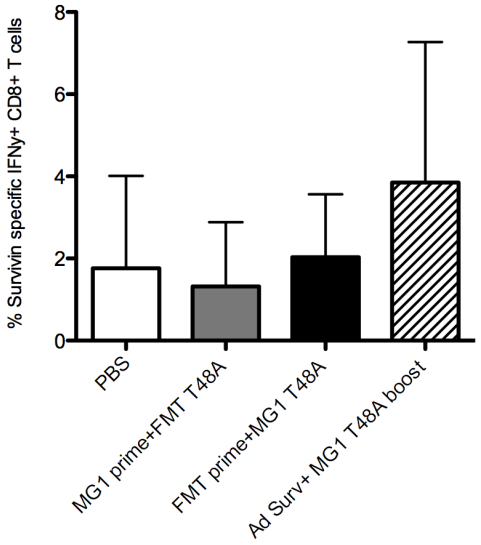



**Figure S4. Frequencies of survivin-specific CD8+ T cells are similar between treatment groups in the 4T1 model.** Groups of 4T1 tumour-bearing mice were treated with either MG1-T48A prime/ FMT-T48A boost, Ad-SurvGFP prime/ MG1-T48A, FMT-T48A prime/ MG1-T48A or PBS. Peripheral blood CD8+ T cells were analyzed 5 days post boost for intracellular IFNy staining in response to stimulation with the immunodominant CD8+ epitopes from survivin via flow cytometry. n=6 per treatment group. No statistical significance.

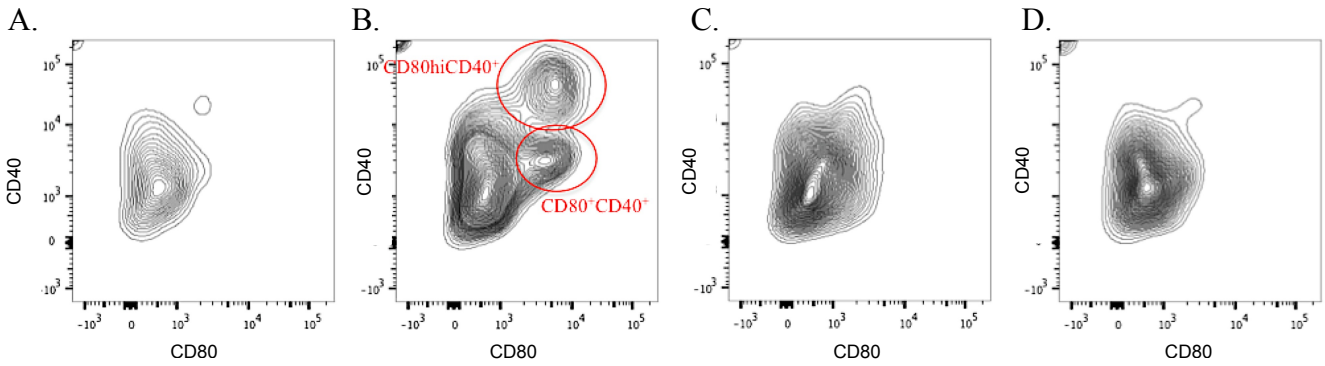



**Figure S5. CD11b+ myeloid cells isolated from 4T1 tumours treated with MG1-T48A prime/ FMT-T48A boost appear to express higher levels of co-stimulatory molecules compared to myeloid cells isolated from tumours of untreated mice.** Groups of 4T1 tumour-bearing mice were treated with either PBS (A), MG1-T48A prime/ FMT-T48A boost (B), FMT-T48A prime/ MG1-T48A (C) or Ad-SurvGFP prime/ MG1-T48A (D). At endpoint, tumours were collected from all the animals and processed in to single cell suspension. Ficoll gradient purification was used to isolate immune cells. Following an overnight incubation, the immune cells isolated from the tumours were stained for CD11b, CD40 and CD80 and analyzed by flow cytometry.

