ONCOLYTIC VIRAL IMMUNOTHERAPY FOR EPITHELIAL CANCER

THE PRECLINICAL DEVELOPMENT OF ONCOLYTIC VIRAL IMMUNOTHERAPY FOR EPITHELIAL CANCER

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Lay abstract

Carcinoma (epithelial cancer) is the most common form of human cancer and two frequently encountered types, namely HPV-associated and prostatic carcinoma are responsible for a substantial worldwide cancer burden. Current therapeutic options show limited clinical benefit and/ or significant long-term side effects for advanced carcinomas, therefore new treatments are urgently required. Oncolytic viruses represent an exciting new form of anti-tumour immunotherapy capable of infecting and killing cancerous cells; here we present a virus called MG1 Maraba that is able to exploit molecular characteristics of these cancers. When MG1 Maraba is engineered to target proteins from HPV-associated cancer and prostatic carcinoma, specific immune attack against these tumours occur in mouse cancer models. MG1 Maraba offers a novel, selective, safe and highly promising therapeutic approach against advanced carcinomas. Based on the information within this thesis human clinical trials assessing MG1 Maraba are due to take place for both HPV-associated and prostate cancer.

Abstract

HPV-associated cancer and carcinoma of the prostate are responsible for significant worldwide morbidity and mortality. The viral transforming proteins E6 and E7 make human papilloma virus positive (HPV+) malignancies an attractive target for cancer immunotherapy however, therapeutic vaccination exerts limited efficacy in the setting of advanced disease. In prostatic carcinoma therapeutic vaccination shows some therapeutic activity but is infrequently curative.

A strategy to induce substantial specific immune responses against multiple epitopes of E6 and E7 proteins based on an attenuated transgene from HPV serotypes 16 and 18, that is incorporated into MG1-Maraba virotherapy (MG1-E6E7), was designed. MG1-E6E7 is able to boost specific immunity following priming with either an adenoviral vector (Ad-E6E7) or customised synthetic peptide vaccines resulting in multifunctional CD8+ T cell responses of an enormous magnitude. MG1-E6E7 vaccination in the HPV+ murine model TC1 is curative against large tumours in a CD8+ dependent manner and results in durable immunologic memory. Using the same adenoviral prime and MG1 boosting strategy targeting the prostatic antigen, STEAP, immunity against multiple CD8+ STEAP epitopes was induced. In a murine prostate cancer model, STEAP specific oncolytic virotherapy significantly improved the survival of mice bearing advanced TRAMP-C2 tumours.

One significant obstacle to therapeutic cancer vaccination is an immunosuppressive tumour microenvironment. MG1 Maraba is able to lethally infect HPV-associated and prostate cancer cells, increase the immunologic activity within the tumour microenvironment *in vivo* and exploit molecular hallmarks of HPV-positive cancer and prostatic carcinoma enabling infection of bulky tumours.

Pre-clinical data generated within this thesis has been instrumental in securing funding for future clinical trials assessing the safety and activity of MG1 Maraba virotherapy for HPV-associated cancer and prostatic carcinoma. This promising approach has the potential to be directly translatable to human clinical oncology to tackle these two highly prevalent and frequently lethal groups of epithelial neoplasia.

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Abbreviations and symbols

α	alpha
β	beta
Δ	delta
γ	gamma
µg	microgram
mg	milligram
g	gram
g	relative centrifugal force
µm	micrometer
µl	microliter
ml	milliliter
kDa	kilodalton
nM	nanomolar
mM	millimolar
M	molar
Ad	adenovirus
ADP	adenovirus death protein
ALL	acute lymphoblastic lymphoma
APC	antigen presenting cell
ATM	ataxia telangiectasia mutated
BCG	bacillus of Calmette and Guérin

BM	bone marrow
CAF	cancer-associated fibroblast
CAR	coxsackievirus-adenovirus receptor
CAR T cell	chimeric antigen receptor T cell
CD4+ T cell	helper T cell
CD8+ T cell	cytotoxic T cell
CD40L	CD40-ligand
cDC	conventional dendritic cell
CRPC	castrate resistant prostate cancer
CTLA-4	cytotoxic T-lymphocyte-associated antigen-4
CTL	cytotoxic T-lymphocyte
CTX	cyclophosphamide
DAMP	damage-associated molecular pattern
DC	dendritic cell
DCT	dopachrome tautomerase
E4orf4	E4 open reading frame 4 protein
ER	endoplasmic reticulum
Fas	Fas receptor
FasL	Fas-ligand
FGF2	fibroblast growth factor 2
Flt3L	Flt3 ligand
GFP	green fluorescent protein

GM-CSF	granulocyte-macrophage colony-stimulating factor
HApRb	HA-tagged pRb
HCC	hepatocellular carcinoma
HDAC	histone deacetylase inhibitor
HNSCC	head and neck squamous cell carcinoma
HPV	human papilloma virus
HSV	herpes simplex virus
ICD	immunogenic cell death
IFN	interferon
IL	interleukin
IP	immunoprecipitation
IRES	internal ribosomal entry site
IV	intravenous
LDL	low-density lipoprotein
MAb	monoclonal antibody
MDSC	myeloid-derived suppressor cell
MIP	macrophage inflammatory protein
MOI	multiplicity of infection
MV	measles virus
NDV	Newcastle disease virus
NK	natural killer
OV	oncolytic virus

PAMP	pathogen-associated molecular pattern
PAP	prostatic acid phosphatase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD-1	programmed death-1
PD-L1	programmed death-ligand 1
PFU	plaque forming unit
PSA	prostate-specific antigen
RAG2	recombination activating gene
RANTES	regulation on activation, normal T cell expressed and secreted
Rb	retinoblastoma
RFP	red fluorescent protein
RIG-I	retinoic acid-inducible gene
RT	reverse transcription
SEM	standard error of the mean
SLAM	signalling lymphocyte activation molecule
SLP	synthetic long peptide
SMC	SMAC mimetic compound
STAT1	signal transducers and activators of transcription-1
STEAP	six-transmembrane epithelial antigen of the prostate
TAA	tumour-associated antigen
TCM	central memory T cell

TCR	T cell receptor
TGF-β	transforming growth factor-β
TK	thymidine kinase
TIL	tumour infiltrating lymphocyte
TLR	Toll-like receptor
TME	tumour microenvironment
TNFα	tumour necrosis factor α
Treg	regulatory T cell
VSV	vesicular stomatitis virus
VV	vaccinia virus
WB	western blot
WT	wild type
WT1	Wilms' Tumour Protein

Statement of academic achievement

Chapters III, IV and VI of this thesis represent three separate manuscripts prepared for submission for publication. I significantly contributed to the design and performed the majority of the subsequently presented studies, however, the completion of this thesis would not have been possible without the following valued contributions:

Dr. Kyle Stephenson- acquisition of data, development of methodology and technical and material support.

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~CHAPTER I~

INTRODUCTION

1.1. Cancer of epithelial tissue

1.1.1. The origin of epithelial malignancies

Epithelial tissue lines both the internal and external surfaces of the organs in the body. During embryogenesis gastrulation leads to the formation of three germ layers, namely endoderm, ectoderm and mesoderm from which all tissue types subsequently arise[1,2]. The majority of neoplasms that are morphologically epithelial in nature are derived from endodermal and ectodermal tissues; however, epithelial tumours such as renal cell carcinoma and adrenal cortical adenoma originate from the mesoderm[3]. Epithelial tumours originate from a broad spectrum of histo-embryologic tissues, which in part explains their diverse biologic behaviour and highlights one of the limitations with previous and somewhat arbitrary morphologic classification systems[3].

Epithelial surfaces act as a functional interface and physical barrier between the body and the environment. Cancer occurrence as a result of familial genetic disease has long been established, for example when associated with inherited mutated tumour suppressors such as retinoblastoma or the BRCA genes, but it should be noted that such presentations are only estimated to account for approximately 5-10% of all cases[4]. Most neoplastic disease is initiated by acquired mutations following exposure of the body to external stimuli in the form of chemical carcinogens, radiation and oncogenic viruses and as such epithelial surfaces are frequently exposed to tumourigenic factors[5]. Whilst cancer is a

diverse group of diseases, there are defined hallmarks that link all subsets with common biologic traits as defined by Hanahan and Weinberg[6]. The hallmarks of malignancy are representative of the fact that cancer is a multi-faceted, complex and progressive group of diseases initiated by intracellular defects and reinforced by acquired changes both intra- and extra-cellularly[6]. Under normal physiological conditions epithelial cells are constantly being lost and replenished by a small population of stem cells and as such epithelium can be considered to be a renewal tissue[7]. The cancer stem cell hypothesis theorises that only a particular subset of cells within the heterogeneous setting of the tumour has the potential to proliferate and effectively maintain the impetus for tumour growth; moreover somatic/ normal tissue stem cells are likely to be the source of cancer stem cells, however some controversy remains around this generalised hierarchical supposition[5]. Evidence also exists supporting a relative propensity of epithelium towards genetic instability and escape from senescence when compared to fibroblasts from the same locale[8]. Both the anatomic and physiologic niche of epithelium predisposes people to the relatively frequent development of epithelial cancer.

1.1.2. Epidemiology, morbidity and mortality of epithelial cancer

Epithelial malignancies are the most frequently diagnosed form of cancer affecting mankind. The most recent global cancer statistics revealed that across male and female populations in developed and developing countries differing

types of epithelial cancer were responsible for the vast majority of both new cases and death related to cancer[9]. Whilst progress with regards to cancer survival rates has been made, cancer, dominated by epithelial malignancies is a leading cause of death for much of the population of America[10]. A similar picture with regards to epidemiology and cancer related mortality is also present within Canada[11]. As such the mandate for ongoing research to generate novel and efficacious therapies for epithelial cancer is obviously compelling.

Our developing comprehension of the remarkable diversity of cancer is driving the rationale for focusing on specific disease subsets using "precision oncology"[12]. In women cervical cancer, with the exception of non-melanoma skin cancer, is both the fourth most prevalent and fourth most deadly type of cancer across all nations[9] with human papilloma virus (HPV) being unequivocally identified as a causal agent[13]. Recently HPV-associated oropharyngeal cancer has been flagged as a growing epidemic with a projection that its prevalence will overtake that of cervical cancer[14]. Carcinoma of the prostate is the fifth most deadly cancer in men worldwide and has the second highest global incidence rate of invasive male tumours (excluding non-melanoma skin cancer)[9]. Whilst an elusive "silver bullet" to cure all cancers is obviously desirable, there is great hope that therapy tailored for distinct groups of neoplasms offers a genuine chance to improve the outcome for many cancer patients[15]. Justified by the prior epidemiologic reasoning this thesis will focus on the development of targeted

novel therapeutics for two highly prevalent and deadly subsets of epithelial neoplasms, namely HPV-associated cancer and prostatic carcinoma.

1.2. Cancer immunology and immunotherapy[16]

1.2.1. Cancer immunology

A recognised hallmark of malignancy is tumour immunoevasion where neoplasms utilise multiple mechanisms to avoid destruction by the host's immune response[6]. Both innate (rapid and non-specific) and adaptive (specific with memory) immunity are required to prevent neoplastic development[17]. The failure of immunosurveillance during tumourigenesis implies that the immune system is ignorant of and/or rendered impotent to the impending danger of tumour formation. Using studies in knockout mice, Shankaran et al. demonstrated that either defective innate or adaptive immunity alone or a combination of both, increase tumour formation[18]. Both recombination activating gene 2 (RAG2) deficient mice −/− in which the development of lymphocytes required for an adaptive response is prevented, and signal transducers and activators of transcription-1 (STAT1) deficient mice −/− in which the STAT1 defect disables interferon signalling pathways that are crucial for innate immunity, are at higher risk for cancer development[18]. Furthermore, these investigations suggest that tumours developing in immunologically intact animals proceed through immunoselection, which facilitates tumour progression in an immune-competent host. Thus cancers that are able to develop in the face of a functional immune

system are shaped by the immune response and become more able to survive, leading these authors to coin the phrase “cancer immunoediting”[18]. The immune system is therefore involved not only in surveillance to try and prevent tumour formation but also subsequently in shaping the progressive immunoevasive tumour cell phenotype.

Neoplasms are able to reduce anti-cancer immune responses (immunoevasion) in a variety of ways primarily within the tumour microenvironment (TME). So called “anti-inflammatory” cytokines that suppress anti-tumour immunity such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) can be produced by tumour cells and also by regulatory T cells (Tregs)[19,20]. Decreased antigen presentation and perturbed antigen presenting cell (APC) function also impede the development of protective immunity[21]. Central and peripheral tolerance (i.e. prevention of an effector response against tumour antigens) play a role in allowing tumour survival and proliferation in hosts with functioning immune systems, an observation that is not unexpected as tumours are derived from self tissues[22]. Recently, the roles of two cell populations, Tregs and myeloid-derived suppressor cells (MDSCs) have been implicated in carcinogenesis by favouring peripheral tolerance towards tumours[23,24]. MDSCs are potent inhibitors of both innate and adaptive immunity and are therefore also a significant problem for cancer immunotherapy[25]. Taken together, these studies imply that for an immunotherapeutic strategy to be effective in cancer therapy, it must be able to

overcome or eliminate the existing tumour tolerance and mechanisms of immune-evasion.

1.2.2. Cancer immunotherapy

1.2.2.1. Innate immunity

Treatments targeting the innate arm of the immune system aim to activate an anti-tumour immune response non-specifically. Bacillus of Calmette and Guérin (BCG) as well as the cytokine interferon-α (IFN-α) have been described to treat human melanoma patients[26]. In human oncology clinics BCG is used for the treatment of superficial bladder transitional cell carcinoma since mechanistically this leads to the infiltration of the bladder with a broad range of immune cell types accompanied by the induction of various cytokines[27]. Interferon-α has direct anti-proliferative effects on neoplastic cells as well as indirectly inciting an anti-cancer immune response, and has been used to treat human patients with melanoma, hairy cell leukaemia and renal cell carcinoma[28], however, the side effects of high dose IFN-α are substantial[28]. The cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) acts as a growth factor for dendritic cells (DCs) and macrophages, both of which are crucial innate cells playing important roles in antigen presentation[29]. Treatment of human patients with a selectively replicative herpes simplex virus designed to express GM-CSF led to durable response rates in recently presented phase III clinical trial data[30]. It is worth noting, however, that APCs such as DCs are able to induce Tregs as well as cytotoxic T-lymphocytes

(CTLs), thereby promoting tumour immunoevasion and thus possible therapeutic failure in certain settings[31]. Various approaches exist for harnessing the innate immune response in an anti-neoplastic manner as per the previous examples; however, the majority of current research is focussed on inducing tumour specific adaptive immunity.

1.2.2.2. Adaptive immunity

Provoking a tumour-specific immune response requires engagement of the adaptive immune system and this can be achieved using specific cytokines, certain monoclonal antibodies, adoptive cell transfer and various vaccination strategies.

Cytotoxic (CD8+) T cells are considered key effector cells in cancer immunotherapy with the majority of therapeutics aimed at provoking specific anti-tumour cell mediated immunity[32]. Killing by cytotoxic T cells is facilitated primarily by the perforin/ granzyme mediated pathway[33], however expression of Fas-ligand (FasL) by CD8+T cells enables binding to the Fas receptor (Fas) on the cognate target cell and induces cell death via apoptosis[34]. Mimicking the role of FasL with gene therapy has been described as a potentially useful approach for treating head and neck cancer[35]. As such CD8+ T cells are able to exert their cytotoxicity using different mechanisms and can be pharmacologically activated using numerous methods that are discussed in the following paragraphs (Fig 1).

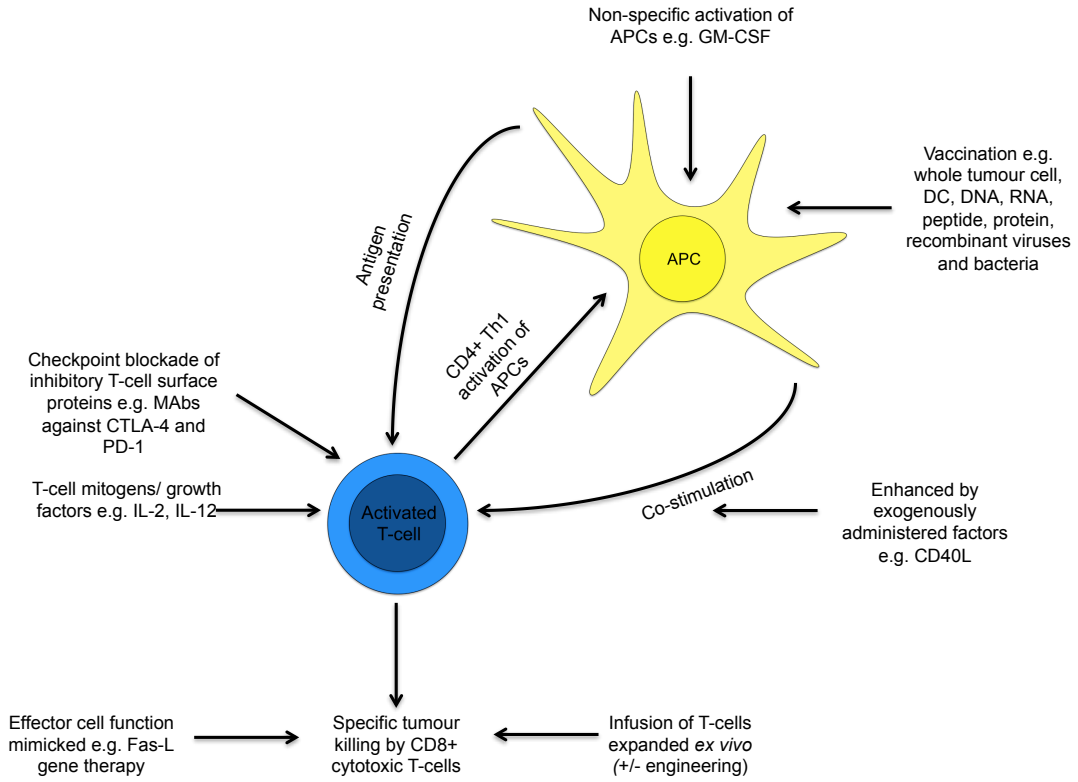


Figure 1. Unleashing the anti-cancer T cell. APC: antigen presenting cell; GM-CSF: granulocyte macrophage colony-stimulating factor; DC: dendritic cell; CD40L: CD40-ligand; CD4+ Th1: CD4+ T-helper type 1 cell; MAb: monoclonal antibody; CTLA-4: cytotoxic T-lymphocyte-associated antigen-4; PD-1: programmed death-1; IL-2: interleukin-2; IL-12: interleukin-12; Fas-L: Fas-ligand. Adapted from "Cancer immunology and canine malignant melanoma: A comparative review"[16]

Co-stimulatory molecules are found on immune cell surfaces and their binding as well as the binding of antigen receptor to complexed peptide: MHC is required to activate immune effectors[36]. When one such molecule CD40, a receptor found on the surface of B cells and APCs binds to CD40L expressed on activated T cells it reciprocally activates both cell types thus enhancing humoural as well as cell

mediated immunity[37,38]. Various strategies using monoclonal antibodies have been used within the co-stimulatory pathways with the aim of enhancing anti-tumour immunity[39]. There is obvious rationale for targeting such molecules within the setting of cancer immunotherapy.

Cytokine therapy is able to promote adaptive immune responses, as well as the previously described innate induction. High dose interleukin-2 (IL-2), a T cell growth factor, has documented efficacy against human melanoma and renal cell cancer; however, its toxicity can be significant and response rates only modest[40]. Another cytokine, the pleiotropic cytokine interleukin-12 (IL-12) exerts multiple downstream effects and promotes both innate and adaptive anti-tumour immunity[41]. Through varying mechanisms cytokine therapy is able to non-specifically activate anti-cancer immunity.

Two recent, exciting developments to reach the human clinic are the use of immune checkpoint inhibitors and adoptive cell therapy. Immune checkpoint molecules are expressed on T cells as an important mechanism to regulate the immune response and maintain self-tolerance[42]. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed death-1 (PD-1) are both proteins found on the surface of cytotoxic T-lymphocytes. Their binding to relevant ligands expressed on immune cell and tumour cell targets dampens effector T cell immune responses and leads to immune evasion, which is

detrimental to anti-tumour immunity[43]. Interestingly checkpoint molecules are also highly expressed on the surface of Tregs where, somewhat paradoxically they activate this subset of T cells; checkpoint blockade is therefore able to inhibit the immunosuppressive activity of Tregs[42]. Clinical trials to prevent normal ligand binding by administering ipilimumab and lambrolizumab, monoclonal antibodies against CTLA-4 and PD-1, respectively, revealed remarkable responses against advanced stage human malignant melanoma[44,45]. In men with prostate cancer that had failed standard therapy, signs of biologic activity of ipilimumab were noted, however, an increase in overall survival was not observed[46]. Treatment of patients with recurrent head and neck cancer (encompassing both HPV-positive and negative tumours) with another PD-1 blocking agent, nivolumab resulted in increased overall survival times compared to standard chemotherapy[47], this same agent also extends survival times for melanoma patients[48]. Blockade of the PD-1 receptor pathway can also be accomplished using monoclonal antibodies against its ligand PD-L1 resulting in durable tumour responses in people suffering from advanced stage malignancies[49]. Checkpoint inhibitors are now an established treatment option for cancer clinicians.

