IMPROVING THE DELIVERY AND REPLICATION OF ONCOLYTIC VIRUSES

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ABSTRACT

The optimal route for clinical delivery of oncolytic viruses (OVs) is thought to be intravenous (IV) injection; however, the immune system is armed with several highly efficient mechanisms to remove pathogens from the circulatory system. To overcome the challenges in trying to deliver OVs IV, cell carriers have been investigated to determine their suitability as delivery vehicles for systemic administration of OVs. Here we demonstrate the utility of a *Drosophila melanogaster* cell platform for the production and *in vivo* delivery of multi-gene biotherapeutic systems. We show that cultured *Drosophila* S2 cell carriers can stably propagate OV therapeutics that are highly cytotoxic for mammalian cancer cells without adverse effects on insect cell viability or cellular gene expression. *Drosophila* cell carriers administered systemically to immunocompetent animals trafficked to tumours to deliver multiple biotherapeutics with little apparent off-target tissue homing or toxicity, resulting in a therapeutic effect. S2 cells provide a genetically tractable platform supporting the integration of complex, multi-gene biotherapies while avoiding many of the barriers to systemic administration of mammalian cell carriers.

Once OVs are delivered to tumour beds, they initiate replication in tumour cells, which often possess defects in antiviral pathways and are thus susceptible to infection. However, not all tumours have defects in their antiviral defenses and thus virus replication in these tumours is rather limited. Identifying and modulating host factors that regulate virus replication in OV-resistant cancer cells, but not normal cells, could lead to increased replication in these tumours and potentially improve therapeutic outcomes. We therefore conducted an RNA interference screen using Sindbis virus (SINV) in order to identify host factors that modulate OV replication in tumour cells. Specifically, serial passage of a SINV-artificial microRNA (amiRNA) library in a tumour cell line followed by deep sequencing of

the selected virus populations led to the identification of several amiRNA sequences that were enriched. Furthermore, the identified amiRNA sequences increased the replication of various OVs both *in vitro* and *in vivo*, ultimately resulting in an enhanced therapeutic effect.

Overall, the work presented here highlights strategies in which both the systemic delivery and tumour-specific replication of OVs can be improved.

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LIST OF ABBREVIATIONS

AGO2	Argonaute 2
amiRNA	Artificial microRNA
ASPN	Asporin
ATCC	American Type Culture Collection
AV	Attenuated Variant
CIK	Cytokine-Induced Killer
EGFR	Epidermal Growth Factor Receptor
EV	Enveloped Virion
FBS	Fetal Bovine Serum
FLuc	Firefly Luciferase
G	Glycoprotein
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GO	Gene Ontology
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
IFN	Interferon
IL	Interleukin
IRF	Interferon Regulatory Factor
ISG	Interferon Stimulated Genes
IT	Intratumoural

IV	Intravenous
IVIS	*In Vivo* Imaging System
L	Large Polymerase
kb	Kilobase
LDLR	Low-Density Lipoprotein Receptor
M	Matrix Protein
MDSC	Myeloid-Derived Suppressor Cell
MFV	Mean Fluorescence Value
MHC	Major Histocompatibility Complex
miRNA	MicroRNA
MOI	Multiplicity Of Infection
mRFP	Monomeric Red Fluorescent Protein
mRNA	Messenger RNA
MSC	Mesenchymal Stem Cell
MV	Mature Virion
N	Nucleoprotein
NDV	Newcastle Disease Virus
NF-κB	Nuclear Factor-κB
NK	Natural Killer
NSC	Neural Stem Cell
OGN	Osteoglycin
OMD	Osteomodulin
ORF	Open Reading Frame
OV	Oncolytic Virus

OVA	Ovalbumin
P	Phosphoprotein
PBS	Phosphate-Buffered Saline
PFU	Plaque-Forming Units
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
PTGS	Post-Transcriptional Gene Silencing
qPCR	Quantitative Polymerase Chain Reaction
Rb	Retinoblastoma
RISC	RNA-Induced Silencing Complex
RNAi	RNA-Interference
S2	Schneider Line 2
SEM	Standard Error of the Mean
shRNA	Short Hairpin RNA
SINV	Sindbis Virus
siRNA	Small Interfering RNA
SLRP	Small Leucine Rich Proteoglycan
TAA	Tumour-Associated Antigen
TK	Thymidine Kinase
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor
VGF	Vaccinia Growth Factor

VSV	Vesicular Stomatitis Virus
VV	Vaccinia Virus
VVdd	Vaccinia Virus Double-Deleted
WT	Wild Type
YFP	Yellow Fluorescent Protein

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CHAPTER 1

1. INTRODUCTION

1.1 Cancer

Cancer is a group of diseases characterized by the uncontrolled proliferation of cells, resulting in their dissemination and invasion into other organs of the body. Despite the complex and heterogeneous nature of cancer, there are "hallmarks", as described by Hanahan and Weinberg, that are common to most types of cancer[1,2]. These features include achieving self-sustained growth, ignoring anti-proliferative signals, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating cell migration and metastasis, as well as evading immune-mediated elimination. The acquisition of these traits is a product of dysregulation in normal gene expression, a process that is governed by heredity, as well as both genetic and epigenetic changes. For example, individuals who inherit germline mutations in the *BRCA* genes, which play a role in DNA repair, are at an increased risk of developing breast and ovarian cancer[3,4]. Also, activation and overexpression of oncogenes and the loss of tumour suppressors can occur as a result of somatic mutations and gene amplifications/deletions[5]. Alternatively, aberrant gene expression can arise as a consequence of epigenetic modifications. Indeed, methylation of promoters results in gene silencing and cancer cells are often hypomethylated on a genome-wide scale, with hypermethylation of specific sites (i.e tumour suppressor genes)[6].

Tumours do not exist as isolated entities but rather within the confines of the tissue in which they originate. The interactions between cancer cells and normal cells within the tumour microenvironment can have both positive and negative impacts on tumour growth. For example, the immune system has the ability to recognize and eliminate malignant cells

from the body; however, tumours also develop mechanisms to escape this immune surveillance[7–11]. Macrophages and fibroblast within the tumour microenvironment are also able to control tumour growth, but are often "reprogrammed" by tumour cells to promote tumour growth by stimulating angiogenesis, inducing immune suppression, and increasing tumour invasion and metastasis[12–15].

Evolution of cancer cells is governed by Darwinian selection in a dynamic and changing microenvironment. This leads to both linear and branched evolution over time, thereby leading to clonal heterogeneity within tumours[16]. This complicates treatment of the disease as therapies may only kill a subset of cells within a tumour. Conventional interventions include tumour resection, radiation therapy, and chemotherapy. In the last two decades targeted therapies and immunotherapies have also been developed, and often are used in conjunction with conventional treatments. According to the National Cancer Institute and the Canadian Cancer Society, survival rates for some cancers, such as non-Hodgkin lymphoma, leukemia and multiple myeloma, have increased over the last few decades; however, for some cancers like bladder and uterine cancer, survival rates have remained stagnant with no improvements over time. Thus, there is still the need for better treatment options.

1.2 Oncolytic Virotherapy

1.2.1 A brief history of virotherapy of cancer

OVs are viruses that have the ability to infect and kill tumour cells without causing significant damage to normal tissues[17]. As early as the late-1800s, there had been case reports of tumour regression coinciding with natural viral infection[18,19]. In a widely cited example, a patient with myelogenous leukemia went into remission following an apparent

influenza infection[18]. However, it was not until the mid-1900s that pre-clinical and clinical testing of cancer therapy with OVs was initiated, largely due to the development of tissue culture systems and rodent cancer models that allowed for such experiments to be conducted[20,21]. In 1949, Hoster *et al*. reported a seminal study in humans where, after observing two cases of remission following natural hepatitis infection in patients with Hodgkin's disease, an additional 21 patients were treated with hepatitis virus from various sources including sera and tissue extracts[22]. Of these, 13 developed viral hepatitis and seven demonstrated signs of improvement in various aspects of their disease. However, deaths attributed to virus infection were also reported in some instances. Over the next 20 years, hundreds of cancer patients were treated with a wide range of non-attenuated viruses with tumour regressions, as well as virus-related complications and even deaths, occasionally reported[23]. The 1970s and 1980s witnessed a decline in the number of clinical studies using OVs, perhaps due to the unfavorable safety profile of the viruses available at the time. Thus, the field was in search of safer and more effective OVs.

1.2.2 Tumour selectivity of OVs

The modern era of oncolytic virotherapy, marked by the engineering of tumour-selective viruses, began in 1991 with the development of a thymidine kinase (TK)-deleted Herpes Simplex Virus (HSV) which demonstrated attenuated neurovirulence yet was still effective in a murine glioblastoma model[24]. The molecular biology revolution, which allowed cancer researchers to gain better understanding of the disease at the molecular and genetic level and also enabled virologists to genetically manipulate viruses, led to the discovery of several candidate OVs with improved safety profiles. Specific targeting of

tumour cells by OVs can be achieved using various strategies, each one capitalizing on inherent differences between normal cells and cancer cells.

Transductional targeting allows for tumour specificity by capitalizing on the presence of cell-surface proteins that are over-expressed or unique to tumour cells. For example, both measles virus and the adenovirus ColoAd1 bind and enter cells via CD46[25–27], which is often over-expressed in a variety of tumours[28]. Similarly, poliovirus and SINV bind CD155[29] and laminin receptor[30], respectively, and these are also highly expressed in solid tumours[31,32]. Another strategy to achieve tumour-specific transduction is to reprogram OVs to bind to specific surface antigens on tumour cells through the display of single-chain antibodies or tumour surface receptor-binding ligands. Indeed, single-chain antibodies targeting epidermal growth factor receptor (EGFR)[33], CD20[34], and CD38[33] have successfully been displayed on measles virus to increase tumour specificity.

Another aspect of the viral life cycle that can be engineered to improve tumour selectivity is viral gene transcription. There are several tumour-specific promoters that have been identified and exploited to achieve this goal[35,36]. Hypoxia[37] and telomere maintenance[38] are common to several cancers and thus promoters of genes involved in regulating these processes, such as hypoxia-induced factor[39] and human telomerase reverse-transcriptase[40], can be harnessed to drive tumour-specific replication of OVs. Likewise, the prostate-specific antigen promoter, which is over-expressed in prostate cancer[41], has been engineered into oncolytic adenovirus[42] and HSV[43] to achieve tissue specific expression. It is important to note that this approach only works with viruses that replicate in the nucleus and rely on the host transcription machinery to drive viral gene expression.

A different strategy to achieve tumour-specific virus replication is to take advantage of microRNAs (miRNAs) that are differentially expressed in tumour cells. Specifically,

miRNAs that are present in normal tissues but absent in tumours can be utilized to de-target virus replication in normal cells by incorporating the target sequence of the miRNA in viral genes critical for successful replication[44]. This approach has been implemented into several OVs including vesicular stomatitis virus (VSV)[45,46], measles virus[47], HSV[48], vaccinia virus (VV)[49] and adenovirus[50].

Importantly, the tumour selectivity of OVs has also been attributed to various molecular features unique to tumour cell biology. One of the more prominent hypotheses is that tumour-specific defects in the interferon (IFN) pathway underlie tumour-selective replication of OVs. Indeed, it has been demonstrated that roughly 70% of tumour cell lines in the NCI-60 panel have defects in the IFN response[51]. Although the drivers of tumour evolution leading to the loss of IFN responsiveness are not completely understood, it is believed that the physiological processes involved in the antiviral response are incompatible with efficient tumour growth. For instance, IFN is known to be anti-angiogenic[52], as well as an inducer of apoptosis[53], cell growth arrest[54] and immune-stimulation[11], all of which are phenomena that cancer cells strive to evade[2]. However, a recent study[55] has found that loss of PTEN, which is one of the most commonly mutated tumour suppressor genes in human cancers[56], results in reduced IFN-induced antiviral immunity, thus shedding some light as to how tumour cells can acquire defects in the IFN response. These defects in the IFN pathway in tumour cells create a window of opportunity for naturally occurring or engineered IFN-sensitive OVs such as Newcastle disease virus (NDV)[57], reovirus[58], SINV[59], measles virus[60] and VSV[51].

Additional examples of rational design of OVs to increase tumour specificity include the deletion of TK gene from HSV[24] and VV[61], which allows for selective replication in tumour cells which often have elevated nucleotide pools. Similarly, VV encodes vaccinia

growth factor (VGF), a secreted protein that binds EGFR and primes surrounding cells for subsequent viral infection[62,63]. Given that EGFR is often constitutively activated in several cancers[64], such tumours are rendered hypersensitive to infection by VV lacking VGF, whereas normal cells are protected from infection[61]. Lastly, tumour specific adenoviruses have been engineered by partial deletion of the E1A gene[65], which interacts with the tumour suppressor gene retinoblastoma (Rb) in order to release the breaks on cell cycle progression and promote virus replication. Since Rb function is often disrupted in cancer cells[66], an E1A-deleted adenovirus is rendered tumour-specific. Overall, identification of molecular and genetic differences between normal and tumour cells, coupled with an understanding of how viruses interact with these altered pathways, has led to the generation of a vast panel of safer, tumour-selective OVs.

1.2.3 OVs are multi-mechanistic anti-cancer agents

As the name implies, OVs replication in tumour cells ultimately leads to cell death and lysis. Indeed, the cellular antiviral response results in the induction of several pro-apoptotic genes that, in an altruistic fashion, serve to limit the spread of virus[67]. However, the anti-cancer activity of OVs is not only limited to virus-induced apoptosis of tumour cells. It has been demonstrated in both murine tumour models and in human patients that OVs can infect and disrupt tumour vasculature, subsequently leading to a loss of blood flow to the tumour and consequently tumour necrosis[68–70]. Specific infection of tumour endothelium was shown to be mediated by vascular endothelial growth factor (VEGF)-induced expression of PRD1-BF1/Blimp1, a transcriptional repressor that targets genes involved in the IFN response[71]. In addition to direct infection of tumour vasculature, OV therapy induces a pro-inflammatory response that leads to the expression of the chemokines CXCL1 and CXCL5

within the tumour microenvironment. These serve to recruit neutrophils, which, through an unknown mechanism, severely compromise blood flow to the tumour thereby leading to tumour necrosis[70].

Another facet of OV therapy that is increasingly recognized as being critical for anti-tumour activity is the potent immune-stimulating properties of viruses. The immune system plays an important role in cancer prevention and tumours must acquire mechanisms to avoid immune destruction in order to thrive[7,9]. Moreover, the role of IFNs in anti-tumour immunity is well established[8,10,11]. In addition to being potent inducers of IFNs, OVs also induce the up-regulation of major histocompatibility complex (MHC) molecules as well as the production of chemokines and cytokines that recruit and activate immune cells, all of which favor enhanced anti-tumour immune responses[70,72–75]. Furthermore, infection and lysis of tumour cells can lead to immunogenic cell death and also in the production of pathogen-associated molecular patterns and danger-associated molecular patterns, thus leading to increased activity of antigen presenting cells and improved adaptive immune responses[76–78]. Clinical evidence that OVs can trigger effective anti-tumour immune responses comes from the finding that in patients with non-resectable melanoma, IT injection of virus in some lesions led to responses in non-injected lesions[79–82].

In addition to being self-replicating therapeutic agents, OVs possess the ability to function as gene expression vectors. Several approaches have been explored to improve the potency of OVs through transgene expression. Increased tumour killing has been achieved through virus-mediated expression of pro-apoptotic genes[83] and prodrug converting enzymes[34,84] specifically within the tumour microenvironment. Arming OVs with matrix metalloproteinases, which are enzymes that can degrade components of the extensive network of macromolecules that make up the extracellular matrix (ECM), improves virus

spread within tumours[85,86]. Similarly, viruses engineered to express immune evasion molecules that blunt the IFN response[87,88] and limit the recruitment of innate immune cells to the tumour[89] have been shown to replicate to higher titers, leading to an increased therapeutic effect. Given that the induction of anti-tumour immune responses is an important mechanism of OV therapy, encoding immune stimulating cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ or interleukin (IL)-12, is another strategy that has been demonstrated to improve tumour control[72,73,90–93]. In fact, T-VEC, an HSV-based OV that is the first and only OV approved by the United States Food and Drug Administration for use in patients, as well as Pexa-Vec, a VV-based OV currently in clinical testing, encode GM-CSF. Lastly, expression of tumour-associated antigens (TAAs) by OVs has resulted in the induction of anti-tumour immune responses far more effective than vaccination with TAAs alone[94–96].

1.3 Oncolytic rhabdoviruses

VSV is a member of the *Rhabdoviridae* family of viruses belonging to the genus *Vesiculovirus*. VSV is a bullet-shaped, enveloped, negative-sense RNA virus with an unsegmented 11-kilobase (kb) genome that consists of five genes encoding the five major viral proteins: nucleoprotein (N), phosphoprotein (P), matrix (M), glycoprotein (G), and large polymerase (L). VSV, with its trimeric G protein, binds to and enters cells via the low-density lipoprotein receptor (LDLR)[97], leading to clathrin-mediated endocytosis of the virus particle[98,99]. Once in the endocytic pathway, acidification of endosomes triggers conformational changes in the G protein that drive fusion of the viral and cellular membranes[100], resulting in the release of the ribonucleoprotein core into the cytoplasm. The polymerase complex, which consists of L, P and N-coated genomic RNA, initiates primary

transcription of viral messenger RNAs (mRNAs)[101–103]. Because the viral polymerase stutters at each intergenic region during polyadenylation, and re-initiation does not always occur, there is a gradient in the amounts of each viral gene being produced, with N being more abundant than P, which is more abundant than M, and so on[104]. Once these are translated, the resulting viral proteins initiate replication of the negative-sense genome through positive-sense intermediates[105]. After a secondary round of transcription, the viral proteins and genomes are assembled at the cell membrane, from which new virions bud.

The M protein of VSV plays a major role in inhibiting host gene expression at multiples levels, thereby promoting translation of viral gene products and also antagonizing the host antiviral response. The M protein is able to potently inhibit host transcription directly[106]. It also inhibits the nuclear to cytoplasmic transport of host mRNAs, thereby preventing the translation of important antiviral factors such as IFNs. It does so by forming a complex with Nup98, a nucleoporin, and Rae1, a poly(A) binding export factor, both of which play a role in the nuclear export of mRNAs[107,108]. It is also believed that M protein-mediated inhibition of host gene expression is in large part responsible for the cytopathic effects induced by VSV[109].

As discussed above, the tumour specificity of VSV as an OV arises from the fact that it is exquisitely sensitive to the antiviral action of IFNs, coupled with the finding that tumours often have defects in their IFN response[110,111]. Indeed, wild type (WT) strains of VSV can replicate and kill several tumour cell lines, but virus replication is attenuated in normal cells, especially in the presence of IFN[111]. Our group and others have capitalized on these findings to further increase the safety profile of VSV. Stojdl *et al.* have isolated naturally occurring VSV attenuated variants (AV), AV1 and AV2, that have lost the ability to block host mRNA export to the cytoplasm due to mutations in the M gene[51]. The net effect

is that, unlike WT strains of VSV, these mutants do not block the production of IFN in normal cells and thus are highly attenuated in normal cells. However, since tumour cells often do not produce or respond to IFN[51,111], these mutants retain their capacity to infect and kill tumour cells. In order to ensure that the mutants do not revert back to WT, a recombinant strain of VSV was generated, VSVΔ51, in which methionine at position 51 of the M protein is deleted[51]. An alternate strategy that has been investigated is to encode the IFN-β gene in WT-VSV[112]. Following the same principles, this virus displays an attenuated phenotype in normal cells but retains its oncolytic activity in tumours.

VSV has several advantages as a therapeutic virus. It replicates rapidly and to high titers, and is highly lytic in tumour cells. It has broad tissue tropism given the expression of its entry receptor, LDLR, on several cell and tissue types. VSV also has a favorable safety profile. Even WT-VSV causes relatively mild disease in animals and humans, and the virus has been further attenuated for use as a cancer therapeutic. Since it replicates exclusively in the cytoplasm and does not produce any DNA intermediates, there is virtually no risk of integration into the host genome. Since a majority of the human population, at least in North America, has not previously been exposed to the virus, there is no pre-existing immunity that could hamper its use. It is also very immunogenic and therefore can promote anti-tumour immune responses[72,113]. Finally, there exists the means to engineer recombinant viruses in order increase the safety or therapeutic activity of the virus[114–116].

In an effort to identify more potent oncolytic rhabdoviruses, Brun *et al.* performed a screen with a variety of WT viruses against a panel of tumour cell lines and identified Maraba virus as the most potent one[117]. Similar to VSVΔ51, two mutations that blunted the ability of the virus to antagonize cellular antiviral defenses but maintained robust replication in tumour cells were engineered into the virus, which was named MG1[117,118]. Interestingly,

for reasons not yet understood, the two mutations created a virus that was safer than WT-Maraba in normal cells yet hypervirulent in cancer cells. Importantly, MG1 expressing the tumour antigen MAGE-A3 is currently undergoing clinical testing both as a stand-alone therapy and also in a prime-boost setting in combination with adenovirus also expressing MAGE-A3 (NCT02285816).

1.4 Oncolytic VV

VV is the prototypic virus of the genus *Orthopoxvirus*, which belongs to the *Poxviridae* family. VV is a large, brick-shaped, enveloped virus that has linear double-stranded DNA genome that is approximately 190kb in length and encodes over 200 genes. Structurally, the virions contain over 80 virally-encoded proteins, 30 of which are found on the surface of the virion[119,120]. The mechanism of entry of the virus into cells is not completely understood. It is believed that VV can enter cells via direct fusion with the plasma membrane and also through endocytosis, a process that involves 12 viral proteins[121]. Once VV enters its host cell, it initiates its replication cycle, which occurs exclusively in the cytoplasm. Amongst the 80 proteins that make up the virion is a complete early transcription system that enables the synthesis of early viral mRNAs soon after infection. The early mRNAs encode enzymes and factors needed for synthesis of viral DNA and for transcription of the intermediate genes, which encode enzymes and factors for late gene expression. Finally, the products of the late genes include the early transcription factors, which are packaged with RNA polymerase and other enzymes into progeny virions[122,123]. VV exists in two infectious forms. The more abundant and basic infectious form is known as the mature virion (MV) and is formed intracellularly. The other form, known as the enveloped virion (EV), has an additional membrane and exists extracellularly. The MV is involved in cell-to-

cell spread whereas the EV is important for virus dissemination through the host[124]. Given its large genome and numerous open reading frames (ORFs), it is not surprising that VV encodes a wide array of genes that modulate host antiviral immunity, both innate and adaptive. These include antagonists of pattern recognition receptors, inhibitors of the IFN, nuclear factor-κB (NF-κB), and tumour necrosis factor (TNF) pathways, cytokine and chemokine binding proteins, complement regulatory proteins, apoptosis inhibitors, and modulators of antigen presentation[125,126].

VV has several attributes that make it suitable as an OV. Importantly, it has a favorable safety profile as demonstrated by its successful use as a smallpox vaccine. In addition, VV possesses the ability to infect a wide spectrum of cell types and it can be genetically engineered to express large banks of transgenes given its large genome. Oncolytic versions of VV have been generated for use as cancer therapeutics. Similar to VSV, miRNA-detargeting[49] and engineering of IFN-sensitive variants[127] are strategies that have been explored to increase the tumour specificity. Another approach that has been investigated is the deletion of TK from VV[90,128]. Building from these initial findings, McCart *et al*. have further attenuated VV replication in normal cells by deleting both TK and VGF[61].

Clinical experience with VV expressing GM-CSF has yielded promising results thus far. The virus is well tolerated and some tumour responses have been demonstrated[129,130]. Importantly, delivery of the virus to tumours following IV administration has been shown, as well as evidence of tumour-specific viral replication[131].

1.5 Systemic delivery of OVs

1.5.1 Obstacles to successful OV therapy

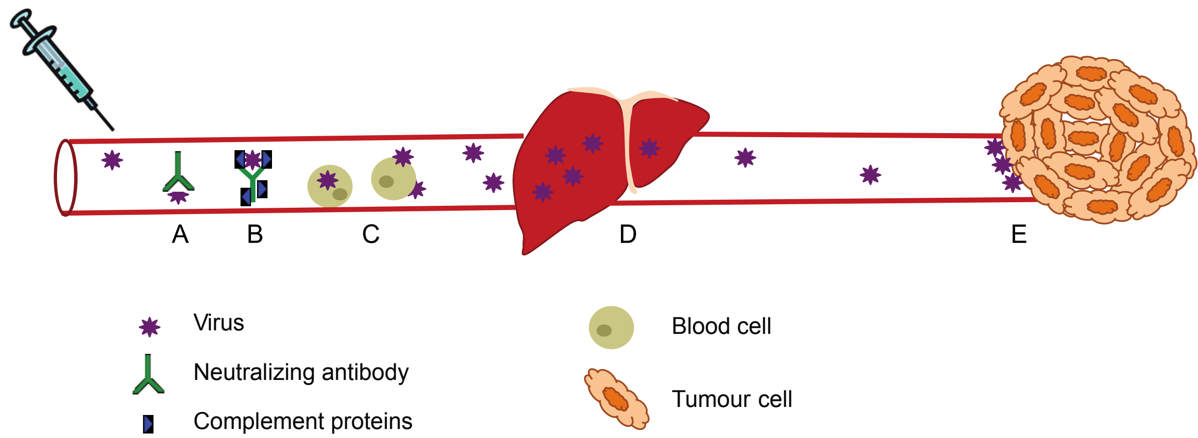
OVs, along with all the strategies employed to increase their safety and potency, are

of limited utility if they cannot be delivered to tumours. Although direct intratumoural (IT) injection is likely the most efficient route of delivery, there are several advantages to IV administration. Firstly, not all tumours are amenable to direct IT injection since they may consist of several small nodules spread out over a large area, or are in an anatomical location that is inaccessible by direct injection. Systemic delivery has a greater chance of reaching disseminated metastases as well as the primary tumour. Although not thoroughly investigated, the ability of some OVs to stimulate anti-tumour immune responses may be greater when administered systemically[95].

Although there is a need for efficient systemic delivery of OVs, there exist several barriers to efficient systemic delivery of OVs (Fig. 1.1). The immune system has evolved several mechanisms to prevent the systemic spread of microorganisms and does not discriminate between pathogens and therapeutic OVs. IV delivery exposes OVs to circulating factors such as antibodies, which either bind to and neutralize virus directly or mark them for destruction by complement and various immune cells[132]. Virus is also neutralized by non-specific binding to serum proteins and circulating cells present in the bloodstream[132]. Organs such as the lung, spleen, and especially the liver, also play a significant role in clearing virus from the bloodstream as these tissues contain resident macrophages, like Kupffer cells, whose role is to scavenge the blood for circulating pathogens[133]. These mechanisms are present in virus-naïve individuals as part of the innate immune system. The neutralization of OVs is even greater if previous exposure has occurred, since adaptive immunity is able to mount a significantly more potent and specific immune response compared to its innate counterpart. This represents an additional and significant hurdle to an efficacious repeat dosing regimen using OVs. Indeed, a significant proportion of the human population has already been exposed to and thus has developed immunological

Figure 1.1: Virus neutralization during systemic delivery.

A) Circulating antibodies and B) complement bind to virus and neutralize them, as well as marking them for destruction by immune effector cells. C) IV administered virus also interacts with circulating blood cells, leading to virus sequestration. D) Liver macrophages, which are part of the reticulo-endothelial system, filter viruses from the blood. E) Viruses that do reach the tumour encounter extensive tumour extracellular matrix and high interstitial fluid pressure, which limit their extravasation into the tumour.


A
B
C
D
E
Virus
Neutralizing antibody
Complement proteins
Blood cell
Tumour cell

memory to many of the OVs currently undergoing clinical development and testing, including reovirus[134], VV[129], and measles virus[135]. Apart from neutralization of virus in the bloodstream, physical barriers such as tumour ECM, as well as limited extravasation of OVs into the tumour bed due to high interstitial fluid pressure within the tumour, can also result in fewer virions being delivered to tumour cells[136].

Despite all of these existing barriers to systemic delivery of OVs, recent clinical trials suggest that IV administered virus can reach the tumour and replicate if administered at sufficiently high doses[131,137], presumably saturating the neutralizing mechanisms present within the human body. Although this was a milestone achievement in the field, there is still much room for improvement as several preclinical[138–140] and clinical[134,137] data demonstrate that pre-existing, virus-specific neutralizing antibodies dramatically reduce the amount of infectious virus that can be delivered to the tumour.

1.5.2 Cell carriers: playing virus hide and seek

To overcome some of the challenges presented in delivering OVs systemically, several strategies have been explored including polymer shielding[141,142], depletion of neutralizing serum factors[143,144], blocking uptake by liver macrophages[145,146], and using cells as delivery vehicles[147,148]. This approach mimics what some viruses have evolved to do in order to spread systemically or gain access to various tissues within their host. For example, human immunodeficiency virus (HIV) is known to bind to circulating dendritic cells and macrophages, which then naturally migrate to lymph nodes and pass on the virus to its target cell population, CD4 T cells[149,150]. The utility of cells as delivery vehicles for OVs has therefore been investigated for several OVs using a variety of cell carrier types and different tumour models in numerous preclinical studies. Notably, in recent clinical trials, IV

administered virus was found to bind several types of circulating cells[131,137] and in the case of reovirus, the cell-bound virus was still infectious and could deliver virus to tumour cells *in vitro* despite the presence of neutralizing antibodies in patients[137].

In theory, the ideal cell carrier would not only protect its viral cargo from neutralization and direct it specifically to the tumour, thus limiting toxicity to normal tissues, but would also have anti-tumour activity of its own. Additional properties such as a favorable safety profile and ease of isolation and propagation/manufacturing are important features to consider when deciding what carrier cell type is most suitable for clinical application. There are several pre-clinical studies demonstrating that carrier cells can deliver a variety of OVs to tumours. Indeed, successful delivery *in vivo* has been achieved with several OV platforms using a wide range of carrier cell types[139,140,147,148,151–171] (Table 1.1). Clearly this approach is not limited to a single virus platform or to a single carrier cell type. In choosing a suitable cell carrier for the delivery of OVs, one must consider the susceptibility of the cell to the virus, the kinetics of viral replication and release, as well as the kinetics of trafficking of the cell carrier from the site of injection to the tumour location. All of these parameters must be optimized to ensure that virus remains unseen by the immune system and therefore protected from neutralization. After a virus enters a cell, there is a period of time before progeny virions are released from the infected cell. Ideally, the cell carrier should reach the tumour during this stage of virus replication. For some rapidly replicating viruses, such as VSV, this eclipse phase can be as short as an hour[147,172], whereas for slower replicating viruses, such as VV, this period of time is much longer[148,173].

Although using a cell carrier that supports virus replication has its advantages, delivery of OVs can occur in the absence of any appreciable replication in the carrier cell. It was demonstrated that VSV[158], reovirus[139], HSV[159], NDV[154], and retrovirus[155] particles could

Table 1.1: Cell carriers used to deliver virus vectors

Cell carrier / Virus
Transformed cells
Adenovirus [152]
Vesicular stomatitis virus [148]
Measles virus [141]
Herpes simplex virus [153]
Vaccinia [154]
Parvovirus [171]
Newcastle disease virus [155]
T cells/cytokine-induced killer cells
Reovirus [140]
Retrovirus [156]
Measles virus [157,158]
Vesicular stomatitis virus [159]
Herpes simplex virus [160]
Vaccinia [149]
Newcastle disease virus [155]
Dendritic cells
Reovirus [140]
Measles virus [161]
Macrophages
Adenovirus [163]
Measles virus [162]
Newcastle disease virus [155]
Peripheral blood mononuclear cells
Reovirus [165]
Myeloid-derived suppressor cells
Vesicular stomatitis virus [164]
Mesenchymal stem cells
Adenovirus [166]
Measles virus [167]
Myxoma [168]
Neural progenitor cells
Adenovirus [170]
Herpes simplex virus [169]
Endothelial progenitor cells
Retrovirus [171]

‘hitchhike’ on the surface of the T cells and could be delivered to tumour cells through various mechanisms, either passively or involving cellular synapses between the carrier cell and the tumour cell. With retrovirus particles, it was found that the virus was handed off either passively or via intracellular perforin-containing cytotoxic granules released from activated T cells when they engaged their target cells[174]. Importantly, surface adsorbed virus was protected from neutralizing antibodies. With VSV-loading of T cells[158], it was observed that at a high multiplicity of infection (MOI), the T cells could not deliver virus to tumours in passively immunized mice but that this could be achieved if loaded at a low MOI. This suggests that at high MOIs, surface bound virus was still accessible to neutralizing antibodies, thus emphasizing the importance of optimizing infection conditions when preparing cell carriers.

1.5.3 Cell carrier types

Based on the pre-clinical studies that have been completed so far, most of the cell types that have been studied as delivery vehicles for OVs fall under one of the following three categories: transformed cells, immune cells or progenitor cells. Each of these cell types has its unique advantages and potential disadvantages.

Transformed cells were amongst the first cell carriers tested for OV delivery to tumours[152,175]. They are relatively easy to grow to sufficiently large numbers and are more readily infected with OVs than normal cells, thus capable of delivering large payloads of virus to tumours. The use of cell carriers that supports viral replication is an attractive approach as it has the added advantage of *en route* dose amplification as the cell carrier produces virus as it makes its way to the tumour. Given that for some viruses, the current doses used in clinical trials are at their maximum due to limits in production of the clinical

grade stocks, a cell carrier with the capacity to produce hundreds or thousands of virus particles per cell can dramatically increase the total amount of therapeutic virus that is delivered to the tumour and thus increase the likeliness of overcoming neutralizing mechanisms. Tumour cells can also traffic, or home, to specific organs as is seen with metastatic disease. This ability can be exploited to achieve targeted delivery to metastatic tumour beds. For example, myeloma cells, which have been used as cell carriers for delivery of oncolytic measles virus, express high levels of CXCR4 and thus often metastasize to the bone marrow[176,177]. Therefore, it has been proposed that myeloma cells themselves can be used to achieve delivery of measles virus to metastatic tumour beds in the bone marrow[176,178]. Although the possibility of an ‘off the shelf’ cell carrier is possible when using transformed cells, the use of a patient’s own tumour cells as cell carriers for OV delivery is also attractive from an immunological standpoint since infected tumour cell vaccines have been demonstrated to enhance anti-tumour immune responses in murine models[72,113,132]. Finally, even though transformed cells have been used in clinical trials[179], proper safety measures must be considered when using any transformed cell line as a cell carrier. However, it has been demonstrated that irradiation of the cell before administration can halt its ability to grow, and thus its tumourigenic potential, while still maintaining virus infectivity[175,178].

Several immune cell types, including T cells[139,154,155,158,180], cytokine induced killer (CIK) cells[148,156], monocytes/macrophages[161,162], and myeloid-derived suppressor cells (MDSCs)[163] have been investigated as carrier cells since they can circulate systemically, they have the ability to specifically recognize tumours or tumour-associated features, and may also possess anti-tumour activity on their own, therefore delivering a one-two punch to the tumour. Adoptive transfer of tumour antigen-specific T cells has been extensively

investigated as a cancer immunotherapy in both pre-clinical and clinical settings. Since this cell type has direct anti-cancer effector functions and can also protect and deliver OVs, it seems like an ideal combination. In pre-clinical studies using OT-1 T cells specific for ovalbumin (OVA), it was found that 5-14% of adoptively transferred T cells migrated to B16-OVA tumours, which is quite remarkable[158]. However in this tumour model, all tumour cells, and only tumour cells, express the highly immunogenic ovalbumin antigen and this does not mirror what is seen in human patients where truly tumour specific and highly immunogenic tumour antigens are rare. Adoptively transferred T cells that do not migrate to the tumour are often found in the spleen and lymph nodes, thus having the potential to target any metastatic disease in these locations or conversely, leading to off-target toxicities. One advantage of combining adoptively transferred T cells loaded with OVs is that the highly pro-inflammatory nature of a virus infection in the tumour milieu can help to prevent T cell silencing and inactivation as a consequence of the immune suppressive microenvironment of the tumour, which is often seen with adoptive T cell therapy[181]. Despite the attractiveness of this approach, its clinical application is challenging and expensive and thus limits its overall feasibility.

CIK cells, which are a population of CD3+, CD56+ NK-like T cells obtained from human peripheral blood or mouse splenocytes after *ex vivo* expansion with IFN-γ, CD3-specific antibody, and IL-2, are another type of cell carrier that possess direct anti-cancer functions[182,183]. However, unlike tumour antigen-specific T cells, they are not restricted to one single antigen. Rather, they recognize NKG2D ligands, which are often up-regulated on tumour cells as a result of stress[184], thus increasing the chances of targeting a larger percentage of tumour cells within the tumour mass as compared to T cells. These cells are also easier to obtain from patients and expand *ex vivo* as compared to antigen-specific T

cells, thus clinical application of this strategy may be a simpler alternative than tumour antigen-specific T cells.

Macrophages are another class of immune cells that have been associated with tumours and tumour stroma, exhibiting the ability to either enhance or inhibit tumour growth depending on their cytokine expression profile. Tumour cells often secrete CCL2, M-CSF, and VEGF, which promote migration of monocytes to the tumour, where they differentiate into tumour-associated macrophages and localize to hypoxic regions within the tumour[12]. Because of the natural homing of macrophages to tumours and the ease of clinical translation of this approach, the delivery of oncolytic measles [161] and adenovirus[162] has been explored in pre-clinical studies with promising results. Interestingly, using a similar approach, a relatively recent study has investigated the use of MDSCs as cell carriers for VSV delivery[163]. MDSCs are a heterogeneous population of immature myeloid cells that play a critical role in the development of tumour-induced immune tolerance[185]. However, it was found that VSV infection of this cell population promoted their conversion from a M2 immune suppressive phenotype to an M1-like tumour killing phenotype[163].

Progenitor cells that have been utilized to deliver OVs include mesenchymal stem cells (MSCs), neural stem cells (NSCs), and vascular progenitor cells, with MSCs being the most extensively studied progenitor cell type. MSCs are multipotent stromal cells that can differentiate into a variety of cell types, including adipocytes, chondrocytes and osteoblasts. They are readily obtainable from various tissues including bone marrow, adipose tissue, umbilical cord, and peripheral blood and they proliferate fairly rapidly in culture making it possible to expand to sufficiently large numbers for clinical application[186]. MSCs naturally home to areas of inflammation, stress, and tissue injury and thus the tumour microenvironment, which is often viewed as a wound that never heals, also provides the

necessary signals to direct MSCs to traffic to their location. Apart from the ability to migrate to tumours, factors released by MSCs are known to have anti-tumour properties, reducing the proliferation of glioma, melanoma, lung cancer, and breast cancer cells[187]. NSCs are another progenitor cell type that has been investigated specifically for intra-cranial delivery of OVs to brain tumours[168,169,188]. Their ability to migrate to tumours resembles that of MSCs, however, it is quite difficult to obtain sufficient numbers of these cells for clinical studies. Advancements in the isolation, propagation, or generation of these cells will immensely benefit future studies.

1.6 Rationale

Despite the preclinical validation of cell-mediated delivery of OVs, there remain significant obstacles to using any mammalian cell type for systemic delivery in the clinical setting. Adherent solid tumour cells and MSCs are unable to traverse capillary beds and generally arrest within the vessels of the first organ they encounter[147,189], while leukocyte-based carriers are able to re-circulate but still exhibit receptor-mediated homing to lymphoid organs and bone marrow[138,180,190]. Thus interactions between mammalian carrier cells and off-target host tissues interfere with systemic tumour targeting. Secondly, primary cell types are often cumbersome to isolate and culture, while systemic administration of permanent cell lines carries the risk of tumourigenicity. Finally, OV infection is by design cytotoxic to mammalian host cells. This precludes the opportunity to genetically modify virus-laden carriers and complicates clinical delivery.

In order to move beyond the limitations of mammalian cells, we have investigated the potential of Dipteran insect cells as novel vehicles for systemic biotherapeutic delivery. Given that VSV establishes a persistent, non-cytolytic infection in insect cells[191,192], we

believe that this might simplify delivery and also enable further manipulation of the cell carriers to improve delivery and therapeutic activity.

1.7 Objectives

1) Characterize VSV infection of *Drosophila* S2 cells.

2) Assess the ability of S2 cell carriers to deliver VSV to tumour cells *in vitro* and *in vivo*.

3) Devise a strategy to express OV therapy-enhancing genes from S2 cell carriers.

4) Determine if insect cell carriers protect OVs from immune-mediated recognition and neutralization.

5) Evaluate the therapeutic potential of insect cell carriers in mouse models of cancer.

2. MATERIALS AND METHODS

Cells

Human (HT29-colorectal adenocarcinoma, U2OS-osteosarcoma, 786-O-renal cell adenocarcinoma, HeLa-cervical adenocarcinoma, SF268-astrocytoma, A549-lung carcinoma, MCF-7-mammary adenocarcinoma, HCT 116-colorectal carcinoma), murine (CT26-WT-colon carcinoma, CT26-lacZ-colon carcinoma, 4T1-mammary carcinoma, L1210-lymphocytic leukemia, A20-B-cell lymphoma), BHK-21 (baby hamster kidney fibroblast) and Vero (African green monkey kidney epithelial cells) cell lines were cultured in Dulbecco's modified eagle medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (FBS) (Hyclone). All mammalian cells were cultured at 37°C under 5% CO_2. *Drosophila melanogaster* Schneider line 2 (S2) cells were cultured in SF900II serum-free medium (Invitrogen) at 25°C under normal atmospheric conditions. Establishment of murine L1210 leukemia cells expressing an integrated firefly luciferase transgene (L1210-FLuc) has been described previously[147]. All cell lines were obtained from the American Type Culture Collection (ATCC). S2-VSVΔ51YFP, S2-VSVΔ51-mRFP, and S2-VSVΔ51-FLuc represent *Drosophila* S2 cells persistently infected with the following viruses: VSVΔ51-YFP, VSVΔ51-mRFP, VSVΔ51-FLuc, respectively (YFP: yellow fluorescent protein; GFP: green fluorescent protein; mRFP: monomeric red fluorescent protein; FLuc: Firefly luciferase). S2-VVdd-mCherry represents S2 cells infected with vaccinia virus VVdd-mCherry and S2-VSVΔ51-YFP/VVdd-mCherry represents persistently infected S2-VSVΔ51YFP cells superinfected with VVdd-mCherry. S2-SINV-GFP represents S2 cells persistently infected with SINV-GFP and S2-SINV-GFP/VVdd-mCherry represents S2-SINV-GFP cells superinfected with VVdd-mCherry.

Viruses

Construction of recombinant strains of VSVΔ51 expressing FLuc or mRFP reporter transgenes (referred to herein as VSVΔ51-FLuc and VSVΔ51-mRFP, respectively) have been described previously[51,147]. Additional recombinants harboring a YFP reporter or miR-30 hairpins were generated by subcloning the YFP coding region or the miR-30 cassette between the XhoI and NheI sites located between the G and L genes of the pXN vector[115] and rescuing recombinant viruses as described previously[114,116]. MG1-GFP has been described previously[117]. All VSV and MG1 stocks were propagated on Vero cells. For animal studies, VSV stocks were further purified from cell culture supernatants by filtration through a 0.22µm Steritop filter (Millipore) and centrifugation at 30,000 x g before resuspension in phosphate-buffered saline (PBS) (Hyclone). Vaccinia (VVdd-mCherry) was made by insertion of mCherry-DNA into the TK gene locus of VVdd, a Western Reserve strain of VV that has both TK and VGF deleted[61]. Successful recombinants were selected by mCherry expression and plaque-purified. VV stocks were propagated in U2OS cells and cell-associated virus was collected by repeat (3) freeze-thaw cycles. Further purification of viral stocks was done by centrifugation at 20,700 x g through a 36% sucrose cushion (in 1mM Tris) before resuspension in 1mM Tris, pH 9. SINV expressing GFP, referred to as SINV-GFP, was propagated in BHK-21 cells. The SINV miR-30-based amiRNA library, composed of approximately 16,000 unique clones, has been described previously[193,194]. Cloning and rescue of recombinant SINV clones expressing GFP or amiRNAs has been described elsewhere[193,195].

Infection and preparation of insect cell carriers

Drosophila S2 cells were initially infected with VSVΔ51 at an MOI of 10 PFU/cell (PFU: plaque forming units). Cultures were passaged continually at a density between 10^6-

10^7 cells/mL to maintain log-phase growth. Doubly-infected carriers were generated by super-infecting S2-VSVΔ51 carriers with VVdd-mCherry at an MOI of 10 for 24h. For delivery to tumour cells, insect cells were washed three times in 10mL SF900II medium, and resuspended in PBS before inoculation onto cell monolayers or injection into animals.

Cell viability assay

Tumour cells were infected with the indicated viruses and cell viability was assessed by alamarBlue (Life Technologies) assay according to the manufacturer's protocol.

Western blot

Cell lysates were collected in NP-40 lysis buffer, run on a NuPAGE Bis-Tris 4-12% polyacrylamide gel, transferred to a PVDF membrane, and probed with polyclonal anti-VSV serum from hyperimmune rabbits. An anti-tubulin (clone YL1/2) antibody (Novus Biologicals) was used as a loading control.

Analysis of cultured insect cells

Cell suspensions were counted on an automated cell viability analyzer (Beckman Coulter), which determined the total cell concentration, viability by trypan blue exclusion, and average cell diameter.

Preparation of insect cell conditioned medium

Drosophila S2 cells were either mock infected or infected with VVdd-mCherry at an MOI of 10 for 24h, harvested and then pelleted by centrifugation. Supernatants were collected and passed through a 0.22μm filter twice to eliminate cell-free VV virions. To test for factors enhancing VSV infectivity, tumour cell monolayers were pre-treated for 2h with conditioned insect cell supernatant diluted into an equal volume of DMEM+10% FBS. Tumour cells were then infected with VSV in the presence of conditioned medium.

Neutralizing antibody assay

Two-fold dilutions of mouse serum were mixed with 1,000 PFU of VSVΔ51-YFP and incubated for 1h before inoculation of Vero cells with the mixture. 72h post-inoculation cells were analyzed for the presence of virus-induced cytopathic effect. Samples were assayed in triplicate and the neutralizing titer was taken as the highest dilution factor of serum that prevented the appearance of cytopathic effects. For experiments comparing infection of U2OS cells in the presence of anti-VV L1R neutralizing antibody[196] (b.e.i. Resources), 1,000 S2 cells infected with VV at an MOI of 10 for 24h or 1,000 PFU of purified VV were incubated with various concentrations of neutralizing antibody for 1h then overlayed onto confluent monolayers of U2OS cells. After a 2h incubation, a liquid overlay was placed over the cells and plates were stained 72h post-infection to visualize and count virus plaques. For *in vivo* experiments, mice were immunized with $5x10^8$ PFU VSVΔ51-YFP administered IV. 2 weeks later, CT26 lung tumours were established by IV injection of $1x10^5$ cells. 10 days after tumour seeding, mice were treated with $5x10^8$ PFU of VSVΔ51-YFP or $1x10^7$ S2-VSVΔ51-YFP cell carriers. Lungs were collected 24h post-treatment and virus was quantified.

Flow cytometry

Mice were sacrificed and the spleens were collected at the indicated time points. The entire experiment was performed on ice. The spleens were mashed through 70µm cell strainers (Fisher) in FACS buffer (PBS+3%FBS). Red blood cells were then lysed with ACK lysis buffer. Upon resuspension in FACS buffer, the splenocytes were incubated for 30 minutes with combinations of the following antibodies: anti-mouse CD122-FITC, CD69-PE, CD3-PECY7, CD11c-APC and CD86-FITC (BD Biosience). Cells were washed, resuspended in FACS buffer and analyzed on a Cyan ADP 9 (Beckman Coulter).

Mice and tumour models

CT26 tumours were established by subcutaneous injection of $1x10^6$ cells into the hind or front flank of female 8- to 10-week old balb/c mice (Charles River Laboratories). Palpable tumours were treated after approximately 10-14 days. HT29 ($3x10^6$ cells) tumours were established subcutaneously in CD1 female nude mice (Charles River). Palpable tumours were treated approximately 14-21 days after injection. IV injections were performed in the tail vein. SINV and VSV were administered at doses of $1x10^8$ and $5x10^8$ PFU, respectively. The formula used to calculate tumour volume is length x $width^2/2$. For the lung tumour model, CT26 lung tumours were established by IV injection of $3x10^5$ cells and were treated IV daily for 5 days, starting on day 3 after tumour seeding. Mice were euthanized 16 days post-tumour seeding and the lungs were collected and perfused with India ink. Briefly, a solution of 50% India ink/PBS was injected in the trachea of mice and lungs were subsequently fixed in Fekete's solution. All experiments were performed in accordance with institutional guidelines review board for animal care (University of Ottawa).

Bioluminescent and *in vivo* imaging

Mice were injected with d-luciferin (Molecular Imaging Products) (200µl intraperitoneally at 10mg/mL in PBS) for FLuc imaging. Mice were anesthetized under 3% isoflurane (Baxter Corp.) and imaged with the *in vivo* imaging system (IVIS) 200 Series (Xenogen Corp.). Data acquisition and analysis was performed using Living Image v2.5 software. For *in vitro* imaging, cells were assayed in black multiwell plates (Sigma-Aldrich). d-Luciferin substrate was diluted directly into the tissue culture medium and then imaged using the IVIS 200. Bioluminescence signal from each well was quantified digitally with the Living Image 2.5 software.

Quantitation of infection in cultured cells, tumours and organs

Tumours were excised from euthanized mice and homogenized in PBS using a tissue homogenizer. Homogenates were serially diluted and plated onto Vero cells for 24h for VSV and MG1 plaque assays, U2OS cells for 72h for VV plaque assays, and Vero cells for 48h for SINV plaque assays.

Fluorescent imaging of cell infection *in vitro*

Fluorescent images of infected cell cultures were acquired using the Axiovert S-100 (Carl Zeiss, Inc.) microscope equipped with an Axiocam camera (Carl Zeiss, Inc.). Axiovision 3.1 software (Carl Zeiss, Inc.) was used for digital image acquisition and Image J was used for post-acquisition image manipulation in order to create 2-color overlays.

Fluorescent imaging of tumour infection *in vivo*

Mice were euthanized and dissected to expose tumours. Brightfield and fluorescent images were acquired using the M205-FA dissecting microscope/imaging system (Leica Microsystems Inc.). The associated LAS FA6000 software was used for all image acquisition and post-imaging manipulations.

Live cell imaging

Time-lapse live-cell imaging was performed on an Axiovert 200M microscope. Cells were maintained at 37°C and 5% CO_2 throughout.

Analysis of circulatory half-life of carrier cells

Mice were injected IV with 10^7 carrier cells expressing luciferase, and blood samples were collected by cardiac puncture from live mice under ketamine anesthesia. Pelleted total blood cells were directly resuspended in the lysis buffer supplied with the Luciferase Assay Kit (Stratagene) and firefly luciferase activity was assayed as per the manufacturer's

protocol. Samples were imaged in a black 96-well plate (Sigma-Aldrich) using the IVIS 200 (Xenogen) and the total signal from each well was digitally quantified using the Living Image 2.5 software.

Virus biodistribution

Mice bearing CT26 subcutaneous tumours were injected IV with $5x10^6$ S2-VSVΔ51-YFP/VVdd-mCherry cells and tumours and the indicated organs (spleen, heart, lung, liver, brain) were collected 24h, 48h, 72h, and 120h post-treatment. Both VSV and VV were quantified by plaque assay as described above.

SINV library passaging

$5x10^6$ CT26WT cells were infected at an MOI of 0.1 with the SINV-amiRNA library. This MOI ensured that all clones were sufficiently sampled. Supernatants were collected 48h post-infection. The output from CT26WT cells was amplified on BHK-21 cells between each passage in order to achieve an input MOI of 0.1 for the next passage.

Sequencing of SINV library

Monitoring of viral populations has been described previously[193]. Briefly, random hexamers were used with Superscript II (Invitrogen) to generate cDNA, and then specific primers with barcoded Illumina linkers were used to amplify the amiRNA hairpin region. Samples were analyzed on the Illumina HiSeq 2000 platform. The amiRNA backbone was then trimmed and distinct sequences were identified. Alignments were used to group highly similar sequences into "families" to control for sequencing error.

Coomassie Blue staining

Cells were fixed with a 3:1 mixture of ethanol:acetic acid for 30 minutes. The fixative was washed off with tap water and cells were stained with a 0.1% solution of Coomassie

Blue for 30 minutes. The stain was rinsed off with tap water and plates were allowed to dry before imaging.

siRNA transfection

Cells were transfected with 50μM siRNA using Lipofectamine RNAiMAX transfection reagent (Invitrogen), according to the manufacturer's protocol. Cells were infected with the indicated viruses 48h post-transfection.

Dual-luciferase reporter assay

Lipofectamine2000 (Invitrogen) was used to transfect BHKs with 500ng psiCHECK-2 plasmid containing the indicated target sites cloned in tandem in the 3' untranslated region (UTR) of Renilla luciferase[197]. 2h prior to transfection, BHK-21 cells were infected with VSVΔ51 expressing an amiRNA matching the target site in the 3'UTR or with a control VSVΔ51 expressing an unmatched amiRNA (MOI=5). Cell lysates were collected 24h post-infection and the dual-luciferase reporter assay (Promega) was performed according to the manufacturer's protocol. Renilla luciferase levels were normalized to FLuc levels and to the unmatched amiRNA virus control.

Virus competition assay

amiRNA-expressing virus was mixed with control virus at a ratio of approximately 1:100 and used to infect CT26WT cells at an MOI of 0.1 for SINV and 0.001 for VSVΔ51. Supernatants were collected 48h post-infection. For SINV, the output from CT26WT cells was amplified on BHK-21 cells between each passage in order to achieve an input MOI of 0.1. This was repeated for a total of 4 passages. After passage 4, 100 isolated viral plaques were randomly selected and the number of GFP-positive plaques was determined by fluorescence microscopy.

Quantitative PCR

Samples were collected 16h post-infection for RNA extraction, which was performed using the RNeasy kit (Qiagen). cDNA was generated using random primers and SuperScript Reverse Transcriptase III (Invitrogen) . Quantitative PCR (qPCR) was performed with SYBR Select master mix (Applied Biosystems) on a Rotor-Gene RG3000A instrument (Corbett Research). Gene expression levels were normalized to GAPDH. Primer sequences can be found in Table 2.1.

ELISA

Samples for the IFN-β ELISA were generated by infecting CT26WT cells that had been transfected with the indicated siRNAs for 48h and subsequently infected with VSVΔ51-YFP at an MOI of 0.01. Culture supernatants were collected 24h post-infection. ELISA (R&D Systems) was performed according to the manufacturer's protocol.

Microarray

Cells were transfected with 50μM siRNA using Lipofectamine RNAiMAX transfection reagent (Invitrogen), according to the manufacturer's protocol. Cells were infected with the indicated viruses 48h post-transfection. Samples were collected 16h post-infection for RNA extraction, which was performed using the RNeasy kit (Qiagen). Samples were analyzed with the GeneChip Mouse Gene 2.0 ST Array (Affymetrix) and data was processed with the Transcriptome Analysis Software (Affymetrix).

Table 2.1: List of primers used for qPCR analysis.

Gene Name	Forward Primer (5'-3')	Reverse Primer (5'-3')
APLN	GTGGATCCTGACATGGTTCTATAC	GTCCTTTGGGCTCTGACTTT
DKK2	GAAGAATGAGGGATGTGGTAAGA	CATAACGGAAGCACTGGTAGTA
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
IFI44	TCCATCCCTTTAGAGTGCTA	GCAAGCAGAACTAAGCTCAT
IFIT3	TCTCAGAAGCTCAGGCTTAC	ACATTTTTGCTCGTTCATTT
IFN-β	GATGACGGAGAAGATGCAGAAG	ACCCAGTGCTGGAGAAATTG
MX2	ATCAATGCTTTTAACCAGGA	TTTTCGATCTCCTTACTCCA
OASL2	TAAGCTGCTTTTCCAGTTTC	AGGACAATGATGTTGTAGGC
OGN	AGCCCAAGAGCACCATTT	CACCTTCCTCTGAAGCTTAGTT
OMD	ATCCAGTACACCATCACCATTT	GTGTATACGAGGGAAGCAGAAG
VSV	GATAGTACCGGAGGATTGACGACTA	TCAAACCATCCGAGCCATTC

3. RESULTS

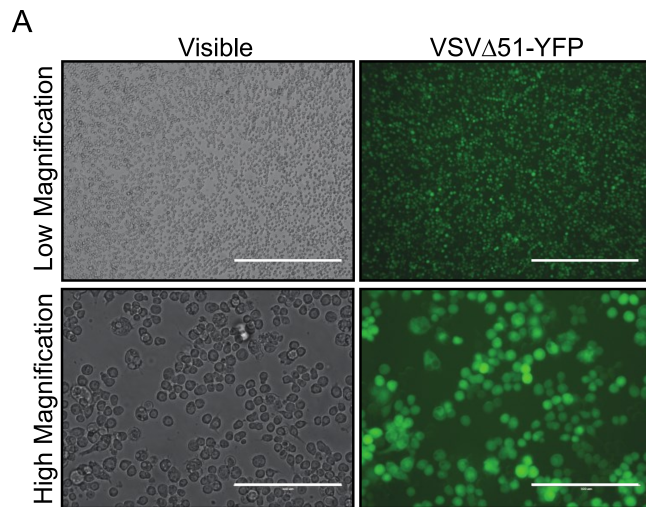
3.1 Establishment of continuous Dipteran cell lines propagating oncolytic VSVΔ51

OV preparations destined for use in research or clinical development are manufactured in mammalian or avian cell cultures. The OVs are propagated in these cell lines via a cytotoxic replicative cycle, in a batch production format followed by extensive purification and safety testing before a clinical grade product can be obtained. In contrast, insect cells can carry similar viruses as persistent infections while suffering little adverse effects[198]. For example, VSV, a highly effective oncolytic agent when administered to mammalian tumour cells[51,111] has been reported to infect and persist in cultured Hemipteran[199] or Dipteran[191] cells without detectable cytopathic effects. We tested whether cultured Dipteran cells could serve as carriers for the continuous growth of VSVΔ51. Suspensions of the *D. melanogaster* S2 cell line were cultured in a minimal, serum-free medium formulation and infected with recombinant VSVΔ51 harboring an YFP reporter transgene to monitor viral persistence. We observed efficient infection of S2 cells with most cells expressing virus-encoded YFP (Fig. 3.1A). Flow cytometry analysis of persistently infected insect cell populations revealed that over 60% of S2 cells had detectable levels of fluorescent transgene expression (Fig. 3.1B). During continued passaging over a period of months, VSV-infected S2 cell cultures showed similar log-phase growth kinetics to uninfected controls (Fig. 3.1C), with only a modest increase in doubling time from approximately 27h to 29h (Fig. 3.1D). No change in the percentage of viable cells or average cell diameter was detected when infected S2 cultures were compared to uninfected controls (Fig. 3.1D). We have continued to monitor virus gene expression at the single cell level during continued passage of infected cell lines,

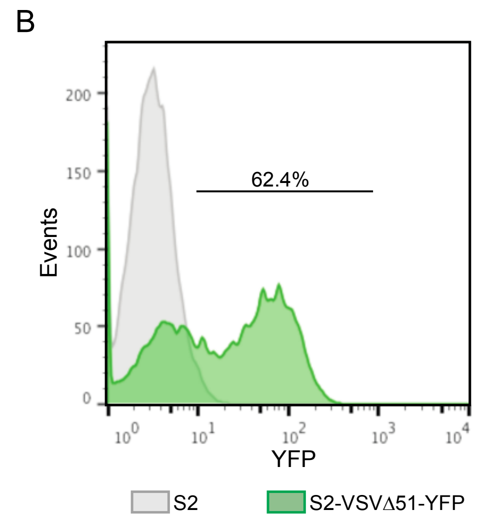
Figure 3.1: Establishment of continuous insect cell lines propagating oncolytic VSVΔ51.

A) Brightfield and corresponding fluorescent image of *Drosophila* S2 cells persistently infected with VSVΔ51-YFP. Scale bars represent 1mm (top panel) and 100μm (bottom panel). B) Flow cytometry analysis of fluorescent transgene expression by S2 cells persistently infected with VSVΔ51-YFP. Indicated values represent percent of cells positive for transgene expression. C) Log-phase growth curves comparing uninfected *Drosophila* S2 cells to two S2 lines persistently infected with VSVΔ51 (VSVΔ51-mRFP or VSVΔ51-FLuc). D) Summary comparing doubling time (t_d), percentage of viable cells, and average cell diameter (d_{av}) of *Drosophila* S2 cultures either left uninfected or persistently infected with VSVΔ51-mRFP or VSVΔ51-FLuc.

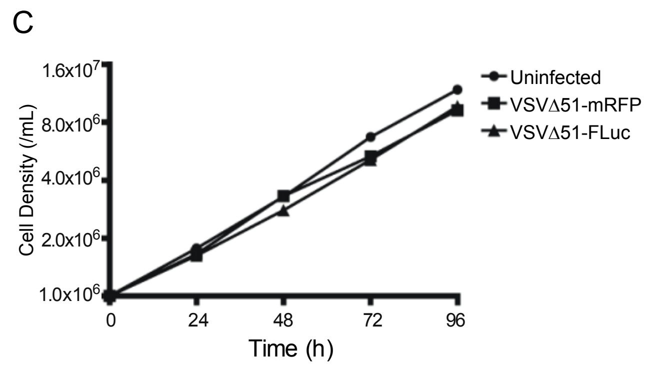
A



B



C



D

	t_d (h)	Viability (%)	d_{av} (μm)
S2^{mock}	27.0	98.5	13.1
S2^{VSVΔ51mRFP}	29.9	98.3	12.9
S2^{VSVΔ51FLuc}	29.3	98.5	12.0

and have found that they maintain a homogeneously high level of infection throughout hundreds of doubling generations (Fig. 3.1A).

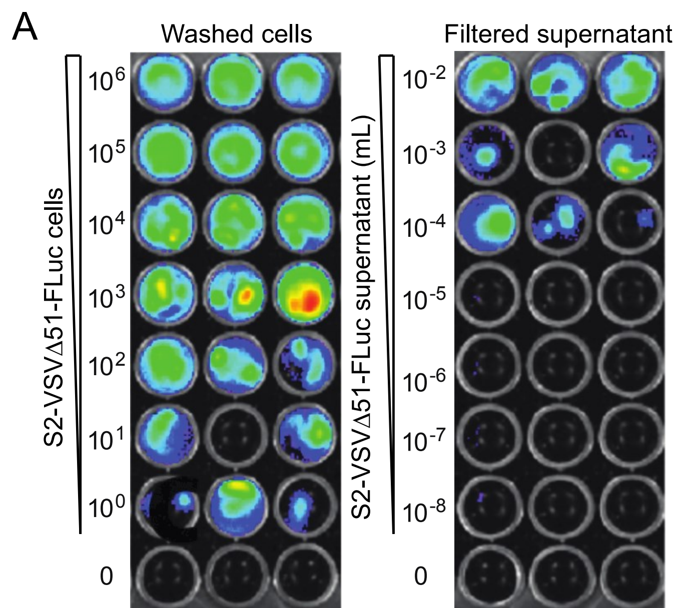
After more than one year of continuous culture and repeated freeze-thaws, we have not observed loss of viral persistence or even a detectable decrease in the percentage of infected Dipteran cells. Furthermore, a recombinant VSVΔ51-GFP lacking the naturally encoded G gene also persisted long-term in S2 cells (data not shown), indicating that spread between cultured Dipteran cells is dispensable for the persistence of viral infection. Thus VSV appears to be maintained as an innocuous passenger in cultured Dipteran cells with continuous propagation of the viral genome in an intracellular form and vertical transmission to daughter cells.

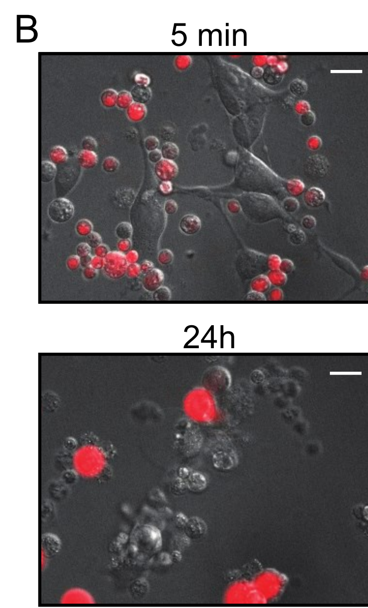
3.2 Persistently infected insect cell carriers deliver oncolytic VSVΔ51 to tumours

We examined whether persistently infected Dipteran cell carriers could deliver OV leading to infection of mammalian tumour cells *in vitro*. Carrier cell cultures harboring VSVΔ51-FLuc (S2-VSVΔ51-FLuc) were harvested and divided into filtered cell-free supernatant and washed cell pellet fractions. Serial dilutions of either cells or supernatant were then added to adherent monolayers of CT26 murine tumour cells. After 48h, the tumour cells were assayed for viral luciferase expression by bioluminescent imaging. As shown in figure 3.2A (right panel), few infectious particles were released into the culture supernatants of insect cell carriers. Limiting dilution analysis revealed approximately 10^4 infectious particles/mL in supernatants harvested from cultures containing 10^6 infected cells/mL. Therefore the quantity of infectious particles released from Dipteran cells was on the order of 0.01/cell, consistent with earlier reports[191]. In contrast, the infected carriers themselves retained the ability to efficiently deliver VSV directly to tumour cells, with as little as a

Figure 3.2: Persistently infected insect cell carriers deliver OV to tumour cells *in vitro*.

A) Limiting dilution assay of insect cell carriers. Serial dilutions of S2 cells persistently infected with VSVΔ51-FLuc or their filtered culture supernatants were overlaid onto monolayers of CT26 murine carcinoma cells. Viral luciferase activity was assayed by IVIS at 48h post-infection. B) Time-lapse images of insect cells delivering VSVΔ51-mRFP to CT26 murine carcinoma cells, at 5min and 24h post-inoculation. Composite images show bright field overlaid with red fluorescence. Scale bars represent 10μm.


A
Washed cells
Filtered supernatant
S2-VSVΔ51-FLuc cells
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
S2-VSVΔ51-FLuc supernatant (mL)
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
10^-8
0

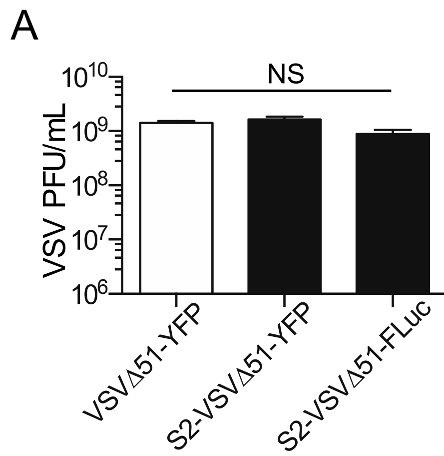

B
5 min
24h

single insect cell able to initiate a productive infection (Fig. 3.2A, left panel). Time-lapse microscopy of this insect cell-mediated delivery process revealed docking of fluorescently labeled Dipteran carriers (S2-VSVΔ51-mRFP) to the surface of tumour cells within minutes of inoculation (Fig. 3.2B). Docking was followed by insect cell death and loss of visible viral transgene (mRFP) expression (Fig. 3.2B). After a short eclipse period, the tumour cells began to show infection-associated rounding within several hours, followed by visible accumulation of the virus-encoded mRFP transgene, and ultimately lysis by approximately 24h (Fig. 3.2B).

We wanted to determine if persistent replication in insect cells compromised the virus's ability to replicate in mammalian tumour cells. We therefore compared virus produced in insect cells with virus produced in mammalian cells and found no difference in the replication and cytotoxicity of insect cell derived VSV when compared to mammalian cell-derived VSV (Fig. 3.3A,B). We next examined whether insect cell carriers could deliver OV to tumours growing in immunocompetent animals. *Drosophila* S2 cells carrying VSVΔ51-FLuc were washed to remove cell-free particles and directly injected into solid subcutaneous CT26 tumours grown in syngeneic balb/c mice. Tumour luciferase expression was followed via longitudinal imaging. As shown in figure 3.4A and B, robust infection was observed specifically within tumours following administration of 10^6 insect cell carriers. Substantial signal was detectable by 24h post-injection and continually increased throughout the duration of the experiment (Fig. 3.4A,B), similar to the previously reported replication kinetics of VSVΔ51 administered as naked virions in this tumour model[70]. Further characterization of the IT dose-response revealed the minimal threshold number of insect cell carriers required to initiate a durable OV infection. Injection of 10^6 cell carriers lead to a detectable signal at all timepoints tested, whereas injection of 10^4 cell carriers led to a

Figure 3.3: Virus derived from persistently infected insect cell carriers is as infectious and cytolytic in tumour cells as virus produced in mammalian cells.

A) VSV titers obtained by plaque assay and B) cell viability assay of CT26-LacZ cells infected at an MOI of 0.01 for 24h with either VSV purified from mammalian Vero cells (VSVΔ51-YFP) or *Drosophila* S2 cells (S2-VSVΔ51-YFP, S2-VSVΔ51-FLuc). Data are presented as means ± standard error of the mean (SEM). Student's unpaired, one-tailed t-test was performed; NS: not-significant, **: $p<0.001$, ***: $p<0.0001$.


A
NS
VSV PFU/mL
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
VSVΔ51-YFP
S2-VSVΔ51-YFP
S2-VSVΔ51-FLuc

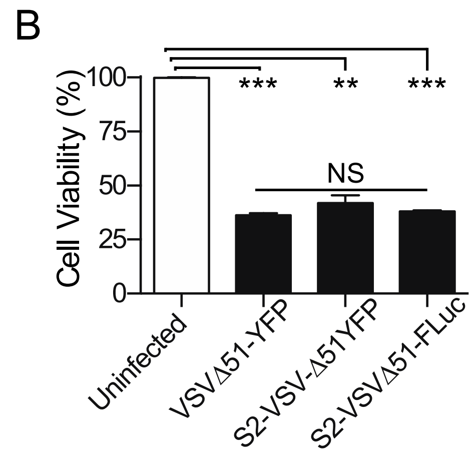
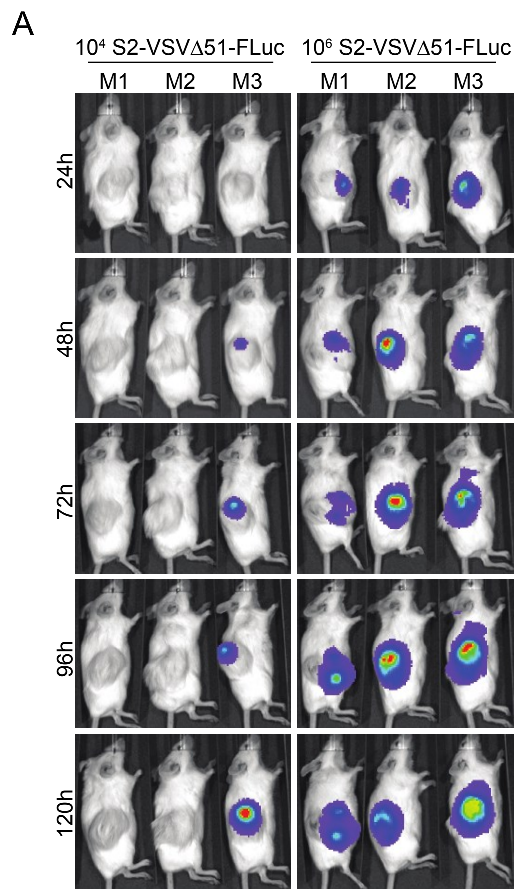
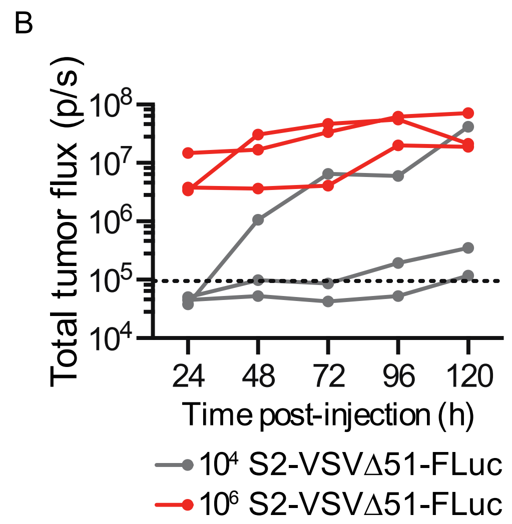

B
***
**
***
NS
Cell Viability (%)
100
75
50
25
0
Uninfected
VSVΔ51-YFP
S2-VSV-Δ51YFP
S2-VSVΔ51-FLuc

Figure 3.4: Persistently infected insect cell carriers deliver OV to tumours *in vivo*.

A) Tumour luciferase activity in triplicate mice bearing subcutaneous CT26 tumours treated by IT injection of 10^4 or 10^6 *Drosophila* S2 cells persistently infected with VSVΔ51-FLuc. B) The total luciferase signal for each tumour was digitally quantified at 24h, 48h, 72h, 96h and 120h post-treatment.


A
10⁴ S2-VSVΔ51-FLuc
10⁶ S2-VSVΔ51-FLuc
M1
M2
M3
M1
M2
M3
24h
48h
72h
96h
120h


B
Total tumor flux (p/s)
10⁸
10⁷
10⁶
10⁵
10⁴
24
48
72
96
120
Time post-injection (h)
10⁴ S2-VSVΔ51-FLuc
10⁶ S2-VSVΔ51-FLuc

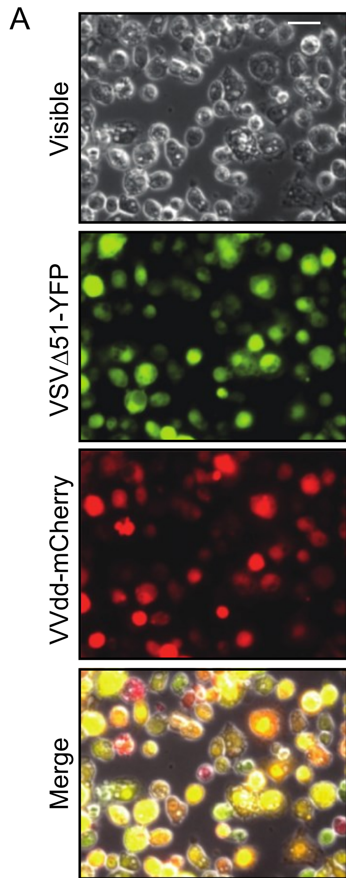
minimally detectable and slow-evolving infection, detectable at levels just over background by day five in two out of three mice tested (Fig. 3.4A,B). Thus, similar to results of previous studies with naked virions[111], a significant threshold dose was required to overcome the innate restrictions to OV growth within the *in vivo* tumour microenvironment.

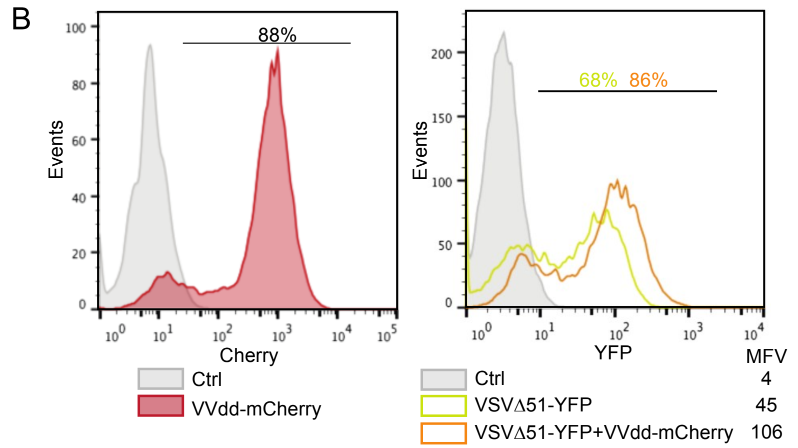
3.3 Insect cells carrying oncolytic VSV can be armed with auxiliary biotherapeutic gene cargoes

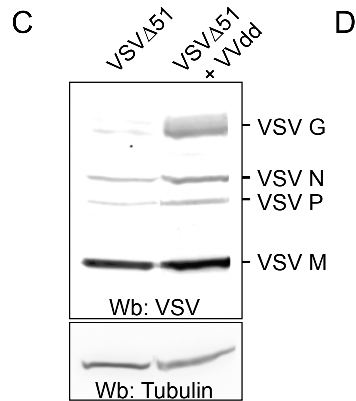
We examined whether insect cells carrying oncolytic VSVΔ51 could be armed with additional biotherapeutic gene products to overcome innate limitations to intratumoural OV growth. Replication competent VV vectors provide a robust platform for biotherapeutic transgene production[82], and their growth and cytopathic effects can be targeted specifically to tumours[90]. Furthermore, they encode an array of secreted immunomodulators[126] with the ability to complement growth deficiencies of heterologous viruses[87,88,200]. We reasoned that insect cell carriers modified to constitutively secrete such immunomodulators should prime the tumour microenvironment to help promote OV growth. To test this idea, we first determined whether insect cells carrying VSVΔ51 (S2-VSVΔ51-YFP) could support super-infection with VVdd tagged with the mCherry fluorescent reporter gene. Indeed, infection of S2-VSVΔ51-YFP carrier cells with VVdd-mCherry led to readily detectable VV gene expression within 24h, with over 85% of cells positive for transgene expression (Fig. 3.5A,B). Insect cells not only maintained VSV gene expression when super-infected with VV, but VSV protein levels as well as VSV production were significantly increased, as assessed by viral reporter gene imaging (Fig. 3.5A,B), anti-VSV western blot (Fig. 3.5C), and VSV growth curves (Fig. 3.5D). Thus we were able to obtain a homogeneous population of carriers with virtually all cells infected with both VSVΔ51-YFP

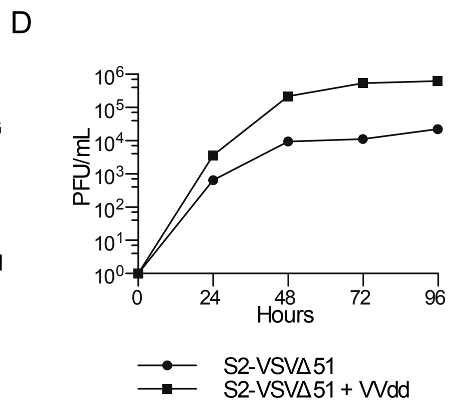
Figure 3.5: Arming *Drosophila* OV carrier cells with additional biotherapeutic cargoes.

A) Persistently infected VSVΔ51-YFP cell carriers were superinfected with VVdd-mCherry at an MOI of 10 and imaged after 24h. Scale bar represents 10µm. B) Flow cytometry analysis of fluorescent transgene expression by S2 cells persistently infected with VSVΔ51-YFP and super-infected with VVdd-mCherry. Indicated values represent percent of cells positive for mCherry (left histogram) and YFP (right histogram) transgene expression. Mean fluorescence value (MFV) of YFP is also indicated below the histograms. C) Expression of VSV proteins in persistently infected S2 carriers in the presence or absence of VV. S2-VSVΔ51-YFP cell carriers were left uninfected or superinfected with VVdd for 24h, then harvested and subjected to immunoblotting with a polyclonal antibody recognizing the VSV proteins. Tubulin was used as a loading control. D) Persistently infected VSVΔ51-YFP cell carriers were washed three times with culture media and then superinfected with VVdd-mCherry at an MOI of 10. Culture supernatants were collected at 24h, 48h, 72h, and 96h post-infection and the amount of VSV produced was quantified by plaque assay.


A
Visible
VSVΔ51-YFP
VVdd-mCherry
Merge


B
88%
Events
Cherry
Ctrl
VVdd-mCherry
68% 86%
Events
YFP
MFV
Ctrl 4
VSVΔ51-YFP 45
VSVΔ51-YFP+VVdd-mCherry 106


C
VSVΔ51
VSVΔ51 + VVdd
VSV G
VSV N
VSV P
VSV M
Wb: VSV
Wb: Tubulin


D
PFU/mL
Hours
S2-VSVΔ51
S2-VSVΔ51 + VVdd

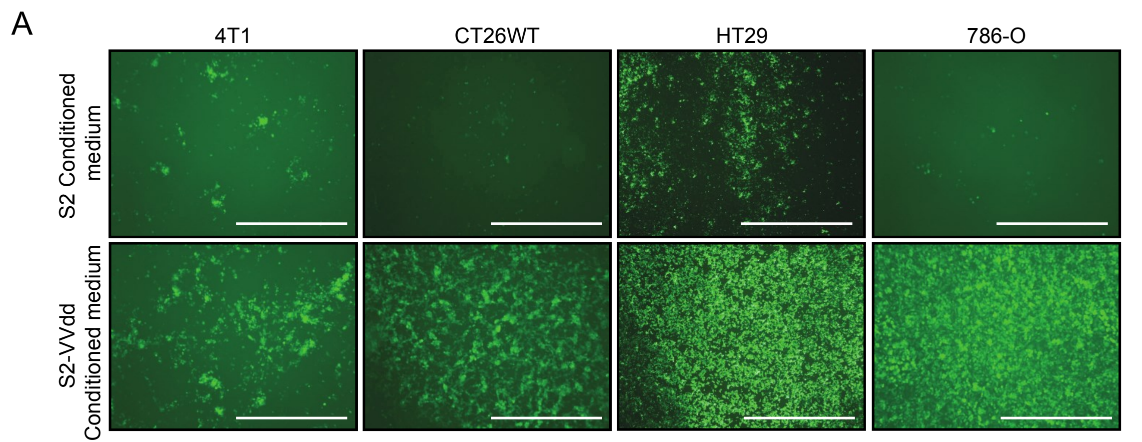
and VVdd-mCherry (Fig. 3.5A,B). To demonstrate that insect cell carriers can be modified to express transgenes that promote OV infection of tumour cells, conditioned media from insect cells, infected only with VV, or from uninfected control S2 cells, were collected and passed through a 0.22µm filter to remove free VV particles. Tumour cell monolayers were subsequently infected with VSVΔ51-YFP viral particles in the presence of conditioned medium and the spread of the VSV-encoded YFP transgene and the production of virus were followed. At the doses employed in this experiment, VSVΔ51-YFP poorly infected tumour cells primed with control medium from uninfected insect cells, with minimal viral gene expression detected (Fig. 3.6A top panel). In contrast, tumour cells primed with conditioned medium from VVdd-infected S2 cells showed a robust VSV infection throughout the monolayer and production of 5 to nearly 500-fold greater titers of infectious virus depending on the tumour cell line tested (Fig. 3.6A bottom panel,B). Thus, these results suggest that insect cell carriers can be engineered to express additional transgenes that increase OV replication.

3.4 Insect cell carriers deliver multiple integrated biotherapeutics to tumour cells

We next examined whether oncolytic VSV and complementing VV immunomodulators could be integrated into insect cell transporters and delivered to tumour cells as a single therapeutic agent, since both OVs have been shown to interact synergistically when co-administered to tumour cells[200]. These doubly-armed cell carriers (S2-VSVΔ51-YFP/VVdd-mCherry) were added to monolayers of mammalian tumour cells and infection was followed by fluorescent imaging and plaque assay. Insect cell carriers were administered at a minimal dose, which resulted in little infection of tumour cells when VSVΔ51 was delivered as a single agent (Fig. 3.7A, top panel). However, the same number

Figure 3.6: VV-loaded insect cell carriers prime tumour cells for infection with VSVΔ51.

A) Fluorescent images obtained from infection of 4T1, CT26WT, HT29, and 786-O with VSVΔ51-YFP at an MOI of 0.01, 0.001, 0.0001, and 0.0001 PFU/cell, respectively, for 48h in the presence of conditioned medium from mock- or VVdd-infected *Drosophila* S2 cells. Scale bars represent 1mm. B) Supernatants from A) were collected at the same timepoint and titered for VSV production. Data are presented as means ± SEM. Student's unpaired, one-tailed t-test was performed; *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.


A
4T1
CT26WT
HT29
786-O
S2 Conditioned medium
S2-VVdd Conditioned medium

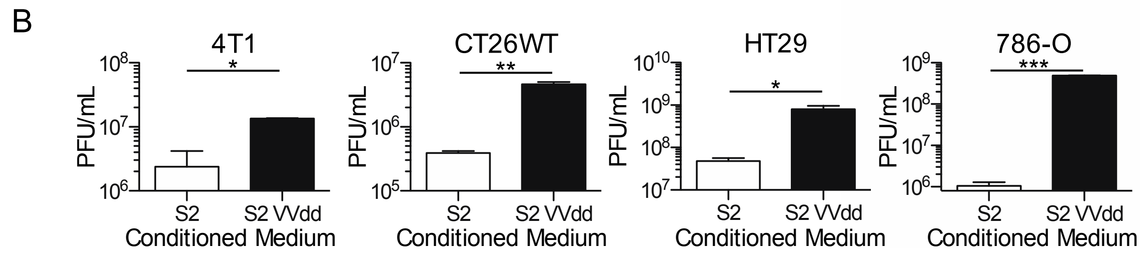
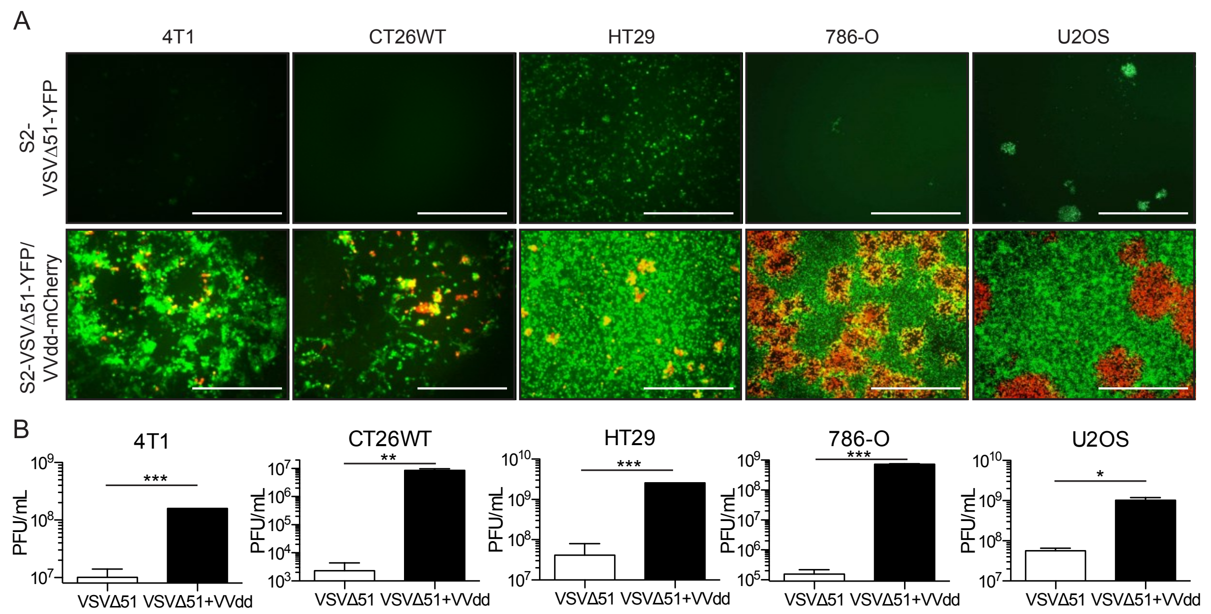

B
4T1
CT26WT
HT29
786-O
PFU/mL
*
**
***
S2
S2 VVdd
Conditioned Medium

Figure 3.7: Insect cell carriers deliver multiple integrated biotherapeutics to tumour cells *in vitro*.

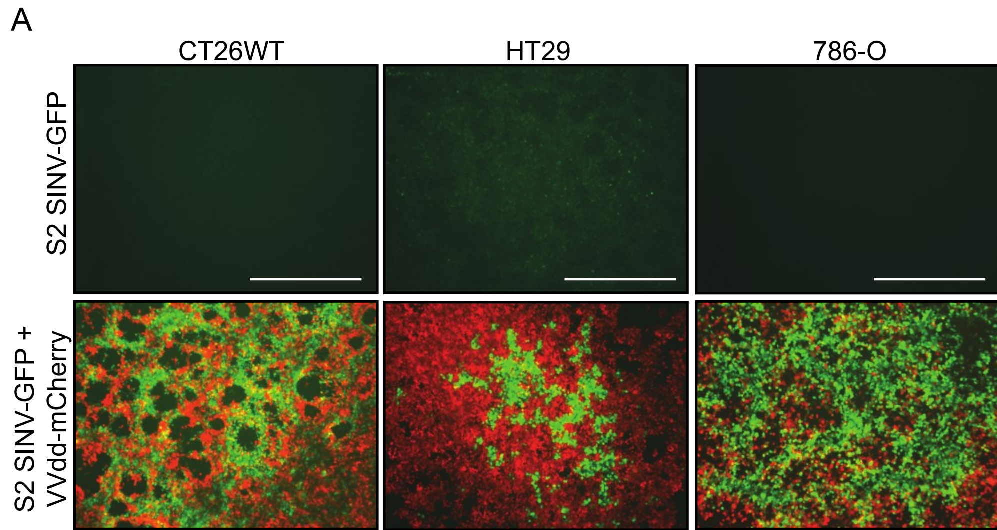
A) *Drosophila* S2-VSVΔ51-YFP or S2-VSVΔ51-YFP/VVdd-mCherry cell carriers were inoculated onto monolayers of the indicated tumour cell lines. Composite YFP/mCherry fluorescent images 48h post-infection are shown. Scale bars represent 1mm. B) Tumour cell supernatants from the same timepoint were titered for VSV production by plaque assay. Data are presented as means ± SEM. Student's unpaired, one-tailed t-test was performed; *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.


A
4T1
CT26WT
HT29
786-O
U2OS
S2-VSVΔ51-YFP
S2-VSVΔ51-YFP/VVdd-mCherry
B
4T1
CT26WT
HT29
786-O
U2OS
PFU/mL
VSVΔ51
VSVΔ51+VVdd
***
**
***
***
*

of doubly-armed S2-VSVΔ51-YFP/VVdd-mCherry carriers constitutively secreting VV immunomodulators could prime the tumour cell monolayers for infection and consequently a much more robust VSVΔ51 infection was observed. Not only were both viruses delivered to the tumour cells, but significantly greater viral spread was also evident with extensive YFP expression throughout the monolayer (Fig. 3.7A, bottom panel), leading to dramatic increases (over 1,000 fold in some cases) in the titer of infectious VSV produced by the tumour cells (Fig. 3.7B). An analogous experiment performed with insect cells persistently infected with SINV, another virus that has applications in gene therapy[201] and can persistently infect insect cells[202], resulted in similar findings (Fig. 3.8A,B). We conducted similar experiments to determine whether insect cells could deliver this integrated biotherapeutic system to tumours *in vivo*. In order to rigorously test its activity within a restrictive microenvironment, the HT29 human colon carcinoma model was used, which has previously been shown to be only partially sensitive to VSVΔ51 *in vivo*[200]. When HT29 tumours were grown as xenografts in nude mice and directly injected with insect cells carrying VSVΔ51 as a single agent, little infection was detected by fluorescent imaging or plaque assay (Fig. 3.9A,B). However insect cells doubly infected with VV and VSVΔ51 led to a profound OV infection within the tumour. Extensive spread of VSV was clear upon fluorescent imaging and we observed a 1,000-fold increase in infectious virus recovered from the tumours (Fig. 3.9A,B). Additionally, VVdd-mCherry was delivered by the doubly infected carriers and also went on to infect the tumours (Fig. 3.9A,B). Thus insect cells carriers can deliver an integrated biotherapeutic system to tumours in order to promote OV growth within a restrictive microenvironment.

Figure 3.8: Insect cell carriers constitutively secreting VV immunomodulators prime tumour cells for infection with SINV.

A) *Drosophila* S2-SINV-GFP or S2-SINV-GFP/VVdd-mCherry cell carriers were inoculated onto monolayers of indicated tumour cell lines. Composite GFP/mCherry fluorescent images 48h post-infection are shown. Scale bars represent 1mm. B) Tumour cell supernatants from the same timepoint were titered for SINV production.


A
CT26WT
HT29
786-O
S2 SINV-GFP
S2 SINV-GFP + VVdd-mCherry

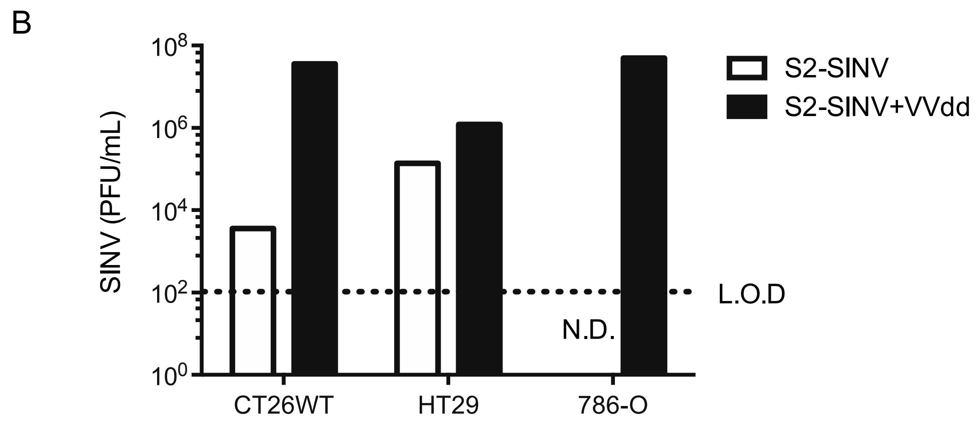
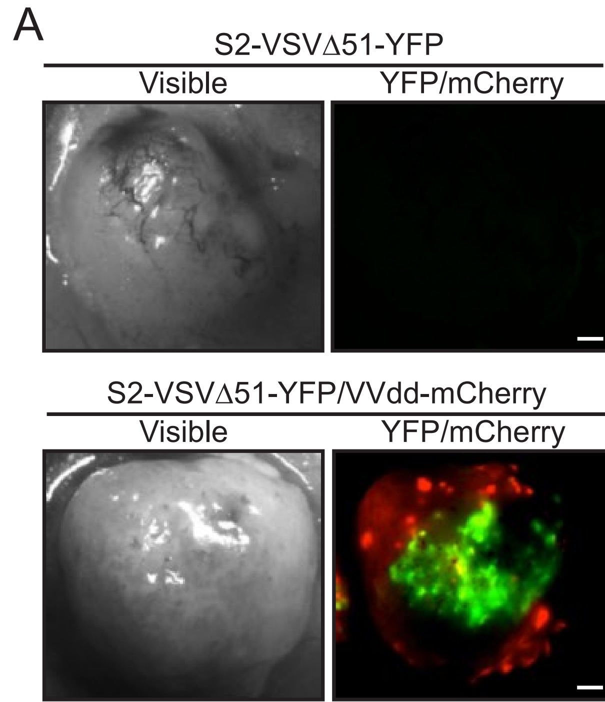
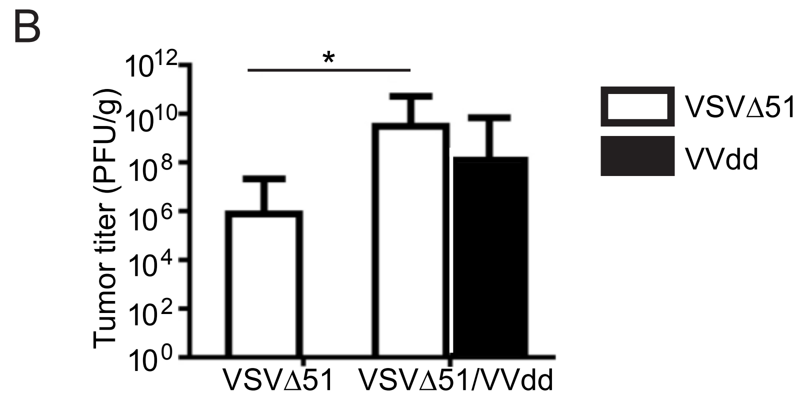

B
SINV (PFU/mL)
10^8
10^6
10^4
10^2
10^0
L.O.D
N.D.
CT26WT
HT29
786-O
S2-SINV
S2-SINV+VVdd

Figure 3.9: Insect cell carriers deliver multiple integrated biotherapeutics to tumours *in vivo*.

A) *Drosophila* S2 cells infected with either VSVΔ51-YFP alone or with both VSVΔ51-YFP and VVdd-mCherry were injected IT into human HT29 tumours grown as xenografts in nude mice. At 3d post-infection tumours were removed and imaged. Brightfield and composite YFP/mCherry fluorescent images are shown. Scale bars represent 2.5mm. B) The same tumours were homogenized and titers of infectious VSV and VV were determined by plaque assay. Data are presented as means ± SEM. Student's unpaired, two-tailed t-test was performed; *: $p<0.05$.


A
S2-VSVΔ51-YFP
Visible
YFP/mCherry
S2-VSVΔ51-YFP/VVdd-mCherry
Visible
YFP/mCherry


B
Tumor titer (PFU/g)
10^12
10^10
10^8
10^6
10^4
10^2
10^0
*
VSVΔ51
VSVΔ51/VVdd
VSVΔ51
VVdd

3.5 Systemic delivery of insect cells carrying multiple integrated biotherapeutics

The treatment of advanced disseminated cancers requires therapies that can be delivered systemically. We observed that mammalian carriers derived from leukocytic cell lineages show much-improved re-circulation in comparison to their counterparts from solid tissues (ref. [147] and Fig. 3.10A,C-D). However, these mammalian carriers still express surface recognition molecules that facilitate their trafficking to, and accumulation in, lymphoid organs (ref. [147] and Fig. 3.10D) limiting their capacity for tumour-targeted biotherapeutic delivery. In contrast, insect cells, which lack these homing receptors and are therefore likely incapable of interacting with mammalian host tissues, could have a longer half-life in the circulation and thus be more available for tumour delivery. To test this idea, we directly compared the circulatory half-life of S2-VSVΔ51-FLuc insect cell carriers to murine leukemia carriers tagged with luciferase (L1210-FLuc [147]). Carrier cells were injected IV into immunocompetent mice, and the cellular component of recovered blood was assayed for luciferase activity at various time-points. Consistent with our imaging studies (ref. [147] and Fig. 3.10D), mammalian leukemia cell carriers rapidly ceased to efficiently circulate soon after IV administration, with a 90% decrease in their recovery from blood between 1min and 5min post-administration (Fig. 3.10A). In contrast, insect cell carriers did not home to or extravasate into non-tumour tissues and circulated at stable levels in the blood throughout the 30min of monitoring in this experiment. This is also consistent with the bioluminescent imaging of systemically administered S2-VSVΔ51-FLuc cells shown in figure 3.10B, which demonstrates that the cells do not lodge in the lungs of the mice. However, it is unlikely that the insect cell carriers persist in the body for an extended period of time (days) since these cells slowly die off when cultured at 37°C (Fig. 3.11). Importantly, systemically

Figure 3.10: Circulatory kinetics and distribution of mammalian and insect cell carriers after IV administration.

A) Circulatory kinetics of *Drosophila* S2 cell carriers administered systemically to immunocompetent balb/c mice. Mice were injected IV with either 10^7 L1210-FLuc murine leukemia cells or 10^7 S2-VSVΔ51-FLuc carriers. Blood samples were collected at the indicated times post-injection, and the cellular fractions were assayed for luciferase activity in triplicate. Each point represents the mean of triplicate measurements ± SEM. B) Balb/c mice were injected IV with each firefly luciferase-tagged cell line and imaged by IVIS at timepoints as indicated. S2-VSVΔ51-FLuc cells imaged at 0.5h post-injection. C) Solid tumour cell lines at 0.5h post-injection, HeLa cervical carcinoma (human), A549 lung carcinoma (human), MCF-7 breast carcino- ma (human), CT26 colorectal carcinoma (murine), SF268 glioblastoma (human). D) Murine L1210 leukemia cells imaged at 0.6h, 5.5h, and 144h post-injection. Ventral and left-side (revealing spleen) views are shown.

A

S2
L1210

Recovery of carrier cells

2.0
1.5
1.0
0.5
0

0 5 10 15 20 25 30

Time (min)

B

S2-VSVΔ51-Fluc

C

Cervical Carcinoma

Lung Carcinoma

Breast Carcinoma

Colorectal Carcinoma

Glioblastoma

D

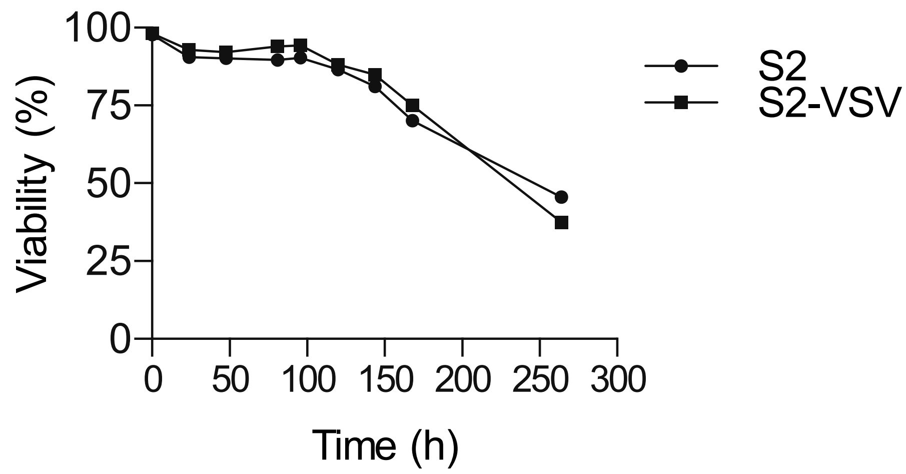
0.6h

5.5h

144h

Figure 3.11: Viability of insect cell carriers cultured at 37°C.

Drosophila S2 cell carriers were cultured at 37°C and cell viability was assessed at the indicated time-points using trypan blue exclusion.


100
75
50
25
0
Viability (%)
0
50
100
150
200
250
300
Time (h)
S2
S2-VSV

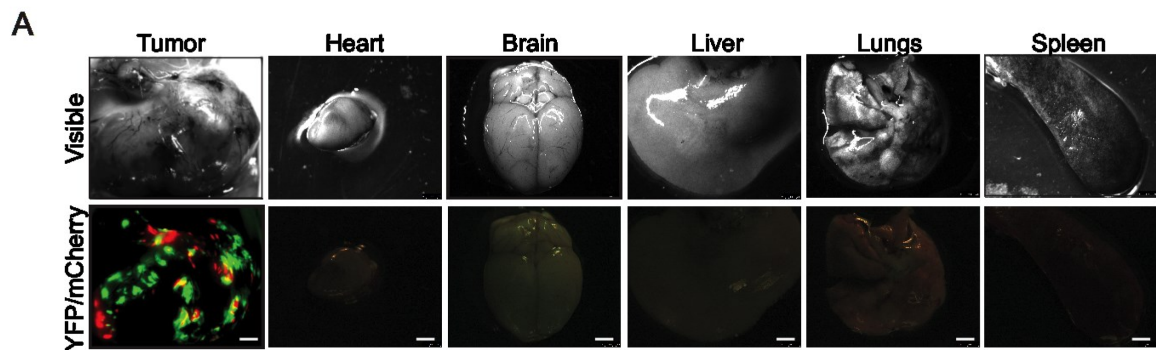
administered insect cell carriers could deliver integrated biotherapeutics to distally located tumour beds. By day 3 following IV administration of doubly-loaded carriers, extensive spread of both VSVΔ51-YFP and VVdd-mCherry was visible in tumours but not in normal organs (Fig. 3.12A,B), and high titers of each virus could be recovered from tumours (Fig. 3.12B). We also looked at the biodistribution of both viruses in tumour and normal organs at various timepoints after systemic delivery with insect cell carriers. Consistent with previous studies performed with naked virus[70], VSV was detected in the spleen, liver, lungs, and tumour 24h post-injection (Fig. 3.12B, left panel). However, by 72h, VSV was undetectable in normal organs and was only detected in the tumour, with high levels of virus maintained for the duration of the experiment (Fig. 3.12B, left panel). Interestingly, we did not detect any VSV in the liver of mice, which is normally seen when naked virus is delivered systemically, suggesting that insect cell carriers may bypass the liver tropism that is often seen with systemic delivery of oncolytic OV[132,203]. As for VV, it was also recovered from the lungs, liver, spleen, heart, and tumour 24h post-injection (Fig 3.12B, right panel). Similarly to VSV and previously published reports[204], virus in normal organs decreased over time while tumour replication was maintained at high levels (Fig. 3.12B, right panel). Therefore, OVs delivered systemically by insect cell carriers maintain a favorable safety profile similar to that of naked virus. Notably, systemic injection of up to 10^8 infected insect cell carriers did not lead to any detectable toxicity in mice (not shown).

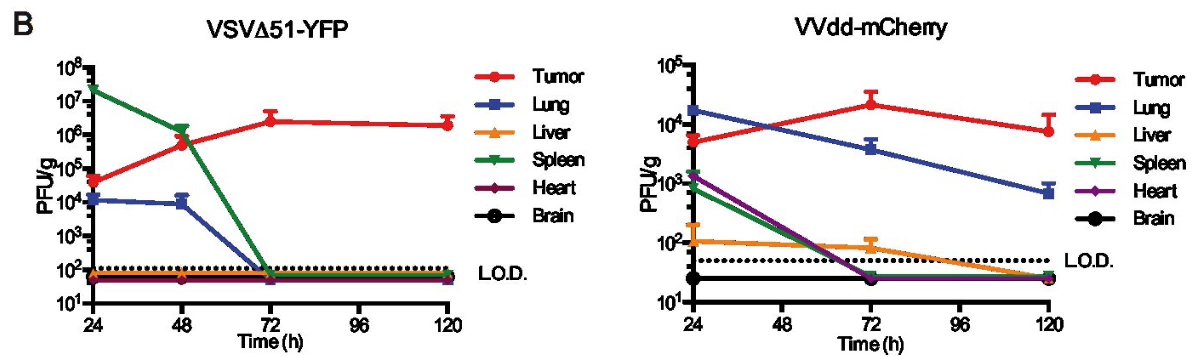
3.6 Insect cell carriers shield virus from immune recognition and neutralization

We sought to determine if insect cells by themselves induced any adverse immune reactions since they are foreign and presumably would be recognized as "non-self" by the immune system. This is especially relevant in the context of natural killer (NK) cells since

Figure 3.12:Virus biodistribution after systemic delivery of insect cell carriers.

A) Triplicate immunocompetent balb/c mice bearing CT26 tumours were treated IV with $5x10^6$ *Drosophila* S2 cells carrying VSVΔ51-YFP and VVdd-mCherry. Tumours and normal organs were collected 24h, 48h, 72h, and 120h post-treatment. Representative brightfield and composite YFP/mCherry fluorescent images of tumour and organs collected 72h post-treatment are shown. Scale bars represent 2.5mm. B) Each tumour and organ was homogenized and titers of VSVΔ51 and VVdd were determined by plaque assay to assess virus biodistribution. Each time point represents the mean + SEM obtained from triplicate mice. The limit of detection (L.O.D) for VSV and VV, indicated by the dashed line, is 100 PFU and 50 PFU, respectively. Samples that were not detected were set to half the limit of detection to facilitate visualization.

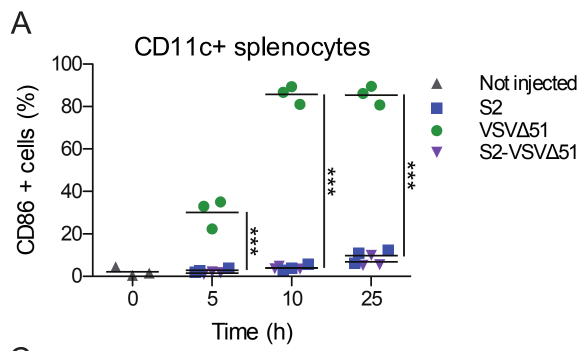

A
Tumor
Heart
Brain
Liver
Lungs
Spleen
Visible
YFP/mCherry

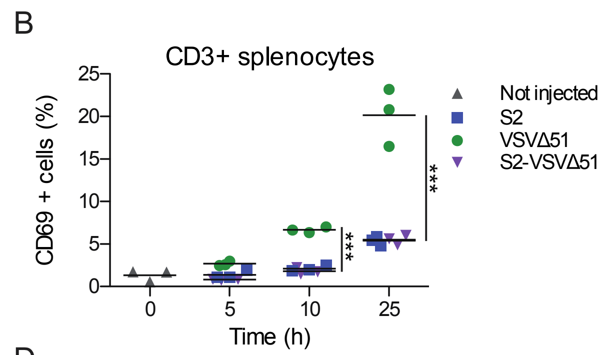

B
VSVΔ51-YFP
VVdd-mCherry
PFU/g
Time (h)
Tumor
Lung
Liver
Spleen
Heart
Brain
L.O.D.

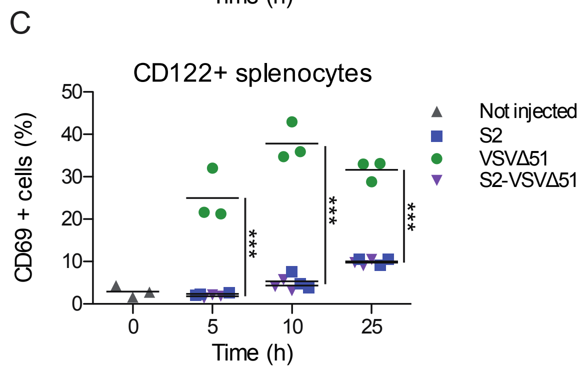
insect cells lack MHC genes and could therefore be targeted for destruction by NK cells. We therefore looked at the activation status of various immune cells after IV injection of insect cells in immunocompetent balb/c mice. Surprisingly, IV injection of uninfected insect cells resulted in a delayed and minimal activation of dendritic cells (CD11c+), T cells (CD3+) and NK cells (CD122+) 25h post-injection (Fig. 3.13A-C). Moreover, systemic administration of persistently infected insect cells also resulted in minimal and delayed activation of immune cells (Fig. 3.13A-C). This is in sharp contrast to free virus particles, where IV administration of VSV resulted in the rapid and robust activation of all the analyzed immune cell types (Fig. 3.13A-C). These results suggest that insect cell carriers, by shielding the virus, minimize immune recognition of VSV. Consistent with these findings, we assessed the neutralizing antibody response generated against VSV after immunization with free virus or with persistently infected insect cells and found that persistently infected insect cells did not induce an anti-VSV antibody response whereas free virus induced high titers of neutralizing antibodies (Fig. 3.13D). A major advantage of cell carriers for the delivery of OVs is their ability to protect the virus from immune-mediated neutralization[205,206]. We therefore assessed whether insect cells could deliver virus in the presence of virus-neutralizing antibodies. In the absence of neutralizing antibodies, both free VV particles and VV-infected insect cells were able to infect U2OS tumour cells (Fig. 3.14A). However, in the presence of VV neutralizing antibodies[196], VV-infected insect cells were 10-20 times more resistant to neutralization than free virus particles at all antibody concentrations tested (Fig. 3.14A,B). We also assessed whether insect cell carriers could deliver VSV to tumours in the presence of VSV-specific neutralizing antibodies. Although both naked virus and insect cell carriers could deliver VSV to lung tumours in virus-naïve mice, immunization of these mice prior to treatment completely ablated the ability of both naked virus and insect cell carriers to initiate

Figure 3.13: Insect cell carriers shield virus from immune recognition.

A) Balb/c mice were injected IV with a single dose of either PBS, VSVΔ51, S2 cells, or S2-VSVΔ51 cell carriers and sacrificed 5h, 10h, and 25h after injection. Spleens were harvested and splenocytes were collected and the number and activation of dendritic cells B) T cells, and C) NK cells was quantified by flow cytometry. Two-way ANOVA with Bonferroni post-test was performed; **: $p<0.01$, ***: $p<0.001$. D) Mice were treated as in A) and 14 days post-injection, blood was collected by saphenous vein puncture and the amount of VSV neutralizing antibodies was quantified as described in materials and methods. One-way ANOVA with Tukey's post-test was performed.


A
CD11c+ splenocytes
CD86 + cells (%)
100
80
60
40
20
0
0
5
10
25
Time (h)
***
***
***
Not injected
S2
VSVΔ51
S2-VSVΔ51


B
CD3+ splenocytes
CD69 + cells (%)
25
20
15
10
5
0
0
5
10
25
Time (h)
***
***
Not injected
S2
VSVΔ51
S2-VSVΔ51


C
CD122+ splenocytes
CD69 + cells (%)
50
40
30
20
10
0
0
5
10
25
Time (h)
***
***
***
Not injected
S2
VSVΔ51
S2-VSVΔ51

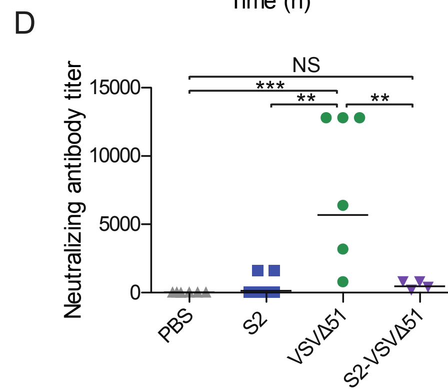
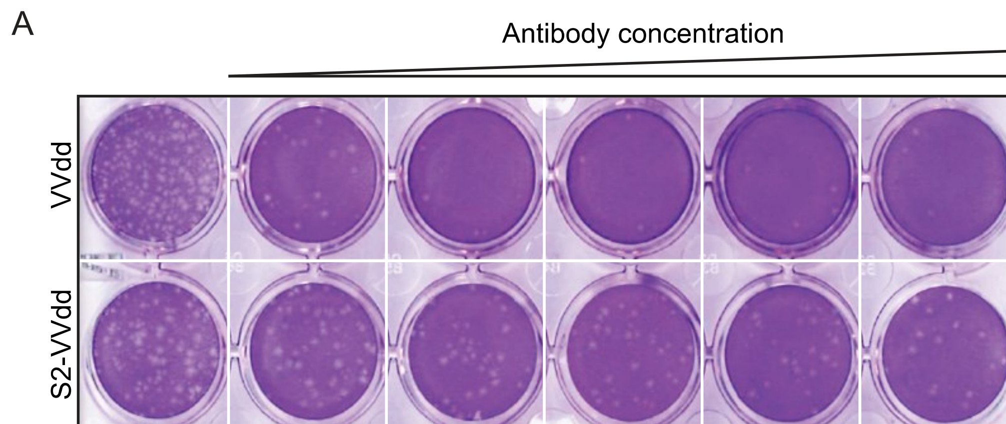
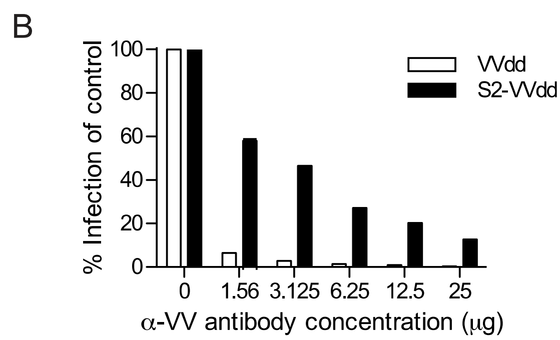
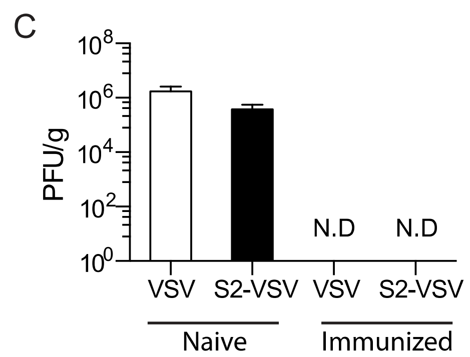

D
Neutralizing antibody titer
15000
10000
5000
0
NS
***
**
**
PBS
S2
VSVΔ51
S2-VSVΔ51

Figure 3.14: Insect cell carriers shield VV, but not VSV, from antibody neutralization.

A) Free VV particles or insect cells infected with VV at an MOI of 10 for 24h were incubated with various concentrations of anti-VV L1R neutralizing antibody for 1h and then titrated on monolayers of U2OS tumour cells. B) Quantification of the experiment shown in C). Naïve or VSV-immunized mice bearing CT26 lung tumours were treated IV with either naked VSVΔ51 or with S2-VSVΔ51 cell carriers and lungs were collected 24h post-treatment for virus quantification. N.D. = not detected. Data are presented as means ± SEM (n=3).


A
Antibody concentration
VVdd
S2-VVdd


B
% Infection of control
100
80
60
40
20
0
VVdd
S2-VVdd
0
1.56
3.125
6.25
12.5
25
α-VV antibody concentration (μg)


C
PFU/g
10^8
10^6
10^4
10^2
10^0
N.D
N.D
VSV
S2-VSV
VSV
S2-VSV
Naive
Immunized

tumour infection (Fig. 3.14C). Overall, these results suggest that insect cell carriers do not induce the rapid activation of immune cells and can shield VV from immune recognition and neutralization.

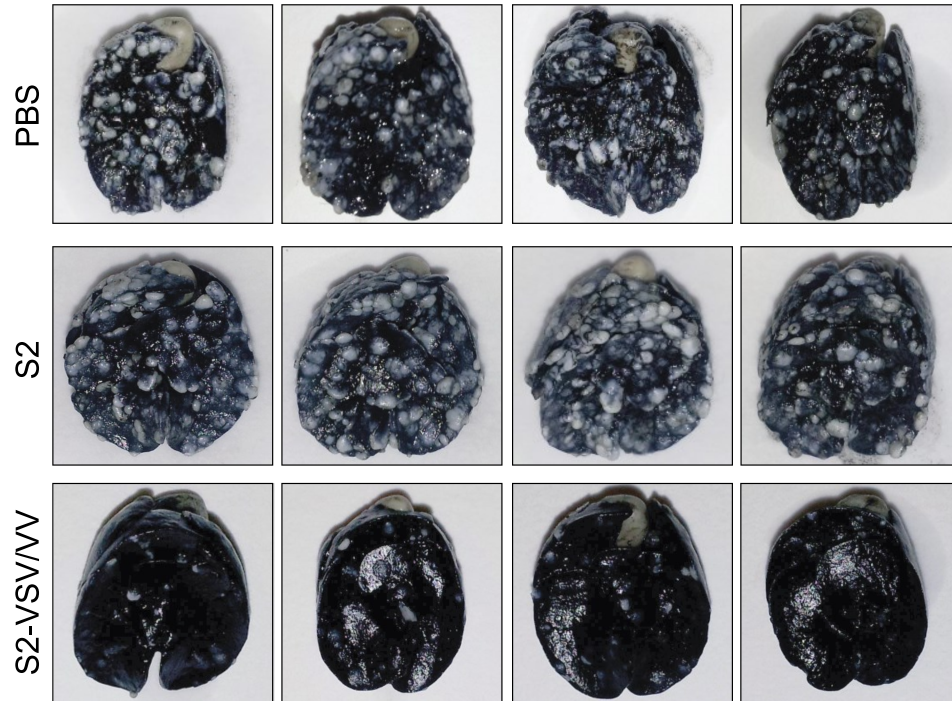
3.7 Systemic delivery of insect cell carriers reduces tumour burden

In order to investigate the therapeutic activity of systemically administered insect cells, we established CT26 lung tumours in balb/c mice by IV injection and treated them IV daily for five days, starting on day three post-tumour seeding with either uninfected S2 cells or with S2-VSVΔ51-YFP/VVdd-mCherry cell carriers. The mice were sacrificed when the first mouse showed signs of respiratory distress, which was an untreated control mouse and occurred on day 16 post-tumour seeding. Figure 3.15A shows representative images of the lungs after staining with India ink in order to visualize tumour burden, in which case tumour nodules appear white in color. While S2 treated mice had similar tumour burden as untreated mice, systemically administered insect cell carriers were able to reduce lung tumour burden, when compared to both groups. We also performed an efficacy study in mice bearing subcutaneous CT26 tumours in order to demonstrate the ability of insect cell carriers to circulate beyond the lungs and still have a therapeutic effect in a different tumour model. Therefore, we treated mice with palpable tumours for 5 consecutive days with either S2 cells or with S2-VSVΔ51-YFP/VVdd-mCherry cell carriers delivered IV and measured tumours every second day. The tumours of mice treated with S2 cells grew at the same rate as untreated control mice, whereas the tumours of mice treated with S2-VSVΔ51-YFP/VVdd-mCherry cell carriers grew significantly slower (Fig. 3.15B). These findings demonstrate the ability of systemically administered insect cell carriers to have a therapeutic effect in immune competent mice.

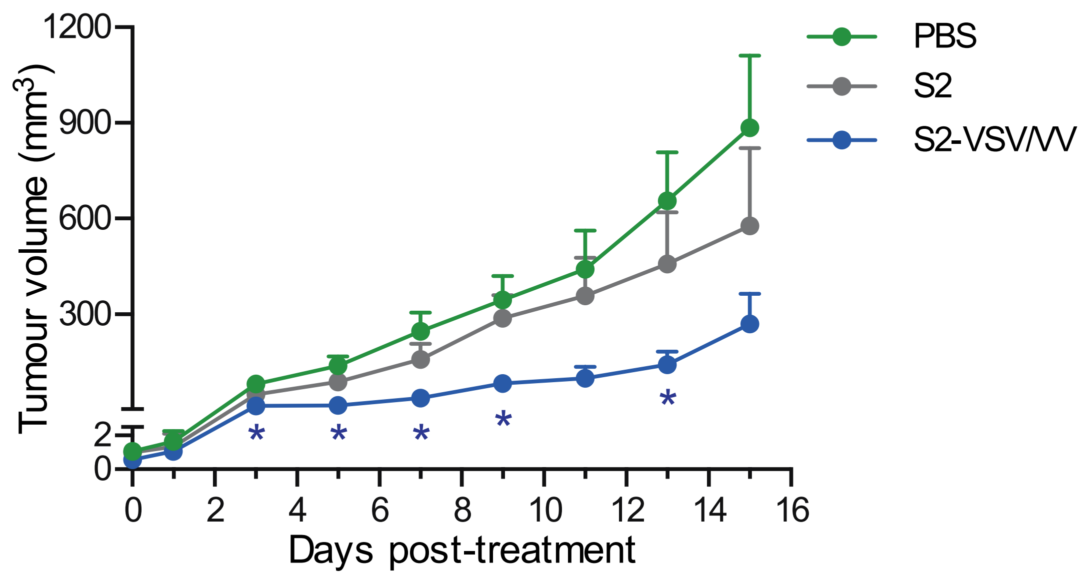
Figure 3.15: Systemic delivery of insect cell carriers reduces tumour burden.

A) Representative images of India ink-perfused lungs from mice bearing CT26 lung tumours. Mice (n=7) were treated IV with $5x10^6$ S2 cells or $5x10^6$ S2-VSVΔ51-YFP/VVdd-mCherry cell carriers for 5 days. B) Tumour volume measures of mice (n=7) bearing CT26 subcutaneous tumours. Mice were treated IV with $5x10^6$ S2 cells or $5x10^6$ S2-VSVΔ51-YFP/VVdd-mCherry cell carriers for 5 days. Values represent mean + SEM. Multiple t-tests with Holm-Sidak correction was performed comparing PBS to S2-VSVΔ51-YFP/VVdd-mCherry; *: $p<0.05$.

A



B



4. DISCUSSION

The current standard of care for cancer, employing conventional chemotherapy and/or radiation therapy is unable to effect cures in the majority of patients with systemic disease. There is little doubt that sophisticated new therapeutic approaches need to be developed if significant success is to be attained in the treatment of metastatic cancers. A great deal of effort has been put toward the development of biotherapeutics including OVs with encouraging recent clinical results[17,131,137,207]. To complement and augment OV therapeutics, there has been an emerging interest in the use of cell carriers to enhance systemic delivery[205,206]. Mammalian carrier cells proposed to date are limited by one or more of the following features: cumbersome isolation and/or manufacturing protocols, limited viral productivity (in the case of non-tumour cells), off-target tissue homing tropisms, and safety concerns (in the case of tumourigenic cell lines).

To improve the clinical feasibility of the carrier cell approach, we have investigated whether insect cells persistently infected with an OV could be exploited for systemic therapy. We observed that cultured *Drosophila melanogaster* cell populations continued to proliferate normally while maintaining a homogeneous infection with VSVΔ51 (Fig. 3.1). Consistent with previous findings[191], these persistently infected Dipteran cell lines released on the order of only 0.01 infectious particles per cell into culture supernatants, much less than the hundreds to thousands typically produced by mammalian cell lines (Fig. 3.2A)[192], and too little to be of therapeutic use. However, insect cells directly harvested from these cultures remained highly infectious and effectively delivered oncolytic VSV to tumour cells through direct contact (Fig. 3.2A,B). Thus, individual VSVΔ51-infected Dipteran cells represent potent therapeutic agents in their own right, and importantly, could deliver virus to tumours *in vivo* following either IT or IV administration (Figs. 3.4 and 3.9). The mechanism

by which VSV is transferred from the S2 cells to tumour cells most likely requires VSV G protein as neutralizing antibodies blocked the transfer of virus to tumour cells (Fig. 3.14C). It is important to note that although S2 cells remained persistently infected for at least a year, we used low passage infected-S2 cells as to avoid adaptation of VSV to the insect host, which has been demonstrated to negatively impact its fitness in mammalian cells[208,209]. However, given that VSV exists as a quasispecies, one round of replication in mammalian cells was shown to be sufficient for mammalian-adapted clones to emerge from the insect-adapted virus population[210].

Unlike the mammalian cell lines typically used to propagate VSV, insect cell carriers remained viable and actively proliferated while infected with their OV cargo, allowing further modification to express additional biotherapeutic gene products. Illustrating this point, we have shown that Dipteran VSV carriers could be armed to constitutively secrete VV-encoded immunomodulators that not only increased VSV levels in the insect cell carriers but also successfully primed the tumour microenvironment to promote OV infection (Figs. 3.5, 3.6, 3.7 and 3.8). Our group has previously demonstrated that the VV gene product B18R, a soluble decoy IFN receptor, is in large part responsible for the increase in VSV replication seen in mammalian cells since IFN is the principal antiviral factor that inhibits the virus[200]. Because invertebrates do not encode IFN genes, the mechanism by VV increases VSV replication in S2 cells is not known. In the case of antiviral immunity against VSV in *Drosophila*, RNAi and autophagy have been shown to exert antiviral activity[211,212]. Since VV encodes VP55, a poly(A) polymerase that also polyadenylates miRNAs and targets them for destruction in mammalian cells[213], a similar mechanism may perhaps be at play here.

Remarkably, the oncolytic VV vector consisting of some 200 genes was also delivered to tumours *in vivo* (Fig.3.9). The mechanism by which this occurs is not fully

understood. Previous studies have found that S2 cells are non-permissive to infection by VV and the block occurs at the level of VV genome replication[214]. However, VV early genes, many of which encode immune-modulatory proteins[215], are still transcribed[214] and therefore allow priming of tumour cells for subsequent VSV infection. Delivery of replication competent VV to tumour cells may occur by un-internalized virions "hitchhiking" on the surface of the insect cells, similar to what is seen with T cell carrier-mediated delivery of a retrovirus[155]. Notably, virus hitchhiking of VSV on T cells also conferred resistance to neutralization, similar to what we observed with VV-infected insect cell carriers (Fig. 3.14A,B). Whether similar mechanisms are at play warrants further investigation. As for VSV, virus was not delivered to tumours in virus-immune mice after systemic administration of infected insect cell carriers (Fig. 3.14C). This likely occurs as a result of the viral glycoprotein being present on the cell surface of the S2 cells, thus making it accessible for antibody binding. This is also true for mammalian cell carriers; however, these have been able to bypass humoral immunity either by viral "hitchhiking" or perhaps simply by saturating the antibody response. Indeed, the transformed cell carriers that have delivered VSV in the presence of neutralizing antibodies typically make thousands of virus particles per cell whereas S2 cells barely release any virus progeny. This being said, pseudotyping strategies to evade antibodies which work for naked virus[216], can also be applied to insect cell carriers. One important finding that warrants further investigation is the observation that we did not detect any VSV in the liver 24h after systemic administration of insect cell carriers (Fig. 3.12B). Although we did not directly compare this to naked VSV in this study, previous work from our lab suggests that naked virus is sequestered in the liver following IV administration, with 10^3 virus particles detected at 24h and 10^3 particles still detected 72h post-injection[70]. This suggests that insect cell carrier delivery of VSV seems to bypass liver

uptake, a major problem that is common to several OVs and can lead to toxicity[132]. Overall, our findings indicate that the insect cell platform has the capacity to simultaneously carry many integrated biotherapeutic genes and viruses to tumours upon systemic administration.

Although we have focused primarily on the delivery of viruses as a "proof of principle" for this cell carrier platform, it is easy to imagine the establishment of stable insect cell lines expressing immune stimulating factors, monoclonal antibodies, imaging gene products, tumour antigens, suicide genes and/or targeting molecules along with viruses to create a sophisticated multi-pronged cancer therapy. Interestingly, a few reports suggest that S2 cells engineered to express MHC class I molecules can be loaded with exogenous peptides and function as antigen presenting cells to generate antigen-specific CD8 T cells[217–219]. Similarly, *Drosophila* S2 cells expressing CD40 ligand have been shown to enhance antigen presentation through engagement of B cells[217,220]. Combining these strategies with S2 cell carrier mediated delivery of OVs could further promote anti-tumour immune responses given that the presence of the virus would provide additional costimulatory signals to the T cells. An important consideration when trying to express exogenous proteins in insect cells is the post-translational modifications in those proteins. Since insect cells do not have the same machinery as mammalian cells, some post-translational modifications in proteins do not occur and this could impact protein stability, solubility, and half-life[221,222].

Importantly, we find that Dipteran cell carriers successfully transported multiple biotherapeutic payloads to tumour beds following systemic administration in immunocompetent animals, resulting in a therapeutic effect (Figs. 3.9 and 3.15). The minimal and delayed immune activation observed upon systemic administration of persistently infected insect cells is somewhat surprising (Fig. 3.13). However, in the case of NK cells, S2 cells lack ligands to engage NK activating receptors and thus minimally

activate NK cells on their own, consistent with previous findings[223]. Perhaps because *Drosophila* cells are not pathogenic, there is no selective pressure on the immune system to evolve mechanisms to detect these cells. In the context of OV therapy, the low immunogenicity of insect cell carriers along with their ability to minimize the recognition of the virus and subsequent immune activation may allow more time for the virus to replicate and spread throughout the tumour before an antiviral response is mounted and the virus is cleared. It is important to note that we only looked at a subset of immune cells at a relatively early time point so this does not exclude the possibility that the immune cells do elicit an immune response. For example, some studies suggest that DNA released by dying S2 cells can activate B cells[217,224], and that human complement rapidly kills S2 cells[223]. In light of this, expressing of complement regulatory molecules such as CD46, CD55 and CD59 may minimize complement-mediated lysis of S2 cells[225].

The delivery of insect cells to tumour beds in our experiments is likely to be non-specific due to the trapping of carrier cells in tumour vasculature, which is known to have intermittent and slow rates of blood perfusion[226]. Interestingly, *Drosophila* hemocytes are capable of recognizing the disrupted basement membrane of fruit-fly tumours and mounting a response against them[227,228]. Since S2 cells are described as hemocyte-like[229], it would be worth investigating if they can recognize features of mammalian tumours. Nonetheless, OVs delivered by insect cell carriers still maintained their tumour specificity, as evident in the biodistribution study (Fig. 3.12). One major advantage of S2 cells that we observed was the ability of S2 cells to circulate at consistent levels for at least 30 minutes, unlike the mammalian leukemia cell carriers where there numbers in the circulation dropped within 5 minutes after administration (Fig. 3.10). Further characterization of the circulatory kinetics, biodistribution and half-life of insect cell carriers will certainly prove to be informative. The

lack of tissue targeting or homing of the insect cells provides a significant area for future development of this platform. Strategies such as the incorporation of targeting molecules, such as artificial ligands that bind to tumour vasculature[230–232], could substantially increase the ability of these cells to deliver biological therapeutics to tumour beds. Magnetic targeting of cells is another applicable strategy that has been successful in preclinical studies using various cell platforms[233–236].

Insect cell lines capable of continuously propagating OVs and other biotherapeutics therefore represent an attractive delivery platform with both immediate clinical utility and long-term potential for continued development. Insect cells stably propagating oncolytic virotherapeutics would be ideally suited to large-scale manufacturing[237], and could be systemically administered to patients without the drawbacks of off-target tissue homing and tumourigenicity that limits the safety of mammalian cell lines. As a standardized clinical vehicle, a continuous insect cell line could serve as a universal chassis for the assembly of any number of different gene products into co-operative systems, thus introducing a novel class of programmable cell biotherapies for cancer.

CHAPTER 2

5. INTRODUCTION

5.1 RNA interference

RNA interference (RNAi) is a conserved biological response to double-stranded RNA molecules which can result in post-transcriptional, sequence-specific inhibition of gene expression[238,239]. In mammalian cells, miRNAs are the most studied mediators of RNAi, with hundreds, and perhaps even thousands of miRNAs regulating 30% to 60% of human protein-coding genes[240–243]. miRNAs are encoded in the genome and are transcribed to yield a primary miRNA (pri-miRNA) transcript[244], which is cleaved by the microprocessor complex, consisting of Drosha and DCGR8[245–248], to yield a short precursor miRNA (pre-miRNA) of approximately 70 nucleotides in length. pre-miRNAs, which form a hairpin structure containing a loop and a duplex stem with interspersed mismatches, are bound by Ran-GTP and exported from the nucleus to the cytoplasm by Exportin 5[249,250], where they are further processed by a complex that contains the endonuclease Dicer and TAR RNA-binding protein[251,252]. This creates miRNA (guide strand) – miRNA* (passenger strand) duplexes approximately 22 nucleotides in length. The duplex associates with Argonaute 2 (AGO2) within the RNA-induced silencing complex (RISC)[251]. The strand within the duplex that has the less stable 5' end is usually chosen as the guide strand, and the passenger strand is removed or degraded[253,254]. The mature RISC containing the guide strand is then directed to target mRNAs for AGO2-mediated post-transcriptional gene silencing (PTGS)[255,256].

Determination of miRNA targets is largely dictated by the "seed" sequence, defined by nucleotides 2-8 at the 5' end of the guide strand, which must be complementary to the target mRNA[257,258]. However, sequences in the middle and 3' regions of miRNAs can also

influence target specificity[259,260]. miRNA target sites often reside in the 3'-UTRs of mRNAs, although they also exist in the 5'-UTRs and coding sequences of mRNAs[241,261,262]. PTGS occurs either by translational repression or via reduction in target mRNA levels[263–266].

Our understanding of RNAi-mediated silencing pathways has facilitated the development of several tools to modulate gene expression, including small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs). siRNAs consist of two RNA strands that form a duplex 19 to 25 base pairs in length with 3' dinucleotide overhangs[267]. Upon delivery into the cell, they are loaded directly into the RISC where guide strand selection occurs followed by mRNA binding to mediate gene silencing. As for shRNAs, they are structurally similar to pre-miRNAs and must therefore be processed by Dicer before loading into the RISC[268]. Cellular expression of shRNA is usually achieved using plasmids or viral vectors[194,269].

5.2 RNAi screens to identify antiviral factors

The ability of viruses to efficiently replicate within their host depends in part on their capacity to exploit various cellular pathways and to suppress and evade the cellular antiviral response. In mammalian cells, the antiviral response is initiated upon detection of molecular and structural components of the virus that are considered foreign [270]. Cellular pattern recognition receptors such as Toll-like receptors (TLRs) and RIG-I-like receptors detect these so-called pathogen-associated molecular patterns and coordinate the activation of antiviral transcription factors, including NF-κB and IFN regulatory factors (IRFs), thereby leading to the expression of IFN[270–272]. Binding of IFN to the type-I IFN receptor activates the expression of hundreds of IFN-stimulated genes (ISGs) that serve to limit virus replication[273–275]. Although IFN is a major player in the antiviral response, other cellular pathways also play a role in virus infection. For example, expression of VGF results in

activation of cell proliferation which promotes VV replication[62]. Metabolic pathways that regulate lipid metabolism and nucleotide biosynthesis can also influence virus replication, as these biomolecules are needed in sufficient quantities to make virus progeny[276,277]. In summary, the reliance of viruses on their hosts for successful replication involves several cellular pathways, many of which can in turn modulate virus replication.

Capitalizing on the power of these tools, RNAi-based screens have uncovered the functions of several antiviral genes[193,274,275]. Such screens have identified genes that regulate various aspects of virus infection including entry, uncoating, replication, and budding for a wide-range of viruses such as VSV, HIV, dengue virus, hepatitis C virus (HCV), and influenza virus[278–282]. Traditional screens have relied on the ability of siRNAs or shRNAs to reduce the expression of genes involved in antiviral pathways prior to virus infection. However, Varble *et al.* recently used a novel approach in which amiRNA-mediated knockdown was coupled with virus replication[193]. Rather than silencing genes by conventional methods, a genome-wide library of approximately 16,000 individual SINV clones was constructed, with each virus encoding a distinct amiRNA. This screening strategy relies on the expression of functional amiRNAs from SINV, a positive-sense RNA virus that replicates in the cytoplasm[283]. While canonical miRNA biogenesis by Drosha normally occurs in the nucleus, processing of the virus-encoded amiRNA is thought to occur via cytoplasmic translocation of Drosha as a consequence of infection[284,285]. Overall, the screening platform has the ability to identify host restriction factors in the context of virus infection by relying on virus competition and selection.

5.3 Rationale

Although RNAi screens have been utilized to discover antiviral factors in normal cells, the technique has not been extensively used to screen for genes that may regulate OV infection in tumour cells, with only a few studies reported[286,287]. This is especially relevant given that resistance to OV infection has been encountered both in preclinical studies as well as in clinical trials[288–290]. Given that tumour cells committed to the malignant phenotype have already differentiated themselves from their normal counterparts with respect to their ability to resist virus replication, they may be uniquely sensitized to OVs by the knockdown of particular antiviral or other gene products. Therefore, screening the SINV-amiRNA library on tumour cells could reveal antiviral gene products that are redundant in normal tissues but indispensible in malignant cells.

5.4 Objectives

1) Passage the SINV-amiRNA library in tumour cells and perform deep sequencing to look for enrichment of specific amiRNAs.

2) Validate the ability of the enriched amiRNAs to enhance SINV replication.

3) Determine if the identified amiRNAs increase the replication of OVs.

4) Investigate the mechanism(s) by which the amiRNAs increase virus replication

5) Evaluate the therapeutic activity of amiRNA-expressing viruses.

6. RESULTS

6.1 *In vitro* passage of SINV-amiRNA library on tumour cells selects for specific amiRNAs

The mammalian antiviral response involves the coordination of extensive signaling networks that result in the production of hundreds of antiviral genes. Since viruses are capable of mediating RNAi-based post-transcriptional gene silencing, we sought to perform a genome-wide RNAi-based screen in which amiRNA is coupled to virus replication with the notion that viruses expressing an amiRNA that targets host antiviral factors will have a growth advantage. Therefore, serial passage of a library of viruses each expressing a unique amiRNA should result in the enrichment of specific amiRNAs that increase virus replication. To this end, we passaged a miR-30-based whole-genome targeting SINV-amiRNA library on CT26WT murine colon carcinoma cells a total of 4 rounds and sequenced the virus population to look for amiRNAs that were enriched. We observed that virus output from the tumour cells increased from passage 1 to passage 4, suggesting that perhaps there was enrichment of amiRNA-expressing virus clones with enhanced replication (Fig. 6.1A). Deep sequencing of passage 4 revealed that several amiRNA sequences were indeed enriched when compared to the unpassaged library (Fig. 6.1B,C and Table 6.1). Importantly, some sequences appeared at frequencies greater than 1% in more than one replicate, indicating that the enrichment of those particular amiRNA-expressing virus clones was likely mediated by the amiRNA and not due to unrelated mutations in the virus backbone that were positively selected for (Fig. 6.1C and Table 6.1). In order to confirm the sequencing results, we also picked 20 plaques at random and performed a PCR screen for the 2 most enriched amiRNA sequences and found that of the 20 plaques picked, 4 plaques (20%) corresponded to

Figure 6.1: Enrichment of specific amiRNA sequences after serial passage of the SINV library in tumour cells.

A) SINV titers obtained after 1 passage and 4 serial passages in CT26WT tumour cells. Data are presented as means ± SEM. Student's unpaired, one-tailed t-test was performed; **: $p < 0.01$. B) Stacked area plot of the frequency of each amiRNA in the SINV-amiRNA library before passaging (P0) and after 4 rounds of serial passaging (P4) in CT26WT cells. Cumulative percent frequencies of all 5 replicates are shown for P4. C) Heat map showing the percent frequency of reads of the 10 most enriched amiRNAs in each replicate before passaging (P0) and after passage 4 (P4).

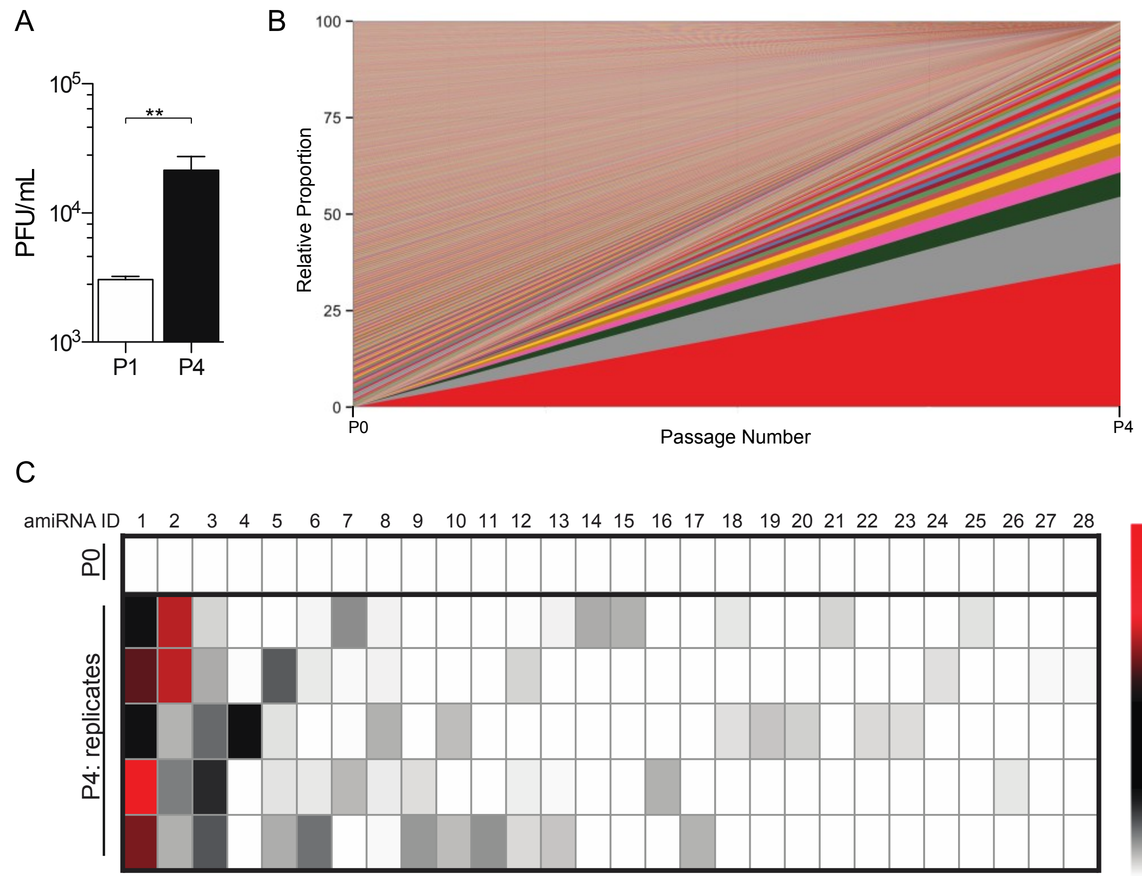

A
10^5
10^4
10^3
PFU/mL
**
P1
P4
B
Relative Proportion
100
75
50
25
0
P0
P4
Passage Number
C
amiRNA ID 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
P0
P4: replicates
50%
40%
30%
20%
10%
0%

Table 6.1: Frequency of reads of the top ten most enriched amiRNAs in each replicate after four rounds of serial passage in CT26WT cells.

amiRNA ID	Sequence	Frequency of Reads (%)					
		P0	P4-R1	P4-R2	P4-R3	P4-R4	P4-R5
1	GTGGGAGAGGAGACATGTTGGC	0.0343	26.1698	34.9893	28.8833	58.5099	36.5393
2	ATGTTGTTGGAGAATAAGACAA	N.D.	39.8990	40.1095	4.2531	7.2182	4.4809
3	ATGCAGTATGACACTCTCTGCA	N.D.	2.4948	4.5950	8.2516	12.0567	9.3752
4	TTAACGTGACAGACATGGCGGC	N.D.	0.0058	0.2513	26.6349	0.0433	N.D.
5	AGGCACAGACAGGTGTCTTCCA	N.D.	N.D.	9.1190	1.7738	1.6772	4.5425
6	ATGAGCAAGAGAGATGGATGTC	N.D.	0.7116	1.2709	N.D.	1.4508	7.8865
7	ATTCCTTGGCTTCATTTGTGCT	N.D.	6.4415	0.3972	0.2905	3.9819	N.D.
8	GTTTCCACCTATGGCCACTGGT	N.D.	0.9696	0.9448	4.3227	1.2053	0.4803
9	TATTGAATGGAGGTCCTAAGGG	N.D.	N.D.	N.D.	N.D.	1.9710	5.7629
10	TTCGATGAACATGGATTCTGCA	N.D.	0.0238	N.D.	3.6999	0.0024	3.7873
11	TAAATGGGTCTGACACTTAGAA	N.D.	N.D.	N.D.	N.D.	0.0075	6.1047
12	TTGGTGTGGGTTCTGGTCAGCA	N.D.	0.2364	2.4662	N.D.	1.0219	2.2367
13	TTTCGGCTTCAGTTCACTCCGG	N.D.	0.9158	0.1483	0.1926	0.4143	3.2441
14	TGGCATTCTGAATGGACCTGCC	N.D.	4.5921	N.D.	N.D.	N.D.	N.D.
15	TGTGAACAGCATGGTTTGGCAC	N.D.	4.3619	N.D.	N.D.	N.D.	N.D.
16	TTTGACTCTGGCTATTAGTCCG	N.D.	N.D.	N.D.	N.D.	4.3512	N.D.
17	TTTCCTGAGGATATTATTGGGG	N.D.	N.D.	N.D.	N.D.	N.D.	4.2352
18	TACTGTAAAGATTTCAAGACCC	0.0250	1.5417	N.D.	1.8560	N.D.	N.D.
19	TTCTGCAGGAATGAGATCACCA	N.D.	N.D.	N.D.	3.2761	N.D.	N.D.
20	GTGGTGACGGAGAGGTAGGGCC	N.D.	N.D.	N.D.	2.6890	N.D.	N.D.
21	TTTGGCACTGAAGTCATGTCCA	N.D.	2.4590	N.D.	N.D.	N.D.	N.D.
22	GATTCATGTGGAACACCAACAC	N.D.	N.D.	N.D.	2.2136	N.D.	N.D.
23	TGTTGGATGCCTCTTCAGGCCC	N.D.	N.D.	N.D.	2.0613	N.D.	N.D.
24	ATGCAGTTGGATTGACTTGCCT	N.D.	N.D.	1.9530	N.D.	N.D.	N.D.
25	TTGCTCAATCGAGTTCAGGGCC	N.D.	1.7812	N.D.	N.D.	N.D.	N.D.
26	TTGGAAGTTGGATACCATGGAG	N.D.	N.D.	N.D.	N.D.	1.4934	N.D.
27	CAGCTCATACTGGAAATCTGGC	N.D.	N.D.	0.4907	0.0092	N.D.	N.D.
28	TTGCAGCAGCATGGGAGTGCCC	N.D.	N.D.	0.4452	N.D.	N.D.	N.D.

P: passage ; R: replicate ; N.D.: not detected

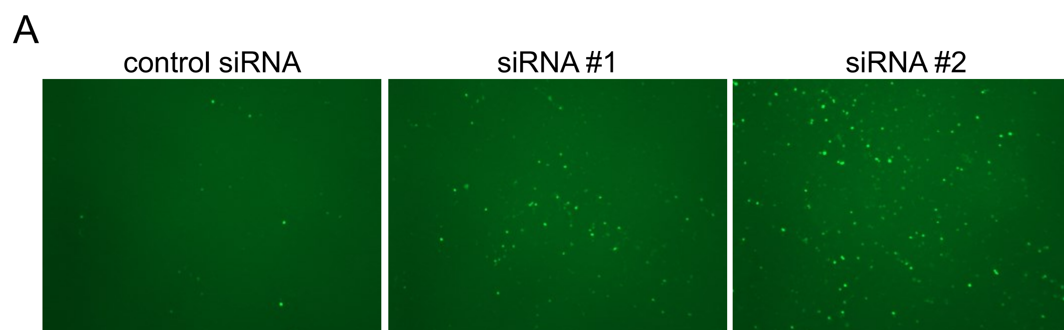
amiRNA#1, and 1 plaque (5%) corresponded to amiRNA#2, which is consistent with the deep sequencing data (not shown).

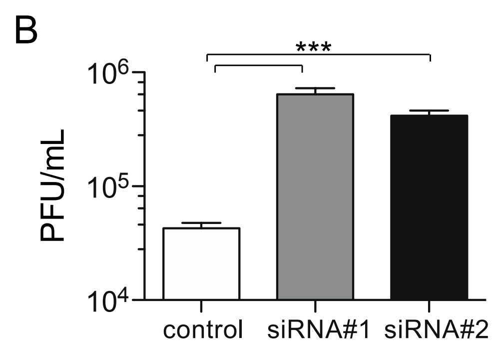
6.2 Enriched amiRNA sequences increase SINV replication in tumour cells

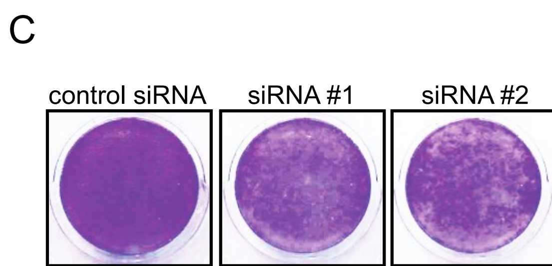
We chose to focus on the two most enriched sequences as these were highly enriched relative to the other sequences in at least 3 biological replicates (Fig. 6.1C and Table 6.1). To confirm that the amiRNA sequences we identified as enriched after serial passaging in tumour cells did in fact increase SINV replication, we transfected CT26WT cells with siRNAs corresponding to the identified hits and subsequently infected them with SINV-GFP. Increased virus replication and spread within the tumour cells was evident as assessed by fluorescence imaging of GFP reporter expression by the virus (Fig. 6.2A). Quantification of virus by plaque assay revealed that replication was increased more than 10-fold in cells transfected with the identified siRNAs compared to a control siRNA targeting luciferase (Fig. 6.2B). This increase in SINV replication also resulted in greater tumour cell killing (Fig. 6.2C). Although the finding that siRNA transfection of the identified sequences can increase virus replication and tumour cell killing, this experimental approach results in knockdown of the target genes before virus infection whereas gene knockdown occurs after virus replication is initiated with the SINV-library screening approach. We therefore sought to determine if SINV-mediated amiRNA expression could also increase virus replication. To answer this question, we cloned the identified amiRNAs back into the parental SINV virus in order to prevent any mutations in the virus backbone that have appeared during passaging from confounding our analysis and examined if the amiRNA-expressing viruses could outperform the parental virus in a competition assay. Despite initially being present at frequencies of only 2% and 5%, after 4 serial passages in CT26WT cells, SINV-amiRNA#1

Figure 6.2: Validation of enriched amiRNA sequences with SINV.

A) Fluorescent images and B) virus titers from CT26WT cells transfected with siRNAs corresponding to the identified hits and subsequently infected with SINV-GFP at an MOI of 0.1. Images were taken and supernatants were collected 48h post-infection for virus quantification by plaque assay (n=4). Data are presented as means ± SEM. Student's unpaired, one-tailed t-test was performed; ***: $p < 0.001$. C) Virus-induced cytotoxicity revealed by Coomassie Blue stain of CT26WT cells transfected with siRNAs corresponding to the identified hits and subsequently infected with SINV-GFP. Cells were fixed and stained 72h post-infection.


A
control siRNA
siRNA #1
siRNA #2


B
***
10^6
10^5
10^4
PFU/mL
control
siRNA#1
siRNA#2


C
control siRNA
siRNA #1
siRNA #2

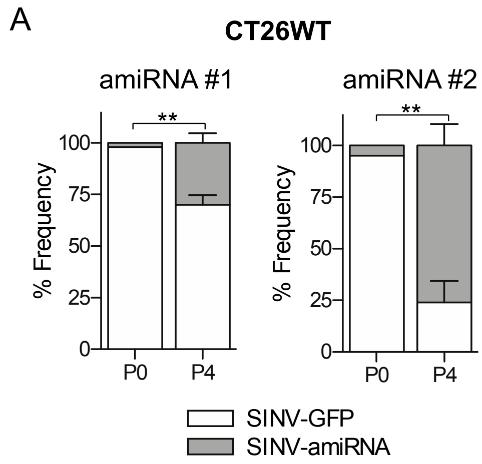
and SINV-amiRNA#2 had increased to frequencies of 30% and 76%, respectively, suggesting that they do indeed confer a growth advantage to the virus (Fig. 6.3A). We performed a similar experiment in HCT 116 cells, which are human colorectal carcinoma cells, and consistent with the competition experiment in CT26WT cells, amiRNA-expressing viruses increased in frequency after 4 rounds of competition (Fig. 6.3B). Overall, these results validate the findings we obtained from our screen.

6.3 Identified amiRNAs enhance replication and tumour cell killing of various OVs

Although it has been demonstrated that SINV has oncolytic properties, we wanted to determine if the amiRNAs we identified in our SINV-library screen could also increase the replication of other OVs [59,291,292]. We therefore performed similar experiments with VSVΔ51, MG1, and VVdd. As was seen with SINV, transfection of siRNAs corresponding to the identified amiRNA sequences followed by infection with virus led to increases in virus replication as assessed by reporter gene imaging (Fig. 6.4A). Quantification of virus output revealed that VSVΔ51 replication was enhanced more than 15-fold for both siRNA#1 and siRNA#2 (Fig. 6.4A bottom-right panel, B), while siRNA#1 and siRNA#2 increased MG1 replication 15-fold and 8-fold, respectively (Fig 6.4A middle-right panel, B). Interestingly, VVdd replication was enhanced approximately 7-fold by siRNA#1, whereas siRNA#2 had no effect (Fig. 6.4A top-right panel, B). The increase in VSVΔ51 replication also led to greater tumour cell killing (Fig. 6.4C). To confirm the positive impact that virus-mediated amiRNA expression has on replication, we cloned the amiRNAs into VSVΔ51. Although it has previously been demonstrated that VSVΔ51 can express amiRNAs, we first tested whether, in our hands, VSVΔ51-mediated expression of amiRNAs resulted in the production of functional amiRNAs. Using a luciferase reporter assay, we were able to convincingly

Figure 6.3: SINV amiRNA-expressing viruses replicate better than the parental virus.

Enrichment of amiRNA-expressing SINV from competition experiments against parental virus SINV-GFP. The percentage of each virus is shown before competition (P0) and after 4 rounds of competition (P4) in CT26WT cells (n=3) and B) HCT 116 cells (n=2). Data are presented as means ± SEM. Student's unpaired, one-tailed t-test was performed; **: $p < 0.01$.


A
CT26WT
amiRNA #1
amiRNA #2
% Frequency
100
75
50
25
0
P0
P4
**
SINV-GFP
SINV-amiRNA

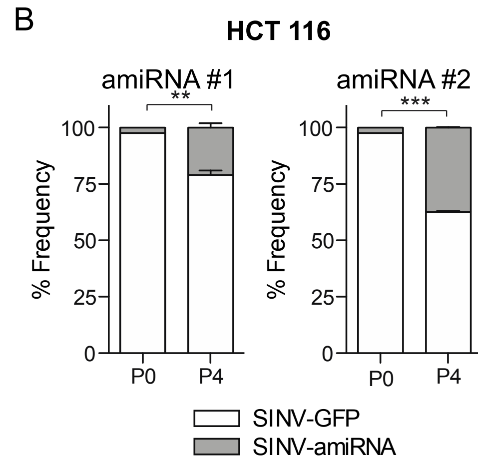
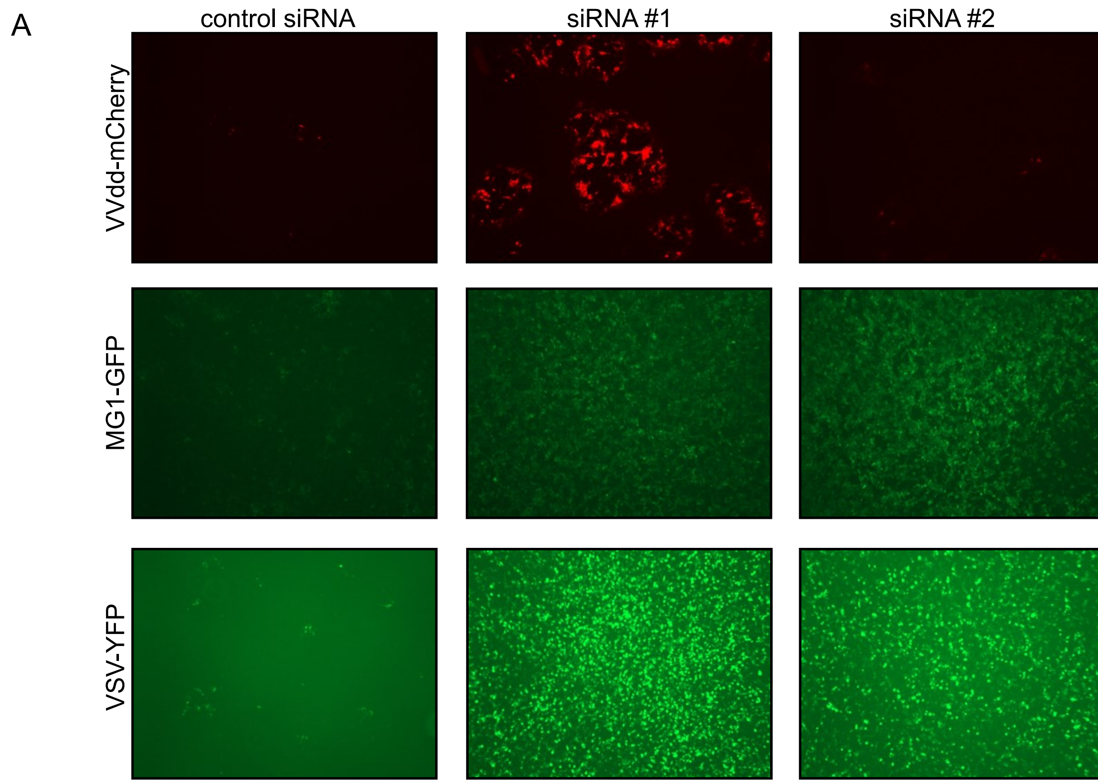
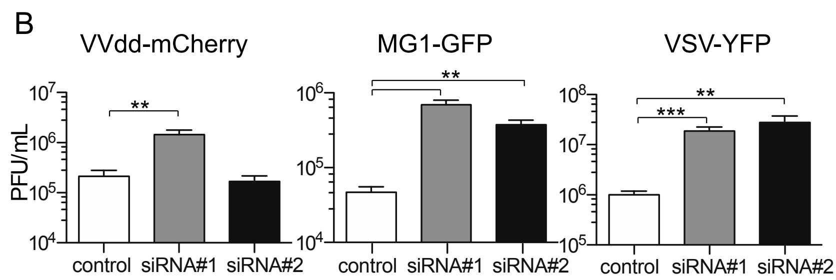

B
HCT 116
amiRNA #1
amiRNA #2
% Frequency
100
75
50
25
0
P0
P4
**
***
SINV-GFP
SINV-amiRNA

Figure 6.4: Identified amiRNAs increase replication of oncolytic VSVΔ51, MG1, and VVdd viruses.

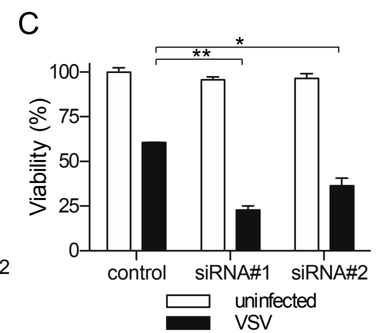
A) Fluorescent images and B) virus titers from CT26WT cells transfected with siRNAs corresponding to the identified hits and subsequently infected with VVdd-mCherry (n=5), MG1-GFP (n=3), or VSVΔ51-YFP (n=6). Images were taken and supernatants were collected for virus quantification by plaque assay 48h post-infection for VSVΔ51 and MG1 and 72h for VVdd. Data are presented as means ± SEM. Student's unpaired, one-tailed t-test was performed;**: $p < 0.01$, ***: $p < 0.001$. C) Virus-induced cytotoxicity as measured by AlamarBlue viability assay of CT26WT cells transfected with siRNAs corresponding to the identified hits and subsequently infected with VSVΔ51-YFP. Cell viability was measured 72h post-infection (n=3). Data are presented as means ± SEM. Student's unpaired, one-tailed t-test was performed; *: $p < 0.05$, **: $p < 0.01$.



A
control siRNA
siRNA #1
siRNA #2
VVdd-mCherry
MG1-GFP
VSV-YFP



B
VVdd-mCherry
MG1-GFP
VSV-YFP
PFU/mL
control
siRNA#1
siRNA#2



C
Viability (%)
control
siRNA#1
siRNA#2
uninfected
VSV

show that amiRNAs expressed by VSVΔ51 were able to silence luciferase expression, whereas control virus expressing an unmatched amiRNA could not (Fig. 6.5). We next performed a virus competition assay to determine if VSVΔ51-mediated amiRNA expression could confer a growth advantage over the parental YFP-expressing virus. Before serial passage, VSVΔ51-amiRNA#1 and VSVΔ51-amiRNA#2 were present at frequencies of 3% and 8%, respectively. After 4 serial passages in CT26WT cells, the amiRNA-expressing viruses had increased to frequencies of 72% and 79%, indicating that they confer a clear growth advantage to the virus (Fig. 6.6). Thus, amiRNAs identified by the SINV-amiRNA library screening approach are capable of increasing the replication of other OVs.

6.4 Identified amiRNAs do not affect the ability to produce or respond to IFN

In order to understand how the identified amiRNAs were enhancing virus replication, we first looked at the ability of the cells to produce IFN after VSVΔ51 infection. As expected, CT26WT cells transfected with control siRNA and subsequently infected with VSVΔ51 were able to induce the expression of IFN-β□at both the mRNA and protein level (Fig. 6.7A,B). Interestingly, when CT26WT tumour cells were transfected with siRNA#1 or siRNA#2 and infected with VSVΔ51, they too were able to produce IFN-β, albeit at higher levels than the control (Fig. 6.7A,B). The enhanced IFN-β production is likely due to the fact that these cells are infected with VSVΔ51 at higher levels (Fig. 6.4A), as we normally observe a positive correlation between VSVΔ51 infection and IFN production (Fig. 6.8). We next assessed whether the cells had functional IFN signaling by looking at their ability to induce the expression of several known ISGs. CT26WT cells transfected with control siRNA and infected with VSVΔ51 were able to up-regulate the expression of all ISGs tested (Fig. 6.7C). Importantly, CT26WT cells transfected with siRNA#1 or siRNA#2 were able to

Figure 6.5: VSV-expression of amiRNAs results in the production of functional small RNAs.

Dual-luciferase reporter assay. BHK cells were transfected with the indicated reporter plasmids possessing target sites for the enriched amiRNAs in the 3’ UTR of the Renilla luciferase reporter and subsequently infected with the indicated viruses. Luciferase activity was measured 24h post-infection. Renilla luciferase activity was normalized to Firefly luciferase activity then subsequently normalized to the unmatched amiRNA expressing control virus (n=3). A pcDNA plasmid expressing the amiRNAs was used as a positive control. Data are presented as means ± SEM. Student's unpaired, one-tailed t-test was performed; **: $p < 0.01$, ***: $p < 0.001$.

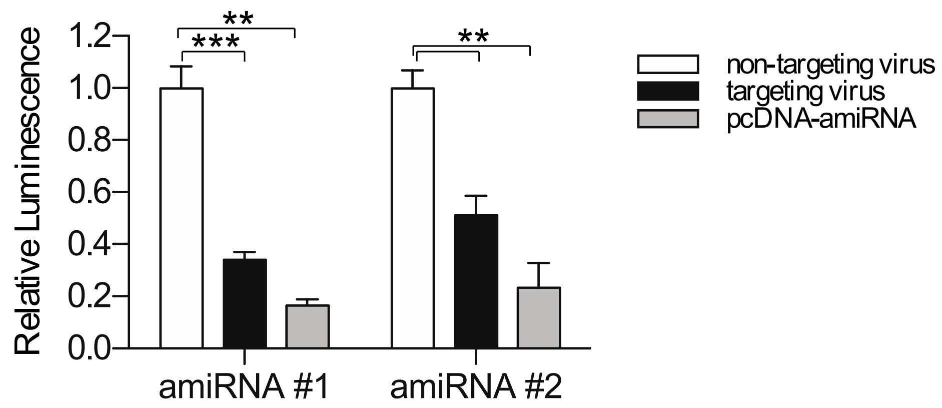

Relative Luminescence
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
***
**
amiRNA #1
amiRNA #2
non-targeting virus
targeting virus
pcDNA-amiRNA

Figure 6.6: VSV amiRNA-expressing viruses replicate better than the parental virus.

A) Enrichment of amiRNA-expressing of VSVΔ51 from competition experiments against parental virus VSVΔ51-YFP. The percentage of each virus is shown before competition (P0) and after 4 rounds of competition (P4) in CT26WT cells (n=3). Data are presented as means ± SEM. Student's unpaired, one-tailed t-test was performed; ***: $p < 0.001$.

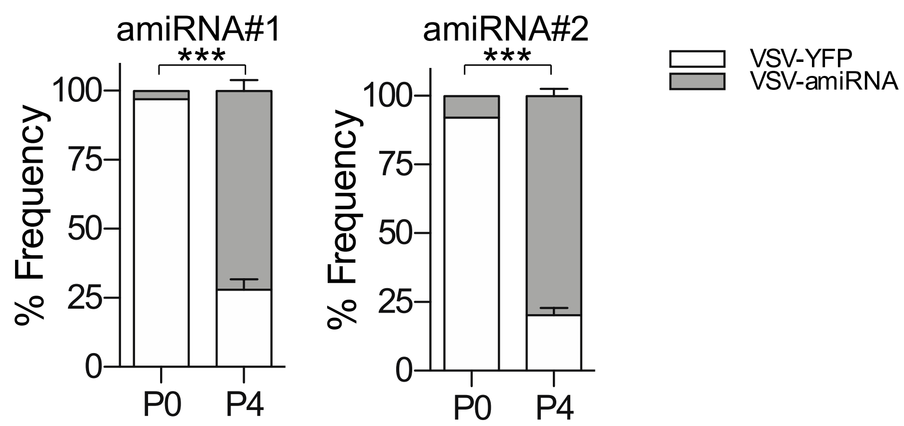

amiRNA#1
***
100
75
50
25
0
% Frequency
P0
P4
amiRNA#2
***
100
75
50
25
0
% Frequency
P0
P4
VSV-YFP
VSV-amiRNA

Figure 6.7: Identified amiRNAs do not target the IFN pathway.

A) IFN-β levels in CT26WT cells transfected with siRNA then infected with VSVΔ51 at an MOI of 0.001 for 24h, as measured by qPCR (n=2) and B) ELISA (n=2). Data are presented as means ± SEM. Student's unpaired, one-tailed t-test was performed; *: $p < 0.05$, **: $p < 0.01$. C) mRNA levels of various ISGs after siRNA transfection and infection of CT26WT cells with VSVΔ51 at an MOI of 0.001 for 24h, as measured by qPCR (n=3). Data are presented as means ± SEM. Student's unpaired, one-tailed t-test was performed; *: $p < 0.05$, **: $p < 0.01$.

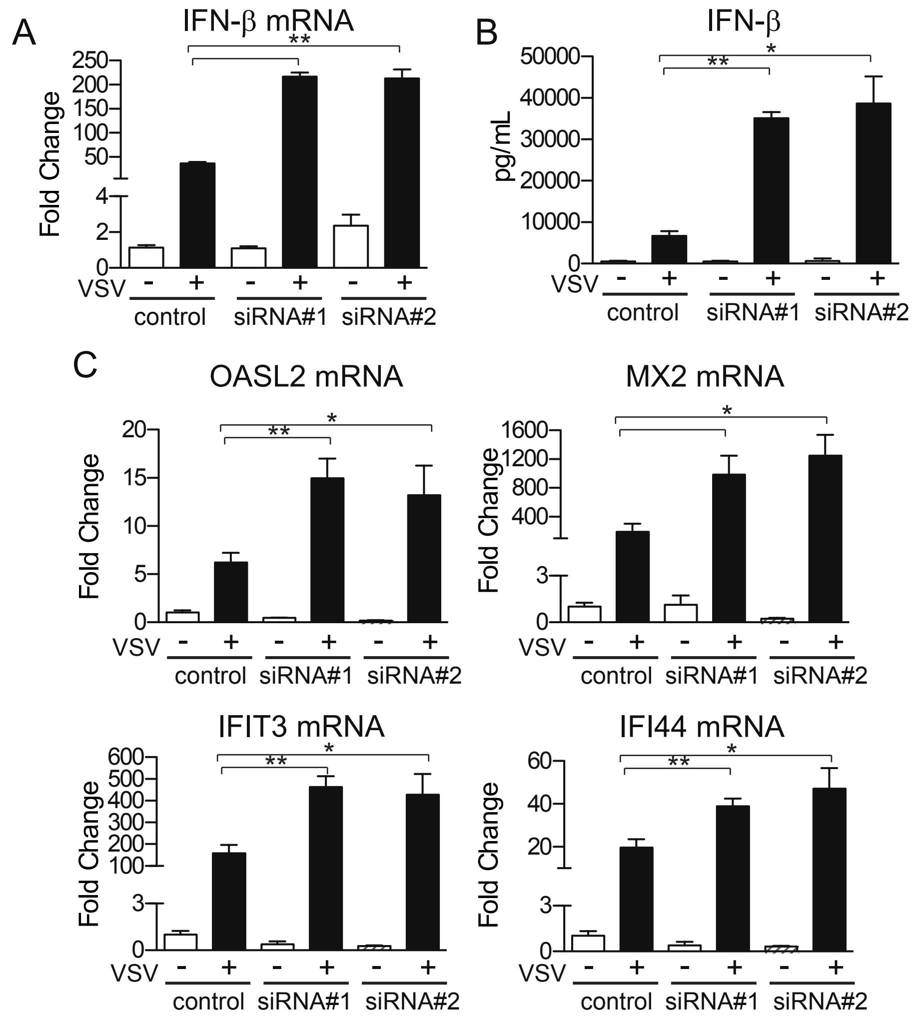
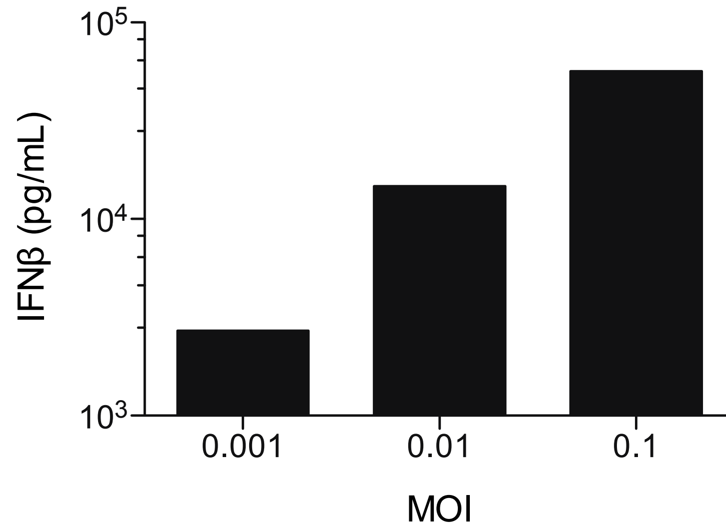

A
IFN-β mRNA
Fold Change
VSV
control
siRNA#1
siRNA#2
B
IFN-β
pg/mL
C
OASL2 mRNA
MX2 mRNA
IFIT3 mRNA
IFI44 mRNA

Figure 6.8: Dose response of IFN-β production after infection with VSVΔ51.

CT26WT cells were infected with the indicated amounts of VSVΔ51 and IFN-β levels in the supernatants 24h post-infection were measured by ELISA.



10^5
10^4
10^3
IFNβ (pg/mL)
0.001
0.01
0.1
MOI

induce all ISGs tested at even higher levels than control siRNA, consistent with the increased levels of IFN-β produced by these cells. Thus, the amiRNAs do not seem to negativelyaffect the ability of cells to produce or respond to IFN.

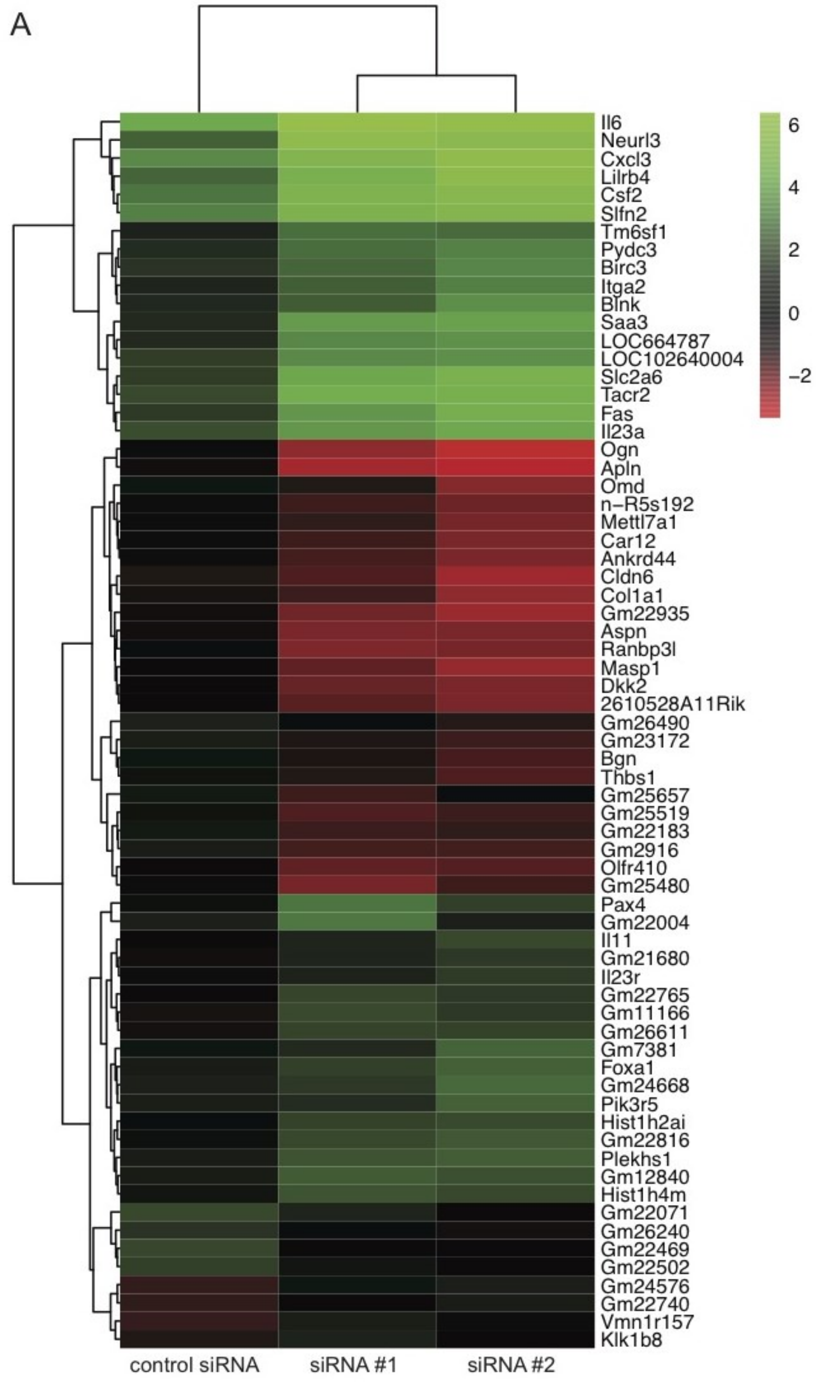
In an effort to identify which genes or pathways are being affected by the siRNAs #1 and #2, we performed microarray analysis of gene expression in CT26WT cells transfected with control siRNA, siRNA#1 or siRNA#2 with and without VSVΔ51 infection (Fig. 6.9A). Remarkably, there is significant overlap ($p < 0.0001$, Fisher's exact test) in the genes differentially expressed (>2-fold) in siRNA#1 and siRNA#2-treated cells (Fig. 6.9A,B). qPCR analysis of select genes confirmed the differences observed in the microarray (Fig. 6.10). We focused our attention to the genes that were the most downregulated in both siRNA#1 and siRNA#2 treated cells. siRNA knockdown experiments revealed that downregulation of ASPN and OGN increased VSV replication 4-fold and 7-fold, respectively, while siRNA knockdown of OMD or RANBP3L had no impact on VSV replication (Fig 6.11). These results suggest that ASPN and OGN are either direct or indirect targets of the identified amiRNAs and that they are involved in regulating VSV replication in CT26WT cells.

6.5 amiRNA-expressing viruses demonstrate enhanced *in vivo* replication within tumours

We next determined if the *in vivo* replication of amiRNA-expressing viruses was also enhanced in CT26WT tumour-bearing mice. We initially looked at SINV-amiRNA replication in subcutaneous tumours after IT injection of virus as well as in the spleen in order to assess if virus-mediated amiRNA expression also impacted virus replication in normal tissues. In line with what we observed in our *in vitro* studies, the amiRNA-expressing

Figure 6.9: Microarray analysis of gene expression changes induced by amiRNA #1 and amiRNA#2 reveals marked overlap of target genes.

A) Microarray analysis of changes in gene expression 16h after VSVΔ51 infection of CT26WT treated with siRNA#1 or siRNA#2. Heat map shows genes that were differentially expressed at least 3.5-fold as compared to cells treated with control siRNA and infected with VSVΔ51. B) Venn diagram showing the number of genes differentially expressed (≥ 2-fold) in VSVΔ51-infected, siRNA#1 or siRNA#2 treated cells, as compared to cells treated with control siRNA and infected with VSVΔ51.


A
Il6
Neurl3
Cxcl3
Lilrb4
Csf2
Slfn2
Tm6sf1
Pydc3
Birc3
Itga2
Blnk
Saa3
LOC664787
LOC102640004
Slc2a6
Tacr2
Fas
Il23a
Ogn
Apln
Omd
n−R5s192
Mettl7a1
Car12
Ankrd44
Cldn6
Col1a1
Gm22935
Aspn
Ranbp3l
Masp1
Dkk2
2610528A11Rik
Gm26490
Gm23172
Bgn
Thbs1
Gm25657
Gm25519
Gm22183
Gm2916
Olfr410
Gm25480
Pax4
Gm22004
Il11
Gm21680
Il23r
Gm22765
Gm11166
Gm26611
Gm7381
Foxa1
Gm24668
Pik3r5
Hist1h2ai
Gm22816
Plekhs1
Gm12840
Hist1h4m
Gm22071
Gm26240
Gm22469
Gm22502
Gm24576
Gm22740
Vmn1r157
Klk1b8
6
4
2
0
−2
control siRNA
siRNA #1
siRNA #2

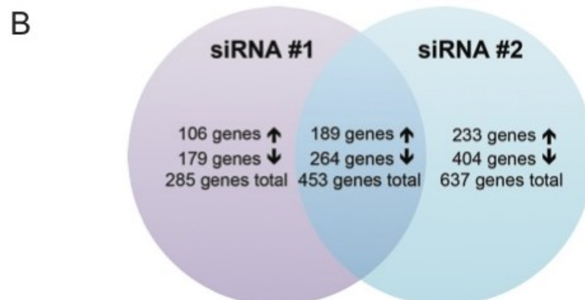

B
siRNA #1
siRNA #2
106 genes ↑
179 genes ↓
285 genes total
189 genes ↑
264 genes ↓
453 genes total
233 genes ↑
404 genes ↓
637 genes total

Figure 6.10: qPCR validation of microarray results.

qPCR of select genes identified as downregulated by microarray analysis of siRNA-treated CT26WT cells (n=3). qPCR of VSVΔ51 genomes was also performed to confirm the increase in VSVΔ51 replication as a result of siRNA#1 and siRNA#2. Data are presented as means ± SEM.

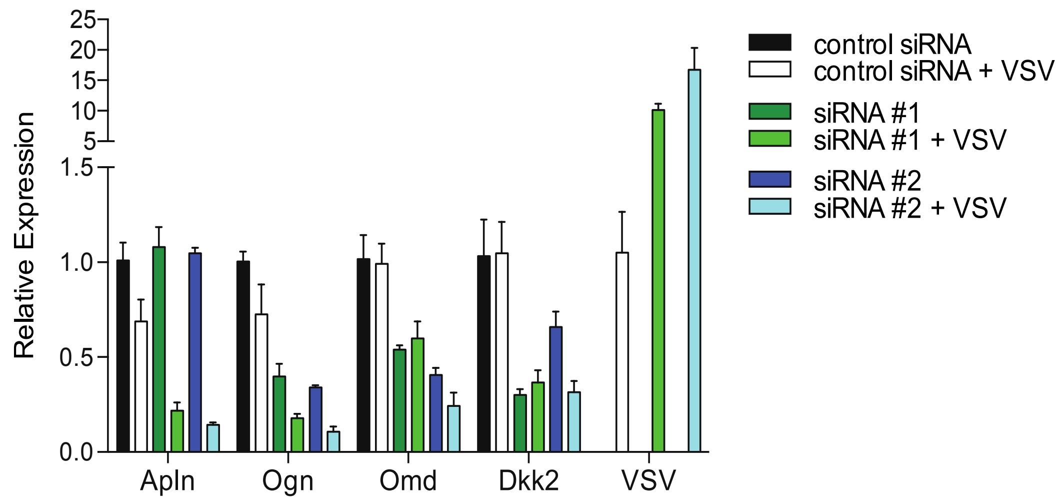
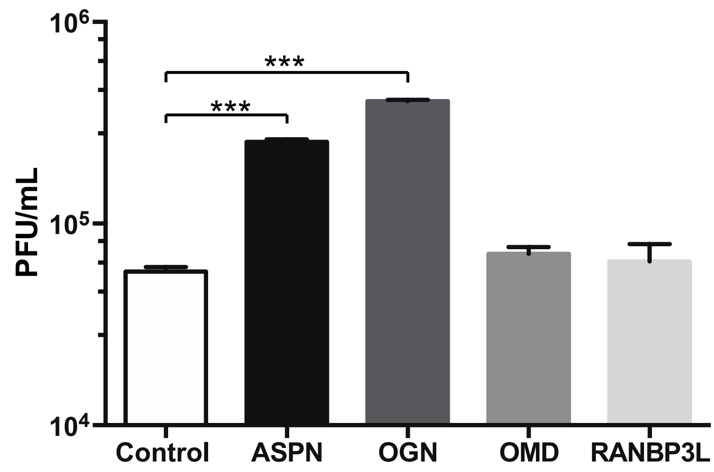

Relative Expression
25
20
15
10
5
1.5
1.0
0.5
0.0
Apln
Ogn
Omd
Dkk2
VSV
control siRNA
control siRNA + VSV
siRNA #1
siRNA #1 + VSV
siRNA #2
siRNA #2 + VSV

Figure 6.11: Knockdown of the SLRPs ASPN and OGN promotes VSV replication in CT26WT cells.

CT26WT cells were transfected with the indicated siRNAs and 48h post-transfection were infected with VSVΔ51. Supernatants were collected 48h post-infection and virus was quantified (n=3). Data are presented as means ± SEM. Student's unpaired, one-tailed t-test was performed; ***: $p < 0.001$.



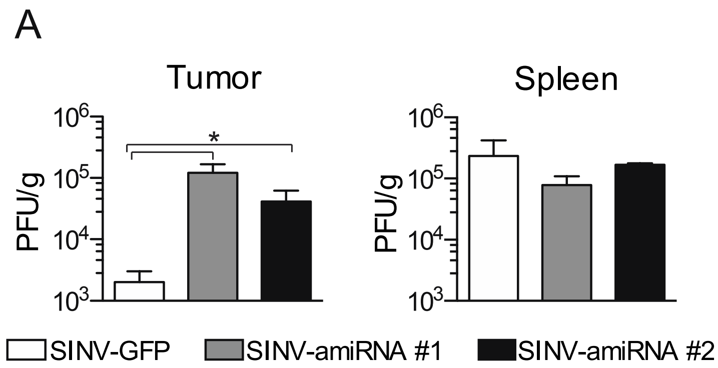
10^6
10^5
10^4
PFU/mL
***
***
Control
ASPN
OGN
OMD
RANBP3L

viruses replicated to titers at least 30 times that of the control virus within 48h after injection (Fig, 6.12A, left panel). Surprisingly, we did not find any differences in virus titers in the spleens of the mice (Fig. 6.12A, right panel). We performed similar experiments with VSVΔ51 amiRNA-expressing viruses but with IV delivery of the virus instead. We looked at virus titers in the tumours 48h after injection and found that the amiRNA-expressing viruses replicated to titers 50 times higher than control virus (Fig. 6.12B, left panel). Once again, we looked at virus titers in the spleen and observed no significant differences between the control virus and the amiRNA-expressing viruses (Fig. 6.12B, left panel).

Finally, we assessed whether the increase in replication we observe translates to an enhanced therapeutic effect. To test this, we established subcutaneous CT26WT tumours in mice and treated them IT daily for five days with the different viruses and monitored tumour growth. Although VSVΔ51-YFP was able to control tumour growth compared to no treatment, the delay in tumour progression was even more pronounced with VSVΔ51-amiRNA#1 (Fig. 6.13). These data demonstrate that the enhanced replication conferred by amiRNA expression translate into better tumour control.

Figure 6.12: amiRNA-expressing viruses show enhanced *in vivo* replication in tumours.

A) Quantification of SINV in tumours (n=6) and spleens (n=2) 48h post-IT treatment of CT26WT tumour bearing mice with $1x10^8$ PFU. Data are presented as means ± SEM. Student's unpaired, one-tailed t-test was performed; *: $p < 0.05$. B) Quantification of VSVΔ51 in tumours (n=14) and spleens (n=3) 48h post-IV treatment of CT26WT tumour bearing mice with $5x10^8$ PFU. Data are presented as means ± SEM. Student's unpaired, one-tailed t-test was performed; *: $p < 0.05$.


A
Tumor
Spleen
PFU/g
10^6
10^5
10^4
10^3
*
SINV-GFP
SINV-amiRNA #1
SINV-amiRNA #2

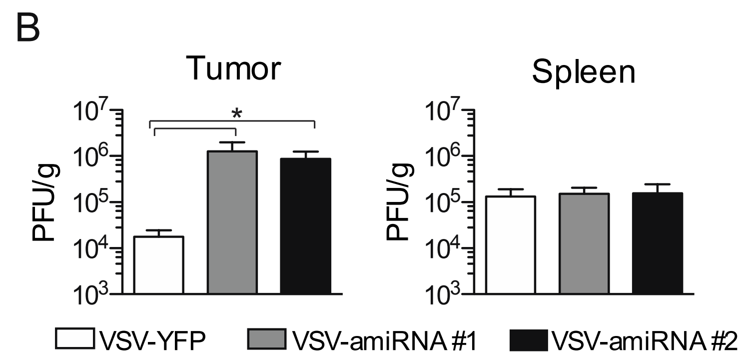
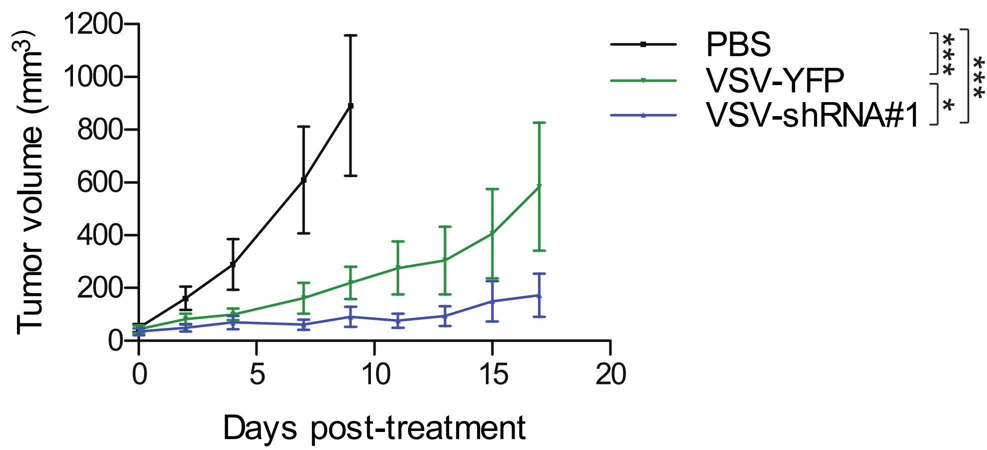

B
Tumor
Spleen
PFU/g
10^7
10^6
10^5
10^4
10^3
*
VSV-YFP
VSV-amiRNA #1
VSV-amiRNA #2

Figure 6.13: VSVΔ51-amiRNA#1 virus controls tumour growth better than the parental virus.

Tumour volume of mice (n=7) bearing CT26 subcutaneous tumours treated IT daily with 5×10^8 PFU for 5 days. Values represent mean ± SEM. A two-way ANOVA test was performed; *: $p < 0.05$, ***: $p < 0.001$.


Tumor volume (mm3)
1200
1000
800
600
400
200
0
0
5
10
15
20
Days post-treatment
PBS
VSV-YFP
VSV-shRNA#1
***
*
***

7. DISCUSSION

Here we show that screening of a replication-competent SINV-amiRNA library results in the selection of amiRNA sequences that increase virus replication specifically in tumour cells. After as little as 4 passages in tumour cells, amiRNAs that were initially present at frequencies below 0.04% in the initial population were enriched to frequencies as high as 58% (Fig. 6.1B,C and Table 6.1). This enrichment is a combination of positive selection for amiRNAs that enhance SINV replication as well as negative selection against amiRNAs that are detrimental to virus replication. Even amiRNAs that have no impact on virus replication are selected against as self-targeting could occur in these virus clones[283]. Notably, secreted antiviral factors such as IFN may also not be positively selected for since knockdown of such genes would benefit the entire virus population as opposed to benefiting an individual clone and would potentially not be uncovered by our screening approach. Also, given that SINV is able to antagonize antiviral responses by shutting off host transcription[293–295], some genes may already be sufficiently repressed that further knockdown would lead to no benefit. It is also important to note that the kinetics of amiRNA expression is governed by SINV's life cycle. This certainly impacts the type of amiRNAs that are enriched during the screen, as amiRNAs that target genes encoding proteins with a half-life longer than the virus life cycle will not be enriched. Similarly, amiRNAs that target genes involved in regulating virus entry or uncoating would not be selected for as these events precede viral gene transcription. This is not the case with classic siRNA or shRNA-based screens where knockdown of genes occurs before virus infection is initiated and thus genes regulating virus entry have been uncovered[296].

We chose to focus on the two most enriched amiRNAs as these were also found in all five biological replicates (Fig. 6.1C and Table 6.1). Some of the amiRNAs were enriched but

in only one or two of the replicates. This may be due to unrelated mutations in the virus backbone that were positively selected for. Another possibility is that, despite our efforts to ensure that all SINV-amiRNA clones were sufficiently sampled, some of the clones are at such low frequencies in the initial inoculum that the probability that they actually infect a cell is low and a stochastic process. It is also important to note that some of the clones at very low frequencies in the initial pool may not have a chance to infect and replicate to high enough numbers to be carried forward to the subsequent passage even if they do provide a growth advantage to the virus.

In our *in vitro* experiments, siRNA knockdown resulted in larger increases in virus replication compared to virus-mediated amiRNA expression (Fig. 6.2A vs Fig. 6.3; Fig. 6.4A bottom panels vs Fig. 6.6). This is most likely because siRNAs delivered prior to infection allow for more efficient knockdown of the relevant target genes. *In vivo*, virus-mediated amiRNA expression resulted in larger increases in virus replication than *in vitro* (Fig. 6.3 vs Fig. 6.12A left panel; Fig. 6.6 vs Fig, 6.12B left panel) most likely due to the fact that selective pressure against the virus is greater *in vivo* and thus increases in viral fitness are more pronounced. Nonetheless, the potential to pre-condition the tumour for viral infection with *in vivo* delivery of tumour-targeted siRNAs[297–299] is an approach worth investigating that may result in even more dramatic increases in virus replication as compared to virus-mediated amiRNA expression. This is particularly relevant for VV for which virus-mediated expression of amiRNAs is not a viable strategy to increase replication since the virus expresses a protein, VP55, which is known to polyadenylate various RNA species including miRNAs, resulting in their degradation[213]. Another strategy worth investigating with VSV-amiRNA expression is to clone the amiRNA upstream of its current position – between the G and L genes – in the viral genome. Due to the discontinuous transcription of VSV genes and

the resulting gradient of mRNAs that is created where VSV-N transcripts are the most abundant and VSV-L transcripts are the least abundant[104], moving the amiRNA upstream of its current position would result in increased expression levels. Since there is an approximately 30% reduction across each gene junction, moving it upstream can result in a two to four-fold increase in amiRNA levels. The only position in which it cannot be placed is between the VSV-N and VSV-P genes as disruption of the ratio of these two genes dramatically attenuates the virus[300].

Although the screen was performed with SINV, a positive-sense RNA virus, the identified amiRNAs were also able to increase the replication of oncolytic VSVΔ51 and MG1, which are negative-sense RNA viruses (Fig. 6.4A). These findings suggest that the amiRNAs target antiviral factors that antagonize both viruses. This is not surprising given that both viruses are IFN-sensitive and that several ISGs can act on a wide range of viruses[275,301,302]. Interestingly, the replication of VVdd, a double-stranded DNA virus that encodes a vast array of immune-modulatory proteins, was also increased but only with siRNA#1, suggesting that a subset of target genes of siRNA#1 are different than those of siRNA#2 (Fig. 6.4A top panels). Importantly, the amiRNA-expressing SINVs also displayed enhanced replication in HCT 116 cells (Fig. 6.3B), suggesting that the relevant mRNA targets are also expressed in human tumour cells and that the amiRNA target sequences are conserved between species. We also conducted similar SINV-amiRNA library screens in various cell lines, including 786-O (human renal cell carcinoma), Mia PaCa (human pancreatic cancer), Panc02 (murine pancreatic cancer) and PANC-1 (human pancreatic cancer) and found amiRNA#1 and amiRNA#2 to be enriched, further supporting the notion that the findings are not specific only to mouse or human colon cancers (data not shown).

The mechanisms by which the amiRNAs are increasing virus replication remain to be determined. We focused our initial analysis on the IFN pathway and actually observed more IFN-β mRNA and protein production with siRNA knockdown in tumour cells, a phenomenon that we attribute to the increase in viral replication (Figs. 6.7A,B and 6.8). We also looked at the expression of ISGs in order to assess the IFN responsiveness of the cells and found that these were also higher with siRNA knockdown (Fig. 6.7C). These results strongly suggest that the IFN pathway is not targeted by the amiRNAs, but does not rule out the possibility that other pertinent ISGs may be affected. Surprisingly, microarray analysis revealed a marked overlap in the genes differentially expressed by siRNA#1 and #2, suggesting that these siRNAs may function similarly to increase virus replication, at least in the case of VSV (Fig. 6.9). Notably, 4 of the 17 members of the small leucine-rich proteoglycan (SLRP) gene family (*OMD, OGN, ASPN, BGN*) were found to be downregulated by both siRNA#1 and siRNA#2 (Figs. 6.9A and 6.10). This suggests that these genes all have target sites in their mRNAs, although miRNA/siRNA target prediction programs did not support this notion, or that they are regulated by a common transcription factor that is also a target of siRNA#1 and siRNA#2. However, only knockdown of ASPN and OGN resulted in increased VSV replication (Fig. 6.11). It is still unclear as to how these genes directly or indirectly regulate virus replication, as SLRPs are involved in many biological processes including development, regeneration, cell proliferation, inflammation, and immunity[303–305]. These proteins reside mostly in the extracellular space and can interact with collagen and other components of the ECM, secreted factors and cell surface receptors to modulate a variety of cellular processes [303,305–308]. Importantly, it has been demonstrated that ASPN, BGN and decorin, another SLRP, can bind to TLR2 and TLR4 to induce an inflammatory response in macrophages[309–311], therefore knockdown of these genes may

promote virus infection by reducing the expression of NF-κB target genes, many of which are involved in the antiviral response[312]. Interestingly, OGN has an IFN-stimulated response element in its promoter[313,314], thus hinting that it may have a still undiscovered role in the IFN response. However, our microarray did not detect any upregulation of OGN as a result of virus infection. Another possibility is that virus replication is increased through modulation of extracellular collagen levels, as it has been demonstrated that collagen and other ECM components negatively impact OV spread[85,86,315]. Thus, the impact of the identified amiRNAs on collagen levels should be investigated in future experiments. Overall, our findings suggest downregulation of OGN and ASPN increases virus replication but there may be other target genes that we did not identify or investigate that may also contribute to the increased virus replication observed.

It is important to note that there are limitations to siRNA transfection followed by microarray analysis when trying to identify target genes. Aside from the fact that transfections may introduce supraphysiological amounts of siRNA which can lead to off-target effects[258,316,317], microarray analysis may not give a complete picture of the cellular changes that occur as a result of the siRNAs since they may also be regulating the translation of target changes as opposed to inducing their downregulation[265]. Thus polysome profiling may be a complimentary technique that would reveal genes whose translation is affected by the siRNAs. Another strategy would be to perform a immunoprecipitation of the RISC complex followed by sequencing of the mRNAs that are associated with the complex, thus providing an insight as to which mRNAs are targeted by the siRNAs[318–320]. The major advantage of this strategy is that it has the potential to identify relevant targets independent of the mechanism of action of the siRNAs.

Our *in vivo* studies revealed that amiRNA-mediated increases in virus replication were tumour-specific (Fig. 6.12) The tumour-specific increases in virus replication are likely a consequence of an already favorable cellular state for virus replication present in cancer cells. This environment enables robust virus replication and therefore high levels of amiRNA expression. In normal cells, virus replication is limited and thus the levels of amiRNA expression are not high enough to impact virus replication. A similar scenario has been mathematically modeled to explain the tumour-specificity of oncolytic VSVΔ51 expressing a soluble IFN-binding decoy receptor[88].

In summary, we have demonstrated the utility of our screening approach in generating more effective OVs. The ease at which this screening strategy can be performed opens the potential to generate ready-to-use OVs tailored to a specific tumour-type or perhaps even personalized for a given patient's tumour. Although we utilized SINV to conduct our initial screen, this strategy can easily be translated to any single stranded positive sense RNA virus as the rescue of these viruses is quite efficient which is an important requirement when generating large libraries of unique clones. The information obtained from these screens may also be applied to manufacturing of large quantities of viruses, such as the production of OVs for clinical use or vaccines.

CONCLUDING REMARKS

The work described in chapter 1 explored the use of insect cells as delivery vehicles for the systemic administration of OVs. Previous studies with various cell carriers revealed that they were limited by their inability to circulate efficiently, their off-target tissue tropisms and the cytotoxic nature of virus on these cell types. We have shown that insect cells can be engineered to express additional therapeutic transgenes that complement oncolytic virotherapy, that they circulate better than their mammalian counterparts, and that they lack any obvious tissue tropism. In chapter 2, we highlight how an RNAi-based screening approach can be utilized to discover amiRNAs that, when expressed by OVs, can substantially increase the virus replication within tumour cells.

As is the case with the development of any new treatment strategy for cancer, the end goal is to discover a therapeutic that will dramatically improve patient outcomes. Given that the field of modern oncolytic virotherapy is still in its infancy, with clinical trials just beginning to provide evidence of therapeutic activity, it is difficult to predict what strategies will be needed to further improve OVs. The initial focus was, of course, on ensuring that OVs were safe, and this is still a major concern when attempting to treat potentially immune-compromised cancer patients with live replicating viruses. However, at least in pre-clinical murine tumour models, it is evident that OVs often cannot cure animals on their own and so there was a push to make OVs more potent either by engineering them or by combining them with other existing treatment modalities. It was also recognized that systemic delivery of OVs is very inefficient. In the last few years, there has been a paradigm shift in the field of oncolytic virotherapy with the realization that their immune stimulating properties are a critical aspect of their mechanisms of action. Current pre-clinical studies are therefore aimed at understanding and improving this important aspect of OV therapy.

Despite all these advances at the pre-clinical level, it is my opinion that the results of ongoing clinical trials with first-generation OVs will be instrumental in guiding the development of future strategies. For example, if dose-limiting toxicities are observed with first-generation OVs undergoing clinical testing, the regulatory bodies may not approve strategies aimed at increasing the replication of OVs. Aside from regulatory hurdles, there are also some important biological questions that remain to be answered. It is not clear at this time as to how much virus replication within tumours is sufficient to result in tumour debulking and to generate anti-tumour immune responses. Along these lines, is systemic delivery of OVs really necessary if local treatment can generate systemic responses? Relevant to the work described in this thesis, clinical trials with various cell carriers are just beginning and so the results of these studies will hopefully serve to validate this approach and perhaps identify aspects in need of improvement. Despite these unknowns, the rapid and exciting developments in the field that have occurred in the last 15 years are evidence that there are no shortages of potential strategies that can applied or combined with OVs and that the next phase of pre-clinical and clinical testing promises to be as exciting.

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CONTRIBUTION OF COLLABORATORS

Dr. Anthony Power initiated the insect cell carrier project and he performed the experiments described in figures 3.1C,D; 3.2B; and 3.10A,C. Dr. Marie-Claude Bourgeois-Daigneault performed the flow cytometry in figure 3.13 A-B.

Dr. Benjamin tenOever and Dr. Andrew Varble constructed the SINV-amiRNA library and performed deep sequencing and initial data analysis of the screen in CT26WT cells. They also provided guidance in the design of the screen. Adrian Pelin assisted in generation of the heat map in figure 6.1 and analysis of the microarray described in figure 6.9.

APPENDICES

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Article

Murine Tumor Models for Oncolytic Rhabdo-Virotherapy

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Abstract

The preclinical optimization and validation of novel treatments for cancer therapy requires the use of laboratory animals. Although in vitro experiments using tumor cell lines and ex vivo treatment of patient tumor samples provide a remarkable first-line tool for the initial study of tumoricidal potential, tumor-bearing animals remain the primary option to study delivery, efficacy, and safety of therapies in the context of a complete tumor microenvironment and functional immune system. In this review, we will describe the use of murine tumor models for oncolytic virotherapy using vesicular stomatitis virus. We will discuss studies using immunocompetent and immunodeficient models with respect to toxicity and therapeutic treatments, as well as the various techniques and tools available to study cancer therapy with Rhabdoviruses.

Key words: biotherapeutic; cancer; efficacy; murine model; oncolytic virus; treatment; tumor; vesicular stomatitis virus (VSV)

Background

The lack of options available for patients with chemotherapy-resistant, advanced, aggressive, or systemic disease is pushing the field of cancer therapy to progress rapidly, with novel strategies being developed. Of the many promising approaches that recently emerged, oncolytic viruses (OVs) have the potential of treating disseminated diseases, targeting various tumor types, and inducing durable systemic antitumor immune responses with minimal side effects compared with current therapies (Singh et al. 2012). OVs are selected or designed to specifically destroy cancer cells (Zeyaullah et al. 2012). Although there are many different OVs currently being investigated around the world, the focus of this review is on the Rhabdovirus family member vesicular stomatitis virus (VSV). VSV is a single-stranded, negative-sense RNA virus that can infect both insects and mammals (Kuzmin et al. 2009). These viruses are highly sensitive to the antiviral activity of interferon (IFN), and thus in normal cells, infection is controlled through the induction of the IFN response (Stojdl, Lichty, et al. 2000). However, several tumor cell lines (SK-MEL3, LNCAP, LC80, and OVCA 420), unlike normal cells

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ARTICLE

Oncolytic vesicular stomatitis virus expressing interferon-γ has enhanced therapeutic activity

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Oncolytic viruses are known to stimulate the antitumor immune response by specifically replicating in tumor cells. This is believed to be an important aspect of the durable responses observed in some patients and the field is rapidly moving toward immunotherapy. As a further means to engage the immune system, we engineered a virus, vesicular stomatitis virus (VSV), to encode the proinflammatory cytokine interferon-γ. We used the 4T1 mammary adenocarcinoma as well as other murine tumor models to characterize immune responses in tumor-bearing animals generated by treatment with our viruses. The interferon-γ-encoding virus demonstrated greater activation of dendritic cells and drove a more profound secretion of proinflammatory cytokines compared to the parental virus. From a therapeutic point of view, the interferon-γ virus slowed tumor growth, minimized lung tumors, and prolonged survival in several murine tumor models. The improved efficacy was lost in immunocompromized animals; hence the mechanism appears to be T-cell-mediated. Taken together, these results demonstrate the ability of oncolytic viruses to act as immune stimulators to drive antitumor immunity as well as their potential for targeted gene therapy.

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INTRODUCTION

The capability to evade the immune system is essential for tumor establishment and has been identified as a hallmark of cancer.[1] Conversely, an effective antitumor immune response against tumor-associated antigens is believed to prevent relapse of the disease.[2] Various proinflammatory cytokines stimulate immunity and their exogenous administration has been used for the treatment of many cancers.[3] Among these, interferon-γ (IFNγ), has a myriad of roles in both arms of immunity.[4] It is mainly produced by T cells, natural killer (NK) cells, and macrophages and has effects on all nucleated cells of the body.[5] It is a powerful immune modulator that has antiproliferative, proapoptotic, and antiangiogenic functions.[5,6] It also induces adaptive immunity through increased antigen presentation and T-cell activation.[7] The pivotal role of IFNγ in tumor immunosurveillance is exemplified by the observation that mice deficient for the IFNγ receptor develop tumors more rapidly and at a higher frequency.[8] Many observations show that the presence of IFNγ might be beneficial in the treatment of various cancers. For examples, in human breast cancer samples, the expression of IFNγ and its receptor were reported to be increased in benign lesions compared to malignant lesions.[9] Because of these observations, along with its many protective functions against cancer and its immune-stimulating properties, IFNγ has long been considered a promising candidate for the treatment of various malignancies.[5] It was demonstrated using the 4T1 murine breast cancer model that intratumoral (IT) administration of IFNγ reduces metastases to the lungs and liver.[10] In the clinic, it has been shown that that local injection of IFNγ in skin lesions of breast cancer patients resulted in partial regression.[11] Also, recombinant human IFNγ-1b has successfully been used to delay progression of microbial infections in chronic granulomatous disease,[12] but its use in human cancer treatment has given mixed results.[13–16] One of the reasons for the limited efficacy was attributed to the very short half-life of IFNγ in serum and therefore, local expression is a more promising approach.[17] One successful strategy to deliver genes to the tumor site is to use viruses. Indeed, the direct IT injection of virus expressing IFNγ demonstrated efficacy in two different xenograft models.[18] Tumor-specific oncolytic viruses (OV) encoding transgenes have also been shown to be an efficient tool to drive sustained local transgene expression.[19,20]

OVs like the vesicular stomatitis virus (VSV) were selected to specifically destroy tumor cells while leaving normal tissues unaffected.[21] While normal cells are protected by type I IFNs, cancer cells frequently acquire defects in this pathway. Thus, normal, but not tumor cells, will be protected against VSV.[22] To further attenuate VSV,

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Programmable insect cell carriers for systemic delivery of integrated cancer biotherapy

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Cell carriers
Gene delivery
Systemic delivery

ABSTRACT

Due to cancer's genetic complexity, significant advances in the treatment of metastatic disease will require sophisticated, multi-pronged therapeutic approaches. Here we demonstrate the utility of a *Drosophila melanogaster* cell platform for the production and in vivo delivery of multi-gene biotherapeutic systems. We show that cultured *Drosophila* S2 cell carriers can stably propagate oncolytic viral therapeutics that are highly cytotoxic for mammalian cancer cells without adverse effects on insect cell viability or gene expression. *Drosophila* cell carriers administered systemically to immunocompetent animals trafficked to tumors to deliver multiple biotherapeutics with little apparent off-target tissue homing or toxicity, resulting in a therapeutic effect. Cells of this Dipteran invertebrate provide a genetically tractable platform supporting the integration of complex, multi-gene biotherapies while avoiding many of the barriers to systemic administration of mammalian cell carriers. These transporters have immense therapeutic potential as they can be modified to express large banks of biotherapeutics with complementary activities that enhance anti-tumor activity.

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1. Introduction

Genetically encoded biotherapeutics with naturally evolved activities can be harnessed for the treatment of disease. In particular, proteins with the ability to regulate cell growth, modulate the host immune system, or recognize tumor-specific antigens are of interest in the treatment of cancer. Although many tested agents demonstrate highly specific therapeutic effects when inoculated or expressed directly in tumors, they often lose potency when administered systemically due to poor stability in blood and/or rapid clearance from the circulation [1]. Incorporation of biotherapeutic genes into viral vectors can help increase the efficiency of delivery to the tumor site. In particular, conditionally replicating oncolytic viruses can be delivered systemically to tumors where they rapidly self-amplify to manufacture the therapeutic transgene specifically at the site of disease [2]. However, host immunity remains a major challenge to the clinical realization of this goal [3–5]. Intravenous delivery exposes oncolytic viruses to circulating factors such as neutralizing antibodies, which bind to and neutralize virus directly or mark them for destruction by complement and by various immune cells [6]. Virus is also neutralized by non-specific binding to serum proteins and circulating cells present in the bloodstream [7]. Organs such as the lungs, spleen, and especially the liver, also play a significant role in clearing virus as these tissues contain resident macrophages whose role is to scavenge the blood for circulating pathogens [7].

We have previously demonstrated that cellular carriers can shield oncolytic virus from neutralization to achieve systemic delivery to tumors in the presence of circulating antibodies [3]. This strategy has successfully been used to improve the delivery of various oncolytic virus platforms including adenovirus [8–10], measles virus [11–13], vesicular stomatitis virus (VSV) [3,14,15], reovirus [16–19], Newcastle disease virus [20], herpes simplex virus [21,22], parvovirus [23], retroviruses [24,25], myxoma virus [26] and vaccinia virus [27,28]. Clinical application and validation of this approach are currently ongoing [19,29].

Our group and others have investigated the ability of numerous mammalian cell types to function as oncolytic virus carriers for systemic therapy in pre-clinical models, including solid [3,23,28] and hematogenous [3,11] cell lines, cytokine-induced killer cells [27,30], T cells [13,21,25], primary monocytes [12,31], myeloid derived suppressor cells [15] and mesenchymal stem cells [32,33]. However, there remain significant obstacles to using any mammalian cell type for systemic oncolytic

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MicroRNAs regulate the immunometabolic response to viral infection in the liver

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Immune regulation of cellular metabolism can be responsible for successful responses to invading pathogens. Viruses alter their hosts' cellular metabolism to facilitate infection. Conversely, the innate antiviral responses of mammalian cells target these metabolic pathways to restrict viral propagation. We identified miR-130b and miR-185 as hepatic microRNAs (miRNAs) whose expression is stimulated by 25-hydroxycholesterol (25-HC), an antiviral oxysterol secreted by interferon-stimulated macrophages and dendritic cells, during hepatitis C virus (HCV) infection. However, 25-HC only directly stimulated miR-185 expression, whereas HCV regulated miR-130b expression. Independently, miR-130b and miR-185 inhibited HCV infection. In particular, miR-185 significantly restricted host metabolic pathways crucial to the HCV life cycle. Interestingly, HCV infection decreased miR-185 and miR-130b levels to promote lipid accumulation and counteract 25-HC's antiviral effect. Furthermore, miR-185 can inhibit other viruses through the regulation of immunometabolic pathways. These data establish these microRNAs as a key link between innate defenses and metabolism in the liver.

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Viruses alter host cellular metabolism in order to meet the material and energy demands of their life cycles. Several viruses induce specific lipid microenvironments to facilitate different stages of their life cycles, including entry[1], replication[2,3] and assembly[4]. Metabolic pathways subverted by viruses for their propagation represent strategic targets for host innate defenses against viral infection, yet only few examples of such regulatory links have been reported[5].

Recent work has shown that cholesterol-25-hydroxylase (CH25H) shows interferon (IFN)-stimulated expression and catalyzes the synthesis of a broadly antiviral lipid effector, 25-hydroxycholesterol (25-HC)[6–8]. Both macrophages and dendritic cells secrete 25-HC in response to IFN stimulation[9]. 25-HC's antiviral activity against several classes of viruses has been linked to inhibition of virus cell-membrane fusion[6,7]. However, 25-HC has membrane-independent effects due to its enantioselective antiviral activity through alterations of cellular signaling pathways[6,7].

MicroRNAs (miRNAs) have emerged as critical post-transcriptional regulators of gene expression[10], acting by binding to the 3′ UTRs of mammalian mRNAs to induce translational repression and/or mRNA destabilization. Through this mechanism, miRNAs are predicted to regulate over 60% of transcripts[11] and to influence diverse processes, including metabolism[12]. Herein we identified a 25-HC–induced miRNA, miR-185, that regulates the antiviral metabolic response to HCV infection in the liver. We demonstrated that miR-185 is an important regulator of the HCV life cycle through its effects on cellular lipid microenvironments crucial to the virus. HCV counteracted 25-HC's antiviral effects through inhibition of miR-185 and miR-130b expression. These findings highlight the importance of miRNAs in controlling the metabolic state of an infected cell as well as the immunometabolic response to viral infection in the liver.

RESULTS

Identifying miRNAs relevant to immunometabolism

25-HC broadly inhibits viruses[6,7], including the flaviviruses hepatitis C virus (HCV; **Supplementary Results**, **Supplementary Fig. 1**) and dengue virus (DENV) (**Supplementary Fig. 2**). In HCV-infected patients, both CH25H expression in the liver and 25-HC levels in the serum are elevated[13]. Therefore, secretion of 25-HC by liver-resident macrophages and dendritic cells is likely to play an important role in the hepatic innate antiviral response to HCV infection. 25-HC acts intracellularly as an inhibitor of sterol response element–binding protein (SREBP) processing[14,15] and agonist of liver X receptor (LXR)-α signaling[16,17], two pathways that regulate hepatic metabolism and the HCV life cycle[8,18,19]. Conversely, 19-HC, an oxysterol that is unable to inhibit the SREBP pathway[15], did not mediate any antiviral effect against HCV (**Fig. 1a**). This demonstrated that 25-HC's inhibition of HCV is linked to modulations in metabolic gene-regulatory networks.

We employed an miRNA profiling strategy termed small molecule–mediated annotation of miRNA targets (SMART) to identify miRNAs stimulated by 25-HC that mediate immunometabolic changes in gene expression (see Online Methods, **Fig. 1b** and **Supplementary Fig. 3**). Huh7.5 hepatoma cells were infected with a high-titer HCV strain (JFH-1_T; genotype 2a)[20] to determine the differential expression of host miRNAs relevant to HCV. Profiling revealed 185 differentially expressed miRNAs (**Fig. 1b**; **Supplementary Table 1**; $P < 0.05$). In parallel, we conducted miRNA profiling on JFH-1_T–infected cells exposed to 5 μM

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Bacterial-Mediated Knockdown of Tumor Resistance to an Oncolytic Virus Enhances Therapy

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Oncolytic viruses (OVs) and bacteria share the property of tumor-selective replication following systemic administration. In the case of nonpathogenic bacteria, tumor selectivity relates to their ability to grow extracellularly within tumor stroma and is therefore ideally suited to restricting the production of bacterially produced therapeutic agents to tumors. We have previously shown the ability of the type 1 interferon antagonist B18R to enhance the replication and spread of vesicular stomatitis virus (VSV) by overcoming related cellular innate immunity. In this study, we utilized nonpathogenic bacteria (*E. coli*) expressing B18R to facilitate tumor-specific production of B18R, resulting in a microenvironment depleted of bioactive antiviral cytokine, thus "preconditioning" the tumor to enhance subsequent tumor destruction by the OV. Both *in vitro* and *in vivo* infection by VSVΔ51 was greatly enhanced by B18R produced from *E. coli*. Moreover, a significant increase in therapeutic efficacy resulted from intravenous (IV) injection of bacteria to tumor-bearing mice 5 days prior to IV VSVΔ51 administration, as evidenced by a significant reduction in tumor growth and increased survival in mice. Our strategy is the first example where two such diverse microorganisms are rationally combined and demonstrates the feasibility of combining complementary microorganisms to improve therapeutic outcome.

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INTRODUCTION

Oncolytic viruses (OVs) promise to improve cancer patient outcomes through their tumor-selective replication and multi-modality attack against cancers.[1,2] Vesicular stomatitis virus (VSV) has a broad cancer cell tropism and is effective when administrated intravenously in murine tumor models. The wild-type strain of VSV expresses a protein, the M or matrix protein, that upon infection acts as an intracellular antagonist of interferon (IFN) production by blocking the transport of IFN mRNAs from the nucleus.[3] Wild-type VSV is neurotoxic in certain mouse strains, while an attenuated version of the virus (VSVΔ51) retains oncolytic activity but is harmless when administered intravenously as the virus can now only productively infect tumor cells that have a defective IFN response.[4] However, one of the major problems with oncolytic virotherapy, is that that some tumors, or regions of tumors, have intact or upregulated antiviral responses, and related intra- and intertumor heterogeneity can result in incomplete oncolysis following VSV therapy. Some human-derived tumor cell lines such as HT29 colon carcinoma retain at least partial responsiveness to IFN and only poorly support the spread of VSVΔ51. We have previously determined the IFN responsiveness of HT29 cells and demonstrated that they could be protected from infection by VSVΔ51 through addition of exogenous IFN-α.[1] Previous studies from our lab have identified that localized expression of *B18R*, a gene from *Vaccinia* virus which encodes a secreted decoy receptor with a broad antagonizing effect against type 1 IFNs, significantly improved the efficacy of the attenuated VSVΔ51 to grow and kill tumors.[1,5,6] A number of studies have explored combination viral strategies,[7–9] but toxicity and safety remain a concern, as knockdown of type 1 IFN in tissues other than tumor presents the risk of susceptibility to unrestricted replication of other infecting viruses. Therefore, a tightly controlled mechanism for tumor-specific B18R production is required to facilitate this strategy.

The phenomenon of tumor-specific bacterial replication has been known for a century. The specificity is believed to be as a result of the uniqueness of tumor physiology resulting from a combination of factors such as local immune suppression, irregular vasculature, relevant nutrient presence in necrotic tissue and the anaerobic nature of hypoxic/necrotic regions within tumors promoting growth of anaerobic and facultatively anaerobic bacteria.[10,11] These regions of tumors are vital to target therapeutically, being a major source of cells responsible for tumor regrowth posttreatment. Many bacteria are readily genetically engineered to produce heterologous proteins. Unlike typical gene delivery vectors, the property of tumor cell invasion is not required, as bacteria themselves can express the gene of interest. As a means of local *in situ* production therefore, noninvasive bacteria (lacking the ability to mediate disease) present an ideal opportunity to act as a "protein factory" within the tumor. Many species of bacteria are health-promoting or probiotic, including certain strains of *Escherichia coli*.[12–14] We have previously demonstrated tumor-specific replication of *E. coli* MG1655 in mice.[15]

We hypothesized that delivery of *E. coli* producing B18R, prior to systemic VSV administration, would safely facilitate tumor-specific knockdown of type 1 IFN and locally enhanced VSV-mediated tumor oncolysis. This study examines the replication

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REVIEW

Cell carriers for oncolytic viruses: current challenges and future directions

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Abstract: The optimal route for clinical delivery of oncolytic viruses is thought to be systemic intravenous injection; however, the immune system is armed with several highly efficient mechanisms to remove pathogens from the circulatory system. To overcome the challenges faced in trying to delivery oncolytic viruses specifically to tumors via the bloodstream, carrier cells have been investigated to determine their suitability as delivery vehicles for systemic administration of oncolytic viruses. Cell carriers protect viruses from neutralization, one of the most limiting aspects of oncolytic virus interaction with the immune system. Cell carriers can also possess inherent tumor tropism, thus directing the delivery of the virus more specifically to a tumor. With preclinical studies already demonstrating the success and feasibility of this approach with multiple oncolytic viruses, clinical evaluation of cell-mediated delivery of viruses is on the horizon. Meanwhile, ongoing preclinical studies are aimed at identifying new cellular vehicles for oncolytic viruses and improving current promising cell carrier platforms.

Keywords: oncolytic virus, cell carrier, systemic delivery, tumor targeting, cancer

Introduction

Oncolytic viruses infect and kill tumor cells while leaving normal tissues unharmed. Specificity toward cancer cells can be a natural feature of the virus, as is the case with reovirus, Newcastle disease virus, and mumps virus, or it can be selected for or engineered into the virus through the use of tumor-specific cell surface molecules,[1] transcription factors,[2] and tissue-specific microRNAs.[3] Similarly, vesicular stomatitis virus, herpes simplex virus, and adenovirus have been genetically attenuated by subduing their ability to antagonize antiviral defenses, thus improving tumor specificity. This strategy leads to enhanced replication in tumor cells, which often possess defects in antiviral pathways,[4] while sparing normal cells. Oncolytic viruses exert their antitumor activities through both direct and indirect mechanisms. Direct infection of tumor cells leads to virus and immune-mediated cytotoxicity, and in some cases, alerting the immune system to the previously tolerated tumor through the recruitment of natural killer (NK) cells and cluster of differentiation (CD)8^{+} cytotoxic T cells.[5] Infection of tumor vasculature can lead to vascular collapse and compromised blood flow within the tumor, thus choking off its access to nutrients.[6] To increase potency, oncolytic viruses have been engineered to express genes that augment virus replication,[7] induce cytotoxicity,[8] promote bystander cell killing,[9] and enhance antitumor immunity.[5] Of overarching concern, however, is that these numerous improvements will provide no benefit to antitumor efficacy unless the virus is successfully delivered to the tumor. Although direct intratumoral injection should deliver all virus particles

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Model-based rational design of an oncolytic virus with improved therapeutic potential

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Oncolytic viruses are complex biological agents that interact at multiple levels with both tumour and normal tissues. Antiviral pathways induced by interferon are known to have a critical role in determining tumour cell sensitivity and normal cell resistance to infection with oncolytic viruses. Here we pursue a synthetic biology approach to identify methods that enhance antitumour activity of oncolytic viruses through suppression of interferon signalling. On the basis of the mathematical analysis of multiple strategies, we hypothesize that a positive feedback loop, established by virus-mediated expression of a soluble interferon-binding decoy receptor, increases tumour cytotoxicity without compromising normal cells. Oncolytic rhabdoviruses engineered to express a secreted interferon antagonist have improved oncolytic potential in cellular cancer models, and display improved therapeutic potential in tumour-bearing mice. Our results demonstrate the potential of this methodology in evaluating potential caveats of viral immune-evasion strategies and improving the design oncolytic viruses.

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

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

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

In order to probe further and overcome the mechanisms of glioma resistance to oncolytic SFV, we combined it with oncolytic vaccinia virus (VV), which itself has shown promise in glioma targeting and also has the capacity to facilitate replication of type I interferon (IFN)-sensitive OVs by antagonizing innate antiviral defenses (6–8). First, we demonstrate efficient killing of DBT mouse glioma cells *in vitro* but not *in vivo* by SFV alone, mirroring the results we observed in our previous rat model (4) (Fig. 1A and B). Lack of efficacy could not be explained simply by the immune competence of the animals, as SFV successfully eradicated another type of syngeneic tumor (CT26LacZ) at a similar dose (not shown). Next, we observed that SFV limits its own spread in DBT cells under spatially restrictive conditions (under agarose) and that this limitation could be lifted by coinfecting cells with VV or neutralizing type I IFN using polyclonal antibody (anti-beta interferon [IFN-β]) or recombinant vaccinia virus-encoded B18R protein (Fig. 1C). Facilitation of SFV spread in DBT cells by VV is dependent on B18R, as we did not see enhancement when B18R-deleted VV was used. While the spread of SFV under agarose was enhanced when SFV was combined with VV, replication of VV itself was strongly inhibited (Fig. 1C), which was also confirmed by quantifying virus output from coinfected DBT cells in free culture (Fig. 1D). DBT cell killing by the virus combinations was synergistic, as calculated by the Chou-Talalay method (Fig. 1E). However, combination of SFV with VV in an orthotopic DBT model did not provide statistically significant improvement in survival compared to the next best therapy, VV alone, by either systemic injection or direct intracranial administration or when viruses were coinjected or given 48 h apart ($P < 0.0729$, log rank test) (Fig. 1F and G). Moreover, systemic delivery of the viruses resulted in one mouse of five displaying hind leg paralysis in two separate experiments. Following intravenous administration, VV is known to reduce the ability of plasmacytoid dendritic cells to secrete type I interferon, thereby increasing systemic infection levels and pathogenesis of at least lymphocytic choriomeningitis virus and Pichinde virus (9, 10).

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

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Video Article

Ex Vivo Infection of Live Tissue with Oncolytic Viruses

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Abstract

Oncolytic Viruses (OVs) are novel therapeutics that selectively replicate in and kill tumor cells[1]. Several clinical trials evaluating the effectiveness of a variety of oncolytic platforms including HSV, Reovirus, and Vaccinia OVs as treatment for cancer are currently underway[2-5]. One key characteristic of oncolytic viruses is that they can be genetically modified to express reporter transgenes which makes it possible to visualize the infection of tissues by microscopy or bio-luminescence imaging[6,7]. This offers a unique advantage since it is possible to infect tissues from patients *ex vivo* prior to therapy in order to ascertain the likelihood of successful oncolytic virotherapy[8]. To this end, it is critical to appropriately sample tissue to compensate for tissue heterogeneity and assess tissue viability, particularly prior to infection[9]. It is also important to follow viral replication using reporter transgenes if expressed by the oncolytic platform as well as by direct titration of tissues following homogenization in order to discriminate between abortive and productive infection. The object of this protocol is to address these issues and herein describes 1. The sampling and preparation of tumor tissue for cell culture 2. The assessment of tissue viability using the metabolic dye alamar blue 3. *Ex vivo* infection of cultured tissues with vaccinia virus expressing either GFP or firefly luciferase 4. Detection of transgene expression by fluorescence microscopy or using an *In Vivo* Imaging System (IVIS) 5. Quantification of virus by plaque assay. This comprehensive method presents several advantages including ease of tissue processing, compensation for tissue heterogeneity, control of tissue viability, and discrimination between abortive infection and *bone fide* viral replication.

Video Link

The video component of this article can be found at http://www.jove.com/video/2854/

Protocol

1. Tissue processing

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

2. Assessment of tissue viability

1. Following tissue processing, add 25µl Alamar blue to well # A1 and # A2 as shown in figure 1 and incubate for 1 hour at 37 degrees in a humidified incubator with 5% CO_2.
2. Following incubation with alamar blue, remove 3 times 100 µl from each A1 and A2 well and transfer to 6 different wells of a 96-well plate. Read the signal using a fluorescence plate reader (530 excitation, 590 emission) and keep the data for your records.
3. After Alamar blue signal has been read, transfer all pieces of tissue using a pipet tip, from well A1 to C1 that contains DMEM, 10% FBS + PS + AmphoB. Be careful not to transfer an excessive amount of media from well A1.

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Targeting Tumor Vasculature With an Oncolytic Virus

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

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INTRODUCTION

The idea of using viruses to attack and destroy cancer cells is gaining momentum as clinical support for the concept continues to mount.[1,2] A variety of clever engineering strategies that lead to selective replication of oncolytic viruses (OVs) in cancer cells have created a remarkably safe therapeutic platform.[3] Although the mechanisms behind restricted virus replication in malignant cells are well established, the complexities of the interplay between the therapeutic virus and the host are still incompletely understood.[4,5] In particular it appears that multiple interactions of the virus with the patient's immune system, blood components, reticuloendothelial system, and the tumor microenvironment all can augment or mitigate the therapeutic efficacy of a particular virus platform.[6] Understanding the mechanism of action of OVs *in vivo* is critical to the design and optimization of therapeutic regimens and combination therapies in future clinical trials as well as optimizing the therapeutic efficacy of the next generation viruses currently in development. Indeed, one key attribute of OV therapeutics is their potential to target the tumor via multiple mechanisms increasing malignant cell killing and decreasing the incidence of therapeutic resistance.[7]

We have been investigating the interaction of OVs with tumor vasculature as this is the key entry point of any systemically administered therapeutic. Attacking the tumor vasculature with a therapeutic virus has some obvious potential advantages as this could lead to destruction of neovasculature, providing a beacon for recruiting the immune system to the infected tumor and of course be an entry point for the virus into the tumor mass.[8] In earlier studies, we have shown that an engineered version of vesicular stomatitis virus (VSV), a prototype OV with activity in a large variety of mouse tumor models, causes catastrophic loss of blood flow in the tumor bed resulting in massive bystander killing of cancer cells following intravenous delivery.[9] This phenomenon was also demonstrated with oncolytic vaccinia virus.[9,10] Furthermore, infection of the tumor resulted in significant increases in the transcription of genes that encode proinflammatory molecules leading to the recruitment of neutrophils and other immune cells to the tumor bed.[9] Here, we have examined the direct interaction of VSV with tumor blood vessels and show for the first time, that limited sites of virus infection of neovasculature correlate with massive cell death within the tumor. We characterized the mechanism behind the massive bystander killing within the infected tumor and found that neutrophil-dependent initiation of microclots within blood vessels led to irreversible damage of tumor vasculature. We demonstrate that intravascular clot formation robustly potentiates the anticancer activity of VSV by reducing proliferation and inducing apoptosis of tumor cells. Most importantly, the infection of vasculature and subsequent initiation of fibrin deposition and clot formation is restricted to tumor beds. Our findings support the idea

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Vesicular stomatitis virus oncolysis is potentiated by impairing mTORC1-dependent type I IFN production

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Oncolytic viruses constitute a promising therapy against malignant gliomas (MGs). However, virus-induced type I IFN greatly limits its clinical application. The kinase mammalian target of rapamycin (mTOR) stimulates type I IFN production via phosphorylation of its effector proteins, 4E-BPs and S6Ks. Here we show that mouse embryonic fibroblasts and mice lacking S6K1 and S6K2 are more susceptible to vesicular stomatitis virus (VSV) infection than their WT counterparts as a result of an impaired type I IFN response. We used this knowledge to employ a pharmacoviral approach to treat MGs. The highly specific inhibitor of mTOR rapamycin, in combination with an IFN-sensitive VSV-mutant strain (VSV$^{\Delta M51}$), dramatically increased the survival of immunocompetent rats bearing MGs. More importantly, VSV$^{\Delta M51}$ selectively killed tumor, but not normal cells, in MG-bearing rats treated with rapamycin. These results demonstrate that reducing type I IFNs through inhibition of mTORC1 is an effective strategy to augment the therapeutic activity of VSV$^{\Delta M51}$.

innate antiviral immunity | malignant gliomas | mTORC1 | oncolytic viruses

Malignant gliomas (MGs) are by far the most frequent, aggressive, and lethal primary brain tumor variants (1, 2). Patients with MGs have a median survival time of approximately 1 y and respond poorly to most available therapeutic modalities (3–5). Thus, more effective treatments are needed.

Recent evidence implicates the PI3K/mammalian target of rapamycin (mTOR) signaling pathway as one of the main oncogenic signaling pathways whose deregulation may underlie gliomagenesis (6, 7). mTOR exists in two complexes: mTOR complex 1 (mTORC1), which is sensitive to the drug rapamycin and regulates mRNA translation, and mTORC2, which is rapamycin-insensitive and regulates the organization of the actin cytoskeleton (reviewed in refs. 8–10). mTORC1 stimulates type I IFN production via phosphorylation of its target proteins 4E-BPs and S6K1/2 (11). Evidence for the critical role of the mTORC1 signaling pathway in innate immunity emerged from the findings that the mTORC1 inhibitor rapamycin suppresses type I IFN in plasmacytoid dendritic cells (pDCs), which are the major producers of systemic type I IFN (12). In addition, genetic deletion of the mTOR downstream target S6K1/2 leads to impaired type I IFN response (see *Results*). In contrast, we recently found that the lack of the translational repressors 4E-BP1/2 leads to enhanced type I IFN production (13).

Oncogenic transformation is associated with a deficient type I IFN response, which constitutes the first line of defense against virus infection (14, 15). Oncolytic viruses are studied as effective anticancer agents because they exploit this selective defect (16–19). One of the best characterized oncolytic viruses, whose replication is extremely sensitive to the inhibition by IFN, is vesicular stomatitis virus (VSV) (20). However, there are several reasons that limit the use of oncolytic viruses for the treatment of MGs. First, some MGs exhibit a robust type I IFN response, which may preclude virus oncolysis in vivo and in vitro. Second, oncolytic viruses are extracellular pathogens, thus the immune system impedes their replication and spread, even within the tumor. Finally, MGs are very heterogeneous, which contributes to their therapeutic resistance. As a consequence, MGs tend to evade single-targeted therapeutic approaches designed to inhibit the proliferation and survival of MGs (4). Therefore, a combinatorial approach that suppresses tumor cell survival and at the same time selectively promotes virus replication in malignant cells should lead to more effective tumor treatments and ultimately boost their translation from the laboratory to the clinic. Thus, we developed a "pharmacoviral" approach to treat MGs. Here, we demonstrate that the combination of rapamycin, which specifically silences mTORC1 activity, with VSV$^{\Delta M51}$ significantly prolongs the survival of immunocompetent rats bearing malignant gliomas. Furthermore, we determine the precise molecular mechanism underlying this process.

Results

Malignant Glioma Cells Respond to and Produce Type I IFN. Although VSV typically replicates in human glioma cell lines (21), MGs are thought to elicit a type I IFN response (16, 22), which could be sufficient to impede the intratumoral spread of the virus. Indeed, when pretreated with human IFN-β, both human glioma cell lines and freshly excised glioma cell are protected against VSV$^{\Delta M51}$ (an exquisitely IFN sensitive VSV-mutant strain and the prototype for VSV-based oncolytic therapies; Fig. 1 *A* and *B* and Fig. S1). In addition, freshly excised glioma cells and human and rat glioma cell lines generate type I IFN, as determined by VSV protection assays and HEK-Blue type I IFN assay (InvivoGen; Fig. 1 *C–E*). Importantly, the generation of type I IFN was prevented when glioma cell lines were pretreated with rapamycin (Fig. 1*F*). These data indicate that inhibition of mTORC1 blocks type I IFN production in vitro.

Rapamycin Potentiates VSV Oncolysis of Gliomas in Vivo. In light of these findings, we reasoned that blocking mTORC1 activity in vivo with rapamycin would inhibit systemic type I IFN production and allow the replication of VSV$^{\Delta M51}$ in IFN-producing MG. To test this hypothesis, we used a pharmacoviral approach

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CURRICULUM VITAE

Dominic G. Roy

EDUCATION

2009 - Present	Ph.D. candidate at the University of Ottawa in Biochemistry with Specialization in Human and Molecular Genetics (accelerated from Masters to Doctoral program in August 2011)
2004 - 2009	University of Ottawa, B.Sc. in Biochemistry (Honours)

WORK/RESEARCH EXPERIENCE

2009 – Present	Ottawa Hospital Research Institute, Lab of Dr. John C. Bell.
2009/05 – 09	Jennerex Biotherapeutics Inc. – Summer Studentship
2007 – 2008	Ottawa Hospital Research Institute, Lab of Dr. John C. Bell- CO-OP
2008/05 – 09	Spartan Bioscience Inc. – Research Technician

PUBLICATIONS

1- Murine tumours models for oncolytic Rhabdo-virotherapy. **(2016).** Falls T, **Roy DG**, Bell JC, Bourgeois-Daigneault MC. *ILAR J*. 57(1) 73-85.

2- Oncolytic VSV expressing interferon-gamma has enhanced therapeutic activity. **(2016).** Bourgeois-Daigneault MC, **Roy DG**, Falls T, Twumasi-Boateng K, St-Germain L, Marguerie M, Garcia V, Selman M, Jennings V, Pettigrew J, Amos S, Diallo JS, Nelson B, Bell JC. *Molecular Therapy-Oncolytics*. Feb 17;3:16001.

3- Programmable insect cell carriers for systemic delivery of integrated cancer biotherapy. **(2015). Roy DG**, Power AT, Bourgeois-Daigneault MC, Falls T, Ferreira L, Stern A, Tanese de Souza C, McCart JA, Stojdl DF, Lichty BD, Atkins H, Auer RC, Bell JC, Le Boeuf F. *J Control Release*. 2015 Dec 28;220(Pt A):210-21.

4- MicroRNAs regulate the immunometabolic response to viral infection in the liver. **(2015).** Singaravelu R, O'Hara S, Jones DM, Chen R, Taylor NG, Srinivasan P, Quan C, **Roy DG**, Steenbergen RH, Kumar A, Lyn RK, Özcelik D, Rouleau Y, Nguyen MA, Rayner KJ, Hobman TC, Tyrrell DL, Russell RS, Pezacki JP. *Nat Chem Biol.* Dec;11(12):988-93.

5- Bacterial-mediated knockdown of tumour resistance to an oncolytic virus enhances therapy. **(2014).** Cronin M, Le Boeuf F, Murphy C, **Roy DG**, Falls T, Bell JC, Tangney M. *Mol Ther*. Jun;22(6):1188-97.

6- Cell carriers for oncolytic viruses: current challenges and future directions. **(2013)**. **Roy DG**, Bell JC . *Oncolytic Virotherapy* 2, 47-56.

7- Model-based rational design of an oncolytic virus with improved therapeutic potential**. (2013).** Le Bœuf F, Batenchuk C, Vähä-Koskela M, Breton S, **Roy D**, Lemay C, Cox J,

Abdelbary H, Falls T, Waghray G, Atkins H, Stojdl D, Diallo JS, Kærn M, Bell JC. *Nat Commun*. 4:1974.

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

9- Ex vivo infection of live tissue with oncolytic viruses. **(2011).** Diallo JS, **Roy D**, Abdelbary H, De Silva N, Bell JC. *J Vis Exp.* Jun 25;(52).

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

11- Vesicular stomatitis virus oncolysis is potentiated by impairing mTORC1-dependent type I IFN production. **(2009).** Alain T, Lun X, Martineau Y, Sean P, Pulendran B, Petroulakis E, Zemp FJ, Lemay CG, **Roy D**, Bell JC, Thomas G, Kozma SC, Forsyth PA, Costa-Mattioli M, Sonenberg N. *Proc Natl Acad Sci US A.* 107(4):1576-81.

12- Application Note AN0028 for Spartan Bioscience Inc: **D Roy**, E Vaillancourt, H Doong, CJ Harder **(2008)**. **DNA extraction with the BuccalQuick™ kit**.

AWARDS/SCHOLARSHIPS

2013-2015	CIHR Doctoral Award - Frederick Banting and Charles Best Canada Graduate Scholarship
2011-2012	Ontario Graduate Scholarship
2011	Canadian Cancer Research Conference Travel Award.
2011	University of Ottawa FGPS Research Travel Grant
2011	University of Ottawa BMI Seminar Day- 3rd Place
2010	Ottawa Hospital Research Institute Poster Award – 1st Place
2009-2015	University of Ottawa Admission Scholarship
2009	University of Ottawa BMI Travel Grant
2009	University of Ottawa GSAED Travel Grant
2008	NSERC Industrial Undergraduate Student Research Award
2006	Canada Millennium Scholarship
2004 - 2009	Queen Elizabeth II Aiming for the Top Scholarship
2004 - 2008	University of Ottawa Admission Scholarship
2004	University of Ottawa Faculty Scholarship

PRESENTATIONS

Oral Presentations

2015/09 ***Invited speaker*** *at the National Research Council* (Ottawa, ON). Insect Cells for Systemic Delivery of Oncolytic Viruses.

2015/09 *OHRI Research Day* (Ottawa, ON). Insect Cells for Systemic Delivery of Oncolytic Viruses.

2011/03 *6th International Meeting on Oncolytic Viruses as Cancer Therapeutics* (Las Vegas, NV). Insect Cells for Systemic Delivery of Oncolytic Viruses (First author. Talk given by Dr. John Bell).

2009/10 *Cold Spring Harbor Laboratory: In Vivo Barriers to Gene Delivery* (New York, NY). Insect Cells for the Systemic Delivery of Oncolytic Viruses (Co-author).

2010/05 *American Society of Gene and Cell Therapy: 13th Annual Meeting* (Washington, DC). Insect Cells for the Systemic Delivery of Oncolytic Viruses.

Poster Presentations

2015/06 *9th International Meeting on Replicating Oncolytic Virus Therapeutics* (Boston, MA). A RNAi Screening Platform to identify host factors that modulate oncolytic virus replication in tumour cells.

2015/05 *8th Annual Canadian Cancer Immunotherapy Consortium* (Vancouver, BC). IFNγ-armed oncolytic virus for cancer treatment (2nd author).

2014/02 *Keystone Symposia: Tumour Immunology: Multidisciplinary Science Driving Combination Therapy* (Banff, AB). Oncolytic viruses for immunotherapy (2nd author).

2014/10 *Cancer Research Institute – 22nd Annual International Cancer Immunotherapy Symposium* (New York, NY). Generation of tumour-specific immune responses and reversion of tumour-driven immune suppression by oncolytic virus treatment (2nd author).

2013/06 *7th International Meeting on Replicating Oncolytic Virus Therapeutics* (Quebec, QC). Insect Cells for the Systemic Delivery of Oncolytic Viruses.

2011/11 *Canadian Cancer Research Conference* (Toronto, ON). Insect Cells for the Systemic Delivery of Oncolytic Viruses.

2011/03 *6th International Meeting on Oncolytic Viruses as Cancer Therapeutics*. (Las Vegas, NV). Insect Cells for the Systemic Delivery of Oncolytic Viruses.

2010/05 *ATGQ: 5th Canadian Gene Therapy and Vaccines Symposium* (Grenville-sur-la-Rouge, QC). Insect Cells for the Systemic Delivery of Oncolytic Viruses.

2010/02 *Ontario Institute for Cancer Research: Annual Scientific Meeting* (Alliston, ON). Insect Cells for the Systemic Delivery of Oncolytic Viruses.