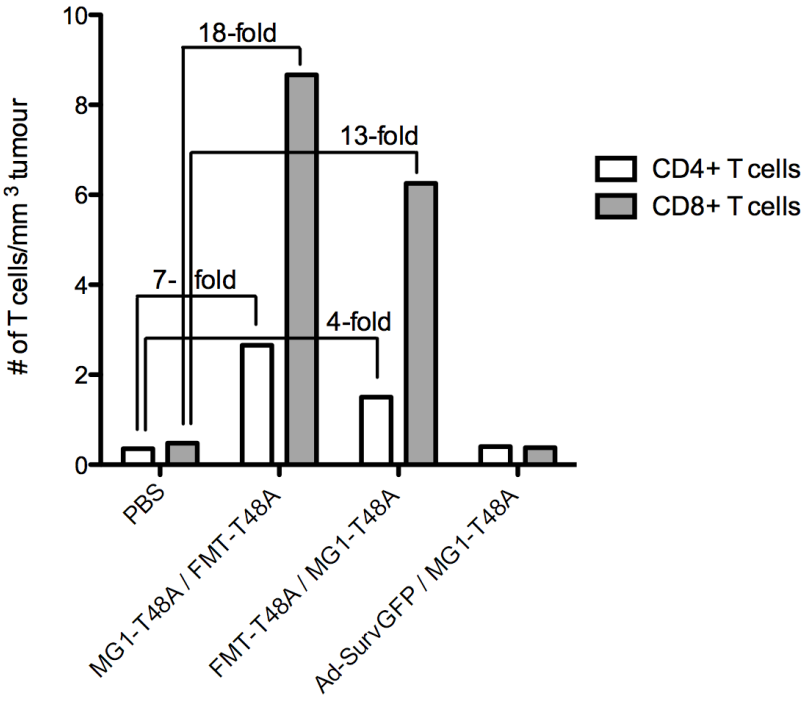



**Figure S6. 4T1 tumour-bearing mice treated with MG1-T48A prime/FMT-T48A boost and FMT-T48A prime/MG1-T48A boost appear to have more CD4+ and CD8+ T cells in their tumours when compared to untreated mice.** Groups of 4T1 tumour-bearing mice were treated with either PBS, MG1-T48A prime/ FMT-T48A boost, FMT-T48A prime/ MG1-T48A or Ad-SurvGFP prime/ MG1-T48A. At endpoint, tumours were collected from all the animals and processed in to single cell suspension. Ficoll gradient purification was used to isolate immune cells. Following an overnight incubation, the immune cells isolated from the tumours were stained for CD4 and CD8 and analyzed by flow cytometry.

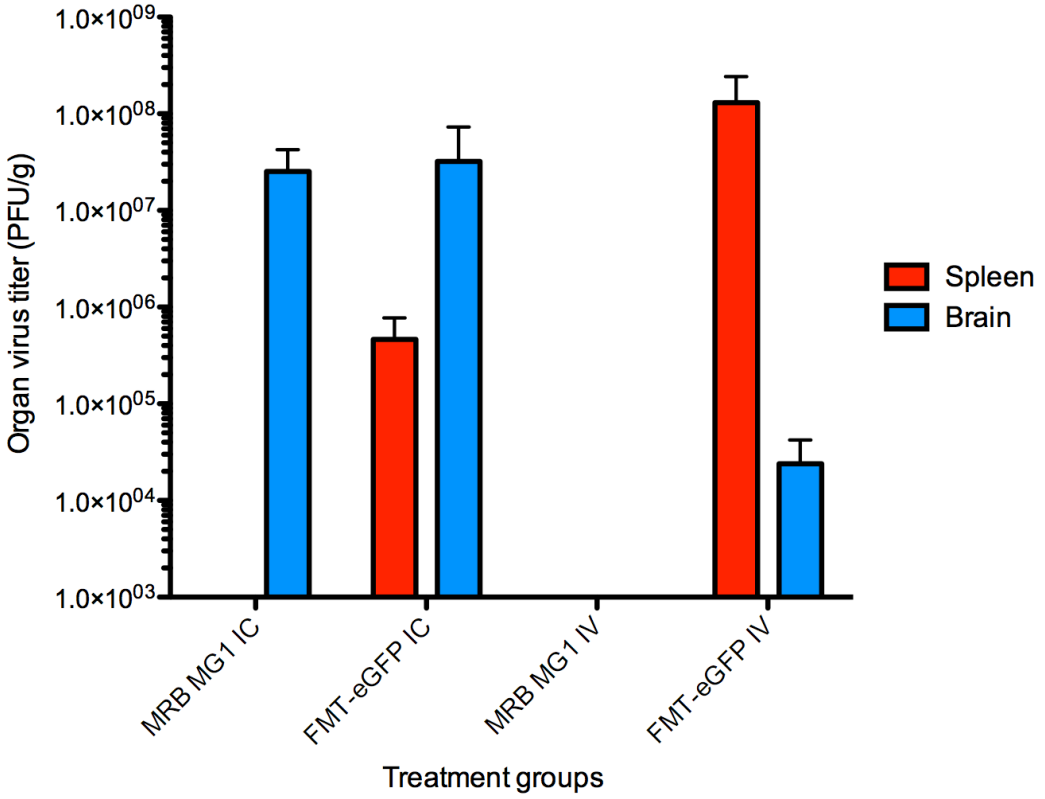
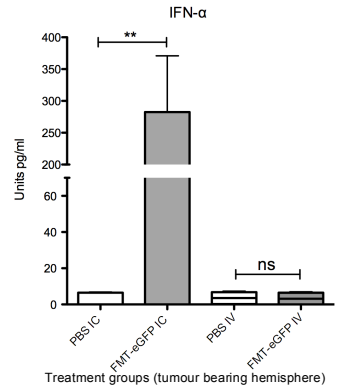



**Figure S7. Replicating Farmington virus is detected in the brain and the spleen post-intracranial and post-intravenous injection.** Naïve female C57BL/6 mice (8-12 weeks) were injected IC with 5E4 CT2A cells. Seven days post tumour seed, mice were injected with 1E8 pfu MRG MG1 IC (n=3), 1E8 pfu FM-eGFP IC (n=4), 1E8 pfu MRB MG1 IV (n=3) or 1E8 pfu FMT-eGFP IV (n=4). After 48 hrs, mice were sacrificed and their brains and spleens were flash-frozen for titering. After weighing, brains and spleens were homogenized with a tissue lyser in a minimal volume of serum free DMEM. After spinning down the debris, supernatant was titered on Vero cells by standard plaque assay.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Units pg/ml
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ns
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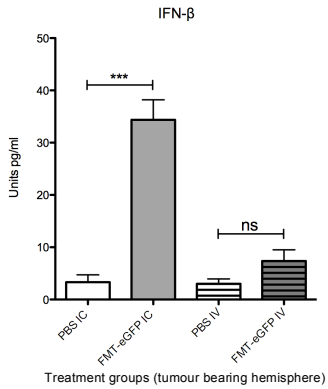
FMT-eGFP IC
PBS IV
FMT-eGFP IV
Treatment groups (tumour bearing hemisphere)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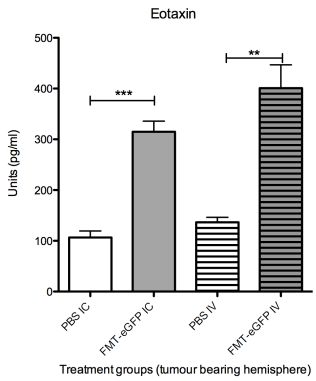
Eotaxin
Units (pg/ml)
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PBS IV
FMT-eGFP IV
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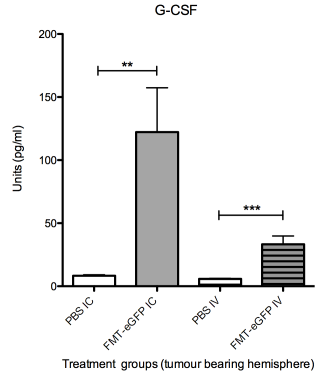
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PBS IV
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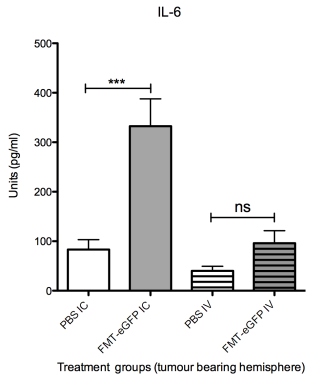
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Units (pg/ml)
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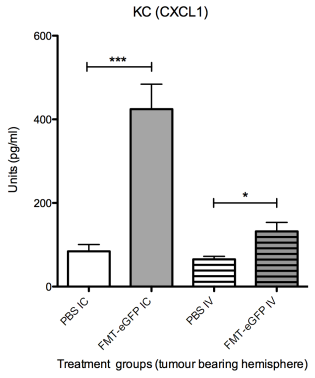
FMT-eGFP IC
PBS IV
FMT-eGFP IV
Treatment groups (tumour bearing hemisphere)


KC (CXCL1)
Units (pg/ml)
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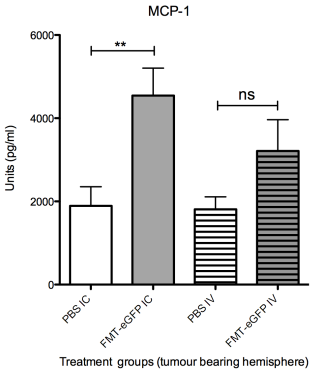
FMT-eGFP IC
PBS IV
FMT-eGFP IV
Treatment groups (tumour bearing hemisphere)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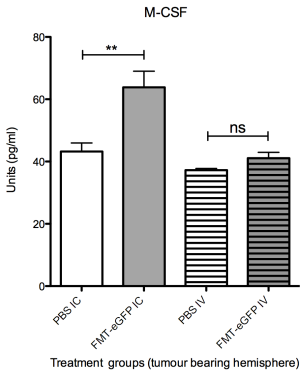
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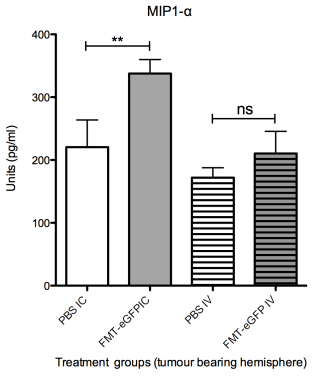
Treatment groups (tumour bearing hemisphere)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PBS IV
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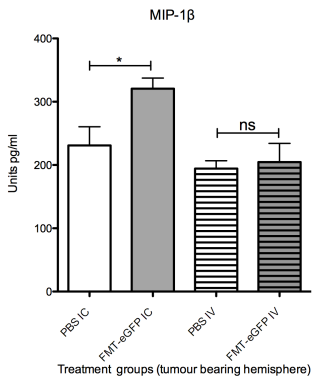
Treatment groups (tumour bearing hemisphere)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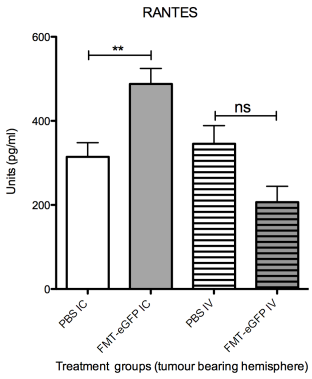
PBS IV
FMT-eGFP IV
Treatment groups (tumour bearing hemisphere)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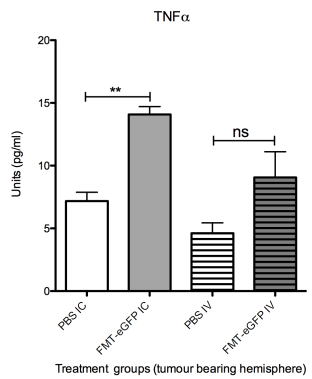
FMT-eGFP IV
Treatment groups (tumour bearing hemisphere)


TNFα
Units (pg/ml)
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PBS IC
FMT-eGFP IC
PBS IV
FMT-eGFP IV
Treatment groups (tumour bearing hemisphere)

**Figure S8. Intracranial injection of FMT-eGFP causes the upregulation of important type I inflammatory chemokines and cytokine detectable 48 hours post injection in the tumour-bearing hemisphere of the brain.** Naïve female C57BL/6 (8-12 weeks) were IC injected with 5E4 CT2A cells. Fourteen days post tumour seed, mice were injected with PBS IC, 1E8 pfu FMT-eGFP IC, PBS IV or 1E8 pfu FMT-eGFP IV. After 48 hrs, mice were sacrificed and brains were harvested, cut into hemispheres (tumour-bearing and non-tumour-bearing) and flash frozen. Tumour-bearing hemispheres of the brain samples were lysed with a tissue homogenizer in 500 uL of RIPA buffer+PI. Debris was spun down and the protein in the supernatant was quantified by Bradford assay. All of the samples were standardized to the same protein concentration before being sent to Eve Technologies© for mouse cytokine/chemokine array. Asterisks indicate significant values compared to PBS as determined by one-way ANOVA with Dunnett's test. *$p<0.05$, **$p<0.01$, ***$p<0.001$. n=5 for each treatment group.