There are various methodologies for treating cancer patients with adoptively transferred cellular therapies. Adoptive therapy in which autologous tumour infiltrating lymphocytes (TILs) are harvested from cancer patients, expanded *ex vivo* and then re-infused intravenously post-lymphodepletion has resulted in

some incredible effects in clinical trials with advanced stage melanoma patients[50]. In a small cohort of cervical cancer patients TIL therapy has demonstrated curative potential against metastatic disease, clinical response was positively correlated with the presence of T cells reactive against the HPV-associated antigens E6 and E7 in the peripheral blood 1 month after treatment[51]. Engineering of autologous T cells *ex vivo* to alter the T cell receptor (TCR) has also been performed enabling T cells to engage with tumour cells in patients where the unaltered autologous cells might be unable to bind to the appropriate targets[52]. Latterly T cells with chimeric antigen receptor (CAR) T cells have also been developed; CAR T cells have proven efficacy in the human clinic with one example being the CD19 CAR for acute lymphoblastic lymphoma (ALL)[53]. Whilst such adoptive cell therapy is hopeful to many, this treatment is very expensive, requires sophisticated techniques and currently is not widely available.

Multiple different vaccination strategies have been devised to induce an anti-tumour immune response against tumour-associated antigens (TAAs). These include allogeneic whole cell tumour vaccines, DC vaccination, DNA vaccination, RNA vaccination, peptide vaccination, protein vaccination, as well as utilising recombinant viral and bacterial vectors[16,54–56]. Recently lots of interest has surrounded one of the best known whole cell vaccines namely GVAX (a killed whole cell vaccine secreting GM-CSF), and more specifically in combination with a boosting vaccine comprising Listeria monocytogenes expressing the TAA

mesothelin for pancreatic cancer; initial success in clinical trials was disappointingly followed by a failure of this therapy to meet subsequent trial endpoints[57,58]. A major clinical breakthrough for cancer vaccination was the demonstration of a modest increase in survival times in men suffering castrate-resistant prostatic cancer when treated with autologous DCs that are engineered *ex vivo*, to express the TAA, prostatic acid phosphatase (PAP) fused to GM-CSF and subsequently re-infused[59]. DNA vaccination has been tested in oncology clinics for many years however, success has been limited; attempts to address the lack efficacy including the use of electroporation for administration and combination with other agents is ongoing[60]. Likewise, novel ways of enhancing the activity of RNA vaccination in a therapeutic setting is continuing. In a preclinical setting RNA-lipoplexes are able to activate DCs and mimic viral responses thereby exerting anti-tumour efficacy in multiple tumour models, the ability to translate this particular regimen to the clinic remains to be seen[61]. Peptide and protein vaccines are at various stages of clinical development with the former being more cost effective to manufacture[62]. Such vaccines can target various TAAs including gp100, MUC-1, HER-2, MAGE-A3 and NY-ESO, however combination therapy with other immunostimulatory therapies will likely be required to capitalise on their potential[62]. Again in the realm of prostatic cancer another promising vaccine candidate, Prostvac is currently in phase III trials[63]. This technology uses a heterologous prime: boost comprising a vaccinia prime and fowlpox boost with both components expressing the TAA, prostate-specific

antigen (PSA) in combination with immunostimulatory molecules; phase II data reveal this treatment was well tolerated and resulted in increased survival[64]. A recombinant Listeria monocytogenes expressing Her2 was able to generate specific anti-tumour immunity and delay progression of disease in a population of dogs suffering from osteosarcoma[65], a similar strategy is currently undergoing assessment using this platform to target the E7 antigen in oncology clinics[66]. Interest in cancer vaccines has been rekindled due to the development of novel and potent platforms as well as the promise of combining these agents with other immunotherapeutics such as the checkpoint inhibitors[58].

As clinical experience with checkpoint inhibitors and other immunotherapies increase the need to accurately predict which tumours are sensitive to such treatment also grows. The presence of CD3+ and CD8+ T cells within the TME has a positive prognostic bearing in various solid tumours[67]. Recently mutational signatures across a variety of different neoplasms have also been mapped with checkpoint inhibitor responsive tumours, such as melanoma, carrying a high mutational burden[68]. Cancers with a high mutational load are posited to be good targets for cancer immunotherapeutics as mutagenesis drives the formation of neo-epitopes that are recognised as antigenically foreign by the immune system and as such are thought of as a “final common pathway” for various cancer immunotherapeutics[69]. On-going research is required to continue to define which patient populations are most likely to respond to current immunotherapy and to

develop strategies to convert patients with immunologically “cold” (resistant) TMEs to the more favourable immunologically “hot” type.

1.3. Oncolytic viruses[54]

1.3.1. Background to viral oncolysis

Oncolytic viruses (OVs) are replicate sufficient viruses that have the ability to selectively and lethally infect tumour cells without causing detriment to other normal host cells. The concept of viruses being of potential therapeutic value is not a new one with a partial remission of myeloid leukaemia following influenza infection in a 42 year old woman being documented over 100 years ago[70]. In the period following this initial observation, research devoted to investigating the molecular and cellular mechanisms of viral oncolysis alongside screening programs for suitable safe therapeutic viruses culminated in the first therapeutic oncolytic virus being licensed for treatment of humans with head and neck cancer in 2005[71]. One of the earliest demonstrations of viral oncolysis was observed when infection of mice with tick-borne encephalitis virus (formerly known as Russian far east encephalitis) led to proliferation of the virus in engrafted sarcoma 180 cells and resulted in interference of tumour growth[72]. Many different families of viruses, encoded by both DNA and RNA genomes have reported oncolytic activity[73]. Oncolytic viruses may be strains that are pathogenic only to animals (oncolytic wild-type viruses); alternatively viruses that have some inherent pathogenicity towards humans have been engineered to prevent their

ability to cause disease whilst maintaining their therapeutic properties. Oncolytic wild-type viruses include Maraba virus, Newcastle disease virus (NDV), vesicular stomatitis virus (VSV), parvoviruses, coxsackievirus and reovirus for example[73–75]. Viruses for which engineering is mandatory to facilitate safety by restricting viral infection to malignantly transformed cells include adenoviruses (Ad), vaccinia viruses (VV), influenza viruses, polioviruses, measles virus (MV) and herpes simplex viruses (HSV)[76–80]. The realm of oncolytic viral research is continually expanding as the potential value of these versatile anti-neoplastic agents is progressively being realised.

1.3.2. Inherent vs. engineered oncotropism

For a virus to be oncotropic it first must be able to enter a neoplastic cell and once inside the target cell it must be able to replicate. To allow cellular entry the tumour cell must express surface receptors to facilitate viral binding. Following entry and uncoating the cell must be permissive to viral replication. The first condition may be modified to focus a virus' tropism but the second condition is dictated by the phenotype of the malignant cell being targeted. For example, Adenovirus gains entry to cells via the coxsackievirus-adenovirus receptor (CAR), however the presence of this receptor is not specific to neoplastic cells and can, in some malignancies, be down regulated thus limiting their susceptibility to adenoviral infection[81]. Other mechanisms for adenoviral entry to neoplastic cells include binding via integrin receptors, which are often abundantly expressed on

tumour cells; this route requires engineering the HI loop of the virus' fibre knob domain[82]. Multiple other methods of engineering CAR independent adenoviral entry into cells have also been described and reviewed by Krasnykh and colleagues[83]. Poliovirus selectively targets motor neurone cells expressing the receptor CD-155 that is a member of the immunoglobulin superfamily[84]. Utilisation of poliovirus as an oncolytic agent in high-grade glioma cells is possible as such tumours also express CD-155[85]. Attenuation of this virus by recombination of poliovirus and human rhinovirus type II prevents neurotoxicity by changing the cell type specific internal ribosomal entry site (IRES) necessary for neurotoxicity whilst maintaining anti-neoplastic activity against gliomas in athymic mice[86]. Herpes viruses are also able to induce oncolysis but have to be engineered to facilitate safe application. Genetic mutations affecting viral replication, neuropathogenicity and immune evasiveness have been described[80]. Measles virus is known to enter lymphocytes via signalling lymphocyte activation molecule (SLAM) allowing Sugiyama and coworkers to exploit this by engineering the rMV-SLAM blind strain that was able to infect and kill human breast cancer cells but did not infect SLAM positive lymphoid cells[87]. VSV primarily enters cells via the ubiquitous low-density lipoprotein (LDL) receptor thereby facilitating broad tropism[88]. Specific binding to tumour cells may be an innate behavioural property of the virus but oftentimes requires genetic alteration of the individual oncolytic agent.

Once viral entry has occurred the neoplastic cellular phenotype further dictates the selective infection of transformed cells. A characteristic facilitating NDV preferentially infecting neoplastic tissue, is reduced or absent type I interferon responsiveness by tumour cells thus allowing selective infection of tumour cells whilst sparing healthy tissue[89]. VSV is also able to exploit this by virtue of its exceptional sensitivity to inhibition by interferon and is therefore able to selectively infect xenografted human melanoma cells in a murine model[90]. The AV1 and AV2 modified strains of VSV were found to be highly attenuated in normal mice but led to durable tumour responses in mouse models of ovarian and colonic cancer demonstrating safety and efficacy *in vivo*[91]. Resistance to interferon allows the influenza virus to counteract the cellular anti-viral response and is achieved by the protein NS1 that is a virulence factor[92]. An NS1 deleted influenza A virus was shown to selectively replicate in interferon deficient melanoma cell lines[92]. The authors of this study also inserted an IL-15 coding sequence in the place of the deleted NS1 leading to natural killer cell mediated lysis of non-infected tumour cells via the immunostimulatory properties of functional IL-15 production[93]. Defects in the type I interferon pathway are of key importance to many OVs.

Impaired apoptotic ability is a well-established feature of many malignant cells[6]. Newcastle disease virus is able to selectively target human non-small cell lung cancer line A549 that over-express the anti-apoptotic protein Bcl-Xl[94]. Deletion of

the anti-apoptotic genes SPI-1 and SPI-2 from vaccinia results in the preferential infection of malignant cells in the murine MC38 colon cancer model thereby enhancing the virus' safety profile[95]. In this way OVs are able to exploit one of the cellular hallmarks of malignancy.

Genetic dysregulation and more specifically oncogene abnormalities in neoplasms may also be advantageous for viruses both in natural and engineered settings. Recent work has shown that melanoma lines are susceptible to various recombinant VSVs with BRAF mutations being predictive for viral infectivity[96]. Parvoviral oncotropism appears to be dependent on cellular replication and transcription factors, such as cyclin A, that are perturbed following neoplastic transformation increasing the efficiency of viral replication in these cells[97]. Reoviral oncotropism is intimately linked with the G-protein Ras that acts to promote cellular proliferation in tumours[98]. The Ras proto-oncogenes are frequently mutated in human cancers making many tumour types potentially susceptible to reoviral oncolysis[98]. Deletion of thymidine kinase (TK), an enzyme required for DNA synthesis and often up-regulated in tumours such as breast cancer[99], from vaccinia is another tactic used to increase selectivity to neoplastic vs. normal tissue[100]. Although NDV, as noted above, is inherently oncotropic further modification to target a specific histotype is possible. This concept is well illustrated by Shobana and coworkers who engineered a recombinant NDV whose F protein was cleaved exclusively in the presence of PSA, thus resulting

only in infection of cells of prostatic lineage[101]. Many oncolytics owe their selectivity to the uncontrolled activation of oncogenes and other lineage specific characteristics.

Ultimately oncolytic viruses target tumour cells selectively by virtue of these cells being permissive for viral replication, often due to cancer-specific defects or alterations to pathways utilized by normal cells in innate antiviral defence. As well, the target cells must bear the binding receptor utilized by the virus for binding and entry; modifying this interaction may further refine oncotropism.

1.3.3. Direct oncolytic cell death

Oncolytic viruses are able to cause tumour cell death independent of immune response by a number of different mechanisms. Replication of adenovirus and herpes simplex virus leads to direct cellular lysis and consequently cell death[102,103]. Transcription of the early adenoviral genes E1A and E1B is necessary for intracellular viral replication and therefore cell death[104]. E1B is required to bind to and inhibit the tumour suppressor gene p53 to allow viral replication. An E1B deficient mutant was developed to abrogate viral infectivity in normal cells however, by the virtue of multiple tumour cells either lacking p53 or containing mutated p53 this virus was still able to replicate in and kill tumour cells[105]. Another tumour suppressor gene that is frequently abnormal or absent in tumours is retinoblastoma (Rb)[106]. The adenoviral product of E1A binds to pRb

allowing G1/S cell cycle progression and hence viral replication even in quiescent cells. The *dl922-947* E1A deleted adenovirus mutant is able to exploit this and is therefore preferentially lethal to neoplastic cells[107]. A further development was the production of a double-restricted E1A/E1B mutant adenovirus shown capable of causing tumour remission with minimal normal tissue effects in a mouse model of gall bladder cancer[108]. Inactivation of tumour suppressor genes such as p53, also dampens innate cellular responses to viral infection thereby promoting viral oncolysis of cancerous cells[109]. In addition to viruses causing direct oncolysis some also exert cytotoxicity via production of proteins that induce both necrotic and apoptotic cellular demise. The E3 adenovirus death protein (ADP) and the type 5 E4 open reading frame 4 protein (E4orf4) are two such examples from adenovirus[110,111]. Genetically modifying OVs facilitates targeted killing of transformed cells.

Engineering viruses to express factors promoting cell death can enhance tumour cell killing. Insertion of the F protein-encoding gene from NDV created a recombinant VSV capable of producing the aforementioned protein[112]. The result was increased oncolytic activity of the recombinant vs. a non-fusogenic control virus achieved by extensive syncytia formation leading to enhanced cytotoxicity[112]. Other examples of engineering leading to increased cell death include transgenic expression of the pro-apoptotic proteins TRAIL[113] and IL-24[114] in oncolytic viruses. Resistance to OV therapy has been identified in glioblastoma

multiforme stem cells but by engineering an HSV to express TRAIL, Tamura and colleagues were able to successfully treat mice engrafted with these resistant cells[115]. Constructing viruses expressing small hairpin (sh)RNA against cellular pro-survival factors such as survivin, hTERT and Ki-67 has also been employed to increase viral tumour killing ability[116–118]. A conditionally replicative adenovirus expressing the tumour suppressor p53 was found to enhance oncolysis in a variety of cell lines by restoring deficient p53 favouring a pro-apoptotic state[119]. Similarly Ma and co-workers constructed a replicate competent adenovirus carrying the p16 gene, another tumour suppressor, and found combined apoptotic and oncolytic effects in a mouse model of gastric cancer[120]. Autophagic cell death is a terminal cellular event mechanistically separate to that of apoptotic or necrotic pathways[121]. Autophagic cell death is induced by the oncolytic adenovirus Δ-24-RGD increasing survival times in a glioma mouse model[122]. OV therapy can result in tumour cell demise in both apoptotic and autophagic forms.

Aside from direct oncolysis of tumour cells OVs are able to infect other populations of cancer-associated cells. Breitbach and colleagues demonstrated increased intra-tumoural inflammatory infiltration as a sequlae to intravascular thrombi induced by VSV[123]. As well as targeting neoplastic cells recent work has revealed that an engineered vaccinia virus is able to selectively replicate in tumour associated vascular endothelial cells culminating in substantial tumour necrosis whilst avoiding toxicity to the normal vasculature and therefore deficits in

wound healing[124]. Subsequent to this work, one study revealed that they key pro-angiogenic factor, VEGF suppresses innate anti-viral responses thereby facilitating selective OV infection of tumour vasculature[125]. Infection of cancer-associated fibroblasts (CAFs) has also been documented and secretion of TGF-β by cancer cells was shown to render CAFs sensitive to infection; in turn the secretion of fibroblast growth factor 2 (FGF2) by CAFs enhanced OV infection of malignant cells by decreasing retinoic acid-inducible gene (RIG-I) expression[126]. Oncolytic viruses are able to target other key cell populations within the tumour microenvironment and therefore have multiple anti-neoplastic functions.

In sum, it can be stated that the interaction between virus and cancer cell is complex and that various forms of cell death have been reported across the range of viruses and tumour types that have been tested (Fig 2). In many cases the form of cell death is characterised by cellular stresses, including endoplasmic reticulum (ER) stress that leads to immunogenic forms of cell death in the presence of viral products leading to robust engagement with the immune system.

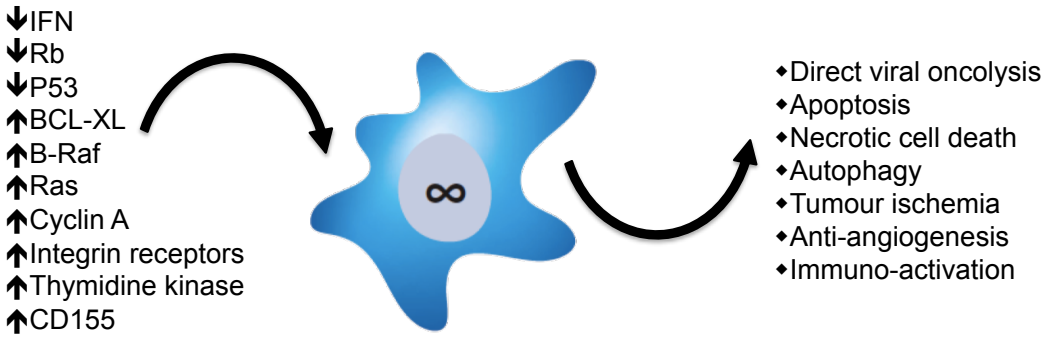


Figure 2. Factors facilitating viral oncotropism and the consequences for the cancer cell, tumour microenvironment and host organism following viral infection. Adapted from "Evolution of oncolytic viruses: novel strategies for cancer treatment"[54]

1.3.4. Induction of an immune response following OV therapy

Stimulation of an anti-tumour immune response as a non-direct means of achieving tumour cell cytopathia is crucial for OV anti-neoplastic efficacy. As OVs are able to invoke immune mediated tumour killing they can be considered a form of immunotherapy[55,127]. OV infection is capable of inciting a favourable immune response against tumours however, immune targeting of the viral vectors also occurs which may limit therapeutic potency.

Infection with OVs act as a source of alarm signals to the immune system. The presence of viral proteins and nucleic acid can be recognised as pathogen-associated molecular patterns (PAMPs) leading to stimulation of the innate

immune system as demonstrated following vaccinia administration to mice[128]. Damage-associated molecular patterns (DAMPs) generated following virus induced cell death in the tumour microenvironment are also implicated in propagating an immune response. Infection with adenovirus has led to release of the danger signal uric acid[129], infections with other OVs leads to the generation of a multitude of other danger signalling molecules including HMGB1, HSP27, HSP 70 and Gp96[95,130,131]. In an attempt to increase the activation of the local innate immune response Rommelfanger et al. injected lipopolysaccharide (LPS), a Toll-like receptor (TLR)-4 agonist intra-tumourally whilst also administering a VSV to melanoma bearing mice. Although this was successful in improving tumour response it resulted in significant systemic toxicity[132]. The release of danger signals is required for activation of APCs[133]. Oncolytic viruses are therefore able to provide signals generating co-stimulation from APCs required for an adaptive immune response and OVs share many similarities with the induction of immunogenic cell death (ICD) secondary to certain chemotherapeutics[55].

Viral oncolysis also liberates TAAs within the TME and an infiltrate of macrophages and natural killer cells follows shortly after tumour OV infection[55,134,135]. The presence of such inflammatory cells in the company of cellular debris and TAAs derived from lysis may act to enhance the phagocytic activity of APCs in the vicinity of the neoplasm[136]. Alterations to the cytokine profile within the microenvironment have also been documented. Errington and

coworkers demonstrated an increase in the pro-inflammatory cytokines IL-6, IL-8, RANTES (regulation on activation, normal T cell expressed and secreted), MIP (macrophage inflammatory protein) 1α and MIP 1β whilst simultaneously noting a decrease in anti-inflammatory IL-10 in reovirus infected xenografted melanoma cells[137]. Alteration of cytokine production has been observed in other studies with increases in IFN-β, GM-CSF, TNF-α as well as other chemokines such as IP-10 and eotaxin recorded[138,139]. It follows that infection of tumours with OVs favours a pro-inflammatory (hot) over an immunosuppressive (cold) TME.

Presentation of TAAs by APCs allows recruitment of the adaptive immune system to further combat the tumour by activating a tumour-specific T cell response. *In vitro* reovirus infection of human melanoma cells has been shown to be immunogenic, recruiting DCs in a dose dependent manner and inducing an expansion of TAA specific CD8+ T cells against the melanoma antigen MART-1 when these cells were co-cultured[140]. Recently responses against neoepitopes have also been demonstrated in a mouse model of lung cancer treated with an oncolytic adenovirus, when the OV was combined with anti-PD1 therapy the breadth of T cell responses against neoepitopes increased[141]. VSV infection in a mouse melanoma model has also demonstrated the importance of tumour specific T cell responses, achieved in this study by encoding TAAs within the virus as well as combining the therapy with adoptive cell transfer treatment resulting in improved efficacy[142]. Furthermore it has been proven that immune

mediated destruction of murine melanoma metastases by reovirus occurs without significant viral oncolysis of tumour cells supporting the role of OVs as direct immunomodulators[143]. The ability of OVs to specifically infect tumour cells and lead to direct oncolysis is complemented by their role as immunotherapeutics making them an attractive weapon against multiple types of cancer.

1.3.5. Enhancing the anti-neoplastic immune response

Not only is an immune response vital with regards to OV anti-neoplastic activity at the initial treatment of tumours, a body of preclinical evidence also highlights the importance of such immunity to prevent disease recurrence. In a murine B16 melanoma model, treatment with reovirus is able to override immune evasion of the tumour and furthermore the immunity derived is protective against future tumour challenge[139]. A similar protective effect against repeat tumour challenge was demonstrated in a mouse mammary adenocarcinoma model treated with a second generation ICP0-null HSV[144]. The same effect with another modified type I HSV was also observed by Liu and co-workers[145]. Somewhat counter-intuitively, oncolytic viruses actually produce fewer durable cures in animal models where the immune system is impaired than when the immune system is intact indicating that the benefit of anti-tumoural immunity can outweigh the negative impact of anti-viral responses[142,146]. It therefore follows that by enhancing the immune response against tumours, increases in therapeutic efficacy and remission

durability are to be anticipated. A number of novel techniques have been developed to capitalise on this theory.

Breaking immune tolerance is crucial for anti-neoplastic immunotherapy but a fine balance must be struck for OV use as to avoid efficacy limiting anti-viral responses[127]. One strategy to overcome this hurdle is to enhance the production of danger signals. By engineering an adenovirus to express immune-stimulatory Cp-G islands, Cerullo and colleagues were able to enhance TLR-9 stimulation translating to improved responses in murine models of melanoma and pulmonary carcinoma[147]. These responses were abrogated when natural killer (NK) cell depleted animals were used and also when TLR-9 was blocked systemically[147]. Improved outcomes in tumour bearing mice were also documented when CpG motifs were incorporated alongside TRAIL in another adenovirus[148] and similarly when expressed by an armed parvovirus[149]. OVs containing transgenes expressing danger signals further enhance the engagement of the innate immune system.

Combining OVs with pro-inflammatory cytokines/ chemokines to further recruit APCs and lymphocytes (NK, T and B cells) is another methodology to enhance immunogenicity. The following paragraph highlights the arming of OVs with the ability to express cytokines, however the roles of chemokines and cytokines in cancer therapy has been reviewed in-depth elsewhere[150,151]. There is ongoing

interest in this area of OV therapy due to the potential of delivering such therapy without encountering toxicities that are seen with high doses of systemically administered cytokine therapy[152]. Early expression of the cytokine IL-2 and the chemokine CCL5 by a double deleted vaccinia engineered to express these molecules inhibited its viral oncolysis and sub-optimally enhanced immune activation as demonstrated by Chen and colleagues[153]. By incorporating a small externally controllable domain to temporally regulate cytokine transgene expression in this OV this group were able to enhance therapeutic activity whilst significantly decreasing toxicity in tumour bearing mice[153]. GM-CSF has been included in multiple OVs to improve APC activation via induction of precursor myeloid cell proliferation and differentiation, as well as stimulating and recruiting dendritic cells. To date GM-CSF has been engineered into HSVs[154–158], VVs[159–163], Ad[164–166], NDV[167,168], VSV[169] and MV[170]. Flt3 (Flt3L) ligand is another cytokine that is capable of exerting anti-neoplastic activity and this ligand is thought to activate both dendritic cells as well as NK cells[171,172]. Incorporation of Flt3L transgenes into Ad and HSV has been used to treat tumour-bearing mice in order to enhance immune mediated tumour destruction[173–175]. The chemokines CCL3 and CCL5 have important roles in the migration of dendritic cells[176], both of these molecules have been expressed in engineered OVs [174,177–179]. Tumour cells infected by OVs expressing cytokines allow precision delivery of these products to the tumour-bed.

Another tactic employed to enhance the anti-neoplastic adaptive immune response is to manufacture viruses expressing cytokines that will more directly stimulate anti-neoplastic lymphocytes. IL-2, IL-12, IFN-α and TNF-α have all been used successfully to treat human cancer[180]. Both NDV and HSV have been manipulated to express IL-2 in order to induce proliferative and cytolytic T cell activity alongside viral oncolysis[181,182]. Another cytokine, IL-12, which stimulates T-helper cells, cytotoxic T lymphocytes and NK cells[183] is expressed by a variety of different transgenic OVs[184–188]. As well as IL-12's immune system potentiation it also exerts an IFN-γ mediated anti-angiogenic effect in an HSV expressing murine IL-12[189]. Zhang and colleagues found that angiogenesis could be further suppressed by manufacturing an HSV co-expressing IL-12 and angiostatin[190]. Insertion of IFN-α and TNF-α transgenes into OVs to instigate neoplastic lymphoid infiltrate has also been achieved[191–193]. In 2012 Stephenson and colleagues inserted an IL-15 transgene into VSV leading to high expression of this cytokine and enhancement of anti-tumoural immunity in a murine model of colonic carcinoma[194]. Local expression of these cytokines via OVs increases anti-cancer lymphoid populations within the tumour microenvironment.

Other techniques to amplify the immune response include insertion of transgenes encoding for co-stimulatory molecules into OVs as well as using combination inserts of different cytokines. Co-stimulatory inserts include 4-1 BBL[195,196], CD40L[197,198] and CD80[199]. Combining pro-inflammatory cytokine expression with

co-stimulatory molecules in single OVs with the aim of improving anti-neoplastic response has been achieved with combinations of IL-12 and 4-1 BBL[196], GM-CSF and CD80[200] as well as IL-12 and B7-1(CD80)[201]. Combinations of inserts including B7-1/ Fc fragment of IgG1 fusion with IL-18[202] and a triple insert IL-12, IL-18 and B7-1/ Fc fragment of IgG1[203]. The last two examples employed B7-1/ IgG1 fusion protein designed to allow tumour cell secretion of this soluble factor to boost immune response beyond that seen with surface expression of B7-1[199]. Thus a variety of strategies have been employed in animal models to enhance the cancer immunotherapeutic potency of many OVs.

1.3.6. Targeting tumour antigens

In the previous paragraphs different mechanisms to enhance the immune response generated by OVs have been discussed. It therefore follows that for OVs to be efficacious anticancer therapeutics this response must be directed against tumour antigens as opposed to the virus itself. Defining a tumour antigen for targeting is dependent on 3 analytical steps[204]. Firstly the antigen must be expressed in the patient's tumour, as neoplasms become more malignant antigens may be lost meaning T cells activated against a TAA will be ineffective. Secondly in order to avoid toxicity against non-malignant tissue, TAAs must be tumour specific. Finally the selected antigen must be immunogenic facilitating immune mediated destruction. Provoking a response against TAAs is a key element for the success of many cancer immunotherapeutics.

Some OVs have been designed to express TAAs and have been successful in recruiting tumour specific immunity translating to clinically relevant tumour remissions. Engineering TAA expressing OVs is a technique that has been adopted to focus immunogenicity against the tumour. Initial experimental work used surrogate (foreign) tumour antigens to validate this approach. One such example involves the incorporation of ovalbumin into B16 melanoma cells and engraftment of these cells into a mouse melanoma model prior to successful anticancer treatment with a VSV expressing the ovalbumin gene[142]. Other examples of using such surrogate targets to successfully induce an immune response have been reported[205,206] and whilst useful as proof of principle direct clinical application is not readily achievable. Bridle and colleagues demonstrated efficacy of OV therapy expressing a natural tumour antigen making use of dopachrome tautomerase (DCT) in malignant melanoma[207]. Another group used the melanoma TAA, gp100 and incorporated this into a VSV platform to treat a mouse melanoma model[208]. Results from the latter study found that intra-tumoural injection of this OV alone was insufficient to stimulate an immune response, however when combined with gp100-specific pmel T cells significantly more mice were cured than when either treatment was used as a sole agent. One possible reason for the success of the DCT targeted therapy was the application of a heterologous prime: boost regime with an Ad-DCT prime followed by VSV-DCT boost causing marked anti-tumoural immunity without eliciting a significant

response against VSV[207]. Using distinct recombinant viral vectors for priming and boosting is thought to favour an anti-neoplastic as opposed to anti-viral response[209]. Various other heterologous viral prime: boost protocols have been reported[210–212]. A novel approach to boost CD8+ memory T cells was recently reported; this study employed viral-vector loaded B-cells with the ability to home in on the follicular regions of secondary lymphoid organs thus bypassing effector T cells which can serve to hinder vaccination boosts[213]. Another mechanism of OV anti-neoplastic activity is their ability to transduce DCs enhancing their profile as immune priming vectors as demonstrated by an M protein mutated VSV[214]. This work revealed that tumour induced immunosuppression was abrogated by using an OV vector and interestingly also revealed that NK cells as opposed to cytotoxic T cells had a crucial effector role[214]. By engineering OVs to express specific TAAs one is able to combine viral oncolysis with a highly potent vaccine platform.

Numerous other tumour antigens that have the potential to be targeted via immunotherapy and vaccination exist and these have been reviewed elsewhere[215]. As well as continuing to identify suitable TAAs to be expressed by OVs work will continue to screen for suitable viruses to act as platforms for this approach. Pulido and colleagues published work in which VSVs expressing cDNA libraries were screened in the treatment of mice with melanoma identifying 3 separate VSV clones which, when used in combination, were able to cure these

tumour bearing mice[216]. Further preclinical investigations and clinical trials are required to assess the promise transgenic OVs have in this setting.

1.3.7. Oncolytic viruses as part of a multi-modal treatment

The increased understanding of the molecular alterations contributing to carcinogenesis has led to the development of therapies targeted against the hallmarks of malignancy, but it is anticipated that the most remarkable future clinical triumphs will be the results of combination therapeutics targeting different tumour defining facets[6]. As with other therapeutic classes there is much interest in establishing where OVs can be most effectively integrated in future medicine. Discussing all the reported combinations of OVs with other therapeutics is beyond the scope of this thesis but some recent exciting examples follow.

Combining OVs with adoptive transfer of immune cells to increase anti-tumoural immunity is one such innovative approach. As mentioned previously combining TAA specific T cells with a TAA expressing VSV in a murine melanoma model elicited a greater immune response compared with either agent given independently[142]. Qiao and colleagues demonstrated that by loading a VSV onto tumour specific T cells in tumour bearing mice the therapeutic efficiency of both modalities was increased, which was in part due to exploiting the recirculation of T cells as a method of delivering an OV[217]. The combination of tumour specific T cells and OVs have also been successful in other studies of melanoma and

glioma mouse models[218,219]. John and colleagues used a different technique to enhance the immune response in work published in 2012; by using an agonist antibody for the co-stimulatory molecule 4-1 BB they were able to enhance the anti-tumour response to an engineered vaccinia virus[220]. In a murine melanoma model combining NDV with CTLA-4 blockade led to rejection of secondary established tumours that were distant to the site of intra-tumoural NDV administration and marked infiltration of these tumours with CD4+ and CD8+ effector T cells[221]. Engeland and colleagues undertook a different approach and made MV encoding antibodies against CTLA-4 and PD-L1, and again in a murine melanoma model survival benefits were observed (a similar improvement was seen when MV was given in combination with systemically administered checkpoint inhibitors)[222]. Combining OVs with adoptive cellular therapy and checkpoint inhibitors is an extremely exciting area of research and data from ongoing studies and trials are keenly awaited.

The use of OVs with traditional cytotoxic chemotherapeutic agents has also been investigated. Concurrent administration of cyclophosphamide (CTX) facilitated dose reduction of an HSV whilst maintaining efficacy in a mouse model of human glioma[223]. In a lung carcinoma model CTX was found to have a synergistic relationship with HSV demonstrated by enhanced anti-tumour effects[224]. One explanation of the cumulative benefit of these treatments is a decreased innate immune response against the virus thus increasing intra-tumoural viral

replication[225]. More recently combination of another alkylating agent, temozolomide with an HSV extended survival times in a preclinical mouse glioma model[226]. Analysis of glioblastoma stem cells published in the same study revealed a synergistic DNA damaging effect of the two agents mediated by activated ataxia telangiectasia mutated (ATM) protein whilst sparing normal neurons[226]. Synergy has been demonstrated between OVs and other chemotherapeutic agents including the microtubule disrupting taxanes (docetaxel and paclitaxel), cisplatin, doxorubicin as well as the vinca alkaloids[227–232]. OVs can also be armed with enzymes to convert non-toxic pro-drugs into active cytotoxic agents and when combined with tumour specific targeting lead to localised delivery of chemotherapy to a tumour as well as oncolytic/ immune mediated killing. One such example is the engineered measles virus strain MV-FCU1-αHMWMAA that expresses the convertases uracil phosphoribosyltransferase and cytosine deaminase thus allowing the pro-drug 5-FC to be given systemically with subsequent activation into 5-FU within the tumour thereby increasing anti-tumour effect[233]. The use of established cytotoxic agents in concert with OVs offers an opportunity to increase the efficacy of both classes of agents.

Of late the importance of immune activation in some novel forms of anti-cancer therapy is being realised. Histone deacetylase inhibitors (HDACIs) are a relatively new form of anti-neoplastic therapy with wide ranging clinical potential[234].

Combining the HDACI valproic acid with an equine herpes virus was found to enhance oncolysis of human glioma cells[235]. HDACIs are able to modulate the innate and adaptive immune response in OV treated tumour-bearing mice by suppressing the vector targeted response and increasing the anti-tumour response thus enhancing therapeutic efficacy[236,237]. Administration of the mTOR inhibitor, rapamycin alongside OVs also has potential value. Rapamycin was able to enhance HSV activity in various tumour cell lines usually considered to be HSV resistant[238]. Bovine herpes virus-1 when used alongside the demethylating agent, 5-azacytidine prevented secondary lesions as well as increasing immune cell infiltrate in a cotton rat model of mammary cancer[239]. Combining the tyrosine kinase inhibitor, sunitinib with a VSV also seemed to have a mutually beneficial effect translating to tumour remission in mice with data indicating that suppression of the innate immune system by sunitinib was the reason behind the synergism[240]. The concurrent use of these targeted medicines enhances the therapeutic profile of OVs.

Radiation therapy in concert with OV treatment also has translational potential for the oncology clinic. This treatment combination is thought to be mutually beneficial for several reasons. Radiation therapy may enhance viral uptake as well as increasing viral replication and gene expression[241]. Another intriguing way of exploiting this relationship is by engineering viruses containing radiation inducible viral promoters as illustrated by a mutated adenovirus containing the

radio-inducible Egr-1 gene in a mouse tumour model[242]. Buckel and colleagues recently published data revealing that increased oncolysis alongside radio-sensitisation of tumour endothelium are effectively achieved when combining external beam radiation therapy with a VSV expressing a single chain antibody against the angiogenic growth factor VEGF[243]. A final example of a combined approach heralded from work by Muthana et al., decreased tumour regrowth was observed in a prostatic mouse carcinoma model treated with macrophages armed with prostate specific adenoviruses after conventional treatment with either docetaxel or external irradiation[244]. More in depth discussions of the anti-neoplastic mechanisms behind combined radiation therapy and OVs as well as other examples of these modalities being combined has been reviewed elsewhere and research is being actively pursued within this exciting field[241,245].

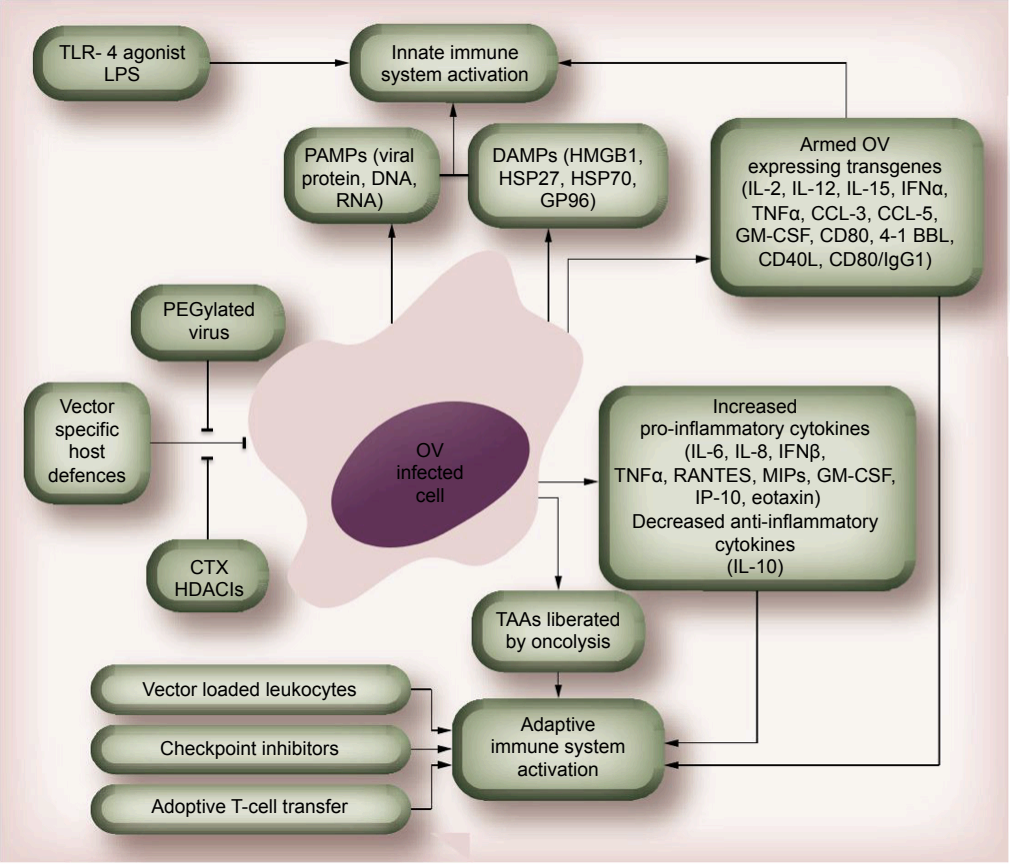


Figure 3. Schematic summarising combined natural and engineered oncolytic viral mechanisms activating the innate and adaptive immune response alongside strategies to reduce host reaction against the viral vector. CTX- cyclophosphamide, HDACIs- histone deacetylase inhibitors. Adapted from "Evolution of oncolytic viruses: novel strategies for cancer treatment"[54]

1.3.8. OVs in the clinic

A new era dawned for OVs at the beginning of this millennium with the first clinical trials involving such viruses. A summary of selected complete clinical trials is found in Table 1 and OVs currently undergoing assessment by clinical trial is found in Table 2. Phase I results for two HSVs treating recurrent malignant gliomas found no significant toxicity whilst revealing preliminary signs of efficacy

in some of the treated patients[246–248]. HSV has also been evaluated in the setting of pancreatic cancer[249]. The Onyx-015 E1B deleted adenovirus was another one of the first OVs to be used in the clinic with a phase I trial in head a neck cancer showing a good safety profile as dose limiting toxicity was not reached at the upper dose range[250]. However, as a sole agent against tumours the ONXY-015 adenovirus lacked efficacy[251]. CG0070 is another adenovirus that has a tolerable safety profile and evidence of anti-cancer activity when treating bladder cancer patients[252]. In advanced gynaecologic malignancies, Ad5-Δ24-RGD also had a favourable safety profile but no overt anti-tumour activity was recorded[253]. Phase I clinical trials have also been conducted for measles virus and NDV. The use of NDV for the treatment of advanced solid tumours resulted in various side effects with the most common being flu like symptoms however, no cumulative toxicities were recorded[254]. No dose limiting toxicities were observed when the Edmonston vaccine strain of measles virus was used in women suffering platinum refractory ovarian carcinoma[255,256]. A non-pathogenic laboratory strain of VSV for the purpose of treating hepatocellular carcinoma (HCC) has also been manufactured in large enough scale and approved for phase I trials in the U.S.A.[257]. The engineered transgenic vaccinia virus, jx-594 was found to be generally well tolerated in a phase I study used against multiple advanced treatment refractory tumours[159]. A phase Ib trial of jx-594 in colorectal cancer patients has also been completed[258]. Reovirus has been evaluated for safety in phase I in patients with

various solid tumours and multiple myeloma[259,260]. Phase I trials are still ongoing for numerous other OVs and will provide important safety data[261,262].

Table 1. Summary of a selection of completed OV clinical trials. Adapted from "Evolution of oncolytic viruses: novel strategies for cancer treatment"[54]

Name of agent	Virus	Indications	Phase	Outcome and comments
Onyx-015	E1B-deleted adenovirus	Head and neck cancer	I	Dose limiting toxicity not reached, mild flu like symptoms observed. No objective responses recorded.
CG0070	Conditionally replicating adenovirus expressing GM-CSF	Superficial bladder cancer	I	Maximal tolerated dose not reached. 48.6% complete response rate.
Ad5-Δ24-RGD	Conditionally replicating adenovirus	Recurrent gynaecologic malignancies	I	Only low-grade toxicities noted. No partial or complete responses seen.
PV701	Naturally attenuated strain of Newcastle disease virus	Advanced solid tumours	I	Primarily mild flu like symptoms recorded. 100 fold intensification from starting dose achieved with objective responses recorded for higher doses.
MV-CEA	Edmonston strain of measles virus engineered to express CEA as a marker antigen	Ovarian carcinoma	I	Dose limiting toxicity not reached. Dose dependent disease stabilisation in 14 of 21 patients.
MV-NIS	Edmonston strain of measles virus engineered to express sodium iodide symporter	Ovarian carcinoma	I	Dose limiting toxicity not reached. Median overall survival of 26.5 months in 16 treated patients.
jx-594	Thymidine kinase deleted vaccinia expressing GM-CSF	Advanced solid tumours	I	Dose limiting toxicity not reached, mild flu like symptoms were the most common adverse effects reported. 87% of tumour biopsies positive for jx-594.

Reolysin	Reovirus	Various advanced solid malignancies	I	Treatment well tolerated and local target tumour response activity in 7/19 patients.
Reolysin	Reovirus	Multiple myeloma	I	No dose limiting toxicities but little evidence of significant oncolytic activity.
G207	Engineered conditionally replicative herpes simplex virus 1	Glioma	I	No adverse events that could be unequivocally related to HSV. Some cases had radiologic and histologic signs of tumour response.
G207	Engineered conditionally replicative herpes simplex virus 1	Glioma	I	G207 given in combination with radiation therapy. Stable disease or partial response seen in 6 of 9 patients
HSV1716	Attenuated conditionally replicative herpes simplex virus 1	Glioma	I	No evidence of encephalitis or other adverse events. 4 of 9 patients alive 14-24 months after OV administration.
HF10	Engineered conditionally replicative herpes simplex virus 1	Pancreatic cancer	I	No adverse side effects. 1/6 patients had partial tumour response
jx-594	Thymidine kinase deleted vaccinia expressing GM-CSF	Hepatocellular carcinoma	II	Randomised dose finding study, significantly longer survival times with higher dose (14.1 vs. 6.7 months).
Reolysin	Reovirus	Malignant melanoma	II	No objective responses but treatment well tolerated. Trials in combination with cytotoxic therapies are ongoing.
Reolysin	Reovirus	Pancreatic adenocarcinoma	II	Treatment well tolerated. No survival benefit of reovirus in combination with chemotherapy compared to chemotherapy alone
T-Vec (originally called OncoVEX-GMCSF)	Herpes simplex virus expressing GM-CSF	Malignant melanoma	II	Overall response rate of 26%. 1 and 2 year survivals of 58% and 52% respectively.

T-vec (originally called OncoVEX-GMCSF)	Herpes simplex virus expressing GM-CSF	Malignant melanoma	III	Significant improvement of durable response rate compared to GM-CSF alone (16% vs. 2%). Trend towards increased survival- data collection ongoing.

Oncolytic viruses are also undergoing assessment of clinical efficacy at more advanced stages[261,262]. Phase II trials of a naturally occurring reovirus against advanced malignant melanoma revealed viral proliferation within neoplastic tissues but in spite of this no objective responses were found, trials of this virus in combination with traditional cytotoxic therapies are ongoing[263]. In pancreatic cancer the addition of reovirus to chemotherapy did not alter progression free survival[264]. In a randomised phase II dose finding study of jx-594 in HCC objective tumour responses to the virus were documented and median survival times correlated with dose rates[160]. A 26% overall response rate was seen in phase II trials of a GM-CSF encoding second-generation oncolytic herpes virus, T-Vec, with durable remissions being recorded[154]. The effectiveness of the GM-CSF herpes virus combined with good therapeutic safety has resulted in the phase III OPTIM trial being conducted for this particular virus[156]. Preliminary results found a significant improvement of durable response rates when compared to GM-CSF given alone subcutaneously[30]. Long term follow up of this trial (median time >4 years) supported a survival benefit for both treatment naïve and advanced stage patients[265]. In 2015 T-Vec became the first FDA approved OV indicated for the treatment of melanoma patients[262]. These are the initial

results from advanced stage trials supporting the use of OVs in the clinic; further results will be published when clinical data fully matures. The presence of OVs in multiple clinical trials throughout the world is a testament to their versatility and promise as novel anti-cancer therapeutics.

Table 2. Oncolytic viruses currently undergoing clinical evaluation in cancer patients. Adapted from "Evolution of oncolytic viruses: novel strategies for cancer treatment"[54] and sourced from www.clinicaltrials.gov

Name of agent	Virus	Combined with	Indications	Phase	Status
CG0070	Conditionally replicating adenovirus expressing GM-CSF	Sole agent	Urinary bladder carcinomas	III	Not yet recruiting
MV-NIS	Recombinant measles virus expressing thyroidal sodium iodide importer	Sole agent	Breast cancer	I	Recruiting
MV-NIS	Recombinant measles virus expressing thyroidal sodium iodide importer	Sole agent or in combination with cyclophosphamide	Multiple myeloma	I/II	Recruiting
ParvOryx	Wild type rodent parvovirus H-1PV	Sole agent	Metastatic pancreatic carcinoma	I/II	Recruiting
GL-ONC1	Attenuated vaccinia	Sole agent	Recurrent gynaecologic malignancies	I	Recruiting
GL-ONC1	Attenuated vaccinia	Sole agent or in combination with eculizumab	Solid organ cancers	I	Recruiting
jx-594	Thymidine kinase deleted vaccinia expressing GM-CSF	Intra-tumoural injection of OV in combination with ipilimumab	Advanced solid tumours	I	Not yet recruiting

jx-594	Thymidine kinase deleted vaccinia expressing GM-CSF	In combination with metronomic cyclophosphamide	Solid tumours, soft tissue sarcoma and breast cancer	I/II	Recruiting
jx-594	Thymidine kinase deleted vaccinia expressing GM-CSF	In combination with sorafenib	Advanced hepatocellular carcinoma	III	Active but not recruiting
Reolysin	Wild type reovirus	Combined with multi-agent chemotherapy and bevacizumab	KRAS mutant metastatic colorectal cancer	I	Recruiting
Reolysin	Wild type reovirus	Combined with pembrolizumab and various chemotherapeutics	Pancreatic adenocarcinoma	Ib	Recruiting
HSV1716	Attenuated conditionally replicative herpes simplex virus 1	Sole agent	Non- central nervous system solid tumours	I	Recruiting
HSV1716	Attenuated conditionally replicative herpes simplex virus 1	Sole agent	Pleural mesothelioma	I/IIa	Active but not recruiting
CAVATAK	Wild type coxsackievirus A21	Intra-tumoural injection of OV in combination with ipilimumab	Malignant melanoma	I	Recruiting
CAVATAK	Wild type coxsackievirus A21	Intra-tumoural injection of OV in combination with pembrolizumab	Malignant melanoma	I	Recruiting
SVV-001	Wild type Seneca valley virus	Sole agent	Small cell lung cancer	II	Active but not recruiting
VSV-IFNβ-NIS	VSV expressing interferon β and thyroidal sodium iodide importer	Sole agent	Malignant solid tumours	I	Not yet recruiting
PVSRIPO	Poliovirus: rhinovirus chimera with tumour specific conditional replication	Sole agent	Glioblastoma	I	Recruiting

MG1-MAGEA3	Attenuated Maraba virus expressing MAGEA3 given subsequently to adenovirus vaccine expressing MAGEA3	Sole agent	Metastatic solid tumours	Phase I/II	Recruiting
MG1-MAGEA3	Attenuated Maraba virus expressing MAGEA3 given subsequently to adenovirus vaccine expressing MAGEA3	In combination with pembrolizumab	Non-small cell lung cancer	Phase I/II	Recruiting

1.3.9. Challenges facing viral oncolytics

Multiple OVs are currently in clinical trials and novel applications of these viruses are continuing to be developed in the lab but several hurdles still remain prior to the consolidation of OVs as a fully established component of the anti-cancer arsenal. Ongoing phase I clinical trials will continue to qualify the safety of administering infectious agents as part of a therapeutic regimen. Clinical data collected so far support the lack of significant toxicity when administering OVs systemically[261]. Further data from efficacy trials are eagerly awaited.

Certain physical and physiologic questions regarding OV administration still need answering. The method in which OVs are delivered can significantly impact upon their activity and is subject to discussion[73,266]. Intra-tumoural injection facilitates high viral delivery to the primary neoplasm but potentially neglects the risk of

disseminated disease and efficacy may be indirectly proportional to tumour size. As such intravenous (IV) administration of these therapeutics may broaden their delivery to target tissues however, doses administered may need to be increased[159]. Complement activation following OV dosing acts as an obstacle to repeat administration; however depletion of complement increases viral delivery to tumours in the pre-clinical setting[267]. Neutralising antibodies can also act as barrier to intravenous therapy however, a recent protocol employing PEGylation of VSV was found to improve viral circulating half-life by inhibiting serum neutralisation[268]. By deglycosylating oncolytic vaccinia viral particles Rojas and colleagues were able to reduce the production of anti-viral neutralising antibodies thereby enhancing OV delivery[269]. Results from ongoing clinical trials will help inform scientists and clinicians as to whether such engineering is required to enhance delivery and if systemic or local delivery is favourable, however, due to the diversity of the therapeutic profile of OVs the route of delivery is likely to vary between platforms.

1.4. MG1 Maraba

1.4.1. MG1 Maraba as a novel oncolytic virus

Maraba virus was initially isolated from Brazilian sandflies and belongs to the genus of vesiculovirus and family of rhabdoviruses, alongside VSV[270,271]. Rhabdoviruses are favourable oncolytic platforms for multiple reasons including lack of pre-existing humoural immunity; broad tumour cell tropism; lack of

pathogenicity against humans (excluding lyssaviruses); the ability to reverse engineer the RNA genome and genetically manipulate these agents with relative ease; rhabdoviruses can be manufactured readily and to high titres and finally as their life cycle does not occur via a DNA intermediate and is entirely extra-nuclear there is no risk of insertional mutagenesis to the host[75,272]. The great potential of Maraba virus as an oncolytic was recognised following the screening of numerous wild type rhabdoviruses for anti-neoplastic activity against a variety of cancer cell lines[75]. Maraba virus was found to be the most potent tumour cell killer of all the viruses tested and was therefore selected for further therapeutic development[75].

An engineered double mutant form of Maraba resulted in enhanced virulence in neoplastic cells whilst decreasing the ability to infect non-transformed cell lines thus increasing Maraba's therapeutic index in the preclinical setting[75]. Oncolytic viruses are susceptible to type I IFN with vesiculoviruses, such as VSV, being highly sensitive to such anti-viral responses[270,272]. The observation that VSV was able to successfully kill a variety of tumour cell lines even in the presence of type I IFN was pivotal in the development of OVs to target many tumours that are defective in IFN signalling[90]. Neoplastic cells with defects in type I IFN signalling therefore act as a selective and hospitable environment for OV replication[270]. The matrix (M) protein from vesiculoviruses is multifunctional and has a key role in blocking the export of mRNA from the nucleus as well as down-regulating

transcriptional activity; this is a crucial mechanism by which VSV attempts to prevent host cell production of type I IFN[272]. Introducing the L123W mutation to Maraba's M protein allowed Brun and colleagues to improve the safety profile of Maraba virus via induction of IFN-β production following viral infection of cells, thus further polarising the ability of Maraba to selectively infect transformed but not normal, diploid cells[75]. A separate mutation (Q242R) was introduced to the glycoprotein (G) that also decreased cytolytic activity in normal cells; the definitive mechanism by which this occurs is not known however, this appears to be IFN-independent[75]. These specific mutations culminated in the creation of MG1 Maraba as a novel OV designed for systemic IV administration. In murine toxicity models the maximal tolerable dose of MG1 was 100 times greater than the wild type and robust anti-tumour responses were demonstrated in both syngeneic and xenogeneic models[75]. Both the excellent safety and potency profiles of MG1 Maraba has resulted in the generation of a substantial body of work aimed at transforming the promise of this preclinical agent into an approved clinical anti-cancer therapeutic.

Since the establishment of MG1 Maraba as an OV research has focussed on interrogating and enhancing this agent's anti-cancer activity. Zhang and co-workers established that both NK cells and conventional dendritic cells (cDCs) are required for MG1's anti-tumour efficacy activity; direct MG1 infection of cDCs facilitates their maturation and in turn they activate NK cells, interestingly this

response does not require viral replication[273]. MG1 Maraba compared favourably to Myxoma virus and a double deleted vaccinia in an *in vitro* model of ovarian carcinoma metastases, after a delayed viral entry to tumour spheroids Maraba was able to spread rapidly throughout these cellular structures leading to significant cytotoxicity[274]. Decreased spheroid surface expression of the low-density lipoprotein receptor was posited as a reason for impaired MG1 viral entry[274]. In tumours that have intact IFN signalling MG1 Maraba virus engineered to express a soluble IFN binding decoy receptor ($MG1^{IDE}$) was able to confer anti-tumour activity in a model lacking sensitivity to MG1 oncolysis[275]. $MG1^{IDE}$ was able exploit the increased rate at which rapidly dividing cancer cells are able to produce viral progeny in comparison to more quiescent healthy cells, which remained protected from infection, thereby maintaining selective infection of neoplastic cells in the absence of IFN defects[275]. Building on the observation that CAF secretion of FGF2 rendered tumour cells sensitive to OV infection MG1-FGF2 was constructed and found to be more efficacious than the original MG1 Maraba strain in a murine pancreatic cancer model[126]. The likelihood of clinical success for MG1 Maraba is bolstered by more preclinical data arising around the use of this platform.

Combinatorial approaches have also been documented to enhance the anti-tumour activity of MG1 Maraba. By using genome-wide RNAi screens Mahoney and co-workers revealed the ER stress pathway as having an important role in

MG1 mediated cytotoxicity and when the ER stress response was inhibited in cancer cells, prior to MG1 infection, the cells were sensitised to caspase-2-dependent apoptosis following infection[276]. Pyrolle derivatives were able to enhance the oncolytic activity of MG1 Maraba as they function to decrease anti-viral defences by inhibiting IFN-β production[277]. Oncolytic viruses including MG1 Maraba are able to induce bystander death of cancer cells when such cells are pre-treated with SMAC mimetic compounds (SMCs); SMCs sensitise cells to apoptotic cell death induced by the production of inflammatory cytokines from OV infected neighbouring cells[278]. Microtubule disrupting agents also enhanced oncolysis by MG1 Maraba decreasing type I IFN mRNA translation as well as protein expression and secretion[279]. When the cytotoxic agent paclitaxel was combined with MG1 Maraba in a preclinical mammary tumour model tumour growth was delayed and survival was prolonged[280]. Further research into how best to integrate MG1 Maraba with other established and novel therapeutics is ongoing.

1.4.2. MG1 Maraba as an oncolytic vaccine

As MG1 Maraba is amenable to engineering and has substantial capacity for transgene insertion a strategy of engineering this OV to express TAAs was devised. By engineering a replicate deficient Ad5 and MG1 Maraba to express the same TAA, namely DCT, Pol *et al.* were able to induce marked tumour-specific CD8+ T cell responses[281]. This strategy was able to cure mice bearing

B16F10 melanoma tumours in both the aggressive intracranial setting as well as an established intra-pulmonary metastatic model[281]. The mechanism by which intravenously administered Maraba MG1 encoding a selected TAA is able to induce such sizeable immune responses was recently elucidated. Rhabdoviral boosting of a primed immune response leads to direct infection of splenic follicular B cells and antigen is subsequently transferred in a non-MHCII dependent manner to local DCs, these local DCs are in an immunologically privileged site whereby they can rapidly expand CD8+ central memory T cells (TCM) in the absence of cytotoxic effector CD8+ T cells thus invoking massive CD8+ T cell responses[282]. To date two clinical trials utilising the Ad prime: MG1 Maraba boost are active as a result of the remarkable preclinical potency of this oncolytic vaccine platform. The first in man MG1 Maraba clinical trial based around Ad and MG1 Maraba encoding the TAA MAGE-A3 is underway for the treatment of advanced/ metastatic MAGE-A3 positive solid tumours and is a combined phase I/II trial (NCT02285816). A subsequent phase I/II trial using the same platform but in combination with pembrolizumab, for MAGE-A3 positive non-small cell lung cancer is also active (NCT02879760). Combining the powerful oncolytic properties of MG1 Maraba alongside the potency of the virus when used as a boosting vector offers a previously unexplored avenue for cancer immunotherapy and in this setting MG1 Maraba offers a genuinely unique and auspicious therapeutic strategy.

1.5. Aims of this thesis

Despite the recent and dramatic progress in the field of cancer therapeutics, particularly cancer immunotherapy, the clinical cure of advanced solid tumours is rarely achieved[283]. This thesis aims to provide pre-clinical bodies of evidence, upon which human clinical trials for the treatment of advanced epithelial malignancies using MG1 Maraba will be founded. Data presented here will focus on the customised design of MG1 Maraba to focus specific immune responses against relevant human antigens in combination with oncolysis for two types of malignancy that cause considerable global morbidity and mortality, these being HPV-associated cancer and carcinoma of the prostate. **We hypothesized that the application of custom designed MG1 Maraba oncolytic viral immunotherapy would be efficacious in preclinical murine models of epithelial cancer.** Three separate chapters will address the following specific areas:

- Customised viral immunotherapy for HPV-associated cancer (Chapter III): A custom made transgene based on specific HPV-associated TAAs is incorporated into the oncolytic vaccination strategy previously described by manufacturing the viral vectors Ad-E6E7 and MG1- E6E7 and appraised in a preclinical murine model of HPV-associated cancer.

- Combining MG1-E6E7 viral immunotherapy with peptide vaccination for the treatment of HPV-associated cancer (Chapter IV): Synthetic long peptide vaccination is combined with the previously described MG1-E6E7 to treat a murine model of HPV-associated cancer.

- Oncolytic viral immunotherapy for carcinoma of the prostate (Chapter V): Two sets of Ad and MG1 Maraba vectors are engineered to express two differing TAAs relevant to murine and human prostatic carcinoma and are interrogated for efficacy in a preclinical model of prostate cancer.

~CHAPTER II~

METHODS

Recombinant viruses

Codon optimised transgenes were specifically manufactured encoding the mutant attenuated E6E7, WT E6E7, STEAP and WT1 (GensScript, Piscataway, NJ) sequences. The E6E7 fusion protein is quadrivalent and based on E6 and E7 from HPV16 and 18. STEAP and WT1 are human sequences. Ad-BHG, Ad-E6E7, Ad-STEAP and Ad-WT1 are human serotype 5 replicate deficient (E1/E3 deleted) adenoviruses. Ad-BHG contains no transgene; Ad-E6E7 contains the transgene encoding the attenuated therapeutic E6E7 construct, Ad-STEAP and Ad-WT1 contain human STEAP and WT1 respectively. The GFP, E6E7, STEAP and WT1 transgenes were inserted between the G and L viral genes of the attenuated MG1 strain of Maraba virus to produce MG1-GFP, MG1-E6E7, MG1-STEAP and MG1-WT1 respectively.

Mutant attenuated E6E7 nucleotide sequence:

CATCAGAAGCGAACTGCTATGTTTCAGGACCCTCAGGAGCGGCCACGCAAA
CTGCCTCAGCTGTGCACCGAACTGCAGACAACTATCCACGACATCATTCTG
GAATGCGTGTACTGTAAGCAGCAGCTGCTGAGGAGAGAGGTCTATGACTTC
GCTTTTCGCGATCTGTGCATCGTGTACCGAGACGGAAACCCATATGCAGTC
GATAAGCTGAAGTTCTACAGCAAGATCTCCGAATACAGGCATTACTGTTACA
GCGTGTACGGGACCACACTGGAGCAGCAGTATAACAAGCCCCTGTGCGAC
CTGCTGATCAGAATTAATCAGAAGCCCCTGTGCCCTGAGGAAAAACAGAGG
CACCTGGATAAGAAACAGAGATTTCATAACATCCGAGGACGATGGACCGGG

CGGTGCATGTCCTGCTGTAGAAGCTCCCGGACTCGACGAGAGACCCAGCT

GGGCGGAGGAGGAGGAGCAGCTTACATGGCACGATTCGAGGACCCTACCC

GAAGGCCATATAAGCTGCCCGACCTGTGCACAGAACTGAATACTTCTCTGC

AGGACATCGAGATTACATGCGTGTACTGTAAAACCGTCCTGGAGCTGACAG

AAGTGTTCGAGTTTGCTTTCAAGGACCTGTTTGTGGTCTACCGGGATTCAAT

CCCTCACGCAGCCCATAAAATCGACTTCTACAGCAGGATCAGGGAACTGCG

CCACTACTCCGACAGCGTGTACGGGGATACACTGGAGAAGCTGACAAACAC

TGGCCTGTACAATCTGCTGATCCGACTGCGACAGAAGCCACTGAACCCAGC

CGAAAAACTGAGACACCTGAACGAGAAGAGACGGTTTCACAATATTGCAGG

CCATTATAGGGGACAGTGCCATAGTTGCTGTAATCGAGCCAGGCAGGAAAG

ACTGCAGCGCCGAAGGGAGACTCAAGTCGGCGGAGGAGGAGGAGCTGCAT

ACATGCACGGCGACACCCCCACACTGCATGAATATATGCTGGATCTGCAGC

CTGAGACTACCGACCTGTACCAGCTGAACGATTCTAGTGAGGAAGAGGACG

AAATCGACGGACCAGCAGGACAGGCAGAGCCTGACCGGGCCCACTATAAT

ATTGTGACATTCTGCTGTAAGTGCGATTCTACTCTGCGGCTGTGCGTGCAGA

GTACTCATGTCGACATCCGCACCCTGGAGGATCTGCTGATGGGGACTCTGG

GCATCGTCCCAATTTGTAGCCAGAAACCAGGCGGCGGCGGCGGAGCAGCT

TACATGCACGGACCCAAGGCTACCCTGCAGGACATCGTGCTGCATCTGGAA

CCTCAGAATGAGATTCCAGTCGACCTGCTGCAGCTGAGTGATTCAGAAGAG

GAAAACGACGAGATCGACGGCGTGAATCACCAGCATCTGCCTGCTAGACGG

GCAGAGCCACAGCGACACACAATGCTGTGCATGTGCTGTAAGTGTGAAGCC

AGGATCAAGCTGGTGGTCGAGTCAAGCGCCGACGATCTGCGCGCCTTCCA

GCAGCTGTTCCTGAATACTCTGTCATTTGTCCCTTGGTGTGCCTCCCAGCAG

WT E6E7 nucleotide sequence:

CATCAGAAGCGAACTGCTATGTTTCAGGACCCTCAGGAGCGGCCACGCAAA

CTGCCTCAGCTGTGCACCGAACTGCAGACAACTATCCACGACATCATTCTG

GAATGCGTGTACTGTAAGCAGCAGCTGCTGAGGAGAGAGGTCTATGACTTC

GCTTTTCGCGATCTGTGCATCGTGTACCGAGACGGAAACCCATATGCAGTCT

GCGATAAGTGTCTGAAGTTCTACAGCAAGATCTCCGAATACAGGCATTACTG

TTACAGCGTGTACGGGACCACACTGGAGCAGCAGTATAACAAGCCCCTGTG

CGACCTGCTGATCAGATGCATTAATTGTCAGAAGCCCCTGTGCCCTGAGGA

AAAACAGAGGCACCTGGATAAGAAACAGAGATTTCATAACATCCGAGGACG

ATGGACCGGGCGGTGCATGTCCTGCTGTAGAAGCTCCCGGACTCGACGAG

AGACCCAGCTGGGCGGAGGAGGAGGAGCAGCTTACATGGCACGATTCGAG

GACCCTACCCGAAGGCCATATAAGCTGCCCGACCTGTGCACAGAACTGAAT

ACTTCTCTGCAGGACATCGAGATTACATGCGTGTACTGTAAAACCGTCCTGG

AGCTGACAGAAGTGTTCGAGTTTGCTTTCAAGGACCTGTTTGTGGTCTACCG

GGATTCAATCCCTCACGCAGCCTGCCATAAATGTATCGACTTCTACAGCAGG

ATCAGGGAACTGCGCCACTACTCCGACAGCGTGTACGGGGATACACTGGA

GAAGCTGACAAACACTGGCCTGTACAATCTGCTGATCCGATGCCTGCGATG

TCAGAAGCCACTGAACCCAGCCGAAAAACTGAGACACCTGAACGAGAAGAG

ACGGTTTCACAATATTGCAGGCCATTATAGGGGACAGTGCCATAGTTGCTGT

AATCGAGCCAGGCAGGAAAGACTGCAGCGCCGAAGGGAGACTCAAGTCGG

CGGAGGAGGAGGAGCTGCATACATGCACGGCGACACCCCCACACTGCATG

AATATATGCTGGATCTGCAGCCTGAGACTACCGACCTGTACTGTTATGAACA

GCTGAACGATTCTAGTGAGGAAGAGGACGAAATCGACGGACCAGCAGGAC

AGGCAGAGCCTGACCGGGCCCACTATAATATTGTGACATTCTGCTGTAAGT

GCGATTCTACTCTGCGGCTGTGCGTGCAGAGTACTCATGTGGACATCCGCA

CCCTGGAGGATCTGCTGATGGGGACTCTGGGCATCGTCTGCCCAATTTGTA

GCCAGAAACCAGGCGGCGGCGGCGGAGCAGCTTACATGCACGGACCCAAG

GCTACCCTGCAGGACATCGTGCTGCATCTGGAACCTCAGAATGAGATTCCA

GTGGACCTGCTGTGCCACGAGCAGCTGAGTGATTCAGAAGAGGAAAACGAC

GAGATCGACGGCGTGAATCACCAGCATCTGCCTGCTAGACGGGCAGAGCC

ACAGCGACACACAATGCTGTGCATGTGCTGTAAGTGTGAAGCCAGGATCAA

GCTGGTGGTCGAGTCAAGCGCCGACGATCTGCGCGCCTTCCAGCAGCTGT

TCCTGAATACTCTGTCATTTGTCTGCCCTTGGTGTGCCTCCCAGCAG

Human STEAP nucleotide sequence:

GAATCACGGAAGGACATCACTAATCAGGAGGAACTGTGGAAAATGAAGCCA

AGAAGGAATCTGGAAGAGGACGACTATCTGCACAAGGACACCGGCGAAACA

AGTATGCTGAAACGACCAGTGCTGCTGCACCTGCATCAGACTGCTCACGCA

GACGAGTTTGATTGCCCCTCTGAACTGCAGCACACCCAGGAGCTGTTCCCA

CAGTGGCATCTGCCCATCAAGATTGCCGCTATCATTGCTTCACTGACATTTC

TGTACACTCTGCTGAGAGAAGTGATCCACCCCCTGGCCACCAGCCATCAGC

AGTACTTCTATAAGATCCCTATCCTGGTCATCAACAAGGTCCTGCCAATGGT

GAGCATCACACTGCTGGCCCTGGTCTACCTGCCTGGAGTGATCGCAGCCAT

TGTCCAGCTGCACAATGGGACAAAGTATAAGAAATTTCCACATTGGCTGGAT

AAGTGGATGCTGACTAGGAAACAGTTCGGACTGCTGTCCTTCTTTTTCGCCG

TGCTGCACGCTATCTACAGCCTGTCCTATCCCATGAGGAGGAGCTACCGGT

ATAAGCTGCTGAACTGGGCTTACCAGCAGGTGCAGCAGAACAAGGAGGAC

GCATGGATTGAACATGACGTGTGGCGCATGGAAATCTACGTGAGCCTGGGC

ATTGTCGGACTGGCCATCCTGGCTCTGCTGGCAGTGACCAGTATCCCTTCT

GTCAGTGACTCACTGACATGGAGAGAGTTTCACTACATTCAGAGCAAGCTG

GGGATCGTGTCCCTGCTGCTGGGCACCATCCATGCACTGATTTTTGCCTGG

AACAAGTGGATCGATATCAAGCAGTTCGTGTGGTATACTCCCCCTACCTTTA

TGATTGCCGTCTTCCTGCCCATCGTGGTCCTGATCTTCAAGTCCATCCTGTT

CCTGCCTTGTCTGCGGAAGAAAATCCTGAAAATTCGGCACGGATGGGAGGA

TGTCACCAAAATCAATAAGACTGAAATCTGTAGCCAGCTG

Human WT1 nucleotide sequence:

CAGGACCCAGCCAGCACTTGCGTCCCCGAGCCAGCCTCCCAGCACACTCT

GAGAAGCGGACCCGGATGCCTGCAGCAGCCCGAACAGCAGGGCGTGAGG

GACCCCGGAGGAATCTGGGCTAAACTGGGAGCAGCTGAGGCATCTGCCGA

ACGGCTGCAGGGCAGGAGAAGTCGCGGGGCAAGTGGCTCAGAGCCTCAG

CAGATGGGGAGCGACGTGAGGGACCTGAACGCACTGCTGCCAGCCGTGCC

TAGCCTGGGAGGAGGAGGAGGATGCGCACTGCCCGTCAGCGGCGCAGCC

CAGTGGGCACCTGTGCTGGATTTTGCCCCCCCTGGCGCTAGTGCATACGGA

TCACTGGGAGGACCAGCTCCACCACCTGCACCACCCCCTCCTCCTCCTCCA

CCCCCTCACTCCTTCATCAAGCAGGAGCCTTCTTGGGGCGGAGCCGAACCA

CACGAGGAACAGTGCCTGAGTGCTTTCACCGTGCATTTTTCAGGGCAGTTC

ACTGGAACCGCAGGAGCTTGTCGATACGGACCTTTTGGACCACCACCTCCA

AGCCAGGCCAGCTCCGGACAGGCTCGAATGTTCCCAAACGCACCCTATCTG

CCTAGCTGTCTGGAGTCCCAGCCAGCCATTCGGAATCAGGGATACTCCACA

GTGACTTTTGACGGGACCCCTTCTTATGGCCACACACCAAGTCACCATGCTG

CACAGTTTCCAAACCACTCTTTCAAGCATGAGGACCCTATGGGCCAGCAGG

GCAGCCTGGGAGAACAGCAGTACTCCGTCCCCCCTCCAGTGTATGGCTGCC

ACACCCCTACAGACTCTTGTACTGGAAGTCAGGCCCTGCTGCTGCGCACCC

CATACTCTAGTGATAATCTGTATCAGATGACTAGCCAGCTGGAATGCATGAC

CTGGAACCAGATGAATCTGGGAGCTACCCTGAAAGGAGTCGCAGCTGGCTC

AAGCTCCTCTGTGAAGTGGACAGAGGGACAGTCCAACCACTCTACTGGGTA

CGAAAGCGACAATCATACCACACCTATCCTGTGCGGCGCCCAGTATAGAAT

CCACACACATGGAGTCTTCAGGGGGATTCAGGATGTGCGGCGCGTCCCAG

GAGTGGCTCCCACCCTGGTGAGAAGCGCATCCGAGACATCCGAAAAACGG

CCCTTTATGTGCGCTTACCCTGGATGTAATAAGAGGTATTTCAAACTGTCAC

ACCTGCAGATGCATAGCAGAAAGCACACTGGGGAGAAACCATATCAGTGCG

ACTTTAAAGATTGTGAACGAAGGTTCTCTAGGAGTGACCAGCTGAAGAGACA

TCAGAGACGGCACACAGGAGTGAAGCCCTTTCAGTGCAAAACTTGTCAGCG

CAAGTTCTCACGAAGCGATCATCTGAAGACTCACACCAGAACACATACTGGC

AAGACATCAGAGAAACCCTTCAGCTGCCGCTGGCCCTCCTGTCAGAAGAAA

TTCGCCCGATCTGACGAACTGGTCCGACATCACAACATGCATCAGAGGAAT
ATGACAAAACTGCAGCTGGCACTG

Cell culture

Murine TC1 cells were grown in RPMI containing 10% foetal bovine serum (Invitrogen, Waltham, MA), 10mmol/l HEPES (Invitrogen, Waltham, MA), 2mmol/l L-glutamine (Invitrogen, Waltham, MA) and 400 µg/ml G418 (Gold Biotechnology, St Louis, MO). Vero, L929 and A549 cells were all cultured in αMEM containing 8% foetal bovine serum and 2mmol/l L-glutamine. SaOS2 cells were cultured in DMEM containing 10% foetal bovine serum and 2mmol/l L-glutamine. HEK293 cells were grown in MEM-F11 containing 10% foetal bovine serum and 2mmol/l L-glutamine. HEK293T cells were cultured in DMEM containing 10% foetal bovine serum and 2mmol/l L-glutamine. TRAMP-C2 cells were cultured in DMEM containing 10% foetal bovine serum, 2mmol/l L-glutamine, 0.005mg/ml human recombinant zinc insulin (Gibco, Waltham, MA) and 10nM dehydroisoandrosterone (MP Biomedicals, Santa Ana, CA). B16 F10 cells were cultured in MEM-F11 containing 10% fetal bovine serum, 2mmol/l l-glutamine, 1mmol/l sodium pyruvate (Invitrogen, Waltham, MA), 1mmol/l vitamin solution (Invitrogen, Waltham, MA), 0.01mmol/l non-essential amino acids (Invitrogen, Waltham, MA) and 50mmol/l β-Mercaptoethanol (Invitrogen, Waltham, MA). All cells were grown at 37°C, 5% CO_2 and 95% relative humidity. Mycoplasma was not detected by PCR in TC1 or TRAMP-C2 cells.

Reverse transcription polymerase chain reaction

RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized using approximately 500ngs of RNA, random primers (NEB, Ipswich, MA) and First-Strand cDNA Synthesis Using SuperScript™ II RT (Invitrogen, Waltham, MA); reactions were also run in the absence of reverse transcriptase to control for genomic DNA contamination. PCR was then performed on cDNA using Taq DNA polymerase (NEB, Ipswich, MA) with the primers found in Table 3.

Table 3. Primers used for detection of tumour-associated antigens.

Primer name	Sequence	Amplicon size (BP)
HPV16E6 forward	ACA GGA GCG ACC CAG AAA GTT AC	413
HPV16E6 reverse	CAG CTG GGT TTC TCT ACG TGT TCT TG	
HPV16E7 forward	GCA ACC AGA GAC AAC TGA TC	182
HPV16E7 reverse	GGT CTT CCA AAG TAC GAA TG	
Murine STEAP forward	CAG GAA GCA GTT TGG TCT CC	195
Murine STEAP reverse	ACA ATC CCC AGG GAC ACA TA	
Murine WT1 forward	GCC TTC ACC TTG CAC TTC TC	271
Murine WT1 reverse	GGT CCT CGT GTT TGA AGG AA	
Murine β-actin forward	TAG GCA CCA GGG TGT GAT GG	265
Murine β-actin reverse	GAC CCA GAT CAT GTT TGA GAC C	

PCR products were run out on 2% agarose (Invitrogen, Waltham, MA) gels and for sequence confirmation STEAP and WT1 bands were dissected out. DNA was extracted using the EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic, Markham, Canada). Extracted PCR products were then cloned using CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Waltham, MA) and amplified using Subcloning Efficiency™ DH5α™ Competent Cells (Thermo Fisher Scientific, Waltham, MA). Plasmid DNA was then extracted using GenElute™ Plasmid

Miniprep Kit (Sigma-Aldridge St Lois, MO) and submitted to the MOBIX Lab (McMaster University, Hamilton, Canada) for sequencing using pJET 1.2 Sequencing Primers (Thermo Fisher Scientific, Waltham, MA).

Immunofluoresence

TRAMP-C2 cells were grown on coverslips and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) at 37°C for 10 minutes. Cells were blocked at room temperature with 1-2% BSA (Equitech-Bio, Kerrville, TX) in PBS. The polyclonal rabbit anti-STEAP antibody (Santa Cruz, Dallas, TX) and the polyclonal mouse sera were applied overnight at 4°C, the polyclonal rabbit anti-WT1 antibody was applied for 1 hour at room temperature. Secondary fluorescent-conjugated donkey anti-rabbit (Thermo Fisher Scientific, Waltham, MA) and goat anti-mouse antibodies (Thermo Fisher Scientific, Waltham, MA) were applied for 1 hour at room temperature prior to staining with DAPI (Molecular Probes, Eugene, OR) for 10 minutes. Immunofluorescent images were captured using EVOS FL Cell Imaging System (Thermo Fisher Scientific, Waltham, MA).

***In vitro* infections**

Six well plates containing confluent TC1 and TRAMP-C2 cells (approximately $1.5x10^6$ cells per well) were infected at decreasing multiplicity of infection (MOI) with MG1 Maraba (from 10 to 0.001 alongside an uninfected control well) in 200µl

of culture medium for 45 minutes, following infection fresh medium was added and at 48 hours post-infection, cells were fixed with methanol and stained with 0.1% crystal violet (Sigma-Aldridge St Lois, MO) in 20% ethanol for viability.

Interferon β response test

The IFNβ responsive L929 were plated alongside TRAMP-C2 cells in a 96 well plate and upon reaching confluence were treated with a dilution series of murine IFNβ overnight. The following day the cells were infected with $5x10^5$ PFU per well of wild type VSV expressing GFP. Fluorescence was detected 24 hours after infection using a Typhoon Trio Variable Mode Imager (GE Healthcare, Buckinghamshire, U.K.).

G deleted VSV assay to determine the effect of E6 and E7 on innate antiviral response in human epithelial tumour cells

A549 human lung adenocarcinoma cells were seeded in a 96 well plate and co-transfected, using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA), with a plasmid of interest in combination with a plasmid encoding the VSV glycoprotein (PSG5-G). Cells were subsequently infected with a G deleted VSV expressing GFP and supernatants were harvested. Supernatants containing any rescued viral progeny were collected and serial dilutions were used to infect confluent Vero cells in a 96 well plate and this was imaged for fluorescence using a Typhoon Trio Variable Mode Imager (GE Healthcare, Buckinghamshire, U.K.).

Only cells that were successfully transfected with PSG5-G and have inhibition of the anti-viral state by the transfected plasmid of interest are able to produce viral progeny as detected by fluorescence[284]. Fluorescence was quantified using ImageQuant TL software (GE Healthcare, Buckinghamshire, U.K.).

Transient transfections

A549 cells plated were in 6 well plates and when 80% confluent, transfected with 2μgs of WT E6E7 from HPV16 and 18, the attenuated E6E7 transgene or GFP in the pShuttle-CMV vector (Agilent, Santa Clara, CA) using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA). SaOS2 cells were co-transfected with HA tagged retinoblastoma in pcDNA 3 (Gift from Joe Mymryk), GFP and one of WT E6E7, attenuated E6E7 or empty pShuttle-CMV.

Protein lysates

Cells were lysed on ice in 100μl of radioimmunoprecipitation assay buffer supplemented with Complete Mini protease inhibitor tablets (Roche, Mannheim, Germany) 24-48 hours after transfection.

Western blotting and antibodies

Equivalent amounts (20 or 30μgs) of protein lysate were loaded per lane onto polyacrylamide gels and separated by SDS-PAGE, transferred to 0.45μm nitrocellulose membrane (Santa Cruz, Dallas, TX). Membranes were blocked with

either 5% fat-free milk in PBS or Odyssey Blocking Buffer (LI COR Biosciences, Lincoln, NE) for 40 minutes at room temperature. Membranes were probed with antibodies raised against p53 (clone DO1, Santa Cruz, Dallas, TX), HA (clone F7, Santa Cruz, Dallas, TX), E7 (clone 8E2, Abcam, Cambridge, U.K.), β-actin (clone 13E5, Cell Signalling, Danvers, MA), GFP (clone D5.1, Cell Signalling, Danvers, MA), STEAP (rabbit polyclonal, Santa Cruz, Dallas, TX) and adenovirus serotype-5 (rabbit polyclonal, Abcam, Cambridge, U.K.). Membranes were then probed with secondary IRDye (LI COR Biosciences, Lincoln, NE) antibodies. Membranes were scanned and had fluorescence quantified using the LI COR Odyssey system (LI COR Biosciences, Lincoln, NE).

Immunoprecipitation

HEK293T cells were transfected with HA-tagged Rb (WT) or Flag-tagged E6E7 (WT and mutants) in pcDNA3.1 using XtremeGENE HP DNA transfection reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions. For western blot (WB), the treated cells were lysed in radioimmunoprecipitation buffer (RIPA) (10 mM phosphate pH 7.4, 137 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitor cocktail II and III (Sigma-Aldridge, St Lois, MO). Total cellular lysates were separated on 7.5-12% SDS-PAGE and transferred to nitrocellulose membrane (Santa Cruz, Dallas, TX). Blots were blocked in Odyssey Blocking Buffer (LI COR Biosciences, Lincoln,

NE) and detection was performed with respective primary antibodies, and bands were visualized with IRDye (LI COR Biosciences, Lincoln, NE) antibodies using Odyssey scanner (LI COR Biosciences, Lincoln, NE). For immunoprecipitations (IP), the treated cells were lysed in IP buffer (20 mM Tris-HCl pH 7.4, 137 mM NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol and protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA.). The cellular lysates were incubated with rabbit anti-HA antibody conjugated to Sepharose beads (Clone C29F4, Cell Signaling, Danvers, MA) at 4°C for 3 hours. The immunoprecipitates were washed thoroughly and boiled in SDS sample buffer for WB using mouse anti-Flag antibody (Clone 9A3, Cell Signaling, Danvers, MA) or mouse anti-HA antibody (Clone 6E2, Cell Signaling, Danvers, MA).

Mice

Six to eight week old C57BL/6 mice were purchased from Charles River (Wilmington, MA) and housed in specific pathogen-free conditions. For TC1 (HPV) studies female mice were used and for TRAMP-C2 (prostate) male mice were used except where stated. All animal studies were approved by McMaster University's Animal Research Ethics Board and complied with guidelines from the Canadian Council on Animal Care.

Tumour challenge

Mice were engrafted with $1x10^6$ TC1 cells for HPV studies (except where stated) or $2.5x10^6$ TRAMP-C2 for prostatic studies subcutaneously under gaseous general anaesthesia. The longest axis of the tumour (length) and the axis perpendicular (width) to this were measured every 2-3 days and tumour volume was calculated using the following formula:

$Volume= 4/3\pi((0.5length)((0.5width)^2))$

Mice reached end point when tumours grew to a volume of $1500mm^3$. For the TC1 model end points also included the mouse losing 20% of its body weight relative to weight recorded prior to tumour engraftment or if the mouse was deemed moribund due to weight loss.

Adenovirus: MG1 vaccination of mice

Adenovirus was administered under gaseous general anaesthesia at a dose of $2x10^8$ plaque forming units (PFU) in 100µl of 0.9% NaCl for injection (Hospira, Lake Forest, IL), the dose was split in 2 and 50µl was injected in the semimembranosus muscle of each hind limb. For HPV studies Maraba MG1-GFP or -E6E7 was administered intravenously at a dose of $1x10^9$ PFU in 200µl 0.9% NaCl as a single dose 9 days after adenoviral vaccination. For prostatic studies initial tumour free immune analysis was performed on female mice boosted with 2 doses of $1x10^9$ PFU MG1-STEAP or MG1-WT1 at 13 and 16 days after priming. For all other prostatic experiments male mice received MG1 boosts of the same

dose at 8 and 11 days after the prime. Priming vaccination was given when tumours reached a mean volume of approximately 250-300mm^3.

Peptide vaccination of mice

For the HPV peptide vaccination experiments mice received either a single dose of peptide or received a peptide/ MG1 prime followed 8 days later by a single boost of either peptide/ MG1. MG1-E6E7 was given intravenously at a dose of 1x10^9 PFU in 200µl 0.9% NaCl. Synthetic 29mer long peptides of greater than 70% purity were custom synthesized by Biomer Technologies (San Francisco, CA). 50µg of each peptide was administered with 50µg of an agonistic antibody against mouse CD40 (clone FGK4.5, Bio X Cell, West Lebanon, NH) and 100µg of polyIC (InvivoGen, San Diego, CA) in a total volume of 300µl 0.9% NaCl delivered intra-peritoneally. In tumour free mice all four peptides were administered concurrently, for mice bearing TC1 tumours only the HPV16 peptides were administered. For prophylactic studies mice were challenged with 1x10^6 TC1 cells subcutaneously 7 days after boosting and for therapeutic studies mice were primed when tumours reached a mean volume of approximately 250-300mm^3. For re-challenge cured mice were engrafted with 2.5x10^6 TC1 cells subcutaneously on the flank contralateral to the site of the cured tumour. Predicted peptide stabilities were calculated as previously described by Guruprasad and colleagues[285].

Direct viral oncolysis

For direct oncolysis studies Maraba MG1 GFP was injected intravenously at a dose of $5x10^8$ PFU in 200µl 0.9% NaCl in 3 doses given 48 hours apart once tumours reached a mean volume of approximately $250mm^3$.

Peptides for immune analyses

Table 4 displays the peptides in crude form that were synthesized by Biomer Technologies (San Francisco, CA) and used to re-stimulate T cells. For tumour bearing experiments WT1 peptides and the muSTEAP peptides were pooled to re-stimulate WT1 and STEAP vaccinated mice respectively.

Table 4. Peptides used for T cell re-stimulation.

Peptide name	Peptide sequence	Final concentration used (µg/ml)	MHC molecule bound in mice
HPV16 E6	EVYDFAFRDL	2	H-2K^b
HPV16 E7	RAHYNIVTF	2	H-2D^b
HPV18 E6	HYRGQCHSCCNRARQ	2	H-2^b
HPV18 E6	HSCCNRARQERLQRR	2	H-2^b
HPV18 E7	KLVVESSADDLRAFQ	2	H-2^b
WT1 NAP	NAPYLPSCL	5	H-2^b
WT1 RMF	RMFPNAPYL	5	H-2^b
muSTEAP 5-13	KDITNQEEL	5	H-2D^b
muSTEAP 186	RSYRYKLL	5	H-2K^b
muSTEAP 327	VSKINRTEM	5	H-2D^b
huSTEAP 327	VTKINKTEI	5	H-2^b

The complete peptide library from the WT sequences of HPV16 and 18, E6 and E7 was also synthesized as 15mer peptides with 9mer overlap and peptides were used at concentrations of 15 μg/ml to re-stimulate splenocytes for epitope mapping.

Intracellular cytokine staining and antibodies

Blood samples were taken for immune analysis of the prime either on the day of boosting or one day preceding boost vaccination. Analyses of boost vaccinations were performed between 5 and 6 days after Maraba MG1/ peptide boosting, spleens were harvested 5 days post Maraba treatment unless otherwise stated. Peripheral blood mononuclear cells and splenocytes were incubated in complete RPMI (containing 10% foetal bovine serum and 2mmol/l L-glutamine) with peptide and, where specified, anti CD107a (clone 1D4B, BD, Franklin Lakes, NJ). Incubations were performed for a total of 5 hours in a 37°C, 5% CO_2 incubator at 95% humidity, 1 μg/ml of brefeldin A (GolgiPlug, BD, Franklin Lakes, NJ) was added for the last 4 hours. Cells were then incubated with anti CD16/CD32 (clone 2.4G2, Mouse BD Fc Block, BD, Franklin Lakes, NJ). T cell surface staining was performed with antibodies against CD8a (clone 53-6.7, eBiosciences, Inc., San Diego, CA) and CD4 (clone RM4-5, eBiosciences, Inc., San Diego, CA). Cells were subsequently fixed and permeabilised (Cytofix/Cytoperm, BD, Franklin Lakes, NJ). Intracellular cytokine staining was then performed using antibodies against IFNγ (clone XMG1.2, BD, Franklin Lakes, NJ), TNFα (clone MP6-XT22,

BD, Franklin Lakes, NJ) and IL-2 (clone JES6-5H4, BD, Franklin Lakes, NJ). Data were acquired using an LSRFortessa cytometer (BD, Franklin Lakes, NJ) and analyzed with FlowJo Mac software (Treestar, Ashland, OR).

T cell counts

A known quantity of fluorescent beads (123count eBeads, eBiosciences, Inc., San Diego, CA) were added to 50µl of whole blood which had been stained with antibodies against CD8a (clone 53-6.7, eBiosciences, Inc., San Diego, CA) and CD4 (clone RM4-5, eBiosciences, Inc., San Diego, CA) and fixed as well as lysed (1-step Fix/Lyse solution, eBiosciences, Inc., San Diego, CA). The cells and beads were re-suspended in FACS after 2 wash steps and absolute cell numbers were calculated. For enumeration of splenocytes, the entire spleen was processed and re-suspended in complete RPMI, 50µl of the re-suspended splenocytes were then analyzed as for peripheral blood. Total blood volume in µl was calculated by multiplying each mouse's body weight in grams by 70 thus allowing a total circulating count of T cells.

T cell memory phenotype and antibodies

PBMCs and splenocytes were incubated with anti CD16/CD32 (clone 2.4G2, Mouse BD Fc Block, BD, Franklin Lakes, NJ). Cells were then stained with antibodies against CD8a (clone 53-6.7, eBiosciences, Inc., San Diego, CA), CD4 (clone RM4-5, eBiosciences, Inc., San Diego, CA), CD62L (clone MEL-14, BD,

Franklin Lakes, NJ), CD127 (clone SB/199, BD, Franklin Lakes, NJ) and the HPV H-2D^b E7 tetramer RAHYNIVTF (Baylor College of Medicine, Houston, TX).

Depletion antibodies

T cells were selectively depleted with 2 doses of anti-CD8a (2.43 clone) or anti-CD4 (GK1.5) clone given 48 hours apart. Mice were injected intra-peritoneally with 200µgs antibody in 300µls 0.9% NaCl. Depletions were assessed flow cytometrically from peripheral blood samples stained for CD8a and CD4.

NanoString® hybridisation

TC1 tumours were removed from 5 mice 24 hours after receiving 1x10^9 PFU of MG1-E6E7 IV, tumours were removed at the same time-point from 3 untreated mice. Tumours were stored in RNAlater (Sigma-Aldridge St Lois, MO) at 4°C for 24 hours prior to storage at -80°C. RNA was extracted using TRIzol (Thermo Fisher Scientific, Waltham, MA). The nCounter® Mouse Immunology panel was used to examine differential expression of mRNAs. Each tumour contributed 1 sample. Profiled data were pre-processed following the manufacturer's recommendations, specifically background was subtracted by using geometric mean of the negative controls; normalisation was performed by using the positive controls and the housekeeping genes. The obtained values were Log2 transformed for further analysis. In order to visualise the distribution of the samples, whole profiles were used for the hierarchical clustering (Euclidean

distance, complete linkage) and for principal component analysis (PCA). Finally, the profiles were analysed for differential expression by using *limma* package in R[286].

Ex-Vivo analysis from human primary tumour samples

Tumour tissue from HNSCC patients undergoing routine surgical procedures were obtained and analyzed, this study was approved by the Ottawa Hospital Research Ethics Board (Protocol# 20120559-01H). HPV+ patients were determined by positive immunohistochemistry for p16 staining, a well-established surrogate marker of HPV presence performed routinely by the Ottawa Hospital Pathology Department[287]. Areas containing tumour were identified by routine gross tissue examinations. Approximately 2mm cores were obtained using a sterile biopsy punch and were further sliced with a scalpel to obtain approximately 2x1mm tumour slices. The slices were randomised and two slices were placed into each well of 24-well plate and cultured in DMEM supplemented with 10% heat-inactivated FBS and 100-units/ml antibiotic/ antimycotic solution (Sigma-Aldridge St Lois, MO). After 48h MG1-GFP infection at $1x10^7$ or $5x10^7$ PFU (or both when adequate tissue was available), the tumour slices were rinsed twice with PBS and processed for viral titration as previously described[288]. Bright field and fluorescent images of tumour slices were obtained employing the EVOS FL Cell Imaging System (Thermo Fisher Scientific, Waltham, MA) at 4x magnification.

Ex-Vivo tumour analysis from TRAMP-C2 tumours

Upon reaching volume endpoint TRAMP-C2 tumours were harvested from untreated male mice. Approximately 2mm cores were obtained using a sterile biopsy punch and were further sliced with a scalpel to obtain approximately 2x1mm tumour slices. Individual slices were placed in a 96 well plate containing 200µl DMEM containing 10% foetal bovine serum, 2mmol/l L-glutamine, 5µg/ml human recombinant zinc insulin (Gibco, Waltham, MA), 10nM dehydroisoandrosterone (MP Biomedicals, Santa Ana, CA), 100U/ml penicillin (Invitrogen, Waltham, MA), 100mg/ml streptomycin (Invitrogen, Waltham, MA), 0.25µg/ml Amphotericin B (Invitrogen, Waltham, MA) and $1x10^6$ PFU of MG1 GFP for each of the slices. A final column of wells contained tumour slices in media without virus as a negative control. Fluorescence was detected 24 hours after infection using ImageQuant TL software (GE Healthcare, Buckinghamshire, U.K.).

Serological analyses

Whole blood samples were collected prior to vaccination with the Ad-STEAP: MG1-STEAP vectors, samples were also collected 15 days after boosting. Samples were allowed to clot for 30 minutes at room temperature prior to serum separation with centrifugation at 2*g* for 15 minutes at 4°C, sera were stored at -80°C. For western blotting HEK293s were infected with Ad-STEAP at an MOI of 10 for 24 hours at 37°C, 5% CO_2 and 95% relative humidity; lysates were

collected as previously described. Pooled pre- and post-immune sera were used as probing antibodies in western blot and immunofluorescence experiments as previously described. Pre- and post-immune pooled sera were used in assessment of antibody-dependent complement mediated cytotoxicity as described previously[289]. Briefly confluent STEAP positive TRAMP-C2 cells were incubated with 3% pooled pre- and post-immune sera in media in 96 well plates for 4 hours at 37°C, 5% CO_2 and 95% relative humidity. Cells were also incubated with control media containing either 3% FBS or 0.5% Triton 100 (Bio-Rad, Hercules, CA). 10µL of CCK-8 viability dye (Dojindo, Kumamoto, Japan) was added per well and cells were incubated for a further 2 hours. Absorbance was read at 450nm using the SpectraMax i3 (Molecular Devices, Sunnyvale, CA).

Immunohistochemistry and histology

Tissues for examination were formalin-fixed and paraffin-embedded. The rabbit EnVision system (Agilent, Santa Clara, CA) was used as per the manufacturers instructions after initial primary incubation with polyclonal anti-Maraba rabbit serum (generated at McMaster Immunology Research Centre) for detection of MG1 using AEC substrate chromogen. Sections were subsequently stained with haematoxylin prior to mounting. Images were obtained using an Olympus VS120 Slide Scanner (Olympus, Tokyo, Japan). Antibodies against CD3 (clone SP7, Abcam, Cambridge, U.K.) and CD8a (clone 4SM15, eBiosciences, Inc., San Diego, CA) were used with the Bond RX IHC auto-stainer (Leica, Wetzlar,

Germany) and the Bond Polymer Refine Detection system (utilising DAB substrate chromogen and haematoxylin) (Leica, Wetzlar, Germany) as per the manufacturers instructions. For assessment of auto-immune associated pathology in STEAP vaccinated animals, 4 male mice received Ad-STEAP IM followed by 2 doses of MG1-STEAP IV prior to being sacrificed 6 weeks after the first dose of MG1-STEAP. As controls 5 untreated mice as well as 5 mice receiving Ad-BHG and MG1-GFP were included. Mice were anaesthetised and perfused with 10% neutral buffered formalin. Prostate, testes, lungs, kidneys and bladder were removed from mice and embedded in paraffin prior to staining with haemotoxylin and eosin.

Viral titration from tumour samples

TC1 tumours were removed from 4 mice 24 hours after treatment with $1x10^9$ PFU of MG1-E6E7 IV and were snap frozen in 1ml of PBS on dry ice prior to storage at -80°C. The tumours were subsequently thawed and weighed prior to homogenisation. The tissue was then centrifuged for 12 minutes at 225*g* at 4°C, the supernatant was then transferred to a fresh microfuge tube, vortexed for 30 seconds and centrifuged at 225*g* for 5 minutes at 4°C. Standard plaque assay was used to determine titres; briefly confluent Vero cells in 60mm tissue culture dishes were infected by a dilution series of supernatants from homogenised tumour tissue for 45 minutes at 37°C, 5% CO_2 and 95% relative humidity. Vero cells were then overlaid with 0.5% agarose in 2xMEM F11 with 8% FBS and

incubated overnight prior to plaque counting. Tumour weights and homogenate volumes were recorded and used to calculate PFU per gram of tumour tissue.

Statistical analyses and data presentation

Data were graphically displayed and analyzed using GraphPad Prism version 6 for Mac (GraphPad Software, San Diego, CA). Unpaired T-tests or Mann-Whitney tests were used when comparing two groups and ANOVA tests were used to compare greater than two groups. Tumour end point survivals were plotted using Kaplan-Meier curves and median survivals were compared using the log-rank tests. Statistical significance was defined as $p \leq 0.05$ (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$).

~CHAPTER III~

CUSTOMISED VIRAL IMMUNOTHERAPY FOR HPV-ASSOCIATED CANCER

3.1. Introduction

Human papilloma virus infection plays a significant role in the pathogenesis of multiple epithelial malignancies including virtually all cases of cervical carcinoma as well as many head and neck cancers[290]. Two high-risk serotypes, HPV16 and 18, trigger the vast majority of all HPV cancers[291]. In 2012, it was estimated that there were over half a million new cases of cervical cancer globally alongside a startling increase in the incidence of oropharyngeal tumours secondary to HPV infection in the developed world[9,14]. This group of malignancies remains a highly significant cause of morbidity and mortality thereby mandating the need for new and efficacious treatment options.

Foreign viral transforming proteins are essential factors in the neoplastic phenotype of HPV-positive cancers. The early genes E6 and E7 encode for proteins that result in loss of cell cycle control and inhibition of apoptosis primarily via interaction with p53 and pRb respectively[292]. In HPV-positive human cells, E6 and E7 also interact with the type I interferon IFN pathway rendering such tumour cells hypo-responsive to type I IFN cytokines[293]. These foreign viral antigens seemingly make HPV-associated cancer an ideal target for therapeutic vaccination however; both preclinical and clinical data currently suggests that tumour-induced immunosuppression associated with advanced disease prevents vaccine monotherapy from being curative[294–297]. Whilst some therapeutic

vaccines are efficacious against early stage HPV-associated cancer they are of minimal benefit in advanced disease.

Oncolytic viruses are a promising new class of therapeutics for cancer patients. Cellular dysregulation, such as type I IFN signalling defects, occur during carcinogenesis and facilitate selective viral oncolysis leading to direct neoplastic cytotoxicity, and consequentially, the induction of an anti-tumour immune response[55,270]. Following an extensive screening process the attenuated, IFN sensitive MG1 strain of Maraba virus was identified as a highly selective and potent rhabdoviral OV capable of infecting and killing various human and murine tumour cells both *in vitro* and *in vivo*[75]. Moreover MG1 Maraba can act as a powerful anti-cancer vaccine platform; mice primed with a replication deficient adenovirus expressing DCT display very potent anti-DCT immune responses when subsequently boosted with MG1-DCT, leading to significantly enhanced survival in a melanoma model[281]. Given this platform's remarkable potency as a vaccine we hypothesized that the application of a custom designed, attenuated tetravalent transgene based on E6 and E7 from HPV16 and 18 would be efficacious against an advanced model of HPV+ cancer and therefore constructed MG1-E6E7. Further rationale for adopting MG1 therapy against HPV+ tumours comes from the observation that xenografted models of HPV+ cancer cell lines are susceptible to rhabdoviral oncolysis[298]. We demonstrate that the induction of a huge specific CD8+ T cell response results in complete tumour

regression in the vast majority of immune-competent mice bearing advanced HPV+ tumours and primary HPV+ human tumour biopsies selectively enhance MG1 replication, thus HPV-associated tumours are bespoke targets for precision designed virotherapy with MG1-E6E7.

3.2. Results

The mutant E6E7 transgene does not degrade p53 or retinoblastoma *in vitro*

A quadrivalent mutated transgene was designed and cloned into the adenoviral and Maraba MG1 viruses based on the E6 and E7 transforming proteins of HPV16 and 18, a linker promoting proteasomal cleavage (GGGGGAAY) was inserted between each of the proteins to avoid the generation of chimeric peptides. In both of the E6 domains, deletion mutations were made to two of the four CXXC motifs that mediate p53 degradation (Fig 4a)[299]. A549 cells containing wild type p53 were transfected with expression vectors encoding the WT E6E7 transgene, the mutated E6E7 transgene and an irrelevant control plasmid (GFP) respectively, subsequently levels of p53 were quantified by western blot. Degradation of p53 was noted with WT E6E7; however, degradation was completely inhibited in the mutant therapeutic transgene using the GFP plasmid as a reference (Figs 4b+c). In both of the E7 domains of the mutated transgene a deletion was applied to one of the carboxy terminus CXXC motifs as well as deletions to the LXCXE sequences responsible for the dysfunction of pRb[300]. The

pRb-null cell line SaOS2 was co-transfected with an expression plasmid encoding HA-tagged pRb (HApRb) alongside a GFP encoding plasmid as well as one of the following three expression vectors encoding either the WT E6E7 transgene, the mutant transgene or a control plasmid. Following transfection levels of HApRb were quantified via western blot with a significant decrease in the WT E6E7 transfected cell lysates, pRb degradation was abrogated in the mutant transgene using control transfections as reference (Figs 4d+e). Immunoprecipitation experiments were undertaken in HEK293T cells and revealed clear interaction between a flag-tagged WT E6E7 transgene and HApRb but no interaction between a flag-tagged version of the mutant transgene and HApRb (Fig 4f). Thus deletions introduced to the therapeutic transgene prevent destabilisation of both p53 and pRb tumour suppressor proteins.

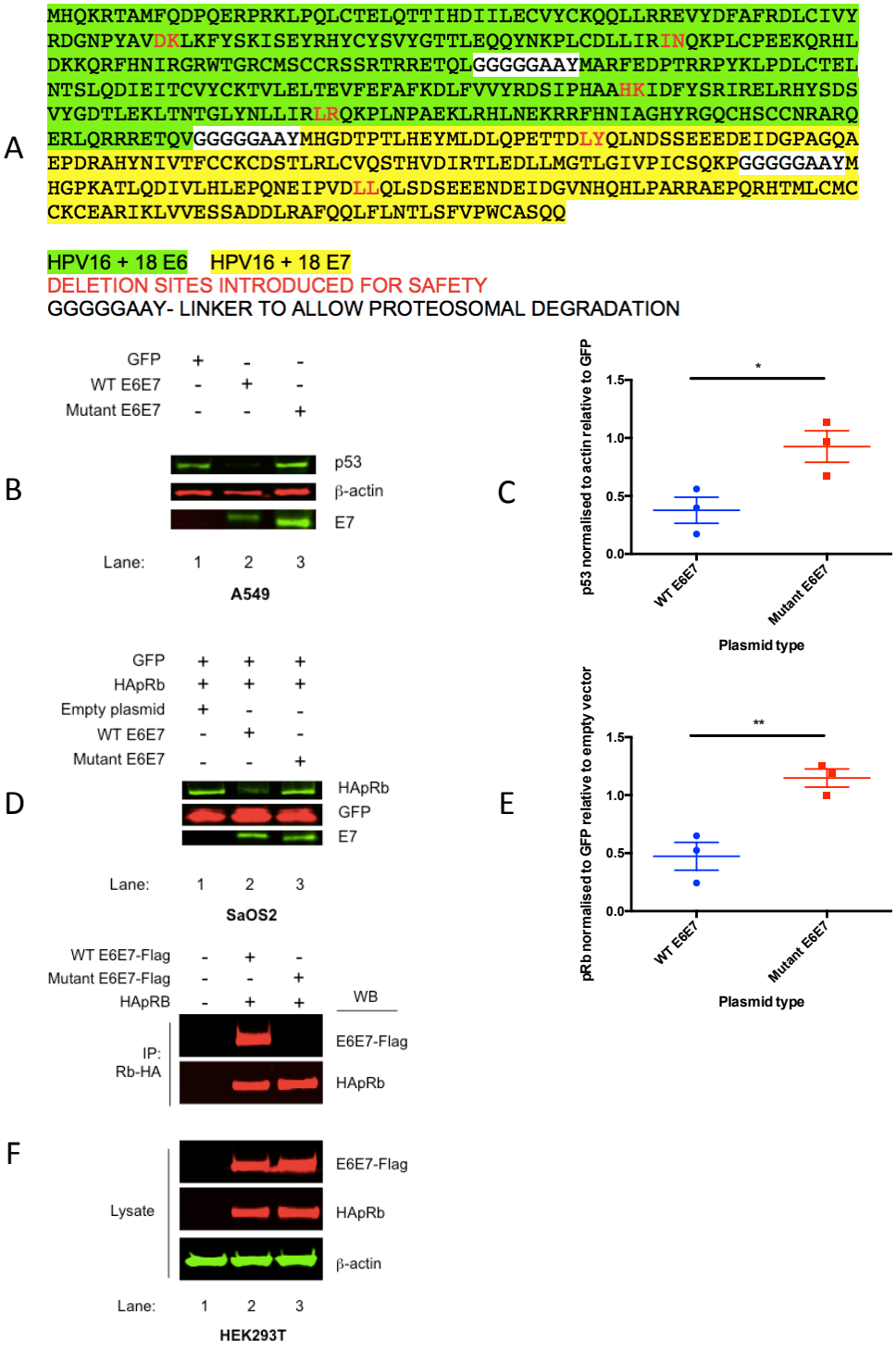


Figure 4. Attenuating mutations for safety of the E6E7 transgene. Design of a therapeutic tetravalent transgene based on the sequences of the E6 and E7 proteins of HPV serotypes 16

and 18 with deletion mutations introduced to prevent their destabilising activity on p53 and pRb respectively. Peptide sequence of transgene cloned into adenoviral priming vector and MG1-E6E7. E6 sequences are highlighted in green, E7 in yellow, linkers on a white background facilitate proteasomal degradation and red characters indicate mutation sites introduced for safety. (A). A549 cells were transfected with plasmids encoding the therapeutic and WT E6E7 transgenes, lysates were probed for p53 (B) and densitometry on western blots was performed (C) (experiment performed in triplicate, mean and SEM displayed, comparison performed using unpaired t test, *p≤0.05). Retinoblastoma null SaOS2 cells were co-transfected with plasmids encoding therapeutic or WT E6E7 alongside HA tagged retinoblastoma and GFP and lysates were probed for retinoblastoma (D) and densitometry on western blots was performed (E) (experiment performed in triplicate, mean and SEM displayed, comparison performed using unpaired t test, **p≤0.01). Immunoprecipitation of flag tagged therapeutic and WT E6E7 sequences were performed using HA tagged retinoblastoma in HEK293T cells (F).

E6E7 vaccination induces specific T cell responses against E6 and E7 epitopes

A vaccination strategy of an adenoviral prime encoding the mutant E6E7 (Ad-E6E7) transgene followed by the MG1 Maraba virus boost (MG1-E6E7) was administered to mice and immune responses were quantified using intracellular cytokine staining (ICS). For comparison sham prime (Ad-BHG) and boost (MG1-GFP) groups were also analysed. Peripheral blood mononuclear cells were re-stimulated with peptides for known E6 (EVYDFAFRDL) and E7 (RAHYNIVTF) C57BL/6 CD8+ epitopes. Blood samples after Ad-E6E7 priming vaccination revealed specific responses generated against both epitopes as indicated by the

production of interferon-γ (IFNγ) from CD8+ T cells, with the E7 epitope producing greater responses, no responses were seen after Ad-BHG (Figs 5a+b). Following MG1-E6E7 a significant increase in IFNγ+ T cell frequency was observed for those mice receiving the E6E7 prime: boost. Again the E7 epitope was dominant with a mean frequency of 68.87% of peripheral CD8+ T cells producing IFNγ following re-stimulation. Small responses were seen after MG1-E6E7 was administered to naïve recipients after sham priming (mean of 0.21% of CD8+ T cells producing IFNγ after E7 re-stimulation) (Figs 5c+d). In order to compare the true magnitude of the responses generated with Ad-E6E7 alone to the prime: boost a subset of mice were sacrificed following boosting, ICS was performed after E7 peptide re-stimulation and the peripheral blood and splenic CD8+ T cells were enumerated using fluorescent beads specifically designed for quantifying cell numbers flow cytometrically. The prime: boost induced a marked and highly significant expansion of total and E7 specific CD8+ T cell populations compared to the Ad-E6E7 alone (Figs 5e+f). When the splenic and circulating pools of E7 specific, IFNγ producing CD8+ T cells were combined a mean absolute count of 4.1×10^7 was generated following the prime: boost (n=10, range=$2.4\text{-}5.1 \times 10^7$). Boosting with MG1-E6E7 was able to generate specific CD8+ T cell responses against E6 and E7 epitopes with an extensive expansion of effector cells.

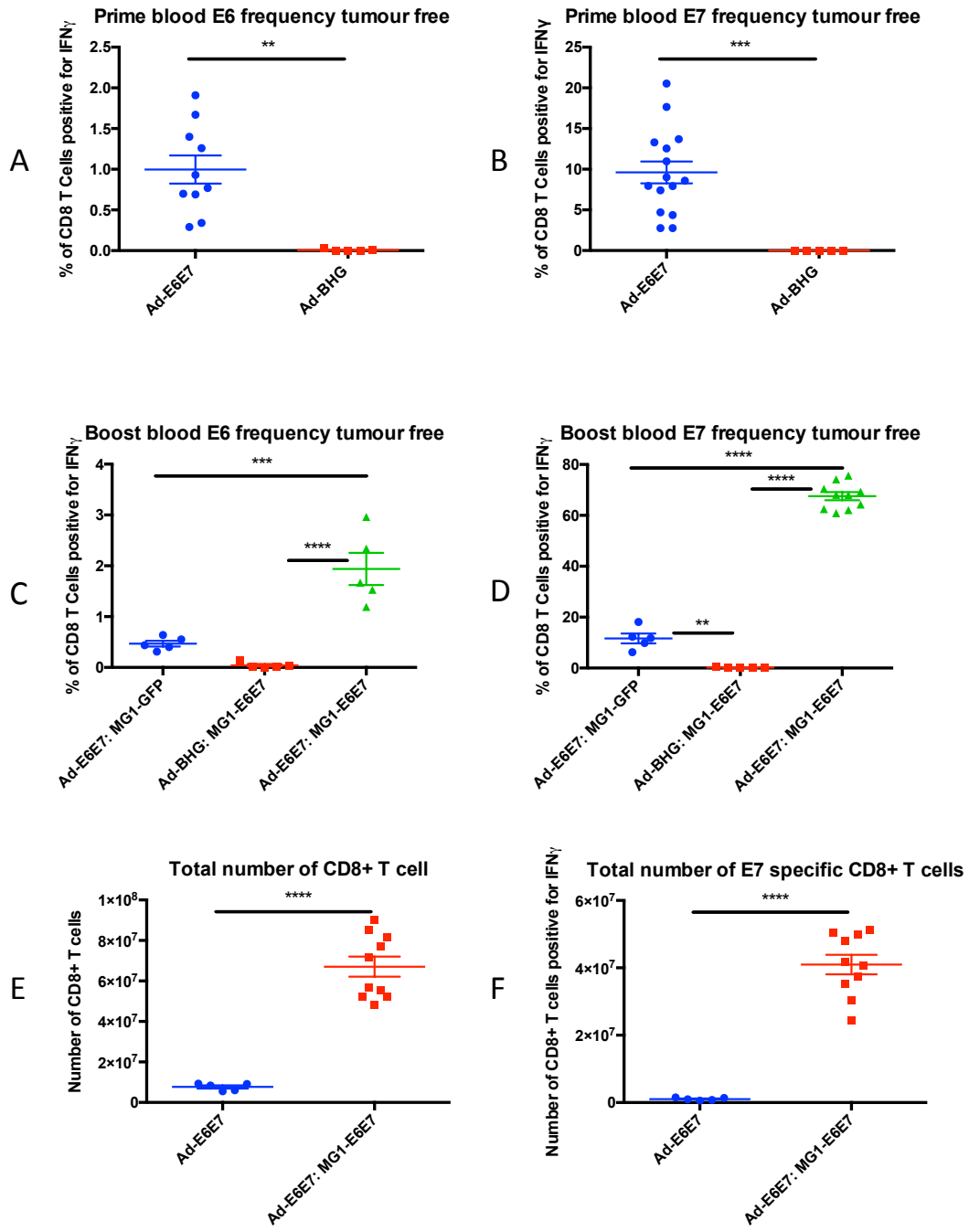


Figure 5. Induction of E6 and E7 specific immune responses. Tumour free C57BL/6 mice were treated with E6E7 vaccination to assess the induction of specific immune responses against

known E6 and E7 peptide epitopes as indicated by IFNγ production from CD8+ T cells. Specific CD8+ T cell responses are seen after adenoviral priming of mice for both E6 (n=10) (A) and E7 (n=15) (B) compared to sham-primed mice (n=5) (mean and SEM displayed, comparison performed using unpaired t test, **p≤0.01, ***p≤0.001). Boosting adenoviral primed mice with MG1-E6E7 leads to significant expansions of the frequencies of both E6 (n=5) (C) and E7 (n=10) (D) specific CD8+ T cells compared to sham-primed mice (n=5) and sham-boosted mice (n=5) (mean and SEM displayed, comparison performed using ANOVA test, **p≤0.01, ***p≤0.001, ****p≤0.0001). Enumeration of total splenic and circulating pools of all CD8+ (E) and E7 specific CD8+ (F) T cells further emphasizes the marked expansion of specific immune effectors post MG1-E6E7 boosted mice (n=10) compared to animals receiving adenoviral priming alone (n=5) (mean and SEM displayed, comparison performed using unpaired t test, ***p≤0.001, ****p≤0.0001).

E6E7 vaccination generates multifunctional T cells

To assess the quality of the immune response, multifunctional T cell analysis was performed on blood and splenic tissue of prime: boost vaccinated mice following E7 re-stimulation, mice receiving Ad-E6E7 alone were used for comparison. In both groups, double (IFNγ & TNFα) (Figs 6a+b) and triple positive (IFNγ, TNFα & IL-2) (Figs 6c+d) CD8+ T cells were found in the circulatory and splenic pools. When double and triple positive CD8+ T cells were quantified mice receiving the prime: boost had significantly more of both populations. The degranulation marker, CD107a (LAMP1), was also included and virtually all cells that produced cytokine were CD107a positive (Fig 7). Vaccination with Ad-E6E7 generated small numbers of E7 specific CD8+ T cells capable of producing multiple effector

cytokines, when mice received the MG1-E6E7 boost a dramatic and significant expansion was seen in these populations.

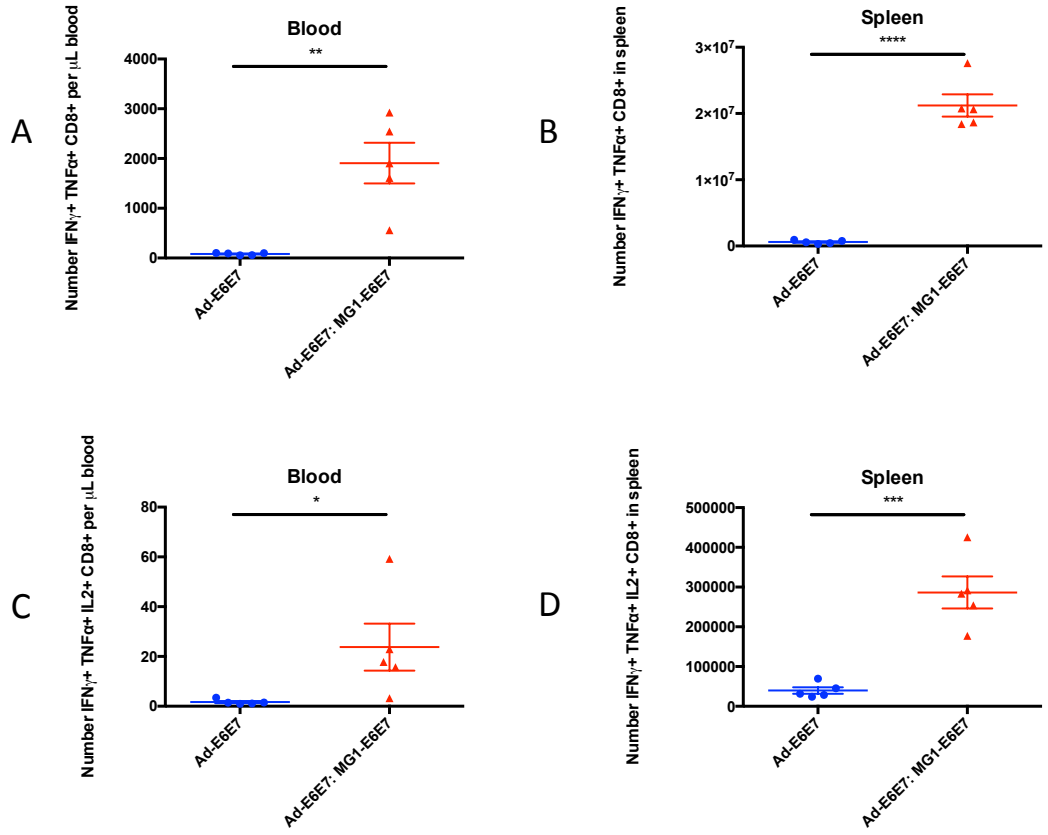
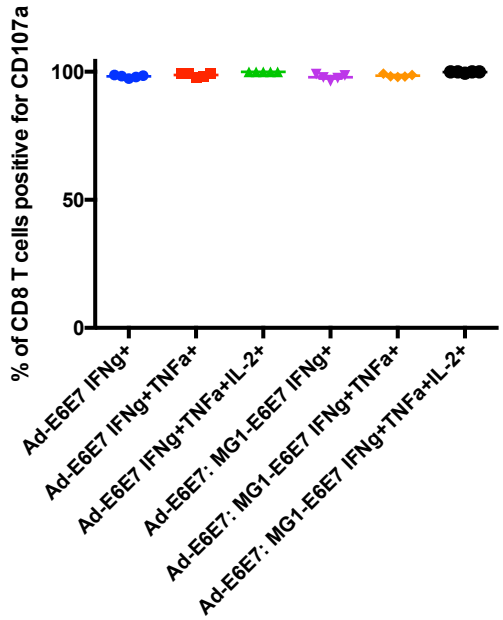


Figure 6. E6E7 vaccination generates multifunctional CD8+ T cells. Tumour free C57BL/6 mice were treated with E6E7 vaccination to assess the induction of E7 specific multifunctional CD8+ T cells post boost. Numbers of double positive (IFNγ & TNFα) CD8+ T cells were significantly higher in the circulating (A) and splenic (B) pools of boosted mice compared (n=5) to mice treated only with the adenoviral priming vector (n=5). Numbers of triple positive (IFNγ, TNFα & IL2) CD8+ T cells were significantly higher in the circulating (C) and splenic (D) pools of boosted mice (n=5) compared to mice treated only with the adenoviral priming vector (n=5) (mean and SEM displayed, comparisons performed using unpaired t tests, *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).

A



B

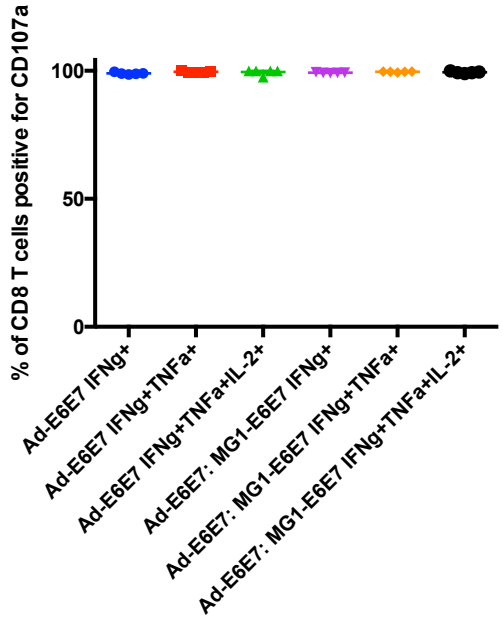


Figure 7. Virtually all E7 specific CD8+ T cells are positive for CD107a. Tumour free C57BL/6 mice were treated with Ad-E6E7 (n=5) alone or boosted with MG1-E6E7 (n=5). Frequency of CD107a positive CD8 T cells in single positive (IFNγ), double positive (IFNγ & TNFα) and triple

positive (IFNγ, TNFα & IL2) populations in the blood (A) and spleen (B) are shown (mean and SEM displayed).

E6E7 vaccination results in complete tumour regression in mice bearing an advanced HPV-positive carcinoma in a CD8+ dependent manner

The HPV16 model cell line was acquired and E6 and E7 expression were confirmed by RT-PCR (Fig 8). Mice bearing subcutaneous TC1 tumours were primed once their tumours reached a large size (approximately 300mm^3). Specific CD8+ T cell responses against both the E6 and E7 were demonstrated in these mice using ICS; a significant expansion of E7 specific T cells was documented after boosting with MG1-E6E7 relative to all other groups. Spontaneous immunity was not detected in untreated animals (Figs 9a+b). Mice were sacrificed when tumour volume reached 1500mm^3 or mice lost 20% of their body mass. All untreated mice succumbed to tumour progression whereas treatment with either a sham prime: boost or prime: sham boost delayed tumour progression and was infrequently curative. In the sham prime: MG1-E6E7 group mice only received a relevant treatment (a single dose of MG1-E6E7) once tumours reached a mean volume of approximately 1000mm^3 and in a single mouse this resulted in complete sustained tumour regression (Fig 10). Treatment of mice bearing advanced TC1 tumours with Ad-E6E7 followed by MG1-E6E7 resulted in complete tumour responses in 75% of mice (n=12) (Fig 9c). In mice treated with the curative regimen, depletion of CD8+ T cells two days before boosting and subsequently forty days after the boost resulted in loss of

control, no such effect was seen when CD8+ cells were depleted at the later time point alone or when CD4+ cells were depleted (Fig 9d). The prime: boost E6E7 vaccine is able to generate specific immunity against E6 and E7 antigens in a murine model of HPV with advanced measurable disease leading to complete regression in a CD8+ dependent manner.

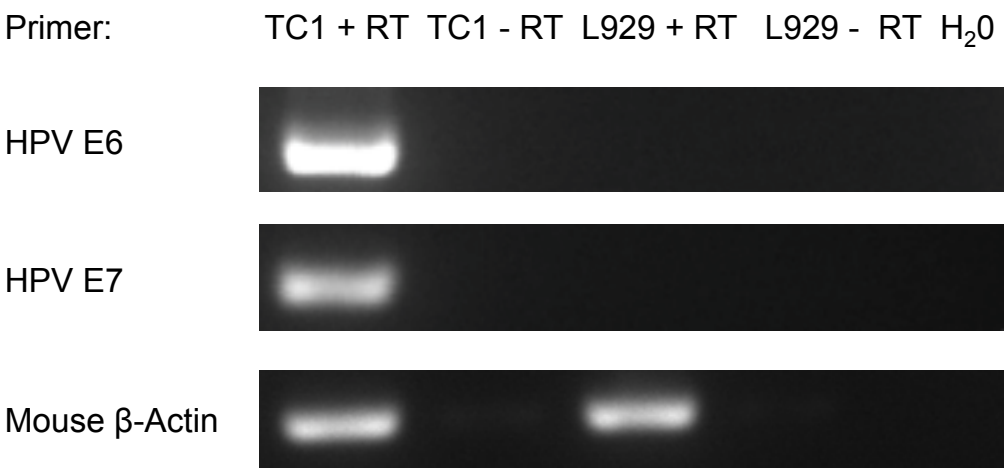


Figure 8. RT-PCR confirms expression of E6 and E7 antigens in TC1 cells. RNA was extracted from TC1 cells and L929 cells as a negative control; standard RT-PCR was performed to amplify cDNA (reactions were also performed without reverse transcriptase to confirm subsequent products were not amplified from genomic DNA contamination). Primers for E6, E7, murine β actin and a reaction containing water in the absence of template were used to complete standard PCR and products were run out on agarose gel and subsequently imaged to confirm gene expression.

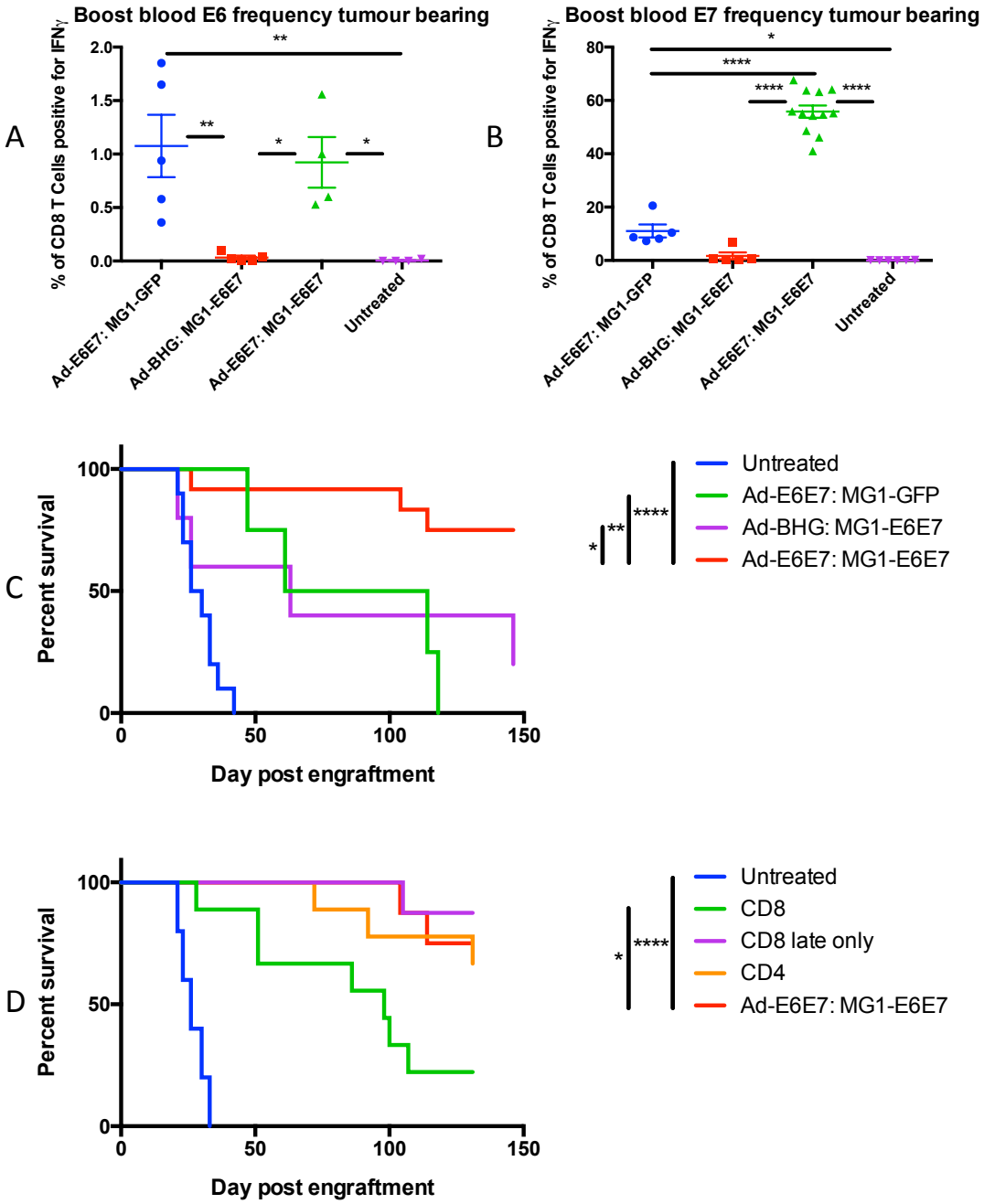


Figure 9. Oncolytic viral immunotherapy induces complete tumour responses in the TC1 model. C57BL/6 were engrafted $1x10^6$ TC1 cells subcutaneously and treated with E6E7 vaccination therapy once tumours reached a mean volume of approximately $300mm^3$. Specific circulating CD8+ T cell responses were seen at peak boost time points against an E6 peptide (A) in mice receiving prime: boost (n=4), sham prime (n=5), sham boost (n=5) but not untreated (n=4) tumour

bearing mice. Specific circulating CD8+ T cell responses were seen at peak boost time points against an E7 peptide (B) in mice receiving prime: boost (n=12), sham prime (n=5), sham boost (n=5) but not untreated (n=6) tumour bearing mice (mean and SEM displayed, comparisons performed using ANOVA test). Treatment with adenoviral prime and MG1-E6E7 boosting (n=12) resulted in long-term survival, which was significantly prolonged in comparison to control mice (n=10), sham boosted (n=4) and sham primed mice (n=5) (pooled data from 2 experiments) (C). Early depletion of CD8+ T cells in prime: boosted mice (n=9) significantly decreased the survival times in comparison to prime: boosted non-depleted mice (n=8), depletion of CD4+ T cells (n=9) or CD8+ at a late time point (n=8) did not adversely affect survival, all untreated mice (n=5) succumbed to disease (D) (log rank tests performed to compare survival curves, *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).

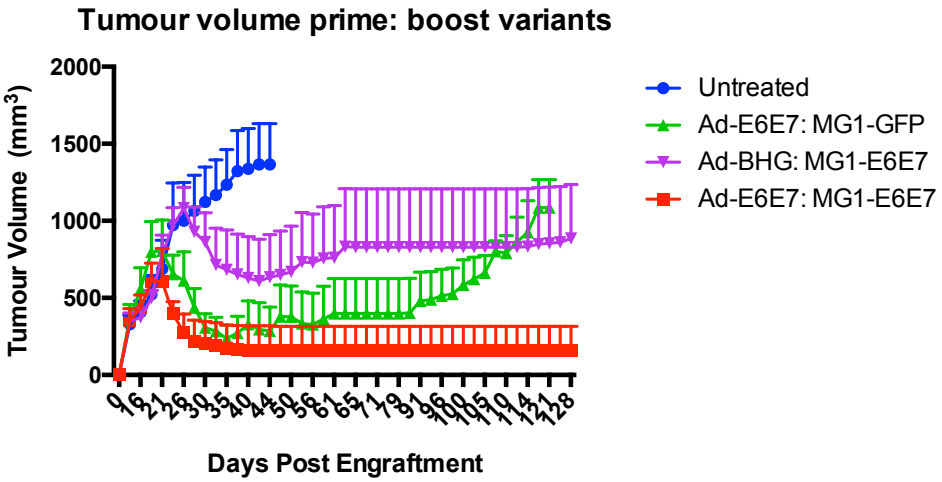


Figure 10. TC1 tumour volume curves of mice treated with oncolytic viral immunotherapy. Mice received adenoviral prime and MG1-E6E7 boosting (n=4), sham boosted (n=4), sham primed mice (n=5) and untreated control mice (n=5). Mice were primed at day 14 when tumours reached a mean volume of approximately 300mm^3 and were boosted with MG1 Maraba at day 23. Tumour growth curves end once all animals from the respective group have reached end point (mean and SEM displayed).

Cured mice have long lasting antigen specific immunity

A subset of mice that were long-term survivors had further analysis to assess T cell memory phenotype. Circulatory and splenic T cells were labeled with an E7 specific tetramer (RAHYNIVTF on H-2D(b)) and stained for CD62L and CD127 at 62 days (Figs 11a,c+e) and 117 days (Figs 11b,d+f) after MG1-E6E7 boosting. Immune analysis revealed a persistence of E7 specific CD8+ T cells at both long-term time points in the blood and spleen. The majority of the specific cells were of the effector memory phenotype, the relative proportions of central memory T cells increased over time (5.1% in blood and 9.4% in spleen at 62 days post boost vs. 11.8% in blood and 18.8% in spleen at 117 days post boost). Oncolytic E6E7 vaccination generates long lasting CD8+ immune memory in mice cured of advanced TC1 tumours.

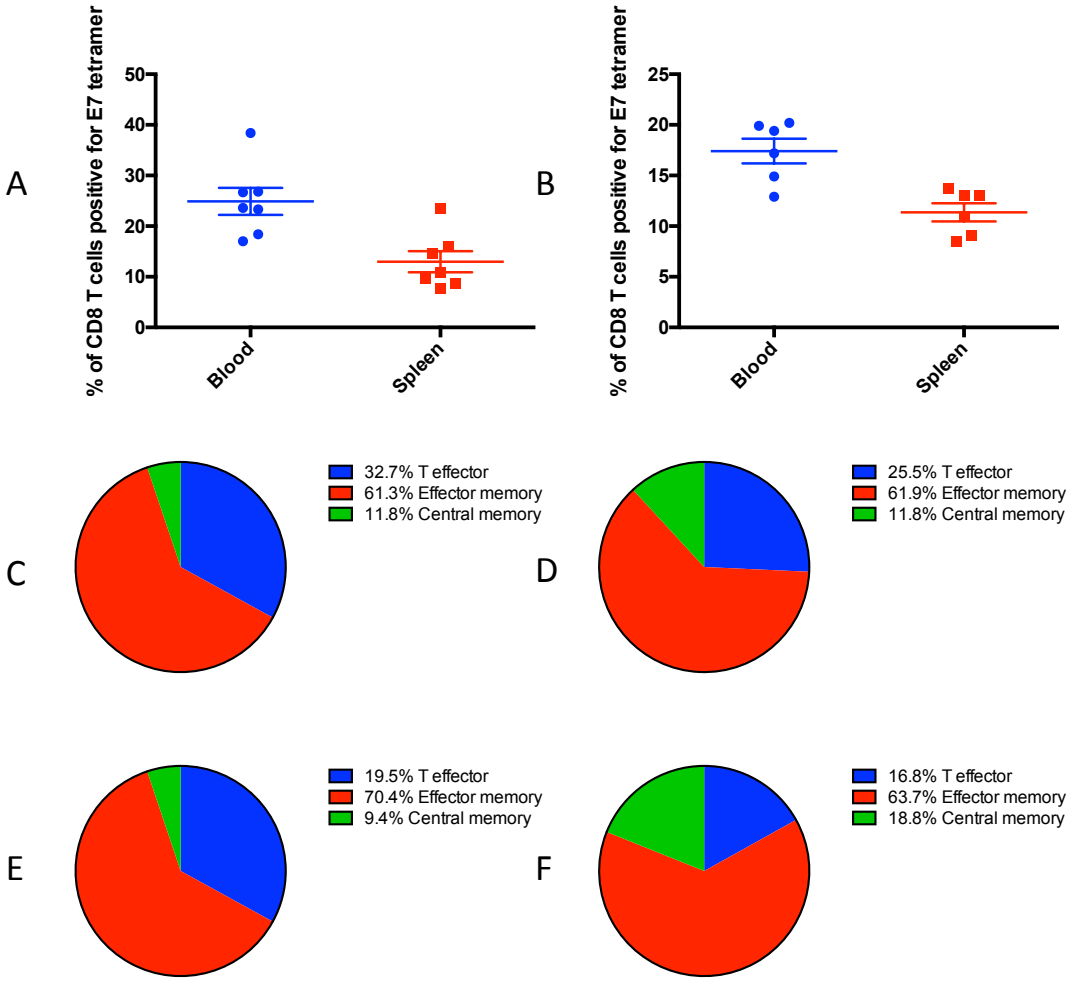


Figure 11. Oncolytic viral immunotherapy results in durable specific immunity. C57BL/6 mice that had complete regression of TC1 tumours following Maraba viral therapy were sacrificed to assess for persistence of specific E7 CD8+ T cells at long term time points as well as memory phenotypic analysis. Experiments were performed at 62 days post MG1-E6E7 boosting (n=7) and 117 days post MG1-E6E7 boosting (n=6) with both circulating and splenic pools evaluated. Frequencies of total tetramer positive CD8+ T cells persisting at day 62 (A) and 117 (B) are shown. Relative proportions of the memory phenotypes of the T cells in the blood (C) at day 62 and spleen at d 62

(E) as well as the blood at day 117 (D) and spleen at day 117 (F) are displayed (mean and SEM displayed for persistence experiments, mean displayed for memory phenotype experiments).

MG1-E6E7 acutely alters the immune transcriptome of TC1 tumours

Within 24 hours of administration of MG1-E6E7 the transcript level of various immune related genes were altered within the tumour. Using an immune panel NanoString® analysis of mRNA isolated from 5 tumours treated with a single dose of MG1-E6E7 alone was performed and revealed increased expression of 17 genes primarily encompassing genes related to antigen presentation and innate viral sensing compared to 3 untreated tumours (Fig 12). IL1b was the only gene in the panel expressed at a lower level than controls following MG1-E6E7 administration. Treatment with systemic MG1-E6E7 rapidly alters the gene expression profile of the immune microenvironment in a murine HPV model.

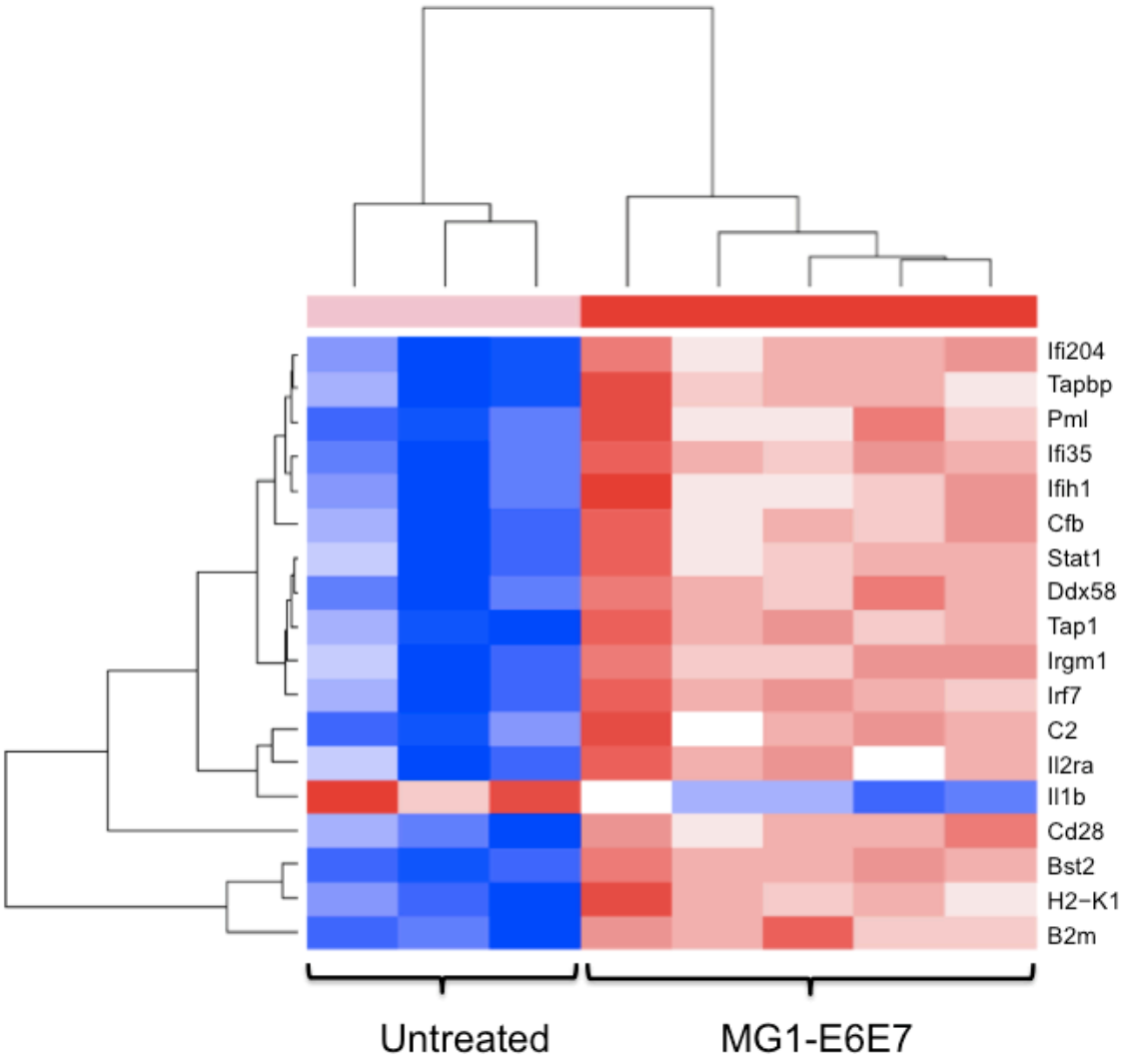


Figure 12. Oncolytic viral immunotherapy acutely alters the intra-tumoural immune transcriptome. Genes differentially expressed within TC1 tumours 24 hours after treatment with systemic MG1-E6E7. mRNA was isolated from TC1 tumours of 5 mice treated with MG1-E6E7 and compared to 3 mice with untreated tumours. NanoString analysis using a murine immunology panel was undertaken and genes with significantly altered expressions between groups (adjusted $p \leq 0.05$) are displayed using a heat map with increased and decreased expressions depicted by red and blue respectively.

E6 and E7 diminish type I IFN responