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Oncolytic Reovirus Combined with Sunitinib as a Novel Multi-Mechanistic Treatment Strategy for Renal Cell Carcinoma

by

Keith Lawson

A THESIS

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Abstract.

Metastatic renal cell carcinoma (RCC) is an incurable disease resistant to both radiation and cytotoxic chemotherapy. Although new molecularly targeted agents have increased the therapeutic options for patients with this disease, 5-year overall survival rates remain below 10%. The aim of this thesis was to determine the preclinical efficacy of reovirus as a monotherapy and in combination with sunitinib, a first line mRCC agent and multi-tyrosine kinase inhibitor, for the treatment of RCC. To assess this, studies employing a panel of RCC cell lines, as well as a syngeneic immunocompetent murine model of RCC were utilized. Collectively, these studies demonstrate that reovirus is both a novel oncolytic and immunotherapeutic agent against RCC. Furthermore, our results provide the first evidence that sunitinib augments the anti-tumour immune response generated by an oncolytic virus. As such, this novel treatment paradigm has immediate clinical applicability for use against multiple tumour histologies, particularly RCC.

Acknowledgements.

I must first thank my supervisor and role model Dr. Don Morris for his unconditional support and belief that I could not only complete this degree but also have success in becoming a surgeon-scientist. Without this, I do not know if I would have been able to stay motivated through the trying times of completing this project during medical school. Additionally, I must my thank my two labmates and friends, Jason Spurrell and Zhong Qiao Shi, who from the beginning of this project have demonstrated an enormous amount of patience and dedication to training me to become a good scientist. In this same regard, I must thank Dr. Frank Jirik for his thoughtful insights into this project as a member of my graduate committee. I would also like to thank the urology group at the University of Calgary, in particular Dr. Jun Kawakami, for accommodating and supporting me in blending my passion for research with my goal of becoming an academic urologist. I have also had the fortune of having an enormous amount of support from my family and friends. In particular my fiancé Ambica Parmar and my two friends Geoff Brin and David Burrowes who have patiently worked to keep me grounded and ensure I stay balanced for which I cannot thank them enough. Last, but not least, I would like to thank all members of the Translational Laboratory at the Tom Baker Cancer Center who always provided a helping hand and made my experience most enjoyable.

Dedication.

To my grandmother Helen Irene Sorensen for always believing in me.

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List of Abbreviations.

mRCC: Metastatic renal cell carcinoma

OV: Oncolytic virus

RCC: Renal cell carcinoma

ccRCC: clear cell renal cell carcinoma

MRD-1: Multidrug resistance protein 1

mTOR: Mammalian target of rapamycin

VEGF: Vascular endothelial growth factor

PFS: Progression free survival

mOS: Median overall survival

VHL: Von Hippel Lindau

HIF: Hypoxia inducible factor

VEGFR: Vascular endothelial growth factor receptor

PDGFR: Platelet derived growth factor receptor

PDGF: Platelet derived growth factor

PI3K: Phosphoinositide 3-kinase

2D-DIGE: Two dimensional gel electrophoresis

IFN-α: Interferon alpha

IL-2: Interleukin two

DC: Dendritic cell

TAA: Tumour associated antigen

CRAds: Conditionally replicating adenovirus

pRB: Protein retinoblastoma

p53: Protein 53

CAR: Coxsackie- and adenovirus receptor

NK: Natural killer

MHC: Major histocompatibility complex

PAMP: Pathogen associated molecular pattern

TGF-βR: Transforming growth factor beta receptor

VV: Vaccinia virus

TK: Thymidine kinase

VGF: Vaccinia growth factor

HUVEC: Human umbilical vein endothelial cells

HSV: Herpes simplex virus

MOI: Multiplicity of infection

SeV: Sendai virus

F: Fusion glycoprotein

uPA: Urokinase proteinase activated

MMP: Matrix metalloproteinase

MV: Measles virus

VSV: Vesicular stomatitis virus

NF-κβ: Nuclear factor kappa beta

EMCV: Encephalomyocarditis virus

MDSC: Myeloid derived suppressor cell

Treg: T regulatory cell

PET: Positron emission tomography

JAM-A: Junction adhesion molecule subtype A

ISVP: Intermediate infectious subviral particle

TRAIL: Tumour-necrosis-factor related apoptosis-inducing ligand

PKR: Protein kinase R

RPMI: Roswell Park Memorial Institute medium

MEM: Minimal essential medium

DMEM: Dulbecco's modified eagle medium

FBS: Fetal calf serum

DMSO: Dimethyl sulfoxide

PBMC: Peripheral blood mononuclear cell

CI: Combination index

FITC: Fluorescein isothiocyanate

PE: Phycoerythrin

ANOVA: Analysis of variance

IFN-γ: Interferon gamma

ELISA: Enzyme-linked immunoabsorbent assay

STAT: Signal transducers and activators of transcription

GM-CSF: Granulocyte maturation-colony stimulating factor

Chapter 1.

Oncolytic virotherapy for renal cell carcinoma: A novel therapeutic paradigm?

Excerpts from this Chapter were published as a review in July 2012 in the Journal Expert Opinion on Biological Therapy. K. Lawson and D. Morris (2012). "Oncolytic Virotherapy for Renal Cell Carcinoma: A Novel Treatment Paradigm?" Expert Opin Biol Ther 2012;12(7):891-903.

1.1 Renal cell carcinoma epidemiology and background.

Renal cell carcinoma (RCC) is the most common malignancy occurring in the kidney, accounting globally for an estimated 209,000 new cases and 102,000 deaths in 2009 [1]. RCC originates in the renal cortex and can be sub-classified based on histology, with the vast majority of cases being of clear-cell (ccRCC) origin [2-3]. Localized RCC is almost exclusively treated by surgical resection and the 5-year survival rate for T1 lesions is estimated to be greater than 97% [4-6]. However, a significant number of patients present with metastatic disease (upwards of 15%) or subsequently develop metastatic recurrence [7-8]. Historically, the treatment of metastatic RCC (mRCC) has been a story of limited success as the disease is highly resistant to both radiation and cytotoxic chemotherapy. While the precise mechanisms of this resistance are still under investigation, it is thought that the high expression of glutathione-redox enzymes and drug efflux pumps (MDR-1) are responsible [9-10]. Because of this and coupled with reports of spontaneous regressions thought to be immune mediated, mRCC has been treated successfully in a minority of cases with immunotherapy (IL-2, IFN-α) [11-12]. Over the last decade however, a better understanding of the molecular biology of mRCC has lead to the development of targeted therapies for this disease. These drugs have revolutionized the management of mRCC and have resulted in a significant increase in the number of therapeutic options available to patients as well as improved clinical outcomes. Since 2007, six targeted therapies have entered onto the market for mRCC. These include the tyrosine kinase inhibitors (sunitinib, sorafenib, pazopanib), mammalian target of rapamycin (mTOR) inhibitors (everolimus, temsirolimus) and anti-vascular endothelial growth factor (VGEF) monoclonal antibodies (bevacizumab) [7]. Despite the

improvements in progression free survival (PFS) and median overall survival (mOS) provided with these therapies, mRCC is still felt to be incurable and the 5-year overall survival rate associated with the disease has not changed significantly, being estimated as less than 10% [7, 13]. As such, a significant research focus remains on the development of novel therapeutics and therapeutic strategies to improve upon the poor outcomes currently associated with this disease.

1.2 Oncolytic virus background.

Oncolytic viruses (OV) are novel therapeutics that are capable of usurping common molecular signalling aberrations present in transformed cells to initiate viral lytic activity [14]. The use of viruses capable of selectively replicating in and destroying transformed cells was described over a century ago. Since this time, a number of OVs have been discovered and our increased knowledge of their mechanisms of action, basis for selectivity and interactions with the host immune system have resulted in significant advances in bringing these agents into clinical use. A diverse range of OVs are currently being developed on both the clinical and preclinical levels for use against a variety of malignancies and include both naturally occurring viruses (ex. encephalomyocarditis virus, reovirus), as well as viruses harbouring genetic modifications facilitating their selectively for transformed cells (ex. adenoviruses). Common genetic modifications to viruses include the insertion of transgenes to “arm” OV for enhanced therapeutic efficacy, as well as the introduction of tissue specific promoters (transcriptional targeting) and/or capsid modifications (transductional targeting) to increase cancer cell selectively. Additionally, OV have also been shown to generate robust intratumoural inflammatory responses allowing for the elimination of bystander cancer cells within the tumour

microenvironment [15]. Moreover, as this activity has recently been demonstrated to prime both innate and adaptive anti-tumour immune responses, OV are now being considered a novel class of potential immunotherapeutics [16].

Given the need for novel therapeutics for use against RCC and the emerging evidence highlighting the safety and efficacy of OV, the literature was reviewed to determine the potential of utilizing these agents against this disease. In the subsequent sections of this thesis the current status of oncolytic virotherapy for RCC will be addressed with emphasis on the molecular and immunological hallmarks of the disease that make it an ideal tumour site for application of these biologic agents.

1.3 Molecular aberrations in RCC: A candidate malignancy for oncolytic virotherapy.

RCC, like many malignancies, is characterized by several oncogenic signalling aberrations that have been demonstrated to be critical in permitting oncolytic virus replication. The association between loss of the tumour suppressor gene Von Hippel Lindau (VHL) with the development of familial and sporadic ccRCC [17-19] is certainly the most extensively studied aberration in RCC molecular biology as nearly all cases of familial ccRCC and upwards of 70% of sporadic ccRCC cases involve inactivation of the VHL locus [20-21]. The VHL gene product, protein VHL (pVHL), plays an integral role in the cellular response to hypoxia by coupling cellular oxygen status with gene transcription. It mediates this activity by serving as the substrate recognition unit of an E3 ligase complex that ubiquitinates and targets members of the hypoxia inducible factor (HIF) family of transcription factors for proteasomal degradation [20, 22]. Because of this role, loss of pVHL through inactivation leads to a decrease in the proteasomal degradation

of HIFs, allowing them to accumulate in the nucleus and drive the expression of genes encoding hypoxia response elements (HRE) in their promoter regions. Additionally, as reviewed by Baldewijns *et al.* [23], oncogenic signalling (i.e., PI3K/Akt/mTOR), tumour hypoxia and constitutive activation (i.e., loss of tuberous sclerosis complex 2) also contribute to HIF upregulation in RCC. Once accumulated in the nucleus, HIF binds DNA as a heterodimer and that consists of an alpha (α) and beta (β) subunit, [23]. Unlike the β subunit (HIF-1β/ARNT), which is constitutively expressed, three α subunit isoforms exist (HIF-1α, HIF-2α, HIF-3α) and their levels are regulated by the proteasome.

To date, the expression of over 200 genes have been shown to be modulated by HIF activation and interestingly a significant number of these genes can be linked to oncolytic virus sensitivity [20, 24]. Included in this are the vascular endothelial growth factor (VEGFR) and platelet derived growth factor (PDGFR) receptors which act to drive RAS/MEK/ERK and PI3K/Akt/mTOR signalling, both of which are known regulators of OV replication. Upregulated ras signalling, for example, is one of the most extensively studied pathways implicated in the selective cancer cell replication of reoviruses, poxviruses as well as herpes viruses [27-29]. Interestingly, utilizing a dominant negative ras mutant (N116Y), Shinohara *et al.* demonstrated the dependence of RCC cell lines on activated ras signalling, highlighting the attractiveness of exploiting this pathway with the aforementioned OVs for this disease [30]. Moreover, active PI3K/Akt signalling has been demonstrated to determine the sensitivity of multiple human malignancies, including RCC, to myxoma virus [31]. As mentioned, this pathway is activated by HIF and involved in the carcinogenesis of RCC as demonstrated by the dependence of RCC growth on Akt signalling both in vitro and in vivo [32]. Hence, HIF mediated PI3K/Akt activation

represents yet another exploitable pathway that facilitates OV sensitivity in RCC. Furthermore, the loss of VHL in RCC also results in the augmentation of nuclear factor kappa beta (NF-κβ) activity, a pathway implicated in the selective replication of encephalomyocarditis virus [33]. Thus, the loss of VHL is a molecular hallmark that is unique to RCC, which not only acts to orchestrate a series of signalling events that regulate RCC carcinogenesis, but also allows for the upregulation of those pathways essential for OV replication.

RCC is characterized by a number of molecular aberrations that extend beyond the HIF axis. These include the hepatocyte growth factor/c-MET, Wnt/β-catenin and E-cadherin pathways, all of which have a growing body of literature to support their role in RCC carcinogenesis [20]. In the context of OV sensitivity, this extensive array of aberrant signalling not only contributes to the natural cancer cell selective replication of OV, but also in the design of genetically engineered viruses, which through transcriptional and/or translational targeting, can be utilized as potent tumouricidal agents. Demonstrating this is the work of Kurida *et al*. who generated a herpes simplex virus that selectively replicates in cells with strong Wnt/β-catenin signalling activity [34]. This virus not only produced robust cytopathic responses *in vitro* but also, facilitated the regression of high β-catenin expressing SW480 xenograft tumours. Hence, beyond the VHL/HIF axis it is clear that other known RCC pathways, such as Wnt/β-catenin activation, are capable of being targeted and exploited by OVs.

Increasingly, through the use of high-throughput biological approaches and systems biology, it is becoming evident that the complexity of RCC is only beginning to be understood. Highlighting this, is a recent study employing fluorescence two-

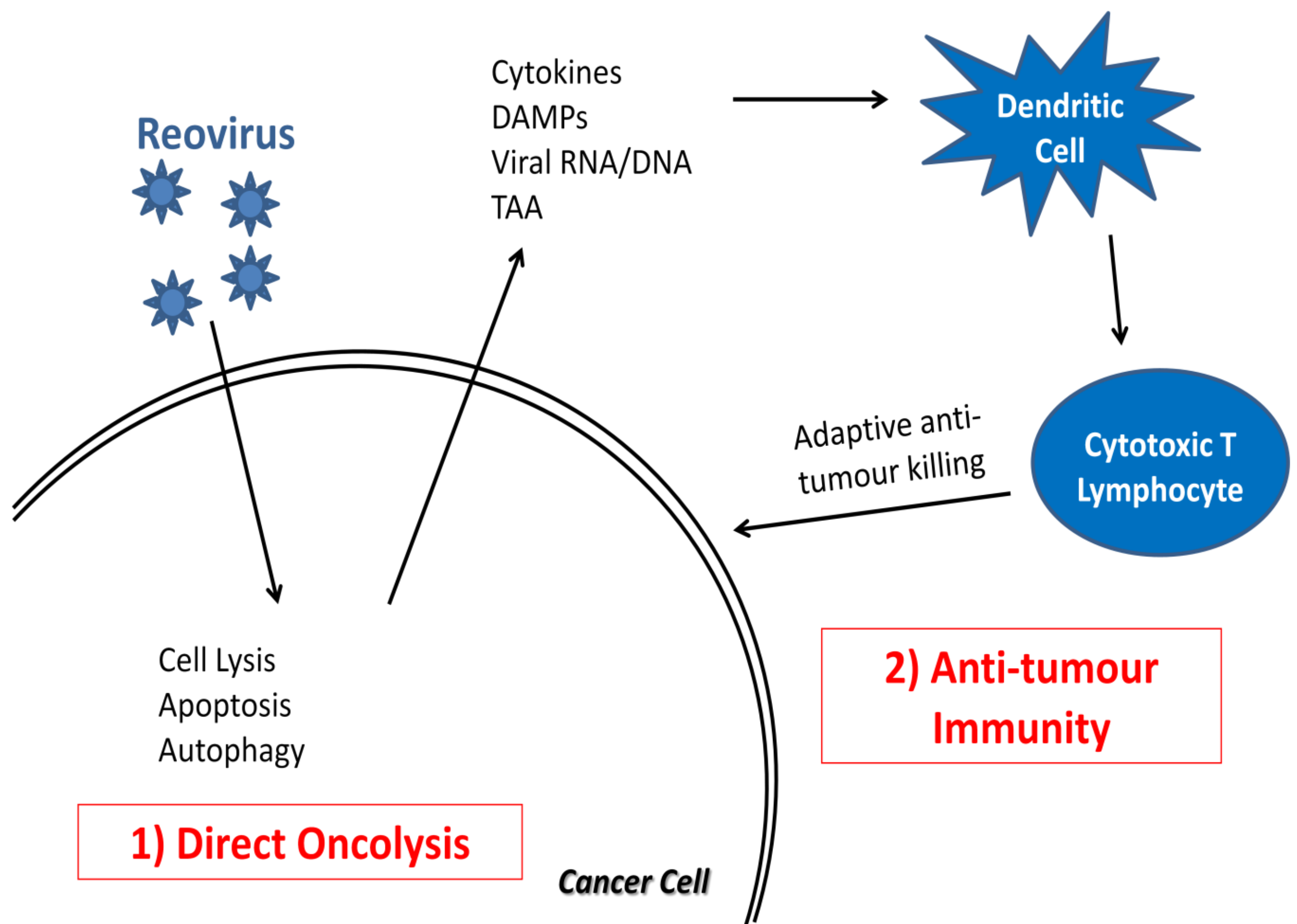
dimensional gel electrophoresis (2D-DIGE) to characterize the stage related alterations occurring at the protein level in RCC [35]. These authors identified a total of 94 novel proteins associated with RCC development and through protein network analysis demonstrated an abundance of alterations in proteins known to mediate cytoskeletal remodelling, mitochondrial dysfunction and lipid metabolism. Beyond this, large scale exome sequencing studies performed on RCC cell lines and tissues have also identified aberrations in histone modifying pathways, which act to regulate gene transcription [36]. The most recent of these reports was conducted by Varela *et al.* who employed an exome sequencing method to identify that 41% of ccRCCs are characterized by truncating mutations of the SWI/SNF chromatin remodelling complex gene PBRM1 [37]. Taken together, the aforementioned studies not only depict RCC as a disease of many signalling aberrations, but also one of significant genetic heterogeneity. Undoubtedly, as this understanding increases, the number of OV exploitable pathways will also increase.

1.4 Immunological responsiveness of RCC: a candidate malignancy for oncolytic virotherapy.

Prior to the introduction of the anti-angiogenic targeted therapies, mRCC was treated with recombinant cytokines, including both IFN-α and IL-2 [11]. Given the anti-tumour immune responses occasionally seen following administration of IFN-α and IL-2 in the form of durable complete responses, RCC is thought to be a prototypical immunotherapy-responsive malignancy [11-12]. Consequently, a significant amount of preclinical research has focused on developing novel immunotherapeutic strategies for this disease. This has included the use of genetically modified T cells, infusion of tumour infiltrating lymphocytes and lymphokine gene-transfected tumour cells, allogeneic stem

cell transplantation, cytokines and dendritic cell (DC) tumour vaccination [11, 38-42]. Interestingly, a recent systematic review and meta-analysis of DC based tumour vaccine therapy revealed an improvement in the combined objective response and stable disease rates (clinical benefit rate of 48%) in patients with mRCC [43]. As such, novel therapeutic strategies incorporating immunotherapy hold promise for use in this disease. OVs should be considered in this regard as these agents are able to release sequestered tumour associated antigens (TAA) in the context of pathogen associated molecular patterns (viral antigens) and danger signals, allowing for the effective priming of DCs and subsequent generation of anti-tumour immunity [16]. Beyond this, OV have also been demonstrated to facilitate cross-presentation of TAAs, stimulate the production of pro-inflammatory cytokines and chemokines and enhance tumoural migration of innate immune effectors, further portraying their immunotherapeutic potential [16, 44] (**Figure. 1**).

Figure 1. Mechanisms of reovirus anti-tumor therapeutic effects. Reovirus infection of cancer cells results in direct cytopathic effects through cell lysis, apoptosis and autophagy. This is known as the direct oncolytic effects of reovirus. This process results in the release of tumor associated antigens (TAA), viral antigens, and danger associated molecular proteins (DAMPs) in the context of pro-inflammatory cytokines/chemokines resulting in the priming of innate and adaptive anti-tumor immune responses. Figure adapted from Prestwich RJ *et. al.* Expert Rev Anticancer Ther 2008;8(10):1581-8.



1.5 Current Evidence for the use of OVs in RCC.

To date, seven OVs have been demonstrated to display therapeutic efficacy against RCC and in the subsequent sections of this review the available preclinical and clinical data highlighting the application of these agents against this disease will be discussed (summarized in **Table 1**).

Table 1. Oncolytic viruses investigated for use against renal cell carcinoma (RCC) and their properties. dsDNA: double strand DNA, nsRNA: negative strand RNA, psRNA: positive strand RNA. *RENCA model: A syngeneic immunocompetent murine model of RCC. Figure adapted from: Lawson KA *et. al.* Expert Opin Biol Ther 2012;12(7):891-903.

	Adenovirus	Vacinnia Virus	Herpes Simplex Virus	Sendai Virus	Measles Virus	Vesicular Stomatitis Virus	Encephalomyocarditis Virus
Family	*Adenoviridae*	*Poxvirus*	*Herpesviridae*	*Paramyoviridae*	*Paramyoviridae*	*Rhabdoviridae*	*Picornavirus*
Genome	dsDNA	dsDNA	dsDNA	nsRNA	nsRNA	nsRNA	psRNA
Genetically Modifiable	Yes	Yes	Yes	Yes	Yes	Yes	No
Immunogenic	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Evidence for use in RCC							
In Vitro	Yes [ref. 46-53]	Yes [ref. 61]	Yes [ref. 64-65]	Yes [ref. 68-69]	Yes [ref. 71]	Yes [ref. 74]	Yes [ref. 33]
Ex vivo	No	No	No	No	No	No	No
In Vivo – Xenograft Model	Yes [ref. 46-53]	Yes [ref. 61]	Yes [ref. 64-65]	Yes [ref. 68-69]	Yes [ref. 71]	Yes [ref. 74]	Yes [ref. 33]
*In Vivo – RENCA Model**	Yes [ref. 54-56]	Yes [ref. 61]	No	Yes [ref. 69]	No	No	No
Clinical	No	Yes [ref 62]	No	No	No	No	No

1.5.1 Adenovirus.

Adenoviruses are non-enveloped dsDNA viruses that have been shown to have oncolytic activity against a wide range of malignancies [45]. The adenoviral genome can be modified with relative ease allowing for the insertion of therapeutic transgenes as well as allow transcriptional and transductional targeting of these agents. Conditionally replicating adenoviruses (CRAds) have also be generated by introducing loss of function mutations into the viral genome. Most often, this involves mutations in the adenoviral E1A and E1B genes as these proteins act to facilitate the transcription of viral gene products through inactivation of the tumour suppressors pRB and p53, respectively [45]. Inactivation of pRB and p53, which are present in the majority of cancers, compensates for the loss of adenoviral E1A/E1B, allowing viral replication to proceed in such cells. In normal cells, which express both pRB and p53, this compensation does not occur and viral replication is inhibited. Adenoviruses further display selective tropism for cancer cells as a result of the interaction between their viral capsid knob and the tumour expressed coxsackie- and adenovirus receptor (CAR) as well as $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrins [45].

Interestingly, the use of genetically modified adenoviruses has been widely studied for use against RCC. By utilizing a promoter containing nine tandem repeats of the HRE to control adenoviral E1A expression, Cuveus *et al.* demonstrated the feasibility of utilizing transcriptional targeting of oncolytic adenoviruses against RCC [46]. Of note, the oncolytic activity of this virus was significantly diminished upon infection of VHL$^{+/+}$ cells, highlighting the specificity of this virus for cells with aberrantly overexpressed HIF. In addition, a recent report of a HIF dependent transcriptionally targeted adenovirus

expressing luciferase displayed a significant replication advantage over non-HIF targeted viruses in RCC xenografts [47]. Transcriptional targeting of RCC has also been demonstrated through the use of a telomerase-specific targeted adenovirus (OBP-301) which was shown to have potent anti-tumour activity in the RENCA syngeneic immunocompetent murine model of RCC [48].

Adenoviruses genetically modified to harbour loss of function mutations for conditional replication have also been described for use in RCC. Moreover, a number of these viruses have been armed to express therapeutic transgenes to enhance their oncolytic efficacy. Highlighting this are studies utilizing ZD55, an adenoviruses deficient in E1A as a result of a 55 kDa deletion that also contains a Bgl II cloning site that allows for insertion of transgenes [49]. In RCC xenografts, intratumoural administration of ZD55 armed to express interleukin-18 as well as short hairpin RNA (shRNA) against Ki-67 and hTERT resulted in the regression of RCC xenograft tumours [50-52]. A similar result was also demonstrated utilizing an infectivity enhanced, dual-targeted adenovirus armed with the VEGFR-1 (Ad5/3-9HIF-Delta24-VEGF-1-Ig) [47]. Treatment of mice with this virus not only lead a marked decrease in tumour volume but also in blood vessel density, highlighting the ability of Ad5/3-9HIF-Delta24-VEGF-1-Ig to function as both an oncolytic as well as anti-angiogenic therapeutic against RCC.

The introduction of genetic modifications to the adenovirus capsid is also an effective strategy for enhancing adenoviral transduction and oncolytic activity against RCC. Guse *et al.* compared the oncolytic efficacy of five capsid modified adenoviruses to wildtype adenovirus and found that the use of an adenovirus expressing a chimeric serotype 5 capsid, containing a fiber knob domain from a serotype 3 adenovirus (Ad5/3),

resulted in the most robust cytotoxic response *in vitro* [53]. Moreover, subsequent RCC xenograft studies demonstrated a significant survival benefit for many of these capsid modified viruses, including Ad5/3.

In order to achieve the maximum therapeutic benefit of oncolytic virotherapy in RCC the anti-tumour immune response must be considered. Interestingly, a number of genetically modified adenoviruses have been generated to produce anti-tumour immune responses against RCC. In an immunocompetent murine model of RCC (RENCA), the use of an adenovirus expressing IL-18 resulted in enhanced anti-tumour activity via the recruitment of cytotoxic T lymphocytes and increased activation of NK cells [54]. Arming of adenoviruses with P16/GM-CSF also results in a similar anti-tumour immune response in the murine RENCA model and the mechanism of this has been demonstrated to be mediated in part by enhanced expression of MHC-1 (H-2Kd) on infected tumour cells [55]. In more recent work, the use of adenoviruses expressing pathogen-associated molecular patterns (PAMPs) has proven to be beneficial in breaking RCC induced tolerance, highlighting the ability to utilize such agents as immunoadjuvants in this disease [56]. In addition to immunostimulatory effects, adenoviruses have also been utilized to reverse the tumour induced immunosuppressive microenvironment, a known barrier to oncolytic virotherapy generated immune responses. This is highlighted by the enhanced anti-tumour immune response that is generated following treatment of RENCA cell line bearing mice with a DC vaccine combined with an adenovirus expressing the TGF-βR to neutralize this immunosuppressive cytokine [57].

To date, a number of armed and unarmed adenoviruses administered intravenously or intratumourally as either monotherapies or in combination with chemotherapy and/or

radiation therapy are in current clinical trials in other histologies [45]. Despite this increasing body of evidence highlighting the efficacy of adenoviruses against RCC at the preclinical level, no clinical trials have been initiated to date. As such, it remains to be determined whether the use of adenoviruses will provide benefit to patients suffering from mRCC.

1.5.2 Vaccinia virus.

Vaccinia virus (VV) is an enveloped dsDNA virus belonging to the poxvirus family. VV has been utilized as an anti-tumour agent due to its ability to serve as a vector for gene therapy, as well as its direct oncolytic properties [58]. VV oncolytic capacity is a result of the virus's ability to selectively and rapidly replicate in transformed cells leading to the efficient cell-to-cell spread of the virus and production of a lytic infection. A number of VV strains exist with the Wyeth, Lister, Copenhaghan and Western Reserve all displaying oncolytic effects [58]. While naturally occurring VV displays a tumour specific tropism, the deletion of both thymidine kinase (TK) and/or vaccinia growth factor (VGF) have been extensively utilized to enhance the selectively of the virus for transformed cells [59]. The VV genome is able to accommodate the insertion of DNA sequences of up to 25 kBPs in length allowing this virus to be "armed" with therapeutic transgenes in order to enhance its anti-tumour efficacy [58]. The Wyeth strain VV, JX-594, which was shown to selectively replicate and deliver the immunostimulatory cytokine GM-CSF to patients with solid organ malignancies when administered intravenously, is a good example of this [60].

On a preclinical level, only one study has investigated the efficacy of oncolytic VV for the treatment of renal cell carcinoma. Guse *et al.* generated a Western Reserve VV strain that harboured mutations in TK and VGF that was armed with VEGFR and demonstrated its potent oncolytic effects against both human (786-0, ACHN, 786-P) and murine (RENCA) RCC cell lines as well as human umbilical vein endothelial cells (HUVEC) *in vitro* [61]. Moreover, intratumoural and intravenous administration of the virus to both xenograft and immunocompetent murine models of RCC resulted in a significant decrease in tumour volume relative to inactivated virus (control). Of note, the use of the VEGFR armed virus lead to a greater reduction in tumour vasculature and volume relative to the unarmed control virus when administered intravenously in the xenograft model, highlighting the value of this approach for RCC. Interestingly, a single case report of a patient with metastatic renal cell carcinoma treated with an attenuated strain of oncolytic VV has also been described [62]. This patient demonstrated a near complete regression in his pulmonary and vertebral metastasis following repeated intravenous injections of the virus. Taken together these studies demonstrate the potential therapeutic efficacy of VV against RCC.

1.5.3 Herpes simplex virus.

Herpes simplex viruses (HSV) belong to the herpesviridae family and are large enveloped dsDNA viruses. These viruses display selective replication in transformed cells as a result of mutations to genes necessary for attenuation of the host PKR responses, as well as viral replication [63]. Inactivation of $\gamma_1$34.5 and UL39 are the most extensively employed and these genes encode a protein that recruits protein phosphatase 1, which is required to inactivate the effect of PKR and a large subunit of ribonucleotide reductase

that is vital for DNA metabolism, respectively [63]. Furthermore, the ability to modify the HSV envelope allows for enhanced tumour selectivity through transductional targeting and also facilitates the introduction of therapeutic transgenes allowing for the enhancement of the direct oncolytic and immunotherapeutic effects of the virus [63].

The use of an oncolytic HSV for the treatment of RCC was first described by Oyama *et al.* who characterized the *in vitro* and *in vivo* efficacy of the replication competent virus, G207, which harbours mutations in both $\gamma_1$34.5 and UL39 [64]. These authors demonstrated that RCC cell lines (ACHN, A498) following 48 hours infection with G207 at 0.1 multiplicity of infection (MOI) facilitated the doubling of viral particles and by one week were completely destroyed. Moreover, the administration of intratumoural G207 significantly decreased tumour burden in RCC xenograft murine models. More recently, two newly constructed HSV, Synco-2D and FusOn-H2, have also demonstrated efficacy against RCC [65]. The Synco-2D construct, is a derivative of type 1 HSV and is engineered, via both random mutagenesis as well as the insertion of the fusogenic gibbon ape leukemia virus glycoprotein, to induce cell membrane fusion upon infection. This allows the virus to mediate cell death via both direct oncolysis as well as synctia formation. The FusOn-H2 construct is a derivative of type 2 HSV and is engineered to conditionally replicate in cells with activated ras signalling in addition to its cell membrane fusion properties. Both Synco-2D and FusOn-H2 have been shown to induce enhanced oncolysis *in vitro* and *in vivo* against RCC [65]. Despite significant preclinical evidence to support the use of oncolytic HSVs against RCC, no clinical trial evaluating the efficacy of these agents against this disease is ongoing.

1.5.4 Sendai virus.

Sendai virus (SeV) is a member of the paramyxoviridae family and consists of a non-segmented, linear, negative strand RNA genome encapsulated by a nucleoprotein [66]. In susceptible cells the virus replicates entirely within the cytoplasm and because of this poses minimal risk for genomic integration and subsequent mutagenesis [66]. Despite the ubiquitous expression of its receptor (saliac acid), SeVs tissue tropism is quite limited due to the required protease cleavage activation of its fusion glycoprotein (F) for host cell penetration [66]. This so called "protease-dependent tropism" has been exploited in the development of recombinant viral strains which display selective anti-tumour activity as a result of mutations in the F gene that render it susceptible to cleavage by tumour produced matrix metalloproteinases [66]. Unlike most OVs, SeV infection does not lead to the rapid production of viral progeny. Instead, this virus facilitates cell-to-cell spread and cell death via membrane fusion and the formation of synctia, which is accomplished through genetic deletions of its envelope matrix protein (dMSeV) [67].

Kinoh *et. al.* were the first to test a recombinant Sendai virus against RCC. These authors optimized their previously constructed dMSeV by replacing the MMP susceptible cleavage site on the F gene with a urokinase (uPA)-sensitive sequence and tested this newly constructed virus for anti-tumour activity *in vitro* and *in vivo* against a panel of cancer cell lines [68]. The RCC cell line utilized in their experiments (Caki-1) displayed high expression levels of uPA and low levels of MMP2 relative to the other cancer cell lines tested and was sensitive to viral oncolysis *in vitro* and *in vivo*, as demonstrated by the regression of Caki-1 subcutaneous tumours following intratumoural administration of virus. As such, the use of urokinase targeted oncolytic Sendai virus vectors appears to have activity in RCC tumours expressing this specific protease; however the number of

RCC patients that this strategy may be applicable to is limited. Beyond this, utilizing the RENCA immunocompetent murine model, Fujihara *et al.* demonstrated the ability to generate potent anti-tumour immune responses through the intratumoural injection of SeV particles against RCC, which was mediated through CXCL10 expression and NK cell activation [69].

1.5.5 Measles virus.

Measles virus (MV) is negative strand RNA virus that belongs to the paramyxoviridae family. This virus was first identified for its oncolytic properties following the observation that patients with various haematological malignancies who contracted MV infections displayed regression of their disease [70]. Most oncolytic MV are derived from the Edmonston vaccine strain (MV-Edm), as wild-type virus has been shown to have deleterious effects when administered to human patients [70]. Similar to Sendai viruses, MVs contain fusion glycoproteins in their envelope allowing them to propagate and induce cell death intratumourally via the formation of synctia. Selective tumour replication of MV results from the enhanced tumour specific expression of its receptor, CD46, relative to normal cells [70]. Moreover, the tumour selectivity and oncolytic potency of MVs can be augmented through the use of reverse genetic approaches, which allow for the insertion of therapeutic transgenes into the virus vector. To date, MV have shown extensive efficacy against a variety of malignancies in preclinical models including breast, hepatocellular, medulloblastoma, glioblastoma, prostate, ovarian, lymphoma and multiple myeloma as reviewed by Galanis [70].

Recently, the application of MV against RCC was demonstrated utilizing an Edmonston strain (MV-NPL), which was reversed engineered to express the N, P and L genes of the wild-type virus [71]. Meng *et al.* highlighted the robust cytopathic effects, viral replication and apoptosis generated with this newly constructed virus against RCC cell lines *in vitro*, by demonstrating its superiority over a MV expressing only the P gene (MV-P), as well as the V gene deficient MV-Etag strain (MV-ETag). The potent therapeutic efficacy of the MV-NPL strain was further demonstrated by the significant regression in tumour volume seen following intratumoural administration of this virus in an RCC xenograft murine model relative to MV-P and MV-Etag. Interestingly, the expression of the wild-type MV genes lead to a protective effect against the host anti-viral interferon response, highlighting the ability of this construct to facilitate viral propagation through preventing immune clearance [71].

1.5.6 Vesicular stomatitis virus.

Vesicular stomatitis virus (VSV) is a negative strand RNA virus whose replication cycle is exclusively cytosolic and is dependent on five proteins encoded in its genome [72]. Ubiquitously expressed cell membrane phosphatidylserine residues facilitate VSV virus spike G protein binding, thereby providing the virus with the ability to infect nearly all animal cells [72]. The resistance of transformed cells to IFN produced during VSV infection results in their selective destruction by allowing viral replication. While VSV has been extensively utilized as both a vaccine vector and experimental agent for studying innate anti-viral immune responses, a number of strains have been developed which display oncolytic properties (reviewed in 72). Furthermore, the ability to utilize this virus as a novel immunotherapeutic was recently demonstrated by Kottke *et al.* who

administered an engineered VSV vector expressing a cDNA in a murine model of prostate cancer to generate a robust anti-tumour immune response [73].

Interestingly, loss of VHL has been shown to mediate resistance to VSV infection [74]. This was demonstrated by Hwang *et al.* to be dependent on HIF, as both a small molecule inhibitor (chetomin) and siRNA against this transcription factor sensitized RCC 786-O VHL$^{-/-}$ cells to VSV. The ability of HIF upregulation to mediate RCC resistance to VSV was subsequently determined to result from the enhanced anti-viral IFN response following virus infection that was initiated in these cells. Thus, while the use of VSV for RCC tumours with intact VHL is promising, strategies to circumvent the strong HIF mediated IFN response in VHL deficient cells will need to be developed for application of this virus to the majority of patients suffering from this disease. One such strategy is to use chetomin, the small molecule HIF antagonist. Additionally, the use of a combined oncolytic virus approach utilizing VSV with reovirus may also be beneficial in this regard, based on a recent study highlighting the ability of reovirus to down-regulate HIF in a RCC cell line [75].

1.5.7 Encephalomyocarditis virus.

Encephalomyocarditis virus (EMCV) is a positive strand RNA virus of the picornavirus family. Interestingly, in various animal populations including mice, the virus is known to cause diabetes and is highly lethal [76]. As infection with EMCV has never been reported to result in deleterious health effects in humans, this virus has been investigated as an oncolytic agent [33]. Notably, the integral role of NF-κβ mediated anti-apoptotic signalling in facilitating the propagation and spread of EMCV was established

through the observation that knockout of the p50 subunit of NF-κβ, which renders it inactive, results in protection of mice from EMCV infection [77]. Roos *et al.* utilized this virus to target RCC, which is known to display aberrant activation of NF-κβ survival signalling as a result of VHL inactivation [33]. These authors demonstrated the ability EMCV to infect RCC cell lines in an NF-κβ-VHL-HIF dependent manner and induce the regression of established xenograft tumours relative to control (irradiated virus), thus highlighting the potential therapeutic efficacy of targeting the NF-κβ pathway in RCC with this virus.

1.6 In vivo barriers to oncolytic virotherapy: Role of the RCC tumour microenvironment in mediating resistance to oncolytic viruses.

Effective oncolytic targeting and destruction of cancer cells by viruses relies on both their ability to selectively replicate and induce cell death, as well as priming of an anti-tumour immune response. However, the lack of success of OVs as cancer monotherapies in early clinical trials suggests that immune clearance of virus, as well as physiochemical tumour microenvironmental barriers to oncolytic virotherapy exist [78]. Interestingly, the RCC specific tumour microenvironment is characterized by OV restrictive factors, which likely act to limit the therapeutic efficacy of these agents through suppression of anti-tumour immunity and prevention of effective intratumoural viral delivery (summarized in **Table 2**).

Table 2. Rational combination approaches involving oncolytic viruses for use against renal cell carcinoma (RCC). VEGFR: vascular endothelial growth factor receptor, PDGFR: platelet derived growth factor receptor, c-kit: proto-oncogene c-Kit, flt3: fms-like tyrosine kinase 3, RET: rearranged during transfection proto-oncogene, CSF-1R: colony stimulating factor-1 receptor. Figure adapted from: Lawson KA *et. al.* Expert Opin Biol Ther 2012;12(7):891-903.

Combination Strategy (OV+)	Targets	Proposed mechanism of synergy	Microenvironment Barriers Overcome	Ref.
Cyclophosphamide	Innate immune effectors	• Decreased viral immune clearance	• Innate anti-viral immune response • Neutralizing antibodies	80-82
Sunitinib	VEGFR, PDGFR, c-Kit, flt3, RET, CSF-1R	• Enhanced anti-tumour immune response • Improved systemic delivery	• Tumour induced immunosuppression • Aberrant vasculature	98, 143
Bevacizumab	VEGFR	• Enhanced anti-tumour immune response • Improved systemic delivery	• Tumour induced immunosuppression • Aberrant vasculature	147-148
Temsirolimus/everolimus	mTOR	• Improved immunosurveillance *(Increased $CD8^+$ memory T cells)*	N/A	143

1.6.1 Host immune response: viral immune clearance and the suppression of anti-tumour immunity.

While the host immune response generated following OV administration allows for protection against a systemic pathologic infection, it also limits tumour specific systemic oncolysis by facilitating viral immune clearance. This is of particular relevance to RCC as this disease is known to respond to immunotherapy (as discussed in section 2.2) yet is characterized by an endogenous immunosuppressive phenotype. Highlighting this is the finding that $CD4^{+}$ T cells isolated from mRCC patients display a blunted Th-1 cytokine response following stimulation with TAA [79]. This potential paradox of active viral immune clearance in the setting of a tumour immune suppressive microenvironment is perhaps why OV monotherapy has only been modestly successful to date. Further, both the innate and adaptive immune responses following OV infection contribute to viral clearance. The involvement of the innate immune response in mediating OV clearance was clearly demonstrated by Fulci *et al.* who highlighted the ability of cyclophosphamide to enhance the therapeutic efficacy of a HSV against a syngeneic rat glioma model through inhibiting HSV mediated NK cell production of IFN-γ and tumour infiltration of $CD68^{+}$/$CD163^{+}$ macrophages [80]. Moreover, depletion of complement has also been demonstrated to enhance glioma virotherapy, further portraying the ability of innate immune response to limit OV efficacy [81]. The involvement of the adaptive immune system in limiting OV efficacy is evidenced by numerous clinical experiences with a variety of OVs, including both reovirus and HSV, which have demonstrated the ability of neutralizing antibodies to mediate viral immune clearance thereby restricting the application of these agents as a monotherapy clinically [82-83].

The host immune system also acts to suppress the therapeutic anti-tumour immune response generated by oncolytic virotherapy by the induction of immunosuppressive cytokines and regulatory cells [84-85]. The induction of anti-tumour "hyperactivated NK cells" upon Treg depletion following treatment of C57Bl/6 mice bearing subcutaneous B16 melanoma tumours with either reovirus or VSV highlights this as does the recent demonstration that VSV induced myeloid derived suppressor cells (MDSC) mediate antagonism between combined oncolytic virotherapy and cyclophosphamide through suppressing antigen-specific T cell activation and NK dependent tumour destruction [84, 86-87]. Interestingly, blood samples from mRCC patients depleted for MDSC with a $CD11b^{+}$ antibody display a reactivation of $CD8^{+}$ T cell proliferation, IFN-γ production and CD3 expression upon antigenic stimulation, highlighting that MDSC regulate the functionality of effector $CD8^{+}$ T cells and contribute to the immunosuppressive phenotype seen in mRCC [88]. Likewise, elevated levels of $CD4^{+}$ $CD25^{+}$ T regulatory cells (Treg) have also been demonstrated to contribute to the immune suppression present in mRCC patients receiving immunotherapy (IL-2) [89]. Moreover, RCC tumours are known to produce immunosuppressive cytokines such as TGF-β which in further contribute to the inhibition of anti-tumour immunity and generation of tumour induced immune tolerance present in this disease [90]. Hence, MDSC, Tregs and immunosuppressive cytokines in the RCC tumour microenvironment are potential barriers to the application of oncolytic virotherapy.

1.6.2 Physical barriers to OV systemic delivery: aberrant tumour vasculature.

Effective treatment of metastatic tumours with OV requires their successful systemic dissemination and penetration into these sites. Similar to treating metastatic

disease with conventional chemotherapy, there are a number of physical barriers that may limit the delivery of OV to tumours [78]. More recently, it is becoming evident that central to this is the aberrant vasculature that characterizes nearly all tumours as a result of neoangiogensis. This process does not result in functional mature blood vessels, but rather, produces a vascular network that is tortuous, dilated, leaky and poorly supported by pericytes [91]. Consequently, despite the relative increase in blood vessel density compared to normal tissues, tumours are poorly perfused. RCC is no exception to this as demonstrated by $H_2^{15}O$ and $C^{15}O$ positron emission tomography (PET) studies which highlight the decreased perfusion characterizing these tumours [92]. Beyond poor perfusion, high intratumoural interstitial fluid pressure resulting from aberrant neo-vascularization may also limit the physical delivery of OV to their target site; as demonstrated by the finding that restoration of normal interstitial fluid pressure results in improved penetration of topotecan into xenografted tumours [93]. Thus, in order to maximize the systemic delivery of OV against RCC, the aberrant vasculature characteristic of this disease must be normalized.

1.7 Conclusions drawn from literature review.

In summary, the treatment of mRCC remains a significant challenge due to the lack of available therapeutic options that have meaningful impacts on overall survival. With an increased knowledge of RCC carcinogenesis/progression it is becoming evident that this disease is characterized by an extensive array of molecular signalling aberrations. Because of this, the development of oncolytic virotherapy for RCC is particularly of interest, as these biologics mediate their selective anti-tumour effects by exploiting the distinct molecular features of transformed cells. Beyond this, OVs are ideal RCC

therapeutics as these agents are capable of generating robust innate and adaptive anti-tumour immune responses, allowing them to be utilized as novel immunotherapeutics against this disease. To date, seven OV have been tested for their therapeutic efficacy against RCC with favourable results *in vitro,* as well as in xenograft and immunocompetent murine models. However, the clinical utility of these agents is largely unknown due to the lack of RCC specific clinical trials. Moreover, investigations with those OVs which have been studied extensively in the clinical setting, namely reovirus, are lacking, highlighting the need for both preclinical and clinical studies utilizing these agents against RCC.

Based on the clinical experience with oncolytic virotherapy in other tumour sites as well as a better understanding of the RCC specific tumour microenvironment it is also apparent that many barriers to the application of these agents exist including viral clearance, tumour induced immunosuppression and aberrant vasculature. As such, it is likely that rationale combination therapies, which overcome these microenvironmental barriers and augment the oncolytic and immunotherapeutic effects of these agents will hold the greatest potential for future clinical use against RCC.

1.8 Project rationale.

To address the gaps in knowledge apparent in the literature a rational combinatorial approach involving the use of oncolytic virus with a currently utilized mRCC therapeutic was developed. The goal of this approach was to achieve direct oncolysis while concomitantly generating an OV mediated anti-tumour immune response augmented through exploiting the well established immune modulatory effects of mRCC

targeted therapies. Theoretically, this approach would allow for the multi-mechanistic targeting of RCC.

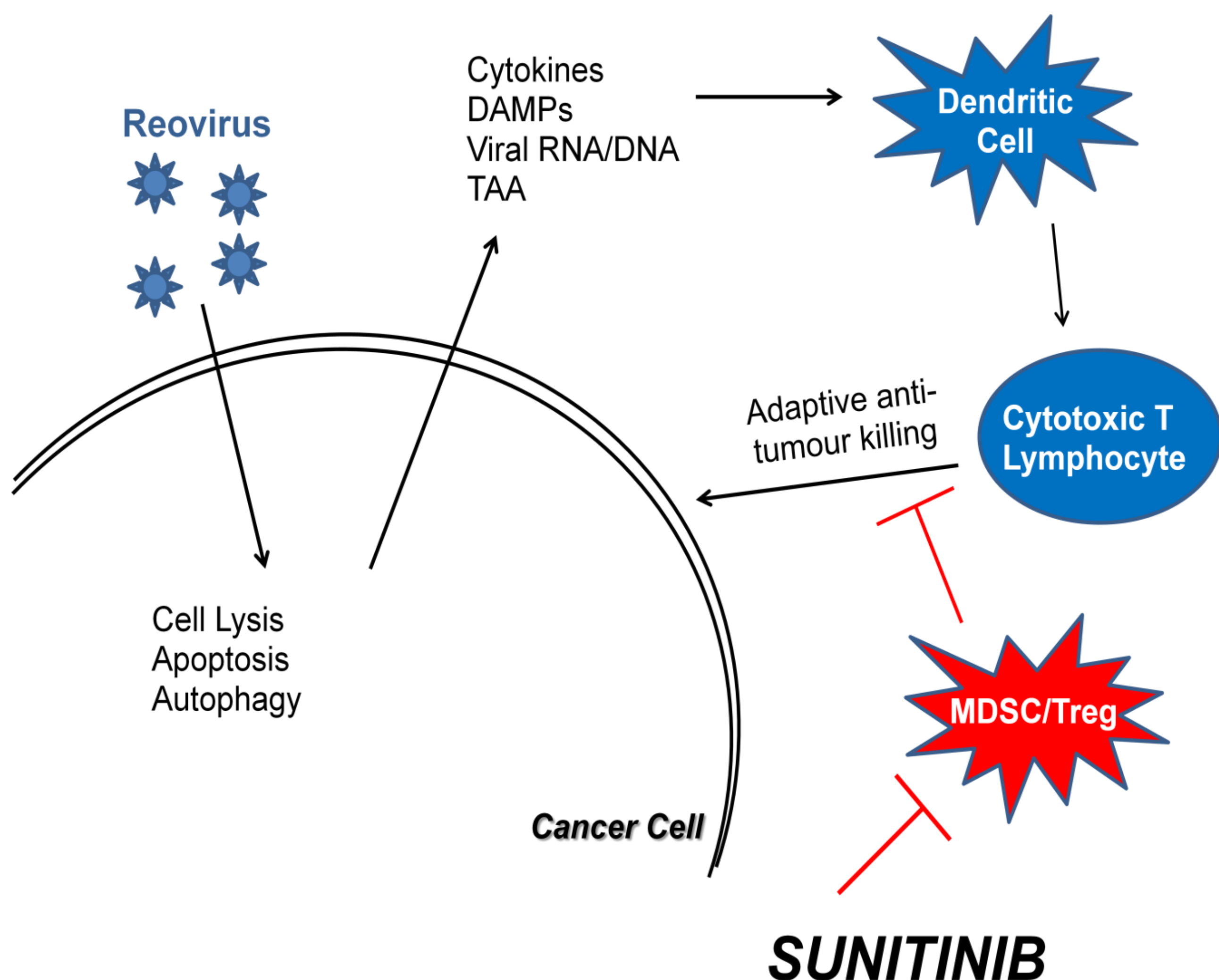
In addition to its novel application against RCC, reovirus was chosen as the OV to include in our combination regimen as it is the most clinically studied OV, demonstrating efficacy in phase II clinical trials in combination with platinum based chemotherapy across multiple solid malignancies [94]. Of the over 600 patients treated thus far with this agent, no maximum tolerated dose has been reported, highlighting its tolerability and safety. Beyond this, reovirus was chosen due its reported widespread efficacy in preclinical murine models of cancer which allows for the generalization of our proposed combination study results to tumour sites beyond renal.

Furthermore, in experimental murine models, reovirus administration is capable of priming innate and adaptive anti-tumour immune responses [16, 44]. This anti-tumour immunity not only contributes to reovirus' therapeutic efficacy, but has also been demonstrated to result in the generation of long term tumour immunosurveillance [95, 96]. Hence, in addition to its direct oncolytic effects, reovirus is also a potent immunotherapeutic, satisfying our goal of utilizing an OV to generate a therapeutic immune response against RCC. The biology of this virus will be covered further in detail throughout Chapters 2 and 3.

Among the targeted therapies utilized in the management of mRCC, sunitinib was chosen in our investigations as it is currently approved for first line therapy. To date, extensive research has demonstrated the anti-angiogenic effects sunitinib has and recently this activity has been shown to mediate the majority of its clinical activity [97].

Interestingly, as highlighted above, sunitinib also acts as a potent immune modulatory agent resulting from its ability to target immune suppressor cells (MDSC, Treg) and reverse the Th-2 bias present in mRCC patients [98]. As this immune modulatory effect of sunitinib was discovered through studying mRCC patients clinically (unlike the mTORi) we believe the use of this agent in our proposed combination approach should give our results more applicability to the clinical scenario. This, together with the increasing evidence that OV are novel immunotherapies, in addition to direct oncolytic agents lead us to focus our investigations on determining whether sunitinib would be able to augment reovirus mediated anti-tumour immunity (**Figure. 2**).

Figure 2. Rationale for augmenting reovirus mediated anti-tumor immunity with sunitinib. RCC patients are characterized by an increased number of immune suppressor cells within their tumor microenvironments such as T-regulatory (Treg) cells and myeloid derived suppressor cells (MDSC), which act to limit the efficacy of immunotherapies, including reovirus. Sunitinib, a multi-tyrosine kinase inhibitor, is capable of downregulating these cell populations. As such, we hypothesis the use of this agent to augment reovirus mediated anti-tumor immunity. Figure adapted from Prestwich RJ *et. al.* Expert Rev Anticancer Ther 2008;8(10):1581-8.



Thus, we propose the use of reovirus in combination sunitinib for the treatment of RCC as a novel therapeutic paradigm that incorporates multiple mechanisms including direct viral oncolysis, anti-tumour immune targeting and anti-angiogenesis. Again our selection of these agents is based on both preclinical and clinical literature that suggests our findings should have immediate translation potential in the form of a clinical trial for patients with mRCC. Our study is novel to the field of OVs in that reovirus has not been investigated as a therapeutic against RCC to date. Beyond this, the use of sunitinib to augment the anti-tumour oncolytic and immunotherapeutic effects of an OV has yet to be reported. In the described work of this thesis, we employ the use of human and murine RCC cell lines as well as a syngeneic immunocompetent murine model of RCC (RENCA model) to determine the oncolytic and immunotherapeutic effects of reovirus as both a monotherapy and in combination with sunitinib against this disease.

1.9 Objectives

- Determine the ability of reovirus to initiate an oncolytic response against RCC *in vitro*.
- Determine the ability of sunitinib to augment reovirus mediated oncolysis *in vitro*.
- Characterization of reovirus and combination therapy effect on tumour burden and overall survival in the RENCA syngeneic immunocompetent murine model.
- Characterization of reovirus and combination therapy effect on anti-tumour adaptive immune response and splenic myeloid derived suppressor cells in the RENCA model.
- Determine the ability of reovirus and combination therapy to prime protective immunity in the RENCA model.

1.10 Hypothesis.

That the combination of reovirus with sunitinib will result in an augmented anti-tumour immune response in addition to the direct oncolytic and anti-angiogenic effects of these agents for enhanced renal cell carcinoma tumour regression and immunosurveillance.

Chapter 2.

In vitro evidence for the use of reovirus as a monotherapy and in combination with sunitinib for the treatment of renal cell carcinoma.

2.1 Introduction.

Reovirus is a common environmental virus and member of the Reoviridae family, which also includes the rotavirus and Colorado-tick fever virus. Unlike the latter two viruses, reovirus infection results in minimal toxic effects in humans, displaying a natural tropism for the respiratory and enteric organs which can result in flu-like symptoms. As such, reovirus is an orphan virus in that it is not historically associated with human disease. The name REO-virus denotes these aforementioned properties: respiratory, enteric, orphan. Three serotypes of reovirus exist including the type 1 Lang, type 2 Jones and type 3 Dearing, being differentiated based on their hemagglutination inhibition activity [99]. The type 3 Dearing strain has been studied most extensively as an oncolytic agent. The reovirus genome consists of 10 dsRNA segments ranging from 1.2 to 3.9 kilobases in length and is surrounded by two isosahedral shaped protein shells which include its outer capsid and inner core. Both the outer capsid and inner core are composed of reovirus proteins which are named after their respective encoding gene segments: lambda (λ), mu (μ), sigma (σ) [100].

The σ-1 protein of the reovirus outer capsid is responsible for cell-surface binding through interactions with sialiac acid on the cell membrane [100]. Alternatively, reovirus σ-1 protein also mediates cell attachment by binding JAM-A receptors, which are members of the immunoglobulin superfamily [101]. Once engaged on the cell membrane, reovirus is internalized through a clathrin-dependent receptor mediated endocytosis mechanism [102]. Within the endocytic compartment its viral capsid undergoes acid-dependent proteolysis to form the disassembly intermediate infectious subviral particle (ISVP). This particle is characterized by the loss of the σ-3 protein and cleavage of the

μ1c protein into its associated delta and circle fragments [100]. Through a series of further conformational changes the ISVP becomes the ISVP* hallmarked by the exposure of hydrophobic residues within the μ-1 fragments and loss of the σ-1 protein [100]. The μ-1 fragment conformational changes results in the rupturing of the endosomal membrane allowing for the release of the reovirus core particles into the cytoplasm where they are transcriptionally active [100]. Importantly, the μ-1 cleavage fragments have also been demonstrated to activate NF-κβ resulting in the initiation of innate immune and apoptosis [103, 104].

Interestingly, efficient reovirus mediated innate immune signalling and apoptosis not only requires NF-κβ, but also relies on activation of the IRF-3 transcription factor. Unlike, NF-κβ however, IRF-3 is not activated by viral protein alone as demonstrated by the finding that reovirus particles lacking genetic material can stimulate the activation of NF-κβ but not IRF-3 [100, 105, 106]. Instead, IRF-3 is activated by a number of dsRNA cytoplasmic binding proteins including RIG-I, RLHs, and Mda5 [107]. These proteins recognize reovirus dsRNA and activate IPS-1 which subsequently results in activation of multiple transcription factors including IRF-3, through the engagement of IKK-α [107]. Once in the nucleus, IRF-3 and NF-κβ act to coordinate the expression of proteins involved in innate anti-viral immunity and apoptosis, however, the newly synthesized proteins resulting from this activity which are vital to the induction of apoptosis remain unknown. Shedding light on this is a recent report by Knowlton *et al.* who determined the levels of the pro-apoptotic protein NOXA rise by 36 fold during a reovirus infection, and that apoptosis induction is significantly diminished in cells lacking this protein [107]. Furthermore, many reports have highlighted the involvement of extrinsic apoptotic

signalling in facilitating reovirus mediated apoptosis such as the engagement of TNF-α and death receptor/TRAIL signalling pathways [108]. As such, the infection of a cell by reovirus results in a coordinated induction of both innate immunity and apoptosis in addition to cell lysis, following viral particle re-assembly, enabling this virus to mediate direct cytotoxicity. This is known as the direct oncolytic effects of a reovirus infection. Importantly, this direct cytotoxicity acts to prime an adaptive immune response which further contributes to the viruses pathogenic effects. The exploitation of this response as a form of cancer immunotherapy will be detailed in Chapter 3.

The ability of reovirus to replicate selectively in transformed cells was first described by Hashiro *et. al.* in 1977 [109]. Subsequently, the work of Dr. Patrick Lee's group at the University of Calgary, shed light on the molecular mechanisms responsible for this selectivity and lead to the discovery of reovirus' oncolytic abilities. In a seminal paper, the established ability of reovirus to replicate in cells with activated ras was exploited to treat cultured glioblastoma cells as well as tumour bearing immune-compromised and -competent mice, highlighting a role for utilizing reovirus as a novel anti-cancer therapeutic for ras activated tumours [110]. In following studies, ras activation was found to negatively regulate the activity of the double stranded RNA-activated protein kinase PKR, an enzyme known to phosphorylate eIF-2 resulting in the inhibition of viral transcripts [111]. Hence, a paradigm was developed in which cancer cells driven by the ras oncogene supported reovirus replication due to inactive PKR [99]. Since this time, the involvement of ras signalling in mediating host cell susceptibility to oncolysis has expanded and a role for this signalling axis has been described for reovirus uncoating, infectivity, and apoptosis [112]. With this however, conflicting reports have also been

published wherein ras signalling status does not predict sensitivity to reovirus oncolysis. Indeed, a recent study in which ras signalling was perturbed by both upstream and downstream inhibitors failed to uncover a correlation between ras signalling and squamous cell head and neck cancer cell lines [113]. As such, despite the well characterized role for ras and intracellular tyrosine kinase signalling in mediating reovirus oncolysis, the precise mechanisms responsible for the selective destruction of cancer cells remain unknown.

Since its initial description as an oncolytic agent, significant progress in establishing reovirus as an anti-cancer therapeutic has been made. Indeed the virus has been demonstrated to replicate and induce a lytic infection in a myriad of cancer cell lines and murine tumour models including breast, ovarian, non-small cell lung, bladder, multiple myeloma, lymphoid, prostate, melanoma, sarcoma and colon cancer [114-123]. Based on the success of this agent preclinically, numerous clinical trials (over 30) have also been initiated with the virus, lead by collaborations with university investigators and the biotech company Oncolytics Biotech Inc. (Calgary, Alberta), which produces and markets reovirus under the trade name Reolysin™. In early phase I and II trials the virus has demonstrated an excellent safety and tolerability profile when administered both intralesionally and intratumourally, with no maximum tolerated dose being reached in the over 600 patients treated to date. The most common side effects reported include local erythema at virus injection sites and self-limiting flu like symptoms [124]. Additionally, as reovirus is genomically stable, the risk of pathogenic mutations occurring once utilized on a widespread basis clinically is minimal. Despite the significant evidence highlighting the safety of reovirus, the efficacy of this agent as an anti-tumour monotherapy is limited

with no durable complete responses being reported. As mentioned above, many barriers to the use of oncolytic viruses exist and these most certainly apply to reovirus. To address these, multiple trials are now investigating the use of combination therapy approaches involving reovirus to overcome such barriers.

To date, no group has studied the use of reovirus as a monotherapy or in a combination regimen for the treatment of renal cell carcinoma. As such in our first line of investigation we sought to characterize the response of RCC to reovirus as a monotherapy and in combination with sunitinib, a first line mRCC therapeutic. This *in vitro* cell culture line of experimentation allows for observations between the interactions of these two agents on RCC cytotoxicity in the absence to the tumour microenvironment to be made. Comparisons of this data to that generated in our *in vivo* model (Chapter 3) will aid in determining the relative contribution of the tumour microenvironment in facilitating the therapeutic interaction between these two agents.

2.2 Methods

2.2.1 Cell lines and virus.

ACHN, A498, 786-0, L-929, and RENCA cell lines were obtained from the American Type Culture Collection (ATCC). ACHN, A498, 786-0 are human clear cell RCC cell lines, RENCA is a murine cortical adenocarcinoma RCC cell line and L-929 is a fibroblast cell line. ACHN and A498 were cultured in minimum essential medium (MEM); 786-0 was cultured in Roswell Park Memorial Institute medium (RPMI); L-929 was cultured in Dulbecco’s modified eagle medium (DMEM); and RENCA was cultured in RPMI. All media was supplemented with 10% heat-inactivated fetal calf serum (FBS).

Reovirus serotype 3 (RV) was grown in L-929 cells then purified and titered as per standard lab protocol. Aliquots of reovirus were UV-irradiated to produce non-replicating dead virus (DV) controls. Sunitinib was purchased from Selleck® chemicals (Houston, TX) and dissolved in 1% dimethyl sulfoxide (DMSO).

2.2.2 Cell viability (WST-1) assay.

Cells were seeded at a density of 3000 (A498, 786-0) or 8000 (ACHN, RENCA) cells/well into 96-well micro-titre plates and incubated for 24 hours in 200 µl of 10% FBS containing media. A hemocytometer and typan blue viability dye were utilized to quantitate the number of viable cells to ensure accurate cell density during seeding. DV, DMSO, RV, sunitinib or a combination of these agents were then added to each well for 48 hours at predetermined concentrations in fresh 10% FBS containing media. Specific to DV and RV infection, aliquoted virus was added to wells in 50 µl of media without FBS for 45 minutes, after which 150 µl of FBS containing media was added (final media contained 10% FBS). Following the 48 hour treatment period, drug containing media was replaced with media containing (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt (WST-1) (Roche, Basel, Switzerland) diluted 10:1 and absorbance (450 nm) was quantified utilizing a Bio-rad® plate reader. Percent viability was calculated as the absorbance ratio of treated/untreated cells multiplied by 100.

2.2.3 Viral progeny assay.

ACHN, 786-0, A498 and RENCA cells were treated with RV plus or minus sunitinib in 6-well plates in their respective media for 48 hours. RV infection was

conducted as described for the cell viability assay. The cultures were then freeze-thawed 3 times and supernatants were harvested for plaque titration on L-929 cells in semi-solid medium for 72 hours. Briefly, L-929 cells were cultured in 6-well plates (4.0 x 10^5 cells/well) and incubated for 24 hours at 37ºC. 100 µl of serial diluted (10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}) supernatants from freeze-thawed RV infected RCC cell lines were then used to infect L-929 cells at 4ºC for 45 minutes. Following this, 25 ml of 2X DMEM containing 10% FBS was mixed with 25 ml of 2% bacto-agar (heated to 46ºC in a water bath) and added in aliquots of 3ml to each well of the 6-well plate and allowed to solidify at room temperature. Plates were then placed upside down and incubated in moisture containers at 37ºC for 3 days. After the 3 day incubation period, 25 ml of 2X DMEM containing 10% FBS and 4% neutral red solution was mixed with 25 ml of 2% bacto-agar (heated to 46ºC in a water bath) and added in aliquots of 3 ml to each well of the 6-well plate and allowed to solidify at room temperature. After a 24 incubation period at 37ºC visible plaques from each well were counted to allow for virus titre calculation. Technical assistance was provided by Zhong Qiao Shi.

2.2.4 Luminex® assay.

RENCA cells were seeded in 6-well plates and treated with RV as per the cell viability assay for 48 hours after which supernatants were collected and centrifuged to remove cells. To quantify levels of RANTES, MIP-1α, IP-10, MCP-1, KC, and MIG, a Mouse Chemokine Multiplex Kit (Invitrogen, Carlsbad, CA) was utilized as per manufacturer's protocol. Briefly, capture analyte (chemokine) beads were added to 96-well plates as per manufacturer's protocol after which 50 µl of kit standards or aforementioned supernatant samples diluted in 50 µl of assay diluent were added to wells.

After a 2 hour incubation period at 37ºC supernatants were removed and 100 µl of 1X biotinylated detection antibody was added to each well. After a 1 hour incubation time at 37ºC antibody was removed, plates were washed and 100 µl of 1X streptavidin-RPE was added to each well for 30 minutes. Plates were then washed and subsequently read on a Luminex® 200 instrument (Austin, TX). Technical assistance was provided by Jason Spurrell.

2.2.5 In vitro synergy assay.

Dose response curves for all cell lines treated with RV or sunitinib were generated as per the cell viability assay. Calcusyn® software (Great Shelford, Cambridge, UK) was utilized to generate effect dose for 50 percent cytotoxicity (ED_{50}) values for each cell line to reovirus and sunitinib from the dose response data. Additionally, ED_{50} values were calculated based on the log plot of the dose response data generated for each cell line with the cell viability assay with an R-value of > 0.95 being considered as acceptable. This data was utilized to verify correct calculation of ED_{50} values generated by calcusyn® software. Cell lines were then seeded into 96-well plates as per the cell viability assay and treated with escalating doses of RV and sunitinib concurrently at a fixed ratio of ED_{50}:ED_{50}. Cell viability was quantified as per the cell viability assay and combination index values were generated from this data using calcusyn® software. For data analysis, a CI value < 1 denoted a synergistic response, > 1 denoted an antagonistic response and = 1 denoted an additive response.

2.2.6 In vitro migration assay

Supernatants were harvested from ACHN cells cultured in RPMI media containing 2% FBS for 48 hours in the presence of DV [1 MOI] or RV [ED_{50} or 1 MOI]. PBMCs were isolated from blood of healthy human subjects by venipuncture with sterile technique followed by centrifugation over a ficoll-hypaque gradient (GE Healthcare, Uppsala, Sweden). PBMCs were cultured in RPMI media with 10% FBS for 48 hours prior to assay to remove adherent PBMC population. 600 µl of supernatants were then placed in bottom chamber of 24 well plates (Invitrogen, Carlsbad, CA) and 1.5 x 10^6 PBMCs suspended in 100 µl of RPMI media (2% FBS) were placed in upper chamber inserts. Plates were incubated at 37ºC and after 6 hours, upper chamber inserts were removed and migratory $CD8^+$ and $CD56^+$ cells were counted via flow cytometry. For flow analysis, migrated PBMCs from bottom chambers were stained with FITC-labeled mouse anti-human CD8 (BD, Franklin Lakes, NJ) or PE-labeled mouse anti-human CD56 (BD, Franklin Lakes, NJ) and re-suspended in 500 µl after which total $CD8^+$ or $CD56^+$ PBMCs were counted utilizing an Attune® flow cytometer.

2.2.7 TUNEL apoptosis assay

ACHN cells (5.0 x 10^5) were seeded into 6-well plates in MEM media containing 10% FBS and incubated at 37ºC for 24 hours. Culture media was then removed and cells were treated with RV [ED_{50} or 100 MOI], sunitinib [ED_{50}] or their combination (concomitantly) for 48 as described for the *in vitro* synergy assay. Cells treated with DMSO [1% in PBS] and DV [100 MOI] or their combination were used as controls for sunitinib and RV, respectively. Apoptosis was subsequently assessed via the APO-BrdU™ TUNEL Assay Kit (Invitrogen, Carlsbad, CA) utilizing flow cytometry. Briefly, supernatants from treated cells were collected and pooled with adherent cells removed

from each well by trypsin digestion. Cells were then washed and fixed with PBS and 1% paraformaldehyde, respectively, followed by re-suspension in 70% ethanol and stored in a -20C freezer. Prior to flow cytometric analysis cells were treated with a BrdU DNA-labeling solution followed by incubation with Alexa Fluor 488 labelled anti-BrdU antibody as per the Invitrogen protocol. Positive and negative controls were provided with Invitrogen APO-BrdU™ TUNEL Assay Kit to serve as relative markers of apoptosis.

2.2.8 Statistics.

Statistical analysis was performed utilizing unpaired two-tailed t-tests. Statistical significance was defined as p-values being < 0.05 unless otherwise stated.

2.3 Results.

2.3.1 Reovirus replicates in human and murine renal cell carcinoma cell lines resulting in oncolysis and chemokine production.

Reovirus is a non-enveloped dsRNA virus that has demonstrated oncolytic activity against a wide variety of malignancies in both preclinical and clinical studies. As this virus has not been investigated to date against RCC our first objective was to determine its oncolytic activity in a panel of human RCC cell lines. Treatment of 786-O, ACHN and A498 cell lines with reovirus resulted in a dose dependent decrease in cell viability as determined by WST-1 assay (**Figure. 3A**). As reovirus oncolysis is dependent on viral replication we also conducted plaque titration assays following reovirus infection of the 786-0, ACHN and A498. These experiments demonstrated a rise in viral titre over 72 hours of reovirus infection, highlighting a direct oncolytic effect of the virus against RCC (**Figure. 3B**). Light microscopy of all cell lines exposed to virus for 48 hours at 40 MOI

confirmed a cytopathic effect, highlighted by plasma membrane blebbing, cell surface detachment and rounding of RCC cells (**Figure. 4**).

Reovirus has been demonstrated to initiate innate immune responses characterized by the production of pro-inflammatory cytokines including RANTES, MIP-1-α, MCP-1, KC, IP-10 and MIG across a variety of melanoma and prostate cancer cell lines in addition to its direct oncolytic effects [96, 125]. Based on these findings we characterized the ability of reovirus to stimulate production of these chemokines during infection of the RENCA murine cell line. This cell line was studied to provide proof-of-principle of inflammatory response generation *in vitro* for our subsequent *in vivo* experiments. Similar to the human RCC cell lines, infection of RENCA cells with reovirus resulted in viral replication and cytotoxicity (**Figure. 5A-C**). Furthermore, following 48 hours of reovirus infection at a concentration of both 0.007 MOI (ED_{50}) and 1 MOI increased chemokine expression was observed as determined by luminex assay, highlighting its ability to generate an inflammatory oncolytic response against RCC (**Figure. 6**).

Figure 3. Reovirus has direct oncolytic effects against human RCC. A) Cell viability of RCC cell lines (786-O, ACHN, A498) treated with escalating doses of RV for 48 hours determined by WST-1 assay. DV at 100 MOI used as control. B) RV titre determined by plaque titration assay over 72 hours following infection of RCC cell lines (786-O, ACHN, A498) with RV at 40 MOI. DV not included as no productive titre. Error bars = SEM of at least three independent experiments.

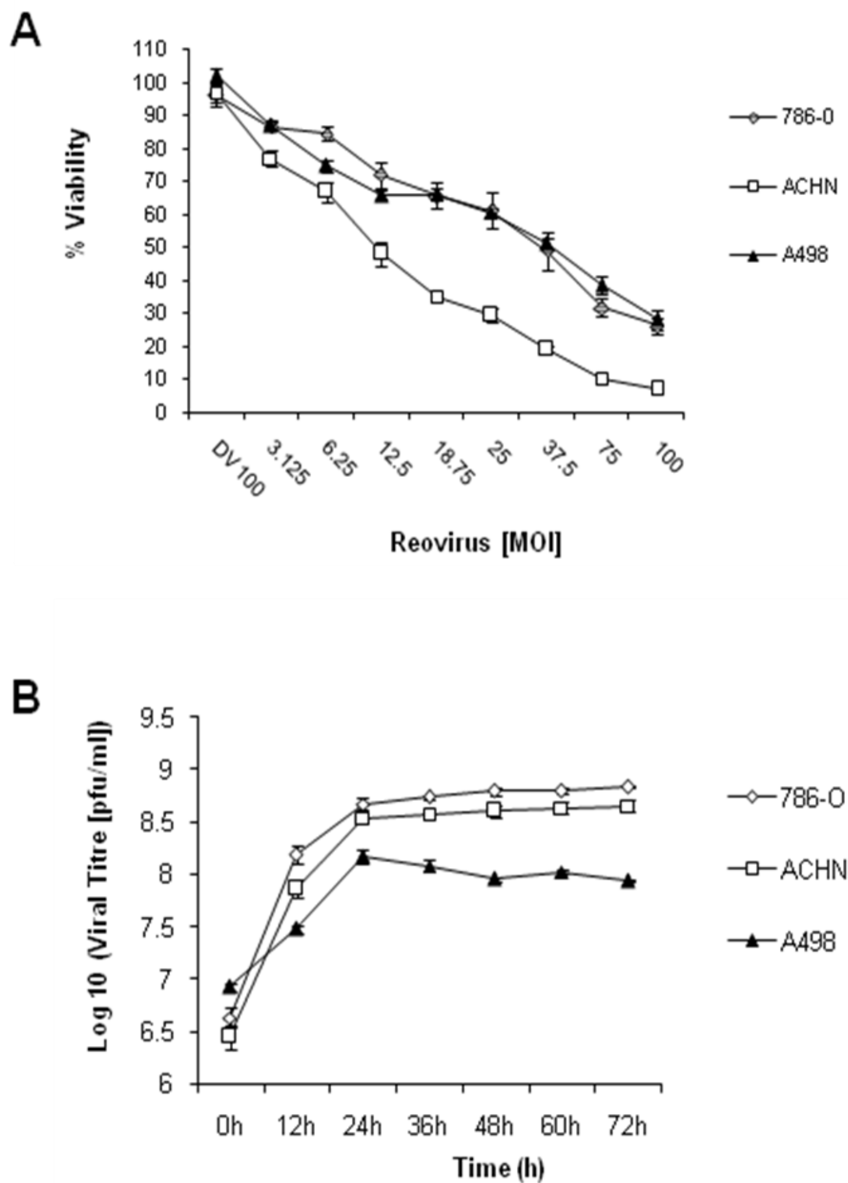


Figure 4. Cytopathic changes in reovirus infected human RCC cells. Pictures of RCC cells (786-O, ACHN, A498) infected with 40 MOI of DV or RV for 48 hours, taken by light microscopy with a ZEISS® Axiovent 200M camera at 10X zoom. Technical assistance was provided by Zhong Qiao Shi.

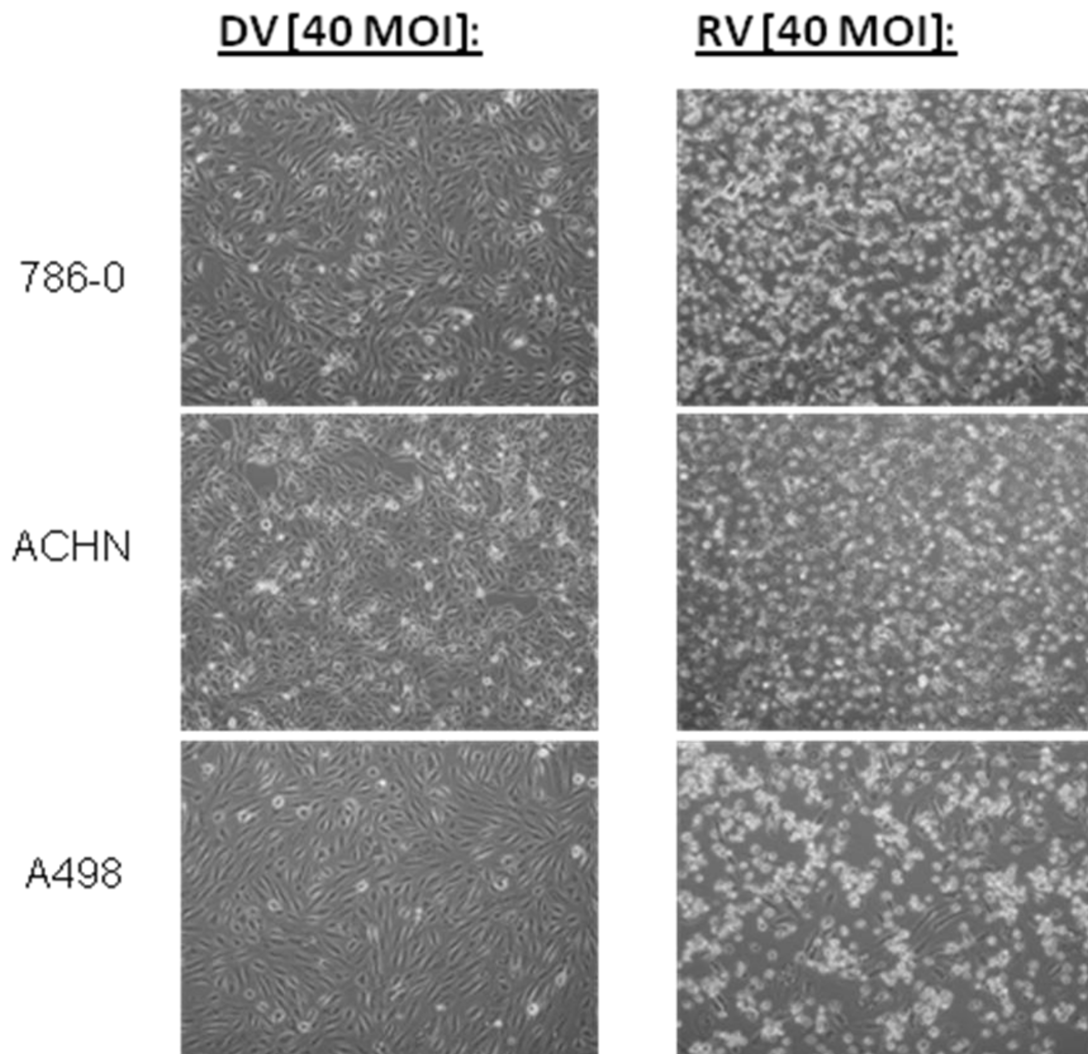
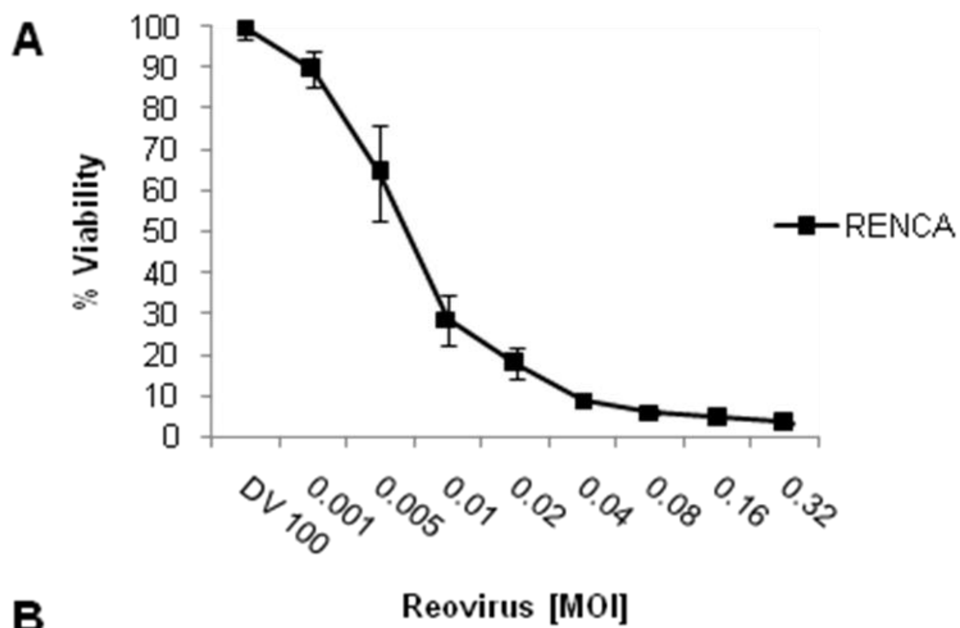


Figure 5. Reovirus induces a cytotoxic response in RENCA cells. A) Cell viability of RENCA cell line treated with escalating doses of RV for 48 hours determined by WST-1 assay. DV at 100 MOI used as a control. B) RV titre determined by plaque titration assay over 72 hours following infection of RENCA cells with RV at 40 MOI. C) Pictures of RENCA cells infected with 40 MOI of DV or RV for 48 hours, taken by light microscopy with a ZEISS® Axiovent 200M camera at 10X zoom.


A
100
90
80
70
60
50
40
30
20
10
0
% Viability
RENCA
DV 100
0.001
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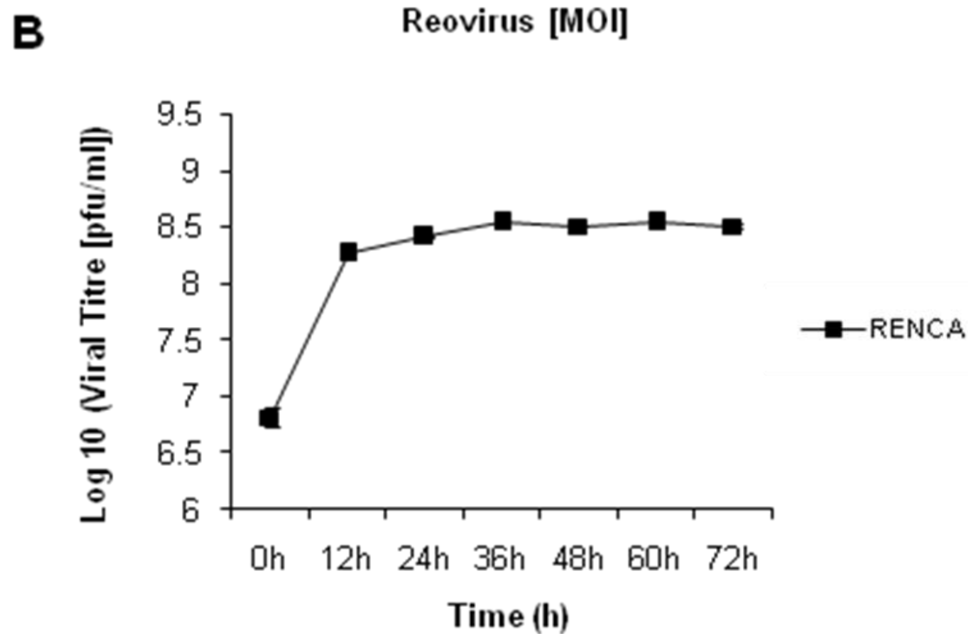
0.01
0.02
0.04
0.08
0.16
0.32
Reovirus [MOI]


B
9.5
9
8.5
8
7.5
7
6.5
6
Log 10 (Viral Titre [pfu/ml])
RENCA
0h
12h
24h
36h
48h
60h
72h
Time (h)

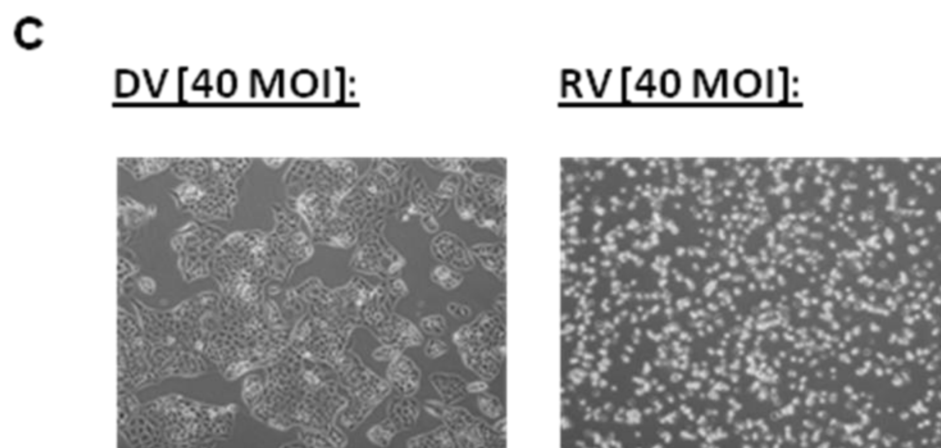
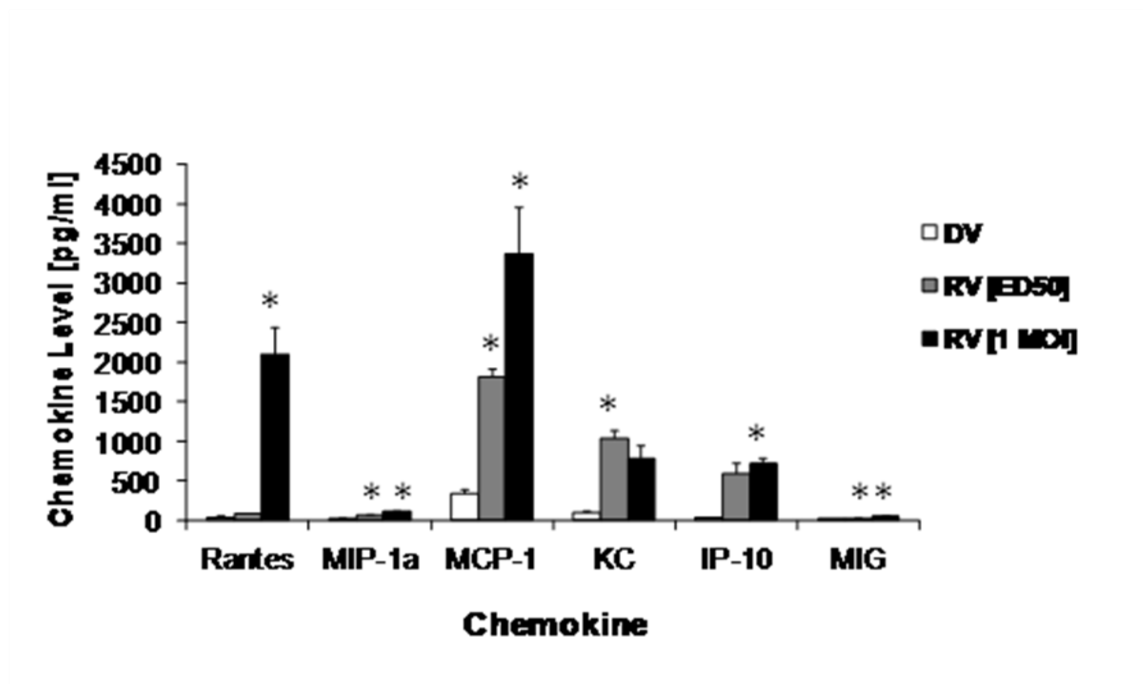

C
DV [40 MOI]:
RV [40 MOI]:

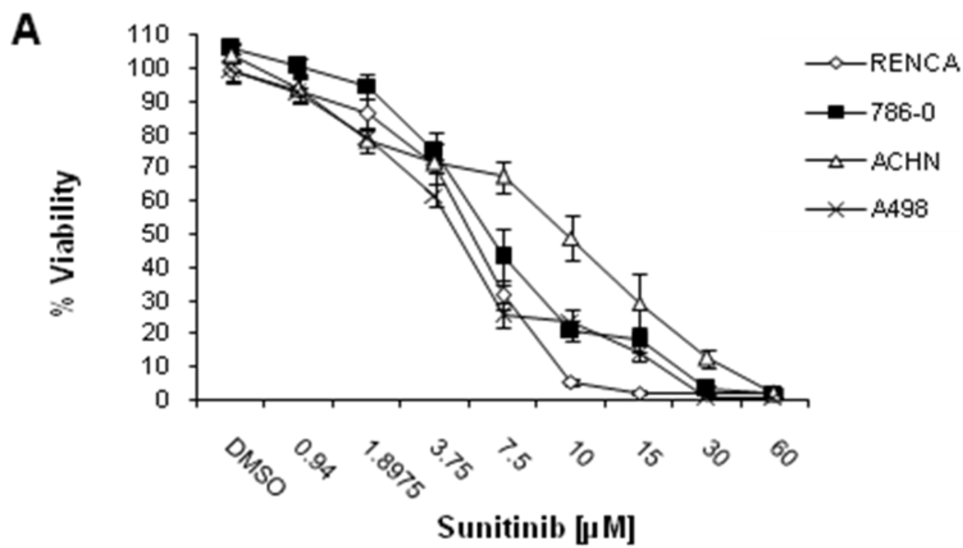
Figure 6. Chemokine expression in reovirus infected RENCA cells. Chemokine levels in supernatants from RENCA cells infected with DV [1 MOI] or RV [ED_{50} or 1 MOI] for 48 hours determined by luminex analysis. * = $P < 0.05$ by t-test relative to DV control. Error bars = SEM of three independent experiments. Technical assistance was provided by Jason Spurrell.

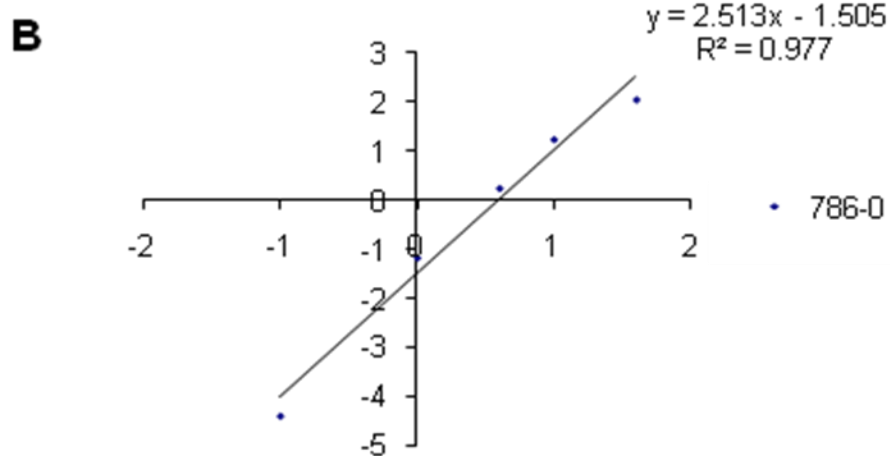


2.3.2 Combination reovirus with sunitinib therapy mediates synergistic cytotoxic responses against RCC in a dose variable replication independent manner.

To assess *in vitro* synergy between reovirus and sunitinib we determined combination index values as determined by the Chou and Talalay method [126]. WST-1 viability assays were conducted in our panel of RCC cell lines treated with escalating doses of sunitinib (0.94-60 μM), demonstrating a dose dependent response for all cell lines (**Figure. 7A**). Utilizing calcusyn® software, the dose response data for reovirus and sunitinib on RCC cells was used to calculate ED_{50} values (**Figure. 7B-C**). Cells were then treated simultaneously with sunitinib and reovirus for 48 hours at increasing doses of these agents held at a fixed ratio of ED_{50}:ED_{50} and cell viability was determined via the WST-1 assay (**Figure. 8A**). Combination index (CI) values calculated with this data revealed a synergistic cytotoxic response (CI < 1) for both the ACHN and A498 cell lines across all doses studied (**Figure. 8B**). Interestingly, both the RENCA and 786-O cells lines also demonstrated synergistic cytotoxic responses to combination reovirus-sunitinib therapy, however, these effects were dose variable (**Figure. 8B**). To determine whether sunitinib augmented reovirus replication to mediate the aforementioned effects, plaque titration assays were conducted on the RENCA, 786-O and ACHN cell lines following treatment with both reovirus and sunitinib. No statistically significant (P<0.05) change in reovirus titre was observed following these experiments as determined by students t-test, highlighting a viral replication independent mechanism underlying these results (**Figure. 9**).

Figure 7. Sunitinib dose response studies and ED50 calculation. A) Cell viability of RCC cell lines treated with escalating doses of sunitinib determined by WST-1 assay. 1% DMSO used as vehicle control. B) Representative log plot used for manual calculation of ED_{50} for 786-0 cell line for validation of ED_{50} value calculated by calcusyn® software. C) Summary of sunitinib and reovirus ED_{50} doses for all RCC cell lines calculated by calcusyn® software. Error bars = SEM of at least three independent experiments.



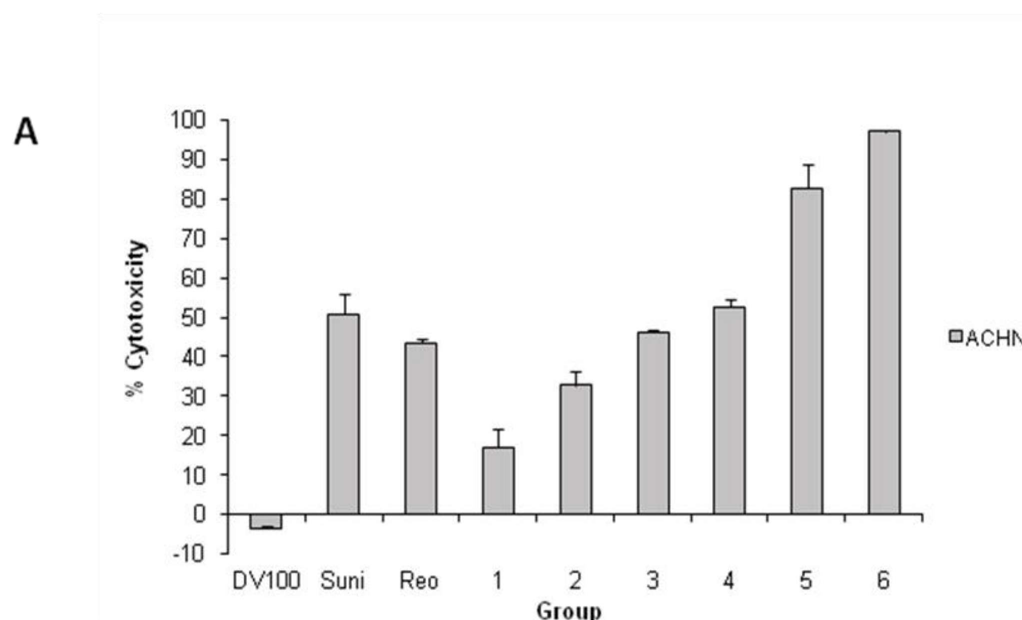


C

ED50 Values:

Cell Line	Reovirus	Sunitinib
786-O	35 MOI	7 µM
ACHN	11 MOI	8 µM
A498	37 MOI	4 µM
RENCA	0.007 MOI	4 µM

Figure 8. Reovirus and sunitinib combination therapy elicits a synergistic cytotoxic response against RCC cell lines. A) Representative WST-1 assay of ACHN cells treated with RV, sunitinib or their combination at fixed ratios (ED_{50}:ED_{50}). DV [100 MOI], 1% DMSO or their combination were used as controls. RV and sunitinib at ED_{50} values used as controls to validate ED_{50}. Group 1-6 indicate escalating RV:sunitinib doses by a factor of 2x starting with ED_{50}/16. B) Summary of combination index values (+/- SEM) for RCC cell lines (786-O, ACHN, A498) treated with sunitinib and RV at a fixed ratio of ED_{50}:ED_{50} as depicted in panel A (groups 2-6) calculated by calcusyn® software. Error bars = SEM of at least three independent experiments.



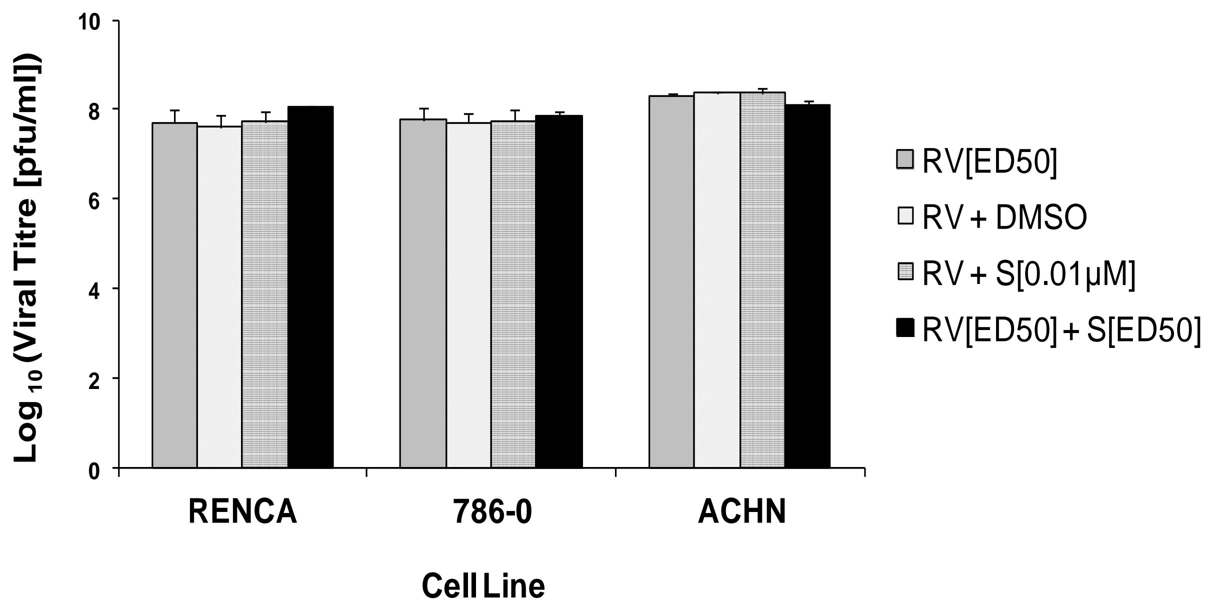
B

Combination Index

<1 : Synergistic =1 : Additive > 1 : Antagonistic

Cell Line	ED50/8	ED50/4	ED50/2	ED50	2 x ED50
ACHN	0.48 +/- 0.01	0.59 +/- 0.02	0.93 +/- 0.06	0.55 +/- 0.20	0.21 +/- 0.01
A498	0.99 +/- 0.07	0.73 +/- 0.07	0.69 +/- 0.07	0.63 +/- 0.12	0.27 +/- 0.05
RENCA	0.53+/- 0.06	0.63 +/- 0.21	0.90 +/- 0.12	1.43 +/- 0.14	1.47 +/- 0.01
786-O	5.97 +/- 2.65	1.63 +/- 0.43	1.23 +/- 0.17	0.33 +/- 0.09	0.23 +/- 0.03

Figure 9. Sunitinib does not effect reovirus replication. RV titre determined by plaque titration assay following treatment of RCC cell lines (RENCA, 786-O, ACHN) with RV, sunitinib (S) or their combination. 1% DMSO was used as vehicle control for sunitinib. NB: no statistically significant change in viral titre demonstrated ($P > 0.05$ by t-test).



2.4 Discussion.

Metastatic renal cell carcinoma is an incurable disease resistant to both radiation and conventional cytotoxic chemotherapy. Despite the revolutionary progress made in the management of this disease with targeted agents, patients invariably progress on therapy and the 5-year overall survival rate remains less than 10% [7, 13]. As such, novel therapeutics are greatly needed. In the current line of experimentation we sought to determine the direct oncolytic effects of reovirus for use against RCC. Furthermore, the ability of sunitinib to augment this activity was determined.

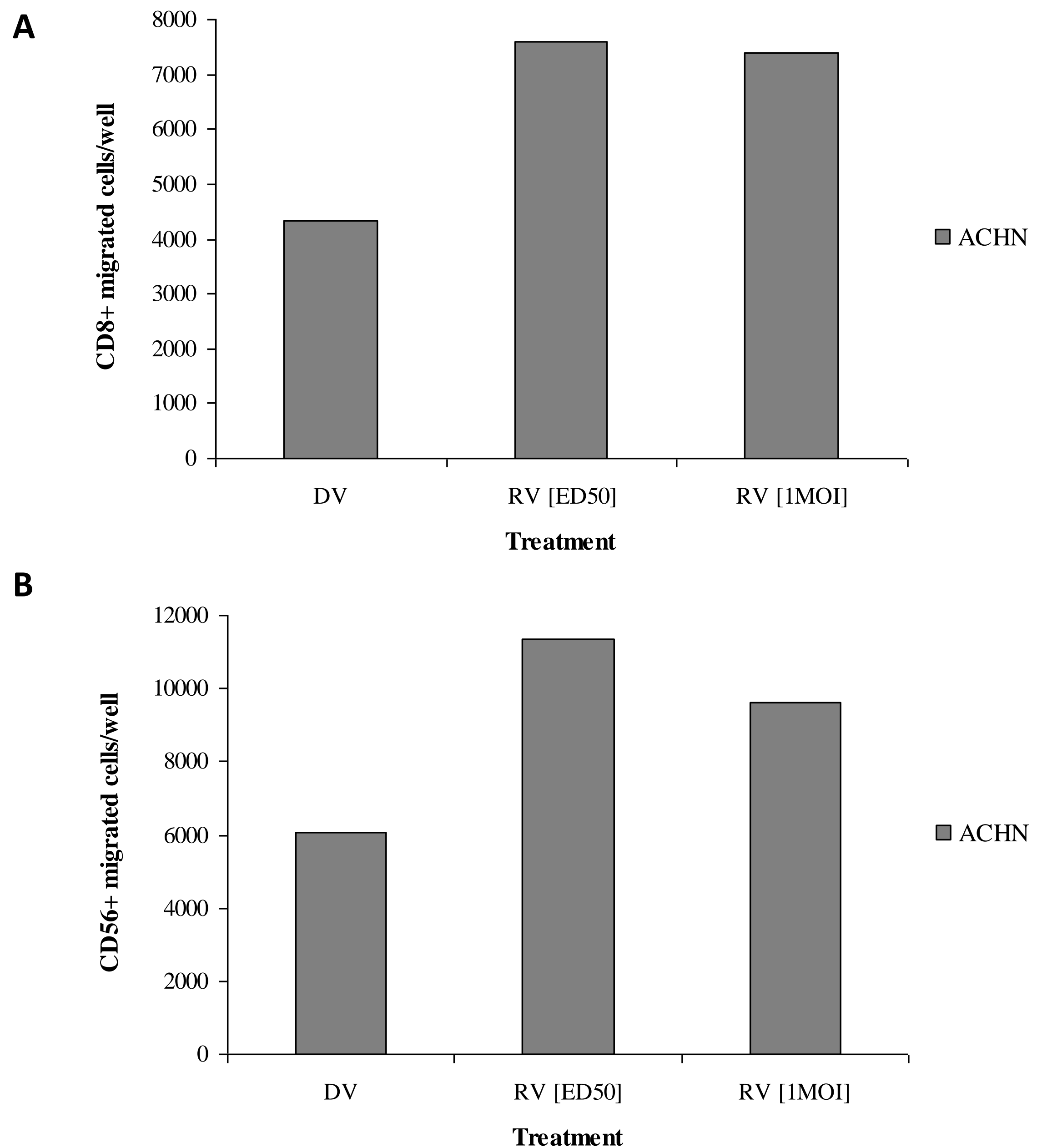
Our *in vitro* studies indeed demonstrate the ability of reovirus to induce oncolysis against RCC as has been described for multiple tumour histologies (**Figures. 3-5**). This activity was observed across all cell lines tested including three human and one murine, highlighting the plausibility of utilizing reovirus to initiate direct oncolysis against RCC clinically. Unfortunately, our experiments employing *ex vivo* RCC tissue specimens did not result in valid data due to technical problems with establishing cell lines. While other groups have established cultured cells for *in vitro* analysis from RCC tumour specimens, this line of experimentation was abandoned due to the considerable time needed for optimization. Nevertheless, such experiments are of great significance to understanding the translational potential of our results as purchased human cell lines have been proven to not be representative of clinical grade specimens [127]. Future work should therefore involve further optimization of protocols to culture RCC cells *in vitro* to study reovirus replication and oncolytic susceptibility.

Interestingly, the ED_{50} values determined for each human cell line was within one log of each other whereas the ED_{50} value for the murine cell line RENCA was 4 log lower, indicating a significantly increased sensitivity to reovirus oncolysis. This cell line also facilitated a more rapid replication of the reovirus, with viral titre reaching plateau at 12 hours post infection as opposed to 24 hours in the human lines, despite peak levels of reovirus titre being similar. Hence, the signalling mechanisms responsible for this rapid reovirus replication may also result in a susceptibility to reovirus mediated cell death. As such, microarray experiments investigating protein expression levels within proliferation and apoptotic pathways between the human and murine cell lines are warranted to identify regulators of our observed differential reovirus sensitivity. Beyond this, baseline characterization of these cell lines with respect to well established reovirus sensitivity pathways (ras, JAM-A status, etc.) may also provide insight into the cellular mechanisms underlying our observed differential sensitivity. This would be accomplished through western blot experiments.

In addition to mediating oncolytic effects against our RCC cell line panel, reovirus infection in RENCA cells also resulted in the production of pro-inflammatory cytokines. In our study the RANTES, MIP-1α, MCP-1, KC, IP-10 and MIG chemokines were chosen to be quantified by luminex analysis as these have all been demonstrated to be upregulated during reovirus infection of prostate cancer and melanoma cell lines and importantly are known mediators of chemotaxis and innate immunity [96, 125]. (**Figure. 6**). As we also observed the upregulation of these chemokines, our results suggest that similar to infection of melanoma and prostate cancer cells, reovirus replication induces an inflammatory cell death response against RCC. Interestingly, pro-inflammatory

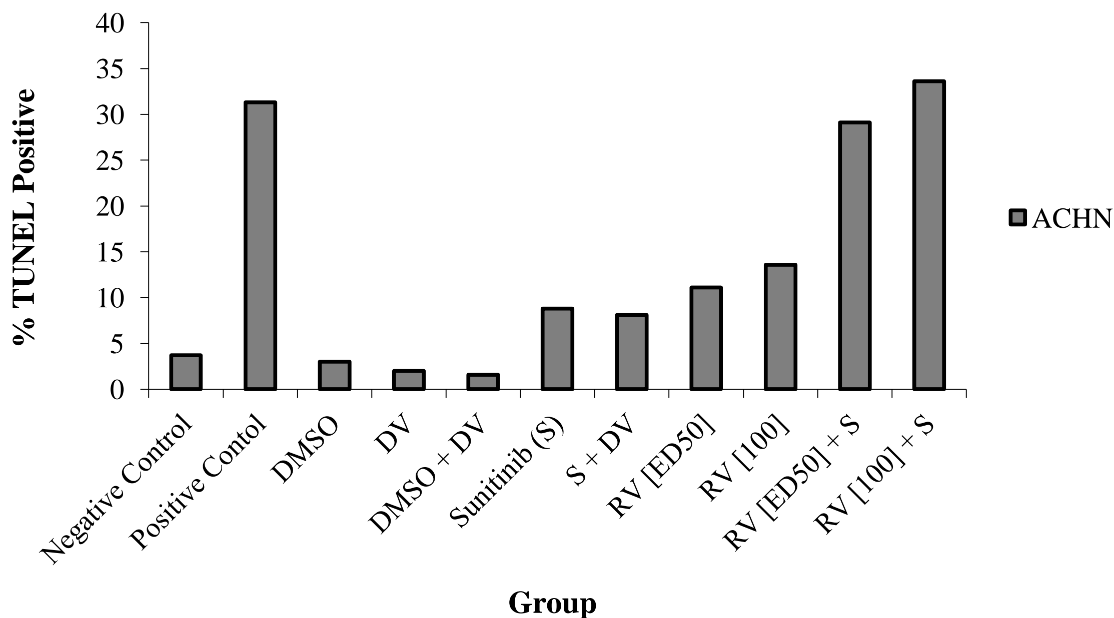
chemokine and cytokine production following reovirus infection of melanoma cells has been linked to NF-κβ and PKR activity, suggesting that similar pathways may also be involved our observed findings in RCC. As such, a natural extension of our work would be to determine this, which could be accomplished through siRNA and pharmacological NF-κβ inhibitors. Beyond this, the reovirus induced chemokine/cytokine repertoire has also been demonstrated to mediate NK and DC chemotaxis as well as adaptive immunity. Indeed, we also observed that supernatants from RCC cells infected with reovirus initiated $CD8^+$ splenocyte and NK cell migration in transwell plates supporting that these chemokines may similarity facilitate innate and adaptive immunity in RCC (**Figure. 10**); however, these results were not replicated nor were *in vitro* cytolysis assays for adaptive immunity conducted. Hence, additional studies are therefore needed to confirm our initial observations but also to determine the precise chemokines/cytokines supporting both innate and adaptive immune responses against RCC. This is of particular importance as such knowledge would allow for strategies to target or utilize these chemokines to augment the anti-tumour reovirus mediated immune response; and likewise damped the anti-viral immune response. Studies employing anti-chemokine/cytokine antibodies would accomplish this.

Figure 10. Supernatant from ACHN cells infected with reovirus for 48 hours induce chemotaxis of CD8^{+} and CD56^{+} peripheral blood mononuclear cells across a 3µm transwell membrane. Supernatants were harvested from ACHN cells cultured for 48 hours in the presence of DV [1 MOI] or RV [ED_{50} or 1 MOI]. PBMCs were harvested from healthy human subjects and cultured for 2 days. For transwell experiments, 600 µl of supernatants were placed in bottom chamber of 24 well plates and 1.5×10^{6} PBMCs were placed in upper chamber inserts. After 6 hours, inserts were removed and migratory CD8^{+} (panel **A**) and CD56^{+} (panel **B**) cells were counted via flow cytometry. N = 1.



To better understand the direct cytotoxic effects of reovirus-sunitinib combination therapy against RCC we utilized the Chou and Talalay method, a widely employed method for assessing synergy [126]. In our studies, we found that in all cell lines a synergistic cytotoxic response was achieved (combination index < 1), however, this was dose dependent (**Figure. 8B**). Given the widespread activity of sunitinib against multiple tyrosine kinases, it is likely that our differential results are secondary to complex signalling interactions dependent on the baseline pathway activation status of each cell line. Likely contributing pathways include those which sunitinib predominantly acts upon including the VEGF and PDGF pathways, which depending on their activation status, may sensitize cells to reovirus oncolysis [128]. From a translational perspective, understanding the molecular biology dictating our observations is crucial as novel biomarkers to predict which tumours would produce this synergistic response would be necessary. Moreover, these details would provide insight into the molecular mechanisms dictating reovirus sensitivity allowing for the development of strategies to circumvent resistance. While such molecular studies were not completed, plaque titration assays following combination treatment of our RCC cell lines demonstrated that sunitinib did not modulate reovirus titer during, suggesting this direct activity is independent of viral replication (**Figure. 9**). As reovirus mediated apoptosis has previously been well characterized in other cancer cell lines [111, 114, 120], we hypothesize this synergy may reside in sunitinib's ability to enhance reovirus induced apoptosis and current experimentation in our laboratory is exploring this as the potential mechanism underlying these findings. Indeed preliminary results support this hypothesis as demonstrated in **figure 11**.

Figure 11. Sunitinib augments reovirus mediated apoptosis in the ACHN RCC cell line. A) ACHN cells were treated concomitantly with RV [ED_{50} or 100 MOI], sunitinib [ED_{50}] or there combination for 48 hours and assessed for apoptosis via TUNEL assay utilizing flow cytometry. DV [100 MOI], 1% DMSO or their combination were used as controls. Positive and negative controls were provided with Invitrogen® TUNEL assay kit to serve as relative markers of apoptosis. N = 1.



Collectively, our *in vitro* results highlight the ability to target RCC with reovirus for direct oncolysis. Moreover, our findings suggest that sunitinib can augment reovirus oncolysis against RCC in a dose and cell line dependent manner. These studies represent the first pieces of evidence to support the clinical use of reovirus against RCC; however, many questions remain unanswered with respect to the mechanisms underlying our observations. Hence, continued work in our laboratory will focus on addressing the aforementioned proposed experiments.

Chapter 3.

In vivo evidence for the use of reovirus as a monotherapy and in combination with sunitinib for the treatment of renal cell carcinoma.

3.1 Introduction.

As highlighted in Chapter 1, many RCC tumour microenviromental factors exist which act to limit the efficacy of OV, including reovirus. Moreover, with an increasing knowledge that oncolytic viruses act to initiate therapeutic anti-tumour immune responses the study of reovirus interaction with the tumour microenvironment is of great significance. Hence, while our *in vitro* results highlight reovirus as a promising therapy for RCC, *in vivo* studies validating these results are necessary.

Many murine tumour model studies have demonstrated the ability of reovirus to reduce tumour burden and improve overall survival when administered either intratumourally or intravenously. Recently, studies employing immunocompetent murine models have demonstrated the importance of virus mediated innate and adaptive anti-tumour immune responses in facilitating reovirus therapeutic efficacy, which has resulted in a paradigm shift in the field of oncolytic viruses to utilizing these agents as novel immunotherapeutics [15, 16]. Interestingly, the generation of anti-tumour immunity during reovirus administration has also been demonstrated clinically in a phase I trial of a single intra-prostatic injection of reovirus that involved 6 patients with localized prostate cancer [115]. In this study, despite the robust anti-viral neutralizing antibody response that was seen, a significant number of intra-prostatic $CD8^+$ T-cell infiltrates were present following reovirus injection. Moreover, in a phase I clinical trial of intravenous reovirus administration to patients who previously received myelotoxic chemotherapy and radiotherapy, an increase in the $CD8^+$ T- and NK-cell population in the peripheral blood was seen [129]. Hence, evidence exists to support the feasibility of achieving reovirus mediated immune targeting of solid malignancies clinically, which is of particular

importance in RCC given the immunological responsiveness of this malignancy as outlined in Chapter 1.

To date, little work has investigated mechanisms to enhance the immunotherapeutic efficacy of reovirus. Instead, novel combination approaches have focused on enhancing reovirus direct oncolysis or systemic delivery through down-regulating neutralizing antibodies as has been achieved with both cyclophosphamide and cisplatin [130, 131]. Hence, novel strategies to augment reovirus mediated innate and adaptive immunity are needed. Interestingly, sunitinib, a multi-tyrosine kinase inhibitor targeting VEGF, PDGFR, C-KIT, RET, CSF-1R and FLT-3 and first line therapy for mRCC, has been demonstrated to augment the adaptive anti-tumour immune response generated by immunotherapies offering promise in this regard [132]. While this drug has traditionally been considered a potent anti-angiogenic agent, recent clinical studies have highlighted its profound immune modulatory effects. Indeed following two 28 day cycles of oral sunitinib therapy at 50 mg, mRCC patients display an increase in the percentage of IFN-γ producing T-cells relative to treatment naive patients [98]. Moreover, the immune suppressive type-2 T-cell cytokine response and accumulation Tregs that is characteristic of mRCC patients is reversed following sunitinib therapy [98]. Interestingly, this reversal of immune suppression correlates with sunitinib's ability to downregulate MDSC, which are also characteristically upregulated in mRCC [133]. Accordingly, this evidence has generated significant interest in targeting myeloid derived suppressor cells with this agent to reverse the mRCC induced immunosuppressive microenvironment to enhance the anti-tumour immune response generated by immunotherapeutics [132]. The feasibility of this combination approach has been demonstrated in an immunocompetent murine model of

RCC (RENCA) in which sunitinib down regulation of MDSC and Treg enhanced intratumoural infiltration and activation of adoptively transferred $CD8^+$ T-cells, leading to a decrease in tumour burden [134]. Furthermore, sunitinib has also been demonstrated to reduce tumour burden and improve overall survival in mouse models of melanoma, hepatocellular carcinoma and colorectal metastasis following immunotherapeutic administration, highlighting the efficacy and widespread utility of this approach [132, 135-136]. Based on this work, the hypothesis that sunitinib enhances the immunotherapeutic efficacy of reovirus was developed.

The studies outlined in this Chapter were preformed to provide preclinical evidence that reovirus has therapeutic activity against RCC *in vivo* and is capable of priming adaptive anti-tumour immune responses against this disease. Additionally, the utility of combining reovirus with sunitinib in order to achieve decreased tumour burden, improved overall survival and enhanced adaptive anti-tumour immunity in a syngeneic immunocompetent murine model of RCC was assessed. Overall, this line of experimentation was undertaken to provide proof-of-principle for the use of reovirus as both a monotherapy and in combination with sunitinib given the known barriers present within the *in vivo* RCC tumour microenvironment.

The RENCA model of renal cell carcinoma was chosen given its ability to represent the immune microenvironment of RCC. This syngeneic immunocompetent murine model is characterized by the subcutaneous implantation of the murine renal adenocarcinoma cell line RENCA. This murine renal cell carcinoma experimental model was first described in 1973 and is still considered to be the gold standard for studying immunotherapeutics against RCC preclinically [137]. Importantly, the use of sunitinib to

downregulate MDSC and reverse tumour induced immunosuppression has been demonstrated in the RENCA model highlighting its appropriateness for our line of experimentation [138].

3.2 Methods.

3.2.1 In vivo studies in a syngeneic immunocompetent murine mouse model.

All mice in these studies were housed in pathogen-free conditions with food and water *ad libitum* and treated within procedural guidelines that were approved by the University of Calgary Animal Care Committee. For all experiments, 2.5 x 10^6 RENCA cells were implanted into the right hind-flank of Balb/c (Charles River, Montreal, QC) mice on day 0. Once tumours were palpable (day 5), therapy was initiated. Mice were grouped into cohorts of 3-6 and treated with PBS [i.p], DV [5 x 10^8 i.t or i.v], RV [5 x 10^8 PFU i.t or i.v], sunitinib [20-60 mg/kg i.p] or a combination of these agents as per each the following experimental protocols (outlined in results section): sunitinib dose response experiment, combination therapy experiment, adoptive transfer experiment. For all studies bi-weekly caliper measurements were taken to monitor tumour burden and mouse weights were recorded to monitor treatment toxicity.

For the sunitinib dose response experiment, mice were implanted with RENCA bearing tumours as described above and treated daily for a total of 12 days with sunitinib at doses of either 20, 40 or 60 mg/kg i.p (6 mice/group) and tumour size was followed. Spleens were then harvested and analyzed for MDSC by flow cytometry as per protocol described below (section 3.2.3). Technical assistance was provided by Zhong Qiao Shi.

For the combination therapy experiment, cohorts of 6 Balb/c mice were implanted with RENCA tumours and treated with PBS [i.p], sunitinib [40 mg/kg i.p], DV [5 x 10^8 pfu i.t or i.v], RV [5 x 10^8 pfu i.t or i.v], or a combination of these agents. Sunitinib was given daily for 14 consecutive days starting on day 5 post RENCA implantation whereas reovirus was administered on post RENCA implantation day 8, 11 and 15. On post RENCA implantation day 18, 3 mice were sacrificed from each group for analysis of $CD8^+$ splenocyte IFN-γ production and MDSC quantification whereas 3 mice were followed for overall survival. This experiment was conducted a second time with 5 mice per group for additional overall survival analysis. Technical assistance was provided by Zhong Qiao Shi.

For the adoptive transfer experiment, spleens were extracted 19 days after RENCA implantation from mice treated with PBS, DV, RV or sunitinib as described for the combination therapy experiment (3 mice/group). Pooled single cell suspensions of mononuclear splenocytes were then generated by passing mechanically separated spleens through 100 µm cell strainers (BD, Franklin Lakes, NJ) followed by centrifugation over a ficoll-hypaque gradient (GE Healthcare, Uppsala, Sweden). 1 x 10^7 mononuclear splenocytes in 100 µl PBS were then administered intravenously via tail-vein into recipient Balb/c mice corresponding to each treatment group (5 mice per group). 4 days later, all recipient mice were challenged with an implantation of 2.5 x 10^6 RENCA cells into the right hind-flank and followed for tumour burden. A schema for this experiment is outlined in **figure 17A** page 84. Technical assistance was provided by Zhong Qiao Shi and Wenqian Chen.

3.2.2 $CD8^+$ cell enrichment.

Spleens were extracted 18 days after implantation from 3 mice in each treatment group of the combination therapy experiment. Pooled single cell suspensions were generated by passing mechanically separated spleens through 100 μm cell strainers (BD, Franklin Lakes, NJ). Splenocyte suspensions were then enriched for $CD8^+$ cells using an EasySep® (Stem Cell Technologies, Vancouver BC) Mouse $CD8^+$ Selection Kit via an EasySep® magnet as per manufacturer's protocol. Briefly, splenocytes were incubated with 50 μl/ml of CD8 PE labelling solution for 15 minutes followed by an second 15 minute incubation period with 100 μl/ml of EasySep® PE selection cocktail. EasySep® magnetic nanoparticles at a concentration of 50 μl/ml were then added to this suspension and after 10 minutes of incubation cells were pelleted by centrifugation and placed in the EasySep® magnet for 5 minutes. After 3 cycles of re-suspension and magnetic separation, the supernatant was discarded completing the isolation $CD8^+$ selected splenocytes. Technical assistance was provided by Jason Spurrell.

3.2.3 Flow cytometry for MDSC enumeration.

For MDSC analysis, spleens were extracted 18 days after implantation from 3 mice in each treatment group of the combination therapy experiment. Pooled single cell suspensions were generated by passing mechanically separated spleens through 100 μm cell strainers. Following this, 1×10^6 splenocytes were stained with FITC-labelled rat anti-mouse CD11b and PE-labelled rat anti-mouse Gr-1 or FITC-labelled rat IgG2b and PE-labelled rat IgG2b (BD, Franklin Lakes, NJ). Flow cytometric analysis was performed after stained cells were washed twice with PBS. Two colour plot analysis on gated cells was used to obtain the percentage of MDSC within the white blood cell population (% MDSC).

3.2.4 IFN-γ ELISA.

Spleens were extracted 18 days after implantation from 3 mice in each treatment group of the combination therapy experiment. Pooled single cell suspensions were generated by passing mechanically separated spleens through 100 μm cell strainers and $CD8^+$ splenocytes were enriched as described above. Culture supernatants from $CD8^+$ enriched splenoctyes incubated for 24 hours with RPMI media, RENCA cells or reovirus-infected RENCA cells were assayed for IFN-γ protein by ELISA as per manufacturer's protocol (R&D, Minneapolis, MN) at room temperature. Briefly, 100 μl of capture antibody at 8 μg/ml was added to wells in a 96-well plate. Following this, capture antibody was washed out and kit standards and experimental samples were added to 96-well plate in 100 μl volumes and incubated for 2 hours. 0.8 μg/ml of detection antibody was then added to wells and plates were incubated for 2 hours after which supernatants were replaced with 100 μl of avidin peroxidise at 1.5 μg/ml. After a 30 minute incubation period, ABTS substrate was added to each well and a Bio-rad® reader was utilized to obtain absorbance (495 nm) values. The sensitivity of the assay was 31.25 pg/ml or higher. Technical assistance was provided by Jason Spurrell.

3.2.5 Statistics.

Statistical analysis was performed utilizing unpaired two-tailed t-tests and ANOVA analysis to determine significance between experimental groups. To determine significant *in vivo* survival benefits log-rank and Kaplan-Meier analysis was preformed. Statistical significance was defined as p-values being < 0.05 unless otherwise stated. Dr. Cay Egan conducted the ANOVA analysis.

3.3 Results.

3.3.1 Reovirus demonstrates therapeutic efficacy as a monotherapy and in combination with sunitinib against an immunocompetent translational model of RCC.

The subcutaneous implantation of murine RCC cells (RENCA cell line) into the hind flank of Balb/c mice is a well described syngeneic immunocompetent model for studying immunotherapies against RCC preclinically [137-138]. Utilizing this model we investigated whether reovirus had therapeutic efficacy against RCC *in vivo* and determined the ability of sunitinib to augment this activity. In order to assess the optimal dose of sunitinib to administer during combination therapy with reovirus mice were treated with escalating doses of sunitinib [20, 40 or 60 mg/kg] and tumour burden was followed. This dose range was chosen as it is clinically relevant and has also been utilized in the RENCA model [138]. A dose of 40 mg/kg of sunitinib significantly decreased tumour burden did not eradicate tumours (**Figure. 12A**) and as such was chosen for subsequent experiments. Importantly, this dose also downregulated tumour induced splenic MDSC, highlighting its potential for augmenting reovirus generated immunity as previously described in this model [138] (**Figure. 12B**).

Administration of sunitinib prior to immunotherapy has been demonstrated to be superior in augmenting the anti-tumour immune response [139]. Hence, in our combination therapy experiments we administered sunitinib prior to treatment of mice with reovirus in order to achieve any potential immune modulatory effects previously described with this agent. For these experiments, RENCA tumour bearing Balb/c mice were treated with sunitinib [i.p], reovirus [i.t or i.v] or a combination of these agents. DV

[i.t or i.v] was administered as a control. Sunitinib treatment was initiated once palpable tumours were present (day 5) and continued for 14 days while reovirus treatment was initiated three days following this and given a total of three times. Importantly, relative to DV and PBS controls, reovirus given as a monotherapy significantly reduced tumour burden when administered either i.t or i.v ($P < 0.05$ by day 18 by t-test) (**Figure. 13**). This effect was more robust with i.t administration. Similarly, a trend overall survival benefit was only observed with i.t reovirus administration when analyzed by Kaplan-Meier analysis (**Figure. 14**). Furthermore, tumour burden in those mice receiving both sunitinib and reovirus (both i.t and i.v) therapy was reduced more significantly than either of these agents used as a monotherapy ($P < 0.05$ by day 18 by t-test), highlighting the superior therapeutic efficacy achieved with this combination approach (**Figure. 13**). Of note, these results were also significant by one-way ANOVA analysis ($P < 0.05$). Additionally, a near significant overall survival benefit was demonstrated for those mice receiving reovirus administered i.t in combination with sunitinib versus either agent used as a monotherapy when analyzed by Kaplan-Meier log-rank analysis ($P = 0.051$) (**Figure. 14**). This overall survival benefit was not demonstrated for reovirus i.v in combination with sunitinib, suggesting durable therapeutic effects in the RENCA model requires i.t administered reovirus.

Figure 12. **Sunitinib downregulates MDSC and decreases tumor burden in the RENCA murine model.** A) Balb/c mice bearing RENCA RCC s.c tumors (2.5 x 10^6 cells) were treated with daily sunitinib [20-60 mg/kg i.p] starting on post RENCA implantation day 5 and lasting for 12 days. PBS (1% DMSO) was used as a vehicle control for sunitinib. Tumor size was measured with calipers. N = 6 mice per group. B) On day 16 post RENCA implantation mice were sacrificed and the percentage of Gr-1^+/CD11b^+ splenocytes (MDSC) were quantified by flow cytometry from pooled spleens of mice from each group in panel A. N = 6 mice per group. Error bars = SEM of tumors within each group. Suni = Sunitinib. A no tumor group was included as negative control for MDSC.

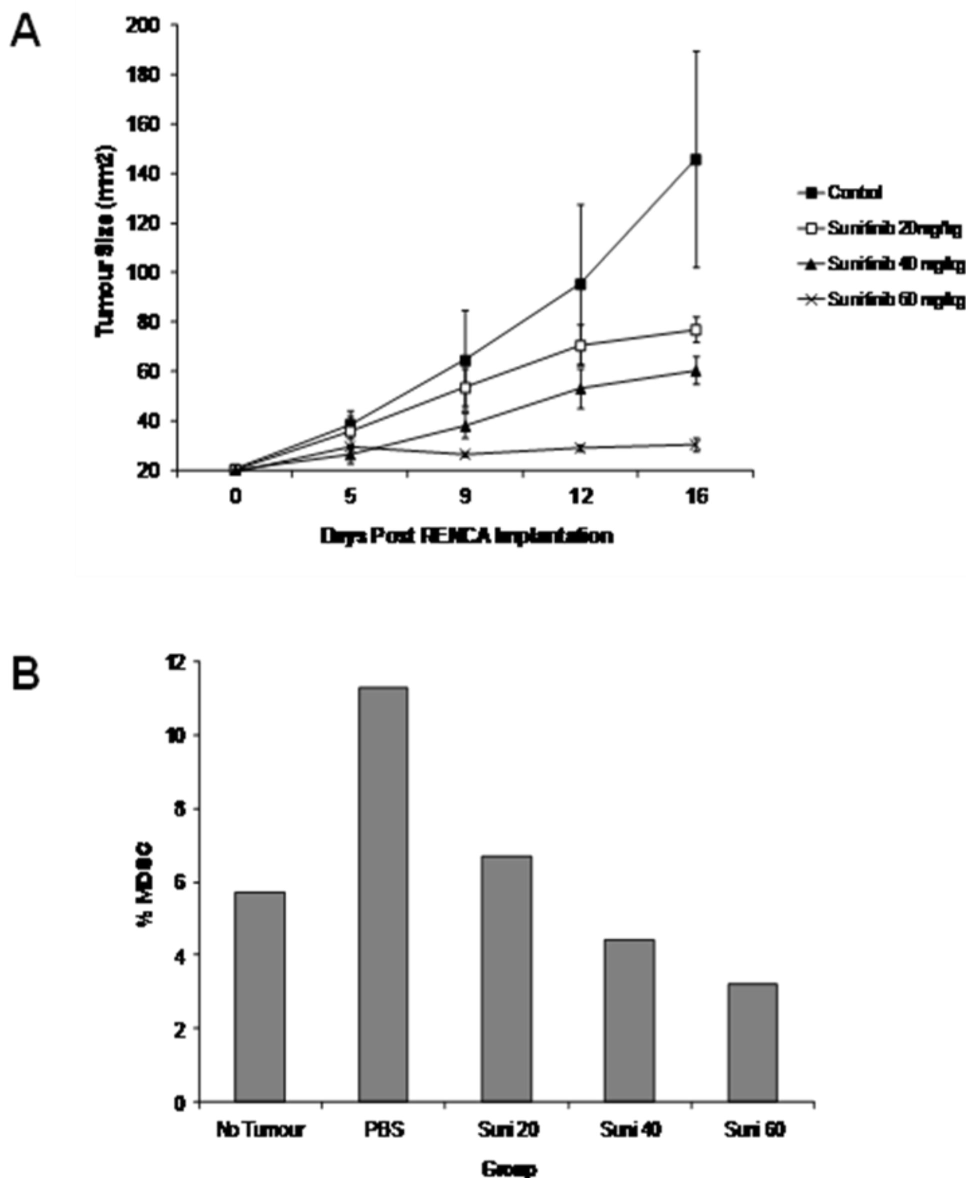


Figure 13. Reovirus combined with sunitinib results in a synergistic decrease in tumor burden in the RENCA murine model. Balb/c mice were implanted with RENCA (2.5 x 10^6 cells) s.c tumors and treated with PBS (1% DMSO) [i.p], sunitinib (S) [40 mg/kg i.p], DV [5 x 10^8 pfu i.t or i.v], RV [5 x 10^8 pfu i.t or i.v], or a combination of these agents. Tumor size was followed with caliper measurements. Sunitinib was given daily for 14 consecutive days starting on day 5 post RENCA implantation (indicated by red arrow). RV was administered three times (indicated by blue arrows). N = 6 mice per group. Error bars = SEM of tumors within each group.

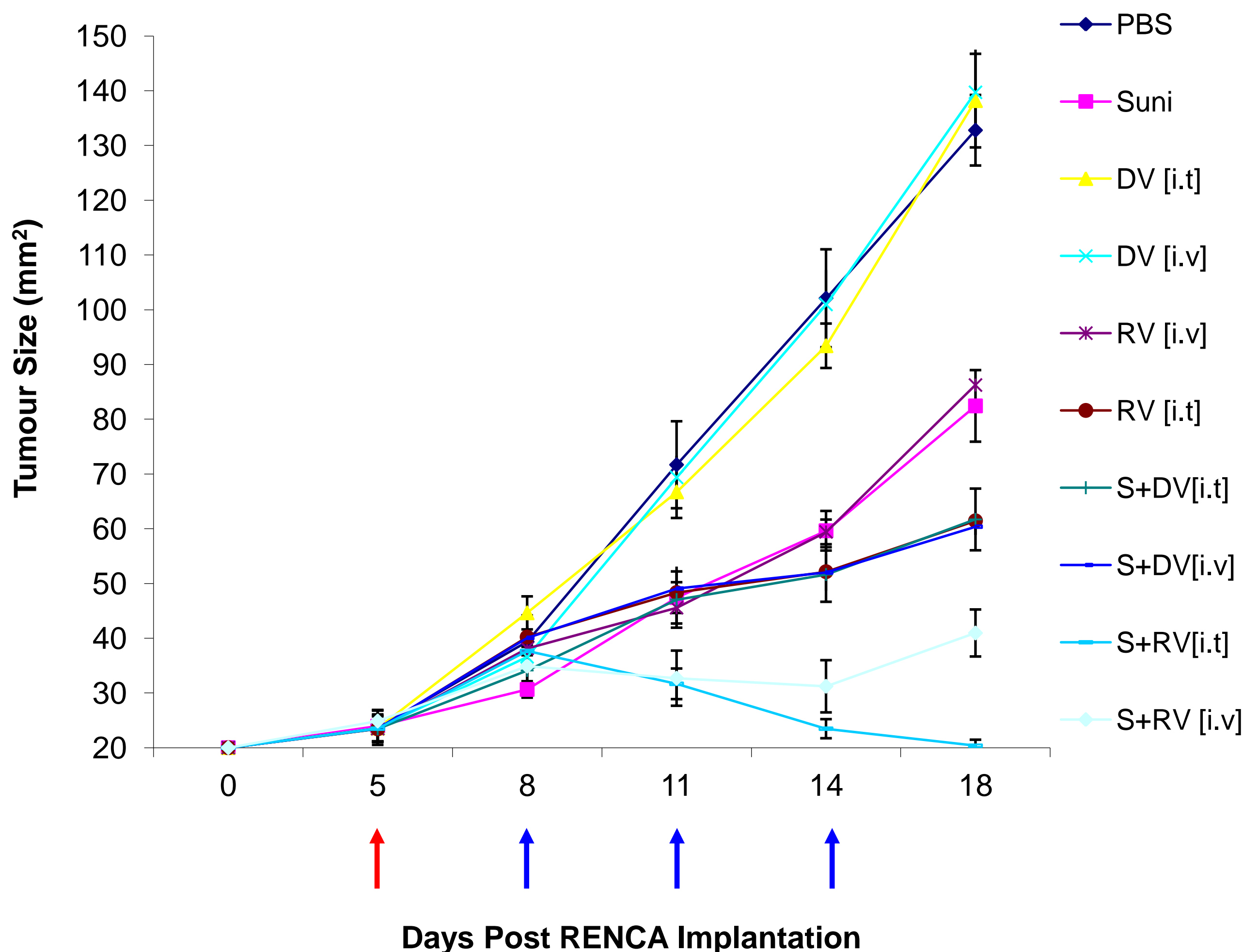
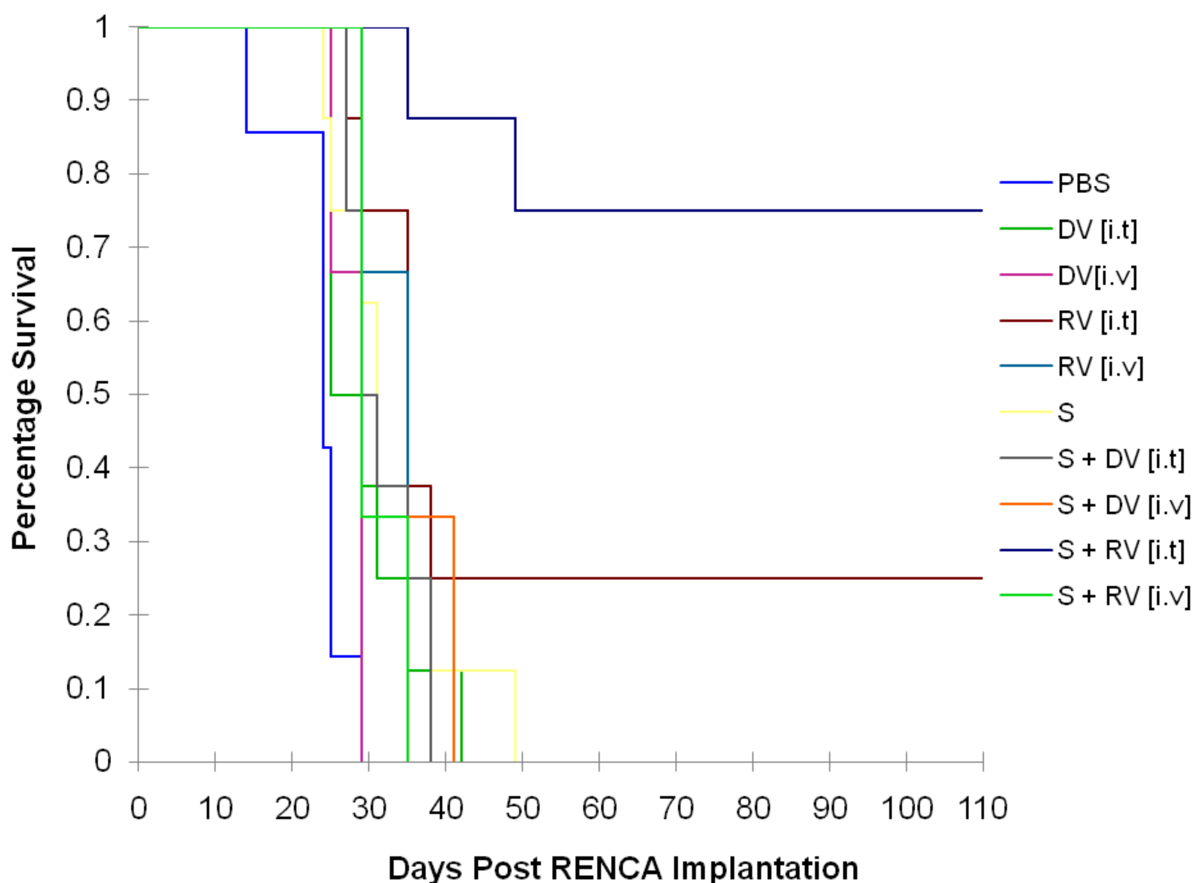


Figure 14. Overall survival analysis for mice receiving reovirus and combination therapy. Balb/c mice were implanted with RENCA (2.5 x 10^6 cells) s.c tumors and treated PBS (1% DMSO), sunitinib (S) [40 mg/kg i.p], DV [5 x 10^8 pfu i.t or i.v], RV [5 x 10^8 pfu i.t or i.v], or a combination of these agents. Sunitinib was given daily for 14 consecutive days starting on day 5 post RENCA implantation. RV was administered three times as shown in figure 13. Overall survival was followed. N = 8 mice per group (3 mice/group for RV or DV administered i.v). Analysis represents data pooled from two independent experiments involving 3 and 5 mice, respectively.

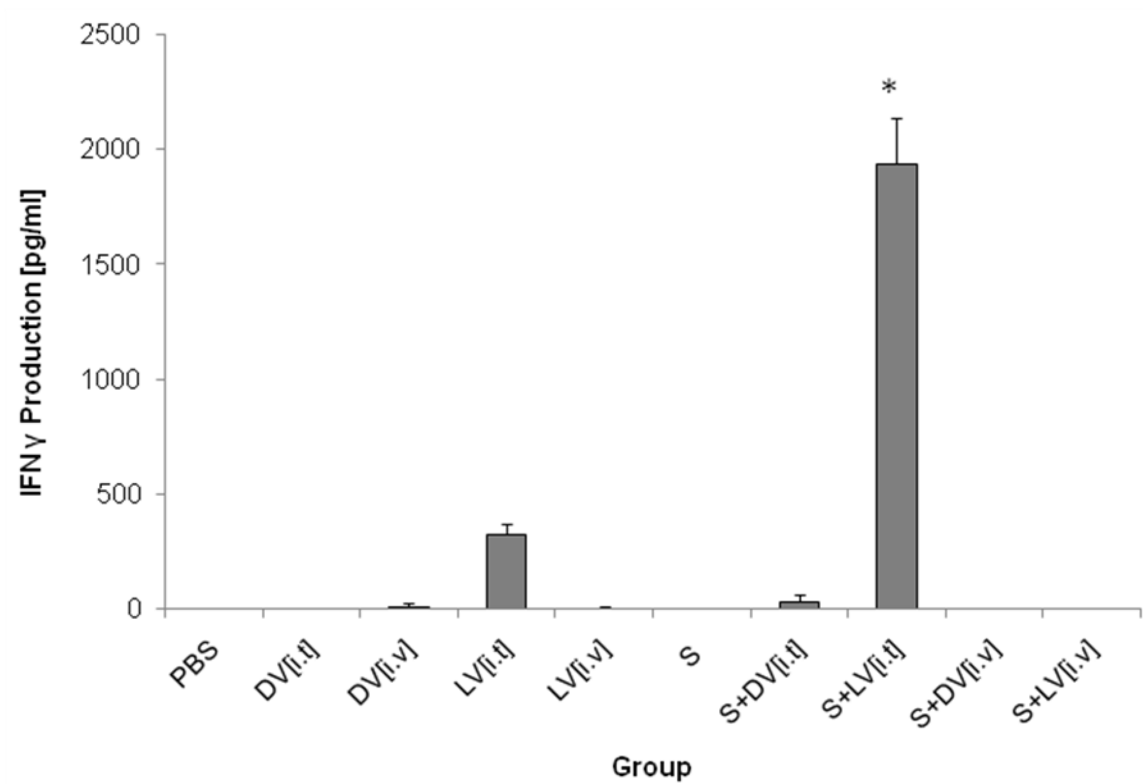


3.3.2 Sunitinib augments reovirus mediated anti-tumour immune response against RCC through reversal of tumour induced immunosuppression.

As reovirus infection of transformed cells results in the production of pro-inflammatory cytokines and priming of innate and adaptive immunity [125], we next determined the anti-tumour immune response generated by reovirus treatment of RCC *in vivo*. Mice receiving sunitinib, reovirus (i.t or i.v) or combination therapy were sacrificed at day 18 post RENCA implantation and $CD8^+$ splenocytes were harvested and co-cultured with reovirus, RENCA cells or reovirus infected RENCA cells for antigenic stimulation prior to IFN-γ ELISA. The spleen was chosen as a source of $CD8^+$ lymphocytes as combination therapy eradicated RENCA tumours. In mice receiving reovirus therapy administered i.t a significant IFN-γ response was demonstrated from harvested $CD8^+$ splenocytes following stimulation with reovirus infected RENCA cells ($P < 0.05$ relative to control by t-test) (**Figure. 15**). This activity was not demonstrated for $CD8^+$ cells stimulated with reovirus or RENCA cells alone (data not shown), nor was significant response seen when reovirus was administered i.v. (**Figure. 15**). Interestingly, sunitinib therapy significantly augmented i.t administered reovirus generated anti-tumour immunity, as a robust increase in IFN-γ production (4-fold) in those mice that received reovirus in combination with sunitinib was observed ($P < 0.05$ by t-test) (**Figure. 15**). Of note, the IFN-γ response seen in $CD8^+$ splenocytes from mice receiving combination therapy was also only observed upon stimulation with reovirus infected RENCA cells, consistent with our findings in the reovirus treated mice. In those mice receiving, sunitinib, DV, reovirus or control minimal or no IFN-γ response was demonstrated

(**Figure. 15**). Taken together, these results highlight that reovirus generates an adaptive anti-tumour immune response against RCC that is augmented by sunitinib.

Figure 15. Anti-tumor splenic CD8+ cell IFN-γ production in mice receiving reovirus, sunitinib or their combination. CD8+ cells were separated from pooled spleens of mice from each group depicted in figure 11 and stimulated with RV infected RENCA cells for 24 hours. IFN-γ production was quantified by IFN-γ ELISA. N = 3 mice. * = $P < 0.02$ relative to RV [i.t]. Error bars = SEM of IFN-γ response within a group. Technical assistance was provided by Jason Spurrell.



As sunitinib is known to reverse tumour induced immunosuppresion by targeting MDSC [138], we quantified levels of splenic MDSC in mice receiving reovirus, sunitinib and combination therapy to determine their correlation to the $CD8^{+}$ splenocyte IFN-γ production. Again, splenic MDSC where chosen as combination therapy eradicated RENCA tumours. In those mice receiving reovirus administered either i.v or i.t a significant increase in splenic MDSC was observed relative to RENCA bearing control mice ($P < 0.05$ by t-test), highlighting an accumulation of these cells following reovirus therapy (**Figure. 16**). Interestingly, in those mice receiving sunitinib or combination therapy, this response was not observed and levels of MDSC were substantially reduced to levels nearing mice bearing no tumour at all (**Figure. 16**). These results suggest that treatment of mice with sunitinib during reovirus administration prevents the accumulation of MDSC, highlighting their potential involvement in the enhanced IFN-γ response demonstrated with combination therapy.

To determine the therapeutic significance of the enhanced adaptive immune response seen with combination therapy, adoptive transfer experiments were conducted to assess for protective immunity as previously described [96]. Mice were treated with reovirus (i.t), sunitinib, DV, control or a combination of these agents for 19 days post RENCA implantation after which splenoctyes were isolated and intravenously transferred into treatment-naïve mice (schema depicted in **Figure. 17**). Mice were not treated with reovirus administered i.v as our previous experiments did not demonstrate an appreciable IFN-γ adaptive anti-tumour immune response in this group. Following treatment, mice were challenged with s.c injection of RENCA cells and followed for tumour burden. Interestingly, only those mice receiving splenocytes from mice treated with combination

therapy demonstrated a reduced tumour growth rate relative to control, highlighting an established protective immune response in this group ($P < 0.05$ relative to all other groups by t-test) (**Figure. 18**). Of note, these results were not significant by one-way ANOVA analysis. No statistically significant overall survival benefits were observed for the other treatment groups relative to control groups in this experiment.

Figure 16. Splenic MDSC in mice treated with reovirus, sunitinib or their combination. Gr-1+/CD11b+ splenocytes (MDSC) were quantified by flow cytometry from pooled spleens of mice from each group as depicted in figure 11. N = 3 mice. Mice bearing no tumors were utilized as negative controls for MDSC. * = P < 0.001 relative to RV [i.t and i.v]. In both panels error bars = SEM of % MDSC within a group.

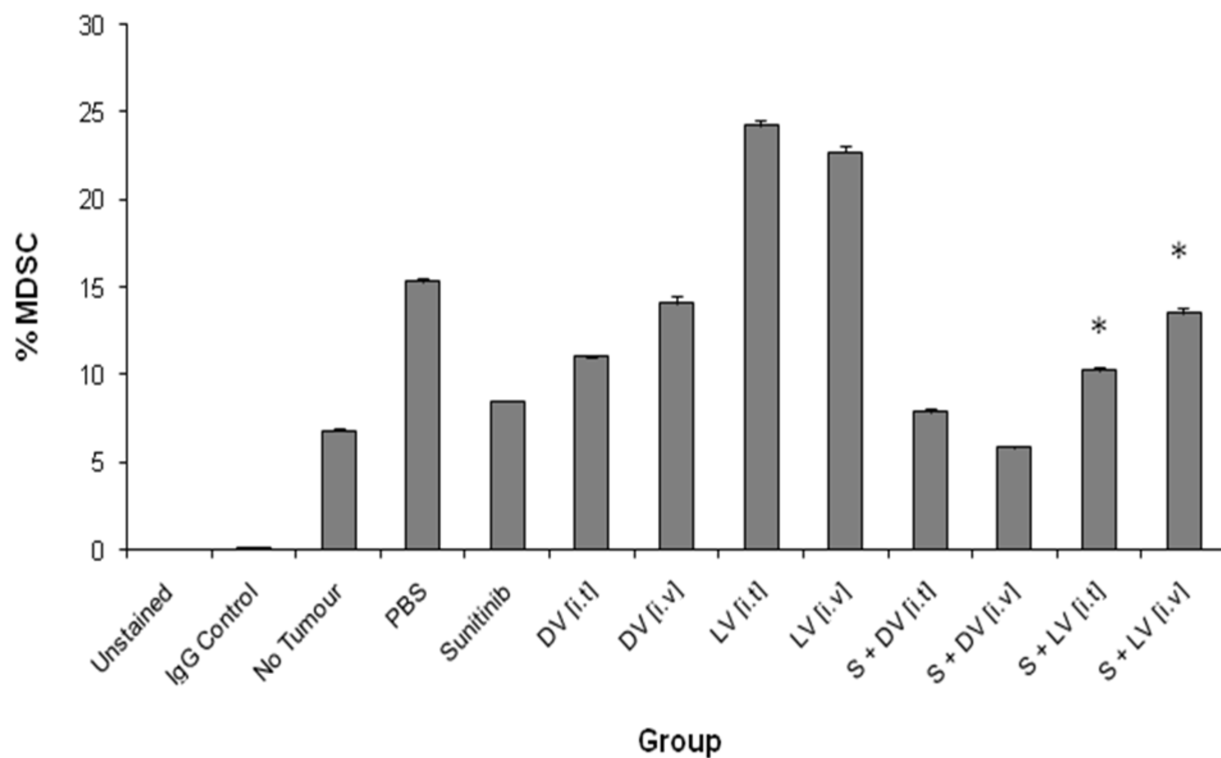


Figure 17. Adoptive transfer experiment schema. Adoptive transfer experimental schema adapted from Gujar et. al. 2011, Molecular Therapy [96]. Briefly, mice (donor) were implanted with RENCA tumors and treated as shown in figure 11. On day 19, splenocytes were harvested and transferred to treatment naive mice (recipient), followed by RENCA (2.5 x 10^6 cells s.c.) tumor challenge. Arrows depict treatment given. X denotes treatment end. 5 x 10^8 pfu RV i.t administered. 40 mg/kg of sunitinib (Sun) administered. PBS (1% DMSO) and DV i.t (5 x 10^8 pfu) administered as controls.

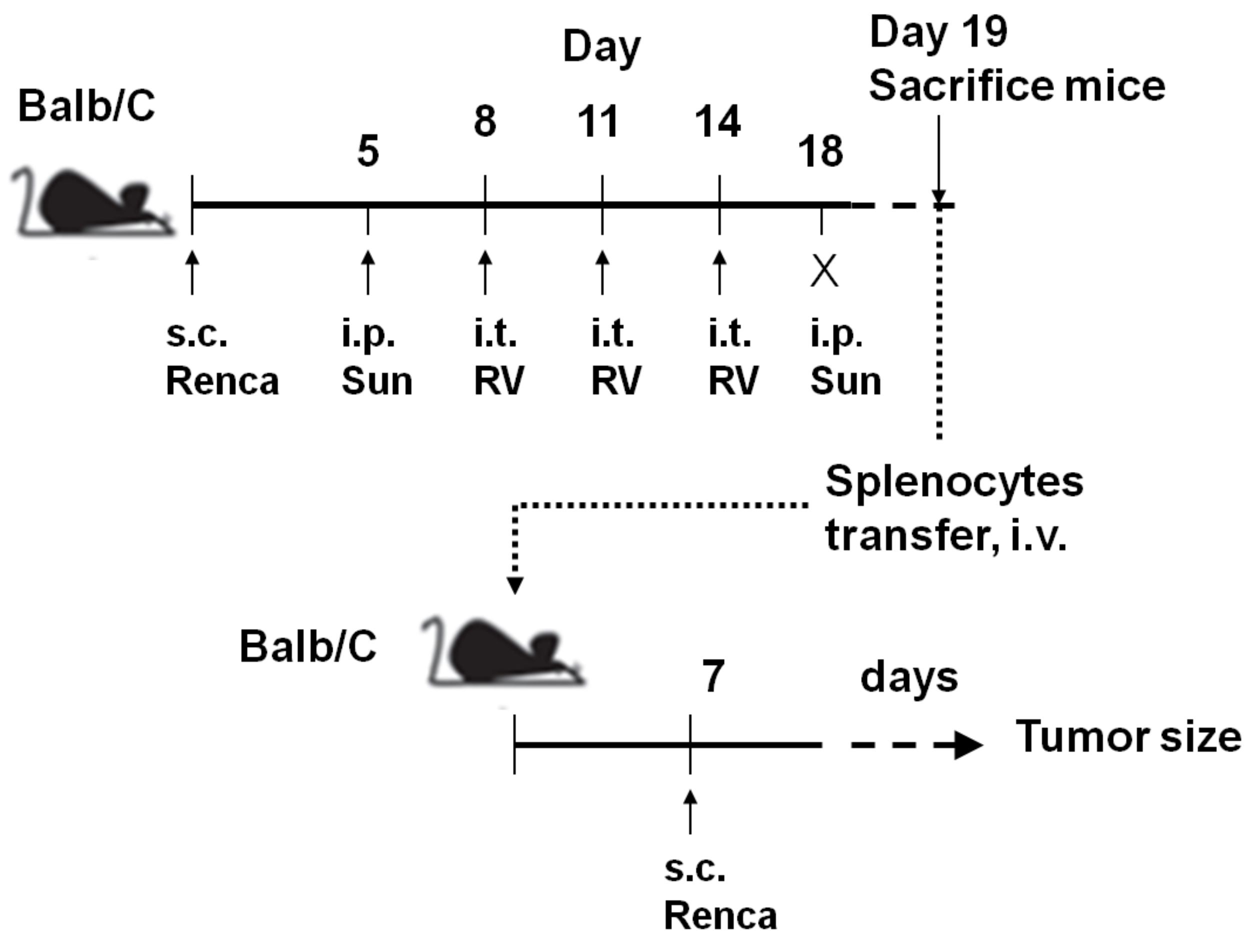
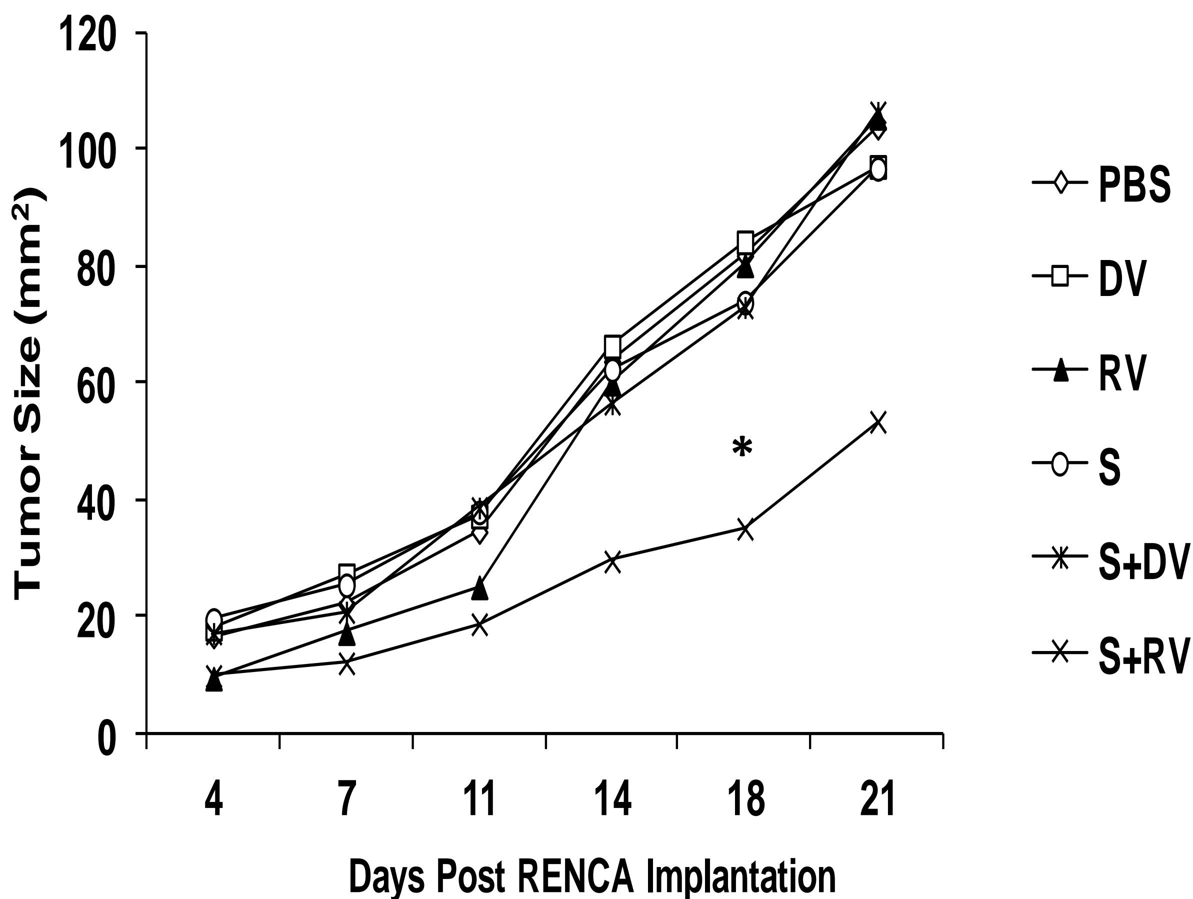


Figure 18. Combination therapy induces a protective immune response against tumor re-challenge. Tumor size in recipient mice challenged with RENCA cells s.c. as per figure 17. * = $P < 0.05$ by two-tailed t-test between all groups. N = 6 mice/group. Technical assistance was provided by Zhong Qiao Shi and Wenqian Chen.



3.4 Discussion.

The therapeutic utility of our *in vitro* studies was confirmed *in vivo* utilizing the RENCA syngeneic immunocompetent murine model of RCC. Here we established that treatment of mice with either reovirus administered i.v or i.t resulted in significant decreases in tumour burden relative to both PBS and DV controls (**Figure. 13**). Furthermore, in those mice receiving reovirus administered i.t, a robust production of IFN-γ was observed following stimulation of $CD8^+$ splenocytes with reovirus infected RENCA cells (**Figure. 15**), highlighting the ability to generate a systemic adaptive anti-tumour immune response against RCC with this agent. Of note, when $CD8^+$ splenocytes from these same mice were stimulated with reovirus or RENCA cells alone as an antigen no IFN-γ response was seen (data not shown). This suggests that the by-products of reovirus oncolysis, such as danger associated molecular pattern molecules (DAMP) and/or sequestered tumour associated antigens (TAA) are required for priming of adaptive anti-tumour immunity. Importantly, these studies also suggest reovirus mediated adaptive immunity in the RENCA model is not dependent on viral antigens alone. Indeed, it has been demonstrated that culture media from reovirus infected melanoma cells filtered to removal viral antigen is capable of priming both innate and adaptive immune responses, supporting this hypothesis [125]. The immunotherapeutic activity of reovirus against RCC described here is of particular translational importance as patients with metastatic RCC have demonstrated complete durable responses following cytokine immunotherapy (IL-2) [12]. This cytokine immunotherapy responsiveness coupled with the success of experimental immunotherapies such as anti-cancer vaccines against RCC [11-12],

highlights the attractiveness of reovirus mediated immune targeting and direct oncolysis as a novel therapeutic strategy against this disease.

The contribution of the reovirus primed adaptive anti-tumour immune response in mediating the observed reductions in tumour burden seen in our experiments was not investigated. This would require $CD8^+$ and $CD4^+$ depletion experiments utilizing antibodies to target and remove these immune cell populations prior to the treatment of tumours. Such experiments would allow the assessment of tumour burden following reovirus administration in the absence of these immune effectors which would allow conclusions to be drawn on their involvement in our observed therapeutic results. Furthermore, as the antigen responsible for the IFN-γ $CD8^+$ splenocyte activity we observed in our experiment is unknown, the use of a RENCA-ova tumour cell line would allow for confirmation of an anti-tumour associated antigen response following reovirus infection. While our results indeed suggest that an anti-tumour associated antigen response is occurring, such experiments would provide further direct evidence to support this hypothesis.

Likewise, investigations confirming reovirus replication and apoptosis induction in tumour specimens were not conducted. As such, the contribution of reovirus mediated direct oncolysis in mediating our observed anti-tumour effects can only be inferred. Immunohistochemical experiments for reovirus proteins as well as activated caspases as has been conducted previously in our laboratory would address these questions. However, these experiments would need to be conducted at serial time points to ensure adequate tumour tissue, given that a number of tumours were eradicated following treatment end.

Interestingly, the adaptive anti-tumour immune response against RCC was only observed when reovirus was administered i.t, despite the inhibition of tumour growth observed upon i.v administration (**Figure. 15**). We hypothesize that higher viral loads in tumours receiving reovirus directly allows for a more robust intratumoural inflammatory response, thereby leading to more effective adaptive immune priming. Our results contrast previous studies in C57BL/6 mice in which adaptive immune responses against melanoma tumours following a single i.v reovirus injection have been described, highlighting the immune response to reovirus differs between murine models [140]. Furthermore, as previous studies have not directly compared differences in the magnitude of reovirus generated anti-tumour immune responses between the i.v and i.t route, our results provide evidence that route of reovirus administration significantly impacts the ability to generate anti-tumour immune responses. As such, methods of optimizing i.v delivery of reovirus such as the use of cyclophosphamide, white blood cell carriers or nanoparticle carriers may need to be utilized if sufficient titers cannot be reached in clinical settings where systemic delivery to solid tumors is vital.

Additionally, experiments employing xenograft murine models utilizing human RCC cell lines and/or *ex vivo* human RCC tissue specimens are required to more accurately determine the applicability of our results to human RCC being treated clinically. Indeed such murine models have been utilized to test therapeutics against RCC preclinically highlighting the plausibility of conducting these experiments [141]. While the RENCA model allows for the study of reovirus-tumour interaction and conclusions regarding proof-of-principle to be made, this model inaccurately reflects human RCC, highlighted by the drastic differences in reovirus sensitivity *in vitro* we observed in

Chapter 2. As such, while our results demonstrate an *in vivo* therapeutic effect of reovirus and generation of an adaptive anti-tumour immune response, it must be considered that the RENCA cell line is far more sensitive to reovirus than the human RCC cell lines. Future experiments in our laboratory aim to address this problem with the use of the aforementioned xenograft models.

Importantly, the RENCA model allowed for testing the hypothesis that sunitinib could augment reovirus mediated anti-tumour immunity and *in vivo* therapeutic efficacy. As mentioned, sunitinib is capable of reversing tumour induced immune suppression in patients with mRCC [98]. This activity resides in its ability to downregulate the levels of circulating MDSC, which have been demonstrated to orchestrate mRCC immune suppression through direct T-cell inhibition as well as stimulating the upregulation of Tregs [98-133]. Accordingly, in our experiments we chose a dose of sunitinib that was capable of downregulating MDSC to determine the effect of sunitinib on reovirus generated anti-tumour immunity (**Figure. 12A-B**). In our study we initiated sunitinib therapy prior to reovirus administration to achieve the greatest augmentation of anti-tumour immunity as has been demonstrated for other immunotherapies [139]. Consistent with an inflammatory cell death, we found that both i.v and i.t reovirus administration *in vivo* induced a marked rise in splenic MDSC (**Figure. 16**). Indeed this rise in MDSC was significantly downregulated by combination of reovirus with sunitinib and a concomitant augmentation in the IFN-γ response from isolated anti-tumour $CD8^+$ splenocytes relative to reovirus monotherapy was achieved. Similar to reovirus monotherapy however, this IFN-γ response was only observed for i.t administration. As demonstrated by **figure 13**, combination therapy also resulted in a statistically significant reduction in tumour burden

highlighting an improved therapeutic effect of combination therapy relative to reovirus or sunitinib as a single agent. Further supporting this is the finding that mice receiving combination therapy also have significantly improved overall survival (**Figure. 14**). Unfortunately, this was not statistically significant by Kaplan-Meier log-rank analysis (P = 0.051), however, given the obvious trend in the data it can be inferred that this is due to the experiment being underpowered. As such, taken collectively these findings highlight the attractiveness of combining sunitinib with reovirus not only for improved therapeutic efficacy but also for augmentation of reovirus generated anti-tumour adaptive immunity against RCC, through downregulation of MDSC.

Although our studies highlight enhanced therapeutic and adaptive anti-tumour immune responses following combination of reovirus with sunitinib, the mechanisms responsible for this activity were not confirmed. Indeed our results suggest an involvement of sunitinib mediated MDSC downregulation as a potential mechanism responsible for the enhanced immune response; however, confirmation of our findings through Gr-1 depletion experiments or through utilizing sunitinib resistance MDSC would be required. Interestingly, MDSC resistance to sunitinib has been demonstrated to result from STAT5 dependent signalling activity as opposed to STAT3 [142]. This activity has been shown to be driven by GM-CSF production within tumours [142]. As such, *ex vivo* treatment of isolated RENCA MDSC with GM-CSF and adoptive transfer into mice receiving combination therapy would allow direct conclusions to be made regarding our observations regarding sunitinib's ability to target native MDSC to reverse tumour induced immunosuppression and enhance adaptive anti-tumour immunity. Furthermore, to address the involvement of direct synergistic effects of reovirus and sunitinib on RENCA

cells *in vivo*, as was demonstrated in our *in vitro* experiments, immunohistochemical studies quantifying cytotoxic/apoptotic cells would be required. Moreover, comparisons of our RENCA *in vivo* experimental results compared to combination therapy experiments in xenograft models would provide insight into the involvement of immune interactions in mediating our observed enhanced therapeutic efficacy.

The aforementioned studies to address enhanced *in vivo* reovirus mediated direct oncolysis are of particular relevance when considering our observed reductions in tumour burden in mice treated with i.v reovirus and sunitinib who did not have appreciable immune responses. Moreover, the ability of sunitinib to enhance systemic delivery of reovirus must be considered as well given that this agent has been demonstrated to normalize aberrant tumour vasculature resulting in improved intratumoural delivery of chemotherapeutics in murine models of RCC [143]. Serial immunohistochemical and viral progeny assays conduced daily on RENCA tumours during combination therapy to assess tumour vasculature and viral titre, respectively would provide indirect insight into the involvement of this process in our observed results.

While reovirus mediated adaptive anti-tumour immune responses following administration of this agent as a monotherapy have been demonstrated to result in protective immune responses in models of melanoma and prostate cancer, this was not seen in the RENCA RCC model [95-96]. However, given the relative aggressive nature of RENCA tumours, this result is not surprising. On the other hand, consistent with the improved anti-tumour adaptive immune response, protective immunity was demonstrated with the use of reovirus when combined with sunitinib. While this result was significant by multiple t-tests, it must be noted that by one-way ANOVA significance was not

reached. Again, similar to the overall survival experiments, given the obvious trend in the data it can be inferred that the experiment was underpowered. As such, our adoptive transfer results do indeed demonstrate that a therapeutic immunosurveillance benefit is achievable through utilizing reovirus in combination with sunitinib.

Overall, the observations made utilizing the RENCA model of RCC provide further evidence to support the use of reovirus as both an oncolytic and immunotherapeutic agent against this disease. Moreover, these results highlight the potential for combining this agent with sunitinib for augmentation of these effects. Indeed, both the application of reovirus against an immunocompetent murine model of RCC as well as the use of sunitinib to enhance the immunotherapeutic potency of an OV have not been reported, highlighting the novelty of this work. As described above, these studies provide the foundation for much research to be conducted to further understand the interaction between OVs and the tumour microenvironment.

Chapter 4.

Concluding remarks and future directions.

Excerpts from this Chapter were published as a review in July 2012 in the Journal Expert Opinion on Biological Therapy. K. Lawson and D. Morris (2012). "Oncolytic Virotherapy for Renal Cell Carcinoma: A Novel Treatment Paradigm?" Expert Opin Biol Ther 2012;12(7):891-903.

With a survival rate of less than 10% at 5 years, mRCC remains a significant health burden necessitating the development of novel therapeutic strategies [7, 13]. To date, nearly all cancer treatment modalities including radiotherapy, cytotoxic chemotherapy, immunotherapy and targeted therapy have failed to significantly improve the overall survival of these patients underscoring the therapeutic resistance of this malignancy. Of the multitude of experimental therapeutics being developed on a preclinical level, the use of oncolytic viruses is particularly appealing given their ability to target tumours in a multi-mechanistic manner. Interestingly, a number of OV have demonstrated efficacy against RCC as reviewed in Chapter 1, making the application of these novel biologic agents against this disease attractive. This thesis aimed to explore the therapeutic utility reovirus, of one of the most clinically advanced OV being investigated to date, against RCC. Moreover, given the current use the multi-tyrosine kinase inhibitor sunitinib in the management of mRCC, we sought to determine the synergistic anti-tumour potential of this agent in combination with reovirus. Relevant *in vitro* and *in vivo* models of RCC were utilized to answer our proposed hypotheses.

The findings presented in Chapter 2 demonstrate that reovirus is capable of inducing a lytic infection in both human and murine cancer cell lines. Given the extensive array of cancer cell lines and tumour sites already demonstrated to be sensitive to reovirus oncolysis, these results further validate the significant potential of oncolytic virotherapy as a cancer modality. Moreover, utilizing the Chou and Talalay method, we demonstrated a cell line specific ability of sunitinib to act synergistically with reovirus to initiate a cytotoxic response against RCC. This work therefore supports ongoing efforts of the field to search for novel combination approaches to enhance *in vitro* OV sensitivity,

particularly targeted agents such as sunitinib. As such, the use of this novel approach against previously reported cancer cell lines resistant to reovirus oncolysis is warranted. Indeed, the proposed molecular mechanism studies highlighted in Chapter 2 would also aid in the application of this novel combination approach to other cancer cell lines.

The observed chemokine upregulation in RENCA cells infected with reovirus and our data highlighting reovirus supernatants promote $CD8^+$ and $CD56^+$ cell migration support the hypothesis that reovirus oncolysis induces an pro-inflammatory innate immune response against RCC. The interaction of OVs with the innate immune system is of growing interest to many researchers in the field given the potential to control anti-viral verses anti-tumour immunity based on the chemokine repertoire. As such, our investigations provide preliminary data for future study into improving therapeutic innate immunity against RCC and further validate findings in prostate cancer and melanoma which demonstrate active chemokine production is a hallmark of reovirus therapeutic efficacy.

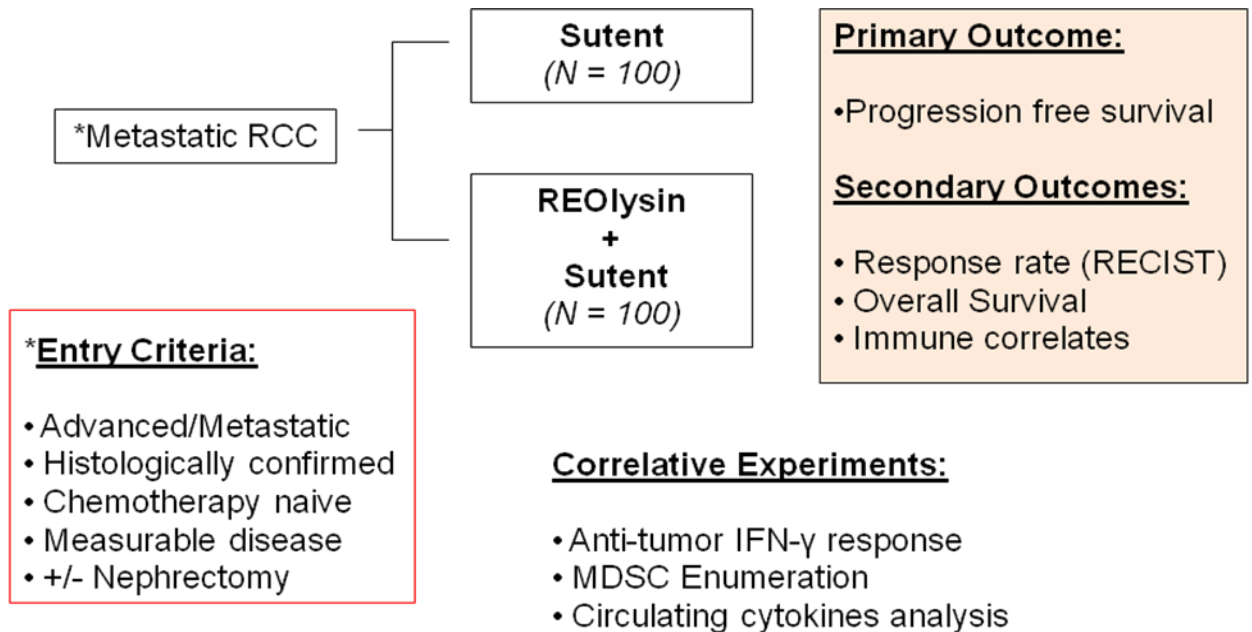
The interaction of the tumour microenvironment with reovirus utilized as a monotherapy and in combination with sunitinib was studied in Chapter 3. These investigations employed a well characterized syngeneic immunocompetent murine model of RCC (RENCA model). Here, reovirus was demonstrated to decrease tumour burden which was associated with the production of an anti-tumour adaptive immune response. Similar to the *in vitro* innate immune studies preformed, this work supports a growing paradigm shift to utilize OVs as novel immunotherapies in addition to their direct cytopathic effects. The ability to target RCC tumours via multiple mechanisms is attractive given the previously stated resistance of this malignancy to many cancer

treatment modalities. Moreover, significant reductions in tumour burden and improvements in overall survival were demonstrated with combination reovirus/sunitinib therapy further demonstrating the potential in the multi-mechanistic targeting of RCC. Interestingly, as originally hypothesized, the ability of sunitinib to downregulate MDSC and augment reovirus mediated anti-tumour immunity was also demonstrated. Given the known immunogenicity of OV other than reovirus, this novel strategy offers significant promise to enhance the immunotherapeutic efficacy of these agents and represents an exciting step forward in the field of oncolytic virotherapy.

Moreover, given the observed success with reovirus in combination with sunitinib, studies involving additional mRCC therapeutics are also warranted. Indeed, the use of mTOR inhibitors such as temsirolimus and everolimus may also prove to be an efficacious treatment strategy for RCC. This is based on recent evidence highlighting the ability to generate $CD8^+$ T cell memory and tumour immunity following inhibition of the mTOR kinase [144]. This strategy was not only utilized to enhance the potency of cancer vaccines in the RCC RENCA model, but also proved efficacious in augmenting the polyfunctionality and quantity of anti-cancer central $CD8^+$ memory T cells following adenovirus administration [145-146]. As such, application of mTOR inhibitors during an intratumoural oncolytic infection may allow for the generation an anti-tumour immune response with an increased proportion of memory $CD8^+$ T cells, thereby providing improved tumour immunosurveillance in addition to the individual tumouricidal effects provided by oncolytic viruses or mTOR inhibitors alone. Hence, future studies investigating the combination of mTOR inhibitors and OVs for RCC are warranted.

Unfortunately, preclinical models of cancer are unable to reliably predict the efficacy experimental therapeutics will have once applied in the clinical setting. Underscoring this is a recent study which demonstrated purchased cancer cell lines from different tumour sites display more similar genetic expression profiles than the *ex vivo* tumour specimens that they are supposed to be representative of [127]. Hence, clinical trials investigating novel therapeutics are the only way to accurately predict efficacy against human cancer patients. Nonetheless, preclinical models, such as the ones utilized in the work outlined in this thesis, provide proof-of-principle data to highlight promising therapies to be tested. Given that both reovirus and sunitinib have extensive clinical data to support their efficacy and safety in human cancer patients, the collective reported findings strongly support further study of these agents in the setting of a randomized clinical trial. Accordingly, future work in our laboratory will focus on translating our findings to the clinic in the form of a randomized phase II trial for patients with mRCC. This trial would investigate the use of reovirus in combination with sunitinib verses sunitinib as a monotherapy (**Figure. 19**). In addition to clinical outcomes such as progression free survival, overall survival and response rate, this trial would involve correlative laboratory experiments on the blood and tumour of patients enrolled in the trial in order to investigate reovirus mediated anti-viral and anti-tumour immunity as well as induction of apoptosis.

Figure 19. Proposed randomized phase II control trial investigating reovirus in combination with sunitinib for mRCC. In the proposed trial patients with mRCC would be randomized to receive sunitinib monotherapy verses sunitinib in combination with reovirus. Clinical endpoints would be followed and correlative laboratory experiments on patient blood and tumour would be conducted.



In summary, the studies outlined in this thesis demonstrate that reovirus has novel oncolytic and immunotherapeutic effects against RCC *in vitro* and *in vivo*. Furthermore this data highlights that sunitinib, a multi-tyrosine kinase inhibitor, augments this activity. Thus, these novel findings provide proof-of-principle for the study of this multi-mechanistic strategy against a broad range of malignancies, particularly RCC. Beyond this, as sunitinib is currently a first line mRCC therapeutic and reovirus is in advanced phase III clinical trials, these findings should be investigated in the setting of a clinical trial.

References.

1. Rini BI, Campbell SC, Escudier B. Renal cell carcinoma. Lancet 2009;373(9669):1119-32

2. Oosterwijk E, Rathmell WK, Junker K, et al. Basic research in kidney cancer. Eur Urol 2011; 60(4):622-33

3. Kovacs G, Akhtar M, Beckwith BJ, et al. The Heidelberg classification of renal cell tumours. J Pathol 1997;183(2):131-3

4. Jeldres C, Baillargeon-Gagne S, Liberman D, et al. A population-based analysis of the rate of cytoreductive nephrectomy for metastatic renal cell carcinoma in the United States. Urology 2009;74(4):837-41

5. Permpongkosol S, Bagga HS, Romero FR, et al. Laparoscopic versus open partial nephrectomy for the treatment of pathological T1N0M0 renal cell carcinoma: a 5-year survival rate. J Urol 2006;176(5):1984-8

6. Lane BR, Gill IS. 7-year oncological outcomes after laparoscopic and open partial nephrectomy. J Urol 2010;183(2):473-9

7. Sun M, Lughezzani G, Perrotte P, et al. Treatment of metastatic renal cell carcinoma. Nat Rev Urol 2010;7(6):327-38

8. Zini L, Capitanio U, Perrotte P, et al. Population-based assessment of survival after cytoreductive nephrectomy versus no surgery in patients with metastatic renal cell carcinoma. Urology 2009;73(2):342-6

9. Alvarez M, Paull K, Monks A, et al. Generation of a drug resistance profile by quantitation of mdr-1/P-glycoprotein in the cell lines of the National Cancer Institute Anticancer Drug Screen. J Clin Invest 1995;95(5):2205-14

10. Lusini L, Tripodi SA, Rossi R, et al. Altered glutathione anti-oxidant metabolism during tumour progression in human renal-cell carcinoma. Int J Cancer 2001;91(1):55-9

11. McDermott DF. Immunotherapy of metastatic renal cell carcinoma. Cancer 2009;115(10 Suppl):2298-305

12. Negrier S, Escudier B, Lasset C, et al. Recombinant human interleukin-2, recombinant human interferon alfa-2a, or both in metastatic renal-cell carcinoma: Groupe Francais d'Immunotherapie. N Engl J Med 1998;338(18):1272-1278

13. Stewart GD, O'Mahony FC, Powles T, et al. What can molecular pathology contribute to the management of renal cell carcinoma? Nat Rev Urol 2011;8(5):255-65

14. Thirukkumaran C, Morris DG. Oncolytic viral therapy using reovirus. Methods Mol Biol 2009;542:607-34

15. Stanford MM, Breitbach CJ, Bell JC, et al. Innate immunity, tumour microenvironment and oncolytic virus therapy: friends or foes? Curr Opin Mol Ther 2008;10(1):32-7

16. Prestwich RJ, Harrington KJ, Pandha HS, et al. Oncolytic viruses: a novel form of immunotherapy. Expert Rev Anticancer Ther 2008;8(10):1581-8

17. Latif F, Tory K, Gnarra J, et al. Identification of the von Hippel-Lindau disease tumour suppressor gene. Science 1993;260(5112):1317-20

18. Foster K, Prowse A, van den Berg A, et al. Somatic mutations of the von Hippel-Lindau disease tumour suppressor gene in non-familial clear cell renal carcinoma. Hum Mol Genet 1994;3(12):2169-73

19. Gnarra JR, Tory K, Weng Y, et al. Mutations of the VHL tumour suppressor gene in renal carcinoma. Nat Genet 1994;7(1):85-90

20. Banumathy G, Cairns P. Signaling pathways in renal cell carcinoma. Cancer Biol Ther 2010;10(7):658-64

21. Kaelin WG Jr. Molecular basis of the VHL hereditary cancer syndrome. Nat Rev Cancer 2002;2(9):673-82

22. Ohh M, Park CW, Ivan M, et al. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. Nat Cell Biol 2000;2(7):423-7

23. Baldewijns MM, van Vlodrop IJ, Vermeulen PB. VHL and HIF signalling in renal cell carcinogenesis. J Pathol 2010 Jun;221(2):125-38

24. Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene 2010;29(5):625-34

25. Shinohara N, Ogiso Y, Tanaka M, et al. The significance of ras guanine nucleotide exchange factor, son of sevenless protein, in renal cell carcinoma cell lines. J Urol 1997;158(3 Pt 1):908-11

26. Elfiky AA, Aziz SA, Conrad PJ, et al. Characterization and targeting of phosphatidylinositol-3 kinase (PI3K) and mammalian target of rapamycin (mTOR) in renal cell cancer. J Transl Med 2011;9:133-133

27. Coffey MC, Strong JE, Forsyth PA et al. Reovirus therapy of tumours with activated Ras pathway. Science 1998;282(5392):1332-4

28. Parato KA, Breitbach CJ, Le Boeuf F et al. The Oncolytic Poxvirus JX-594 Selectively Replicates in and Destroys Cancer Cells Driven by Genetic Pathways Commonly Activated in Cancers. Mol Ther 2012;20(4):749-758

29. Farassati F, Yang AD, Lee PW. Oncogenes in Ras signalling pathway dictate host-cell permissiveness to herpes simplex virus 1. Nat Cell Biol 2001;3(8):745-50

30. Shinohara N, Ogiso Y, Tanaka M et al. The significance of Ras guanine nucleotide exchange factor, son of sevenless protein, in renal cell carcinoma cell lines. J Urol 1997;158(3 Pt 1):908-11

31. Werden SJ, McFadden G. Pharmacological manipulation of the Akt signaling pathway regulates myxoma virus replication and tropism in human cancer cells. J Virol 2010;84(7):3287-302

32. Sourbier C, Lindner V, Lang H, et al. The phosphoinositide 3-kinase/Akt pathway: a new target in human renal cell carcinoma therapy. Cancer Res 2006;66(10):5130-42

33. Roos FC, Roberts AM, Hwang II, et al. Oncolytic targeting of renal cell carcinoma via encephalomyocarditis virus. EMBO Mol Med 2010;2(7):275-88

34. Kuroda T, Rabkin SD, Martuza RL et al. Effective treatment of tumours with strong beta-catenin/T-cell factor activity by transcriptionally targeted oncolytic herpes simplex virus vector.Cancer Res 2006;66(20):10127-35

35. Junker H, Venz S, Zimmerman U et al. Stage-related alterations in renal cell carcinoma – comprehensive quantitative analysis by the 2D-DIGE and protein network analysis. Plos one 2011;6(7)e21867-e21867

36. Dalgliesh GL, Furge K, Greenman Cett al. Systematic sequencing of renal carcinoma reveals inactivation of histone modifying genes. Nature 2010;463(7279):360-3

37. Varela I, Tarpey P, Raine K, et al. Exome sequencing identifies frequent mutation of the SWI/SNF Complex gene PBRM1 in renal carcinoma. Nature 2011;(7331):539-42

38. Chang AE, Li Q, Jiang G, et al. Phase II trial of autologous tumour vaccination, anti-cd3-activated vaccine primed lymphocytes, and interleukin-2 in stage IV renal cell cancer. J Clin Oncol 2003;21(5):884-90

39. Kujawski M, Zhang C, Herrmann A, et al. Targeting STAT3 in adoptively transferred T cells promotes their in vivo expansion and antitumour effects. Cancer res 2010;70(23):9599-610

40. Childs R, Chernoff A, Contentin N, et al. Regression of metastatic renal-cell carcinoma after nonmyeloablative allogeneic peripheral-blood stem-cell transplantation. N Eng J Med 2000;343(11):750-8

41. Lesimple T, Moisan A, Guille F, et al. Treatment of metastatic renal cell carcinoma with activated autologous macrophages and granulocyte-macrophage colony-stimulating factor. J Immunother 2000;23(6):675-9

42. Golumbek PT, Lazenby AJ, Levitsky HI et al. Treatment of established renal cancer by tumour cells engineered to secrete interleukin-4. Science 1991;254(5032):713-6

43. Draube A, Klein-Gonzalez N, Mattheus S, et al. Dendritic cell based tumour vaccination in prostate and RCC: A systematic review and meta-analysis. Plos one 2011;6(4):e18801-e18801

44. Steele L, Errington F, Prestwich R, et al. Proinflammatory cytokine/chemokine production by reovirus treated melanoma cells is PKR/NF-κβ mediated and supports innate and adaptive anti-tumour immune priming. Mol cancer 2011;10:20-20

45. Pesonen S, Kangasniemi L, Hemminki A. Oncolytic adenoviruses for the treatment of human cancer: focus on translational and clinical data. Mol Pharm 2011;8(1):12-28

46. Cuevas Y, Hernández-Alcoceba R, Aragones J, et al. Specific oncolytic effect of a new hypoxia-inducible factor-dependent replicative adenovirus on von Hippel-Lindau-defective renal cell carcinomas. Cancer Res 2003;63(20):6877-84

47. Guse K, Diaconu I, Rajecki M, et al. Ad5/3-9HIF-Delta24-VEGFR-1-Ig, an infectivity enhanced, dual-targeted and antiangiogenic oncolytic adenovirus for kidney cancer treatment. Gene Ther 2009;16(8):1009-20

48. Huang P, Kaku H, Chen J, et al. Potent antitumour effects of combined therapy with a telomerase-specific, replication-competent adenovirus (OBP-301) and IL-2 in a mouse model of renal cell carcinoma. Cancer Gene Ther 2010;17(7):484-91

49. Zhang ZL, Zou WG, Luo CX, et al. An armed oncolytic adenovirus system, ZD55-gene, demonstrating potent antitumoural efficacy. Cell Res 2003;13:481-9

50. Zheng JN, Pei DS, Sun FH, et al. Potent antitumour efficacy of interleukin-18 delivered by conditionally replicative adenovirus vector in renal cell carcinoma-bearing nude mice via inhibition of angiogenesis. Cancer Biol Ther 2009;8(7):599-606

51. Zheng JN, Pei DS, Mao LJ, et al. Inhibition of renal cancer cell growth in vitro and in vivo with oncolytic adenovirus armed short hairpin RNA targeting Ki-67 encoding mRNA. Cancer Gene Ther 2009;16(1):20-32

52. Zheng JN, Pei DS, Sun FH, et al. Inhibition of renal cancer cell growth by oncolytic adenovirus armed short hairpin RNA targeting hTERT gene. Cancer Biol Ther 2009;8(1):84-91

53. Guse K, Ranki T, Ala-Opas M, et al. Treatment of metastatic renal cancer with capsid-modified oncolytic adenoviruses. Mol Cancer Ther 2007;6(10):2728-36

54. Hwang KS, Cho WK, Yoo J, et al. Adenovirus-mediated interleukin-18 mutant in vivo gene transfer inhibits tumour growth through the induction of T cell immunity and activation of natural killer cell cytotoxicity. Cancer Gene Ther 2004;11(6):397-407

55. Wang L, Qi X, Sun Y, et al. Adenovirus-mediated combined P16 gene and GM-CSF gene therapy for the treatment of established tumour and induction of antitumour immunity. Cancer Gene Ther 2002;9(10):819-24

56. Tosch C, Geist M, Ledoux Cet al. Adenovirus-mediated gene transfer of pathogen-associated molecular patterns for cancer immunotherapy. Cancer Gene Ther 2009;16(4):310-9

57. Zhang M, Berndt BE, Chen JJ, et al. Expression of a soluble TGF-beta receptor by tumour cells enhances dendritic cell/tumour fusion vaccine efficacy. J Immunol 2008;181(5):3690-7

58. Guse K, Cerullo V, Hemminki A. Oncolytic vaccinia virus for the treatment of cancer. Expert Opin Biol Ther 2011;11(5):595-608

59. Thorne SH. Immunotherapeutic potential of oncolytic vaccinia virus. Immunol Res 2011;50(2-3):286-93

60. Breitbach CJ, Burke J, Jonker D, et al. Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. Nature 2011;477(7362):99-102

61. Guse K, Sloniecka M, Diaconu I, et al. Antiangiogenic arming of an oncolytic vaccinia virus enhances antitumour efficacy in renal cell cancer models. J Virol 2010;84(2):856-66

62. Arakawa S Jr, Hamami G, Umezu K, et al. Clinical trial of attenuated vaccinia virus AS strain in the treatment of advanced adenocarcinoma. Report on two cases. J Cancer Res Clin Oncol 1987;113(1):95-8

63. Campadelli-Fiume G, De Giovanni C, Gatta V, et al. Rethinking herpes simplex virus: the way to oncolytic agents. Rev Med Virol 2011;21(4):213-26

64. Oyama M, Ohigashi T, Hoshi M, et al. Treatment of human renal cell carcinoma by a conditionally replicating herpes vector G207. J Urol 2001;165(4):1274-8

65. Fu X, Nakamori M, Tao L, et al. Antitumour effects of two newly constructed oncolytic herpes simplex viruses against renal cell carcinoma. Int J Oncol 2007;30(6):1561-7

66. Inoue M, Tokusumi Y, Ban H, et al. A new Sendai virus vector deficient in the matrix gene does not form virus particles and shows extensive cell-to-cell spreading. J Virol 2003;77(11):6419-29

67. Kinoh H, Inoue M, Washizawa K, et al. Generation of a recombinant Sendai virus that is selectively activated and lyses human tumour cells expressing matrix metalloproteinases. Gene Ther 2004;11(14):1137-45

68. Kinoh H, Inoue M, Komaru A, et al. Generation of optimized and urokinase-targeted oncolytic Sendai virus vectors applicable for various human malignancies. Gene Ther 2009;16(3):392-403

69. Fujihara A, Kurooka M, Miki T et al. Intratumoural injection of inactivated Sendai virus particles elicits strong antitumour activity by enhancing local CXCL10 expression and systemic NK cell activation. Cancer Immunol Immunother 2008;57(1):73-84

70. Galanis E. Therapeutic potential of oncolytic measles virus: promises and challenges. Clin Pharmacol Ther 2010;88(5):620-5

71. Meng X, Nakamura T, Okazaki T, et al. Enhanced antitumour effects of an engineered measles virus Edmonston strain expressing the wild-type N, P, L genes on human renal cell carcinoma. Mol Ther 2010;18(3):544-51

72. Lichty BD, Power AT, Stojdl DF, et al. Vesicular stomatitis virus: re-inventing the bullet. Trends Mol Med 2004;10(5):210-6

73. Kottke T, Errington F, Pulido J, et al. Broad antigenic coverage induced by vaccination with virus-based cDNA libraries cures established tumours. Nat Med 2011;17(7):854-9

74. Hwang II, Watson IR, Der SD, et al. Loss of VHL confers hypoxia-inducible factor (HIF)-dependent resistance to vesicular stomatitis virus: role of HIF in antiviral response. J Virol 2006;80(21):10712-23

75. Cho IR, Koh SS, Min HJ, et al. Down-regulation of HIF-1alpha by oncolytic reovirus infection independently of VHL and p53. Cancer Gene Ther 2010;17(5):365-72

76. Craighead JE, McLane MF. Diabetes mellitus: induction in mice by encephalomyocarditis virus. Science 1968;162(3856):913-4

77. Schwarz EM, Badorff C, Hiura TS, et al. NF-kappaB-mediated inhibition of apoptosis is required for encephalomyocarditis virus virulence: a mechanism of resistance in p50 knockout mice. J Virol 1998;72(7):5654-60

78. Wojton J, Kaur B. Impact of tumour microenvironment on oncolytic viral therapy. Cytokine Growth Factor Rev 2010;21(2-3):127-34

79. Tatsumi T, Herrem CJ, Olson WC, et al. Disease stage variation in CD4+ and CD8+ T-cell reactivity to the receptor tyrosine kinase EphA2 in patients with renal cell carcinoma. Cancer Res 2003;63(15):4481-9

80. Fulci G, Breymann L, Gianni D, et al. Cyclophosphamide enhances glioma virotherapy by inhibiting innate immune responses. PNAS 2006;103(34):12873-8

81. Ikeda K, Wakimoto H, Ichikawa T, et al. Complement depletion facilitates the infection of multiple brain tumours by an intravascular, replication-conditional herpes simplex virus mutant. J Virol 2000;74(10):4765-75

82. Ikeda K, Ichikawa T, Wakimoto H, Silver JS, Deisboeck TS, Finkelstein D, Harsh GR 4th, Louis DN, Bartus RT, Hochberg FH, Chiocca EA. Oncolytic virus therapy of multiple tumours in the brain requires suppression of innate and elicited antiviral responses. Nat Med 1999;5(8):881-7

83. White CL, Twigger KR, Vidal L, et al. Characterization of the adaptive and innate immune response to intravenous oncolytic reovirus (Dearing type 3) during a phase I clinical trial. Gene Ther 2008;15(12):911-20

84. Willmon C, Diaz RM, Wongthida P, et al. Vesicular stomatitis virus-induced immune suppressor cells generate antagonism between intratumoural oncolytic virus and cyclophosphamide. Mol Ther 2011;19(1):140-9

85. Woller N, Knocke S, Mundt B, et al. Virus-induced tumour inflammation facilitates effective DC cancer immunotherapy in a Treg-dependent manner in mice. J Clin Invest 2011;121(7):2570-82

86. Kottke T, Galivo F, Wongthida P, et al. Treg depletion-enhanced IL-2 treatment facilitates therapy of established tumours using systemically delivered oncolytic virus. Mol Ther 2008;16(7):1217-26

87. Kottke T, Thompson J, Diaz RM, et al. Improved systemic delivery of oncolytic reovirus to established tumours using preconditioning with cyclophosphamide-mediated Treg modulation and interleukin-2. Clin Cancer Res 2009;15(2):561-9

88. Ochoa AC, Zea AH, Hernandez C, et al. Arginase, prostaglandins, and myeloid-derived suppressor cells in renal cell carcinoma. Clin Cancer Res 2007;13(2 Pt 2):721s-726s

89. Cesana GC, DeRaffele G, Cohen S, et al. Characterization of CD4+CD25+ regulatory T cells in patients treated with high-dose interleukin-2 for metastatic melanoma or renal cell carcinoma. J Clin Oncol 2006;24(7):1169-77

90. Hegele A, Varga Z, von Knobloch R, et al. TGF-beta1 in patients with renal cell carcinoma. Urol Res 2002;30(2):126-9

91. Jain RK. Normalization of tumour vasculature: an emerging concept in antiangiogenic therapy. Science 2005;307(5706):58-62

92. Anderson H, Yap JT, Wells P, et al. Measurement of renal tumour and normal tissue perfusion using positron emission tomography in a phase II clinical trial of razoxane. Br J Cancer 2003;89(2):262-7

93. Dickson PV, Hamner JB, Streck CJ, et al. Continuous Delivery of IFN-B Promotes Sustained Maturation of Intratumoural Vasculature. Mol Cancer Res 2007;5(6):531-42

94. Karapanagiotou EM, Roulstone V, Twigger K et al. Phas I/II trial of carboplatin and paclitaxel chemotherapy in combination with intravenvous oncolytic reovirus in patients with advanced malignancies. Clin Cancer Res 2012;18(7):2080-9

95. Gujar SA, Marcato P, Pan D, Lee PW. Reovirus virotherapy overrides tumour antigen presentation evasion and promotes protective antitumour immunity. Mol Cancer Ther. 2010;9(11):2924-33

96. Gujar SA, Pan DA, Marcato P, Garant KA, Lee PW. Oncolytic virus-initiated protective immunity against prostate cancer. Mol Ther. 2011;19(4):797-804

97. Huang D, Ding Y, Li Y et. al. Sunitinib acts primarily on tumour endothelium rather than tumour cells to inhibit the growth of renal cell carcinoma. Cancer Res; 70(3);1053-62

98. Finke JH, Rini B, Ireland J, et al. Sunitinib reverses type-1 immune suppression and decreases T-regulatory cells in renal cell carcinoma patients. Clin Cancer Res 2008;14(20):6674-82

99. Norman KL, Lee PW. Reovirus as a novel oncolytic agent. J Clin Invest 2000;105(8):1035-8

100. Danthi P, Guglielmi KM, Kirchner E et. al. From touchdown to transcription: the reovirus cell entry pathway. Curr Top Mircobiol Immunol 2010;343:91-119

101. Barton ES, Forrest JC, Connolly JL et. al. Junction adhesion molecule is a receptor for reovirus. Cell 2001;104:441–451

102. Borsa J, Morash BD, Sargent MD et. al. Two modes of entry of reovirus particles into L cells. J Gen Virol 1979; 45:161–170

103. Danthi P, Hansberger MW, Campbell JA et. al. JAM-A-independent, antibody-mediated uptake of reovirus into cells leads to apoptosis. J Virol 2006;80:1261–1270

104. Danthi P, Coffey CM, Parker JS et. al. Independent regulation of reovirus membrane penetration and apoptosis by the m1 f domain. PLoS Pathog 2008;4:e1000248

105. Holm GH, Zurney J, Tumilasci V et. al. Retinoic acid-inducible gene-I and interferon-b promoter stimulator-1 augment proapoptotic responses following mammalian reovirus infection via interferon regulatory factor-3. J Biol Chem 2007;282:21953–21961

106. Connolly JL, Dermody TS. Virion disassembly is required for apoptosis induced by reovirus. J Virol 2002;76:1632–1641

107. Knowlton JJ, Dermody TS, Holm GH. Apoptosis induced by mammalian reovirus is beta interferon (IFN) independent and enhanced by IFN regulatory factor 3- and NF-kB-dependent expression of noxa. J Virol 2011;86(3):1650-1660

108. Clarke P, Meintzer SM, Gibson S et. al. Reovirus-induced apoptosis is mediated by TRAIL. J Virol 2000;74(17):8135-9

109. Hashiro G, Loh PC, Yau JT. The preferential cytotoxicity of reovirus for certain transformed cell lines. Arch Virol 1977;54(4):307-15

110. Coffey MC, Strong JE, Lee PW. Reovirus therapy of tumours with activated ras pathway. Science 1998;282(5392):1332-4

111. Strong JE, Coffey MC, Tang D et. al. The molecular basis of viral oncolysis: usurpation of the ras signaling pathway by reovirus. EMBO J. 1998;17(12):3351-62

112. Marcato P, Shmulevitz M, Pan D et. al. Ras transformation mediates reovirus oncolysis by enhancing virus uncoating, particle infectivity, and apoptosis-dependent release. Mol Ther 2007;15(8):1522-30

113. Twigger K, Roulstone V, Kyula J et. al. Reovirus exerts potent oncolytic effects in head and neck cancer cell lines that are independent of signalling in the EGFR pathway. BMC Cancer 2012;12(368)

114. Norman KL, Coffey MC, Hirasawa K, Demetrick DJ, Nishikawa SG, DiFrancesco LM, Strong JE, Lee PW. Reovirus oncolysis of human breast cancer. Hum Gene Ther. 2002;13(5):641-52

115. Thirukkumaran CM, Nodwell MJ, Hirasawa K, Shi ZQ, Diaz R, Luider J, Johnston RN, Forsyth PA, Magliocco AM, Lee P, Nishikawa S, Donnelly B, Coffey M, Trpkov K, Fonseca K, Spurrell J, Morris DG. Oncolytic viral therapy for prostate cancer: efficacy of reovirus as a biological therapeutic. Cancer Res. 2010 Mar 15;70(6):2435-44

116. Hirasawa K, Nishikawa SG, Norman KL, Alain T, Kossakowska A, Lee PW. Oncolytic reovirus against ovarian and colon cancer. Cancer Res. 2002;62(6):1696-701

117. Alain T, Muzik H, Otsuka S, Chan S, Magliocco T, Diaz R, Forsyth PA, Morris D, Bebb G. Susceptibility of mantle cell lymphomas to reovirus oncolysis. Leuk Res. 2010;34(1):100-8

118. Hanel EG, Xiao Z, Wong KK, Lee PW, Britten RA, Moore RB. A novel intravesical therapy for superficial bladder cancer in an orthotopic model: oncolytic reovirus therapy. J Urol. 2004;172(5 Pt 1):2018-22

119. Wilcox ME, Yang W, Senger D, Rewcastle NB, Morris DG, Brasher PM, Shi ZQ, Johnston RN, Nishikawa S, Lee PW, Forsyth PA. Reovirus as an oncolytic agent against experimental human malignant gliomas. J Natl Cancer Inst. 2001;93(12):903-12

120. Sei S, Mussio JK, Yang QE et. al. Synergistic antitumour activity of oncolytic reovirus and chemotherapeutic agents in non-small cell lung cancer cells. Mol Cancer 2009;8:47

121. Thirukkumaran CM, Shi ZQ, Luider J et. al. Reovirus as a viable therapeutic option for the treatment of multiple myeloma. Clin Cancer Res 2012;18(18):4962-72

122. Errington F, White CL, Twigger KR et. al. Inflammatory tumour cell killing by oncolytic reovirus for the treatment of melanoma. Gene Ther 2008;15(18):1257-70

123. Hingorani P, Zhang W, Lin J et. al. Systemic administration of reovirus (Reolysin) inhibits growth of human sarcoma xenografts. Cancer 2011;117(8):1764-74

124. Morris DG, Feng X, Difrancesco LM et. al. REO-001: A phase I trial of percutaneous intralesional administration of reovirus type 3 dearing (Reolysin) in patients with advanced solid tumours. Invest New Drugs 2012 [Epub]

125. Steele L, Errington F, Prestwich R, Ilett E, Harrington K, Pandha H, Coffey M, Selby P, Vile R, Melcher A. Pro-inflammatory cytokine/chemokine production by reovirus treated melanoma cells is PKR/NF-κB mediated and supports innate and adaptive anti-tumour immune priming. Mol Cancer. 2011;10:20

126. Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res. 2010;70(2):440-6

127. Gillet JP, Calcagno AM, Varma S et. al. Redefining the relevance of established cancer cell lines to the study of mechanisms of clinical anti-cancer drug resistance. PNAS 2011;108(46):18708-13

128. Kottke T, Chester J, Ilett E et. al. Precise scheduling of chemotherapy primes VEGF-producing tumours for successful systemic oncolytic virotherapy. Mol Ther 2011;19(10):1802-12

129. White CL, Twigger KR, Vidal L, De Bono JS, Coffey M, Heinemann L, Morgan R, Merrick A, Errington F, Vile RG, Melcher AA, Pandha HS, Harrington KJ. Characterization of the adaptive and innate immune response to intravenous oncolytic reovirus (Dearing type 3) during a phase I clinical trial. Gene Ther. 2008;15(12):911-20

130. Kottke T, Thompson J, Diaz RM, et al. Improved systemic delivery of oncolytic reovirus to established tumours using preconditioning with cyclophosphamide-mediated Treg modulation and interleukin-2. Clin Cancer Res 2009;15(2):561-9

131. Pandha HS, Heinemann L, Simpson GR et. al. Synergistic effects of oncolytic reovirus and cisplatin chemotherapy in murine malignant melanoma. Clin Cancer Res 2009;15(19):6158-66

132. Ozao-Choy J, Ma G, Kao J, Wang GX, Meseck M, Sung M, Schwartz M, Divino CM, Pan PY, Chen SH. The novel role of tyrosine kinase inhibitor in the reversal of

immune suppression and modulation of tumour microenvironment for immune-based cancer therapies. Cancer Res. 2009;69(6):2514-22

133. Ko JS, Zea AH, Rini BI, Ireland JL, Elson P, Cohen P, Golshayan A, Rayman PA, Wood L, Garcia J, Dreicer R, Bukowski R, Finke JH. Sunitinib mediates reversal of myeloid-derived suppressor cell accumulation in renal cell carcinoma patients. Clin Cancer Res. 2009;15(6):2148-57

134. Kujawski M, Zhang C, Herrmann A, Reckamp K, Scuto A, Jensen M, Deng J, Forman S, Figlin R, Yu H. Targeting STAT3 in adoptively transferred T cells promotes their in vivo expansion and antitumour effects. Cancer Res. 2010 Dec 1;70(23):9599-610

135. Bose A, Taylor JL, Alber S, Watkins SC, Garcia JA, Rini BI, Ko JS, Cohen PA, Finke JH, Storkus WJ. Sunitinib facilitates the activation and recruitment of therapeutic anti-tumour immunity in concert with specific vaccination. Int J Cancer 2011;129(9):2158-70

136. Avella DM, Li G, Schell TD, Liu D, Shao-Min Zhang S, Lou X, Berg A, Kimchi ET, Tagaram HR, Yang Q, Shereef S, Garcia LS, Kester M, Isom HC, Bart Rountree C, Staveley-O'Carroll KF. Regression of established hepatocellular carcinoma is induced by chemo-immunotherapy in an orthotopic murine model. Hepatology 2012;55(1):141-52

137. Hillman GG, Droz JP, Hass GP et. al. Experimental animal models for the study of therapeutic approaches in renal cell carcinoma. In Vivo 1994;8(1):77-80

138. Xin H, Zhang C, Herrmann A, Du Y, Figlin R, Yu H. Sunitinib inhibition of Stat3 induces renal cell carcinoma tumour cell apoptosis and reduces immunosuppressive cells. Cancer Res. 2009;69(6):2506-13

139. Farsaci B, Higgins JP, Hodge JW. Consequence of dose scheduling of sunitinib on host immune response elements and vaccine combination therapy. Int J Cancer 2012;130(8):1948-59

140. Prestwich RJ, Errington F, IIett EJ et. al. Tumour infection by oncolytic reovirus primes adaptive antitumour immunity. Clin Cancer Res 2008;14(22):7358-66

141. Karam JA, Zhang XY, Tamboli P et. al. Development and characterization of clinically relevant tumour models from patients with renal cell carcinoma. Eur Urol 2011;59(4):619-28

142. Cohen PA, Ko JS, Storkus WJ et. al. Myeloid-derived suppressor cells adhere to physiologic STAT3- vs STAT5-dependent hematopoietic programming, establishing diverse tumour-mediated mechanisms of immunologic escape. Immunol Invest 2012;41(6-7):680-710

143. Hillman GG, Singh-Gupta V, Al-Bashir AK et al. Dynamic contrast-enhanced magnetic resonance imaging of sunitinib-induced vascular changes to schedule chemotherapy in renal cell carcinoma xenograft tumours. Transl Oncol 2010;3(5):293-306

144. Li Q, Rao RR, Araki K, et al. A central role for mTOR kinase in homeostatic proliferation induced $CD8^+$ T cell memory and tumour immunity. Immunity 2011;34(4):541-53

145. Wang Y, Wang X-Y, Subjeck JR, et al. Temsirolimus, an mTOR inhibitor, enhances anti-tumour effects of heat shock protein cancer vaccines. Br J Cancer 2011;104:643-652

146. Bassett JD, Swift SL, VanSeggelen H, et al. Combined mTOR inhibition and OX40 agonism enhances CD8+ T cell memory and protective immunity produced by recombinant adenovirus vaccines. Mol Ther 2012;20(4):860-9

147. Dickson PV, Hamner JB, Sims TL, et al. Bevacizumab-induced transient remodeling of the vasculature in neuroblastoma xenografts results in improved delivery and efficacy of systemically administered chemotherapy. Clin Cancer Res 2007;13(13):3942-50

148. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol. 2009;9(3):162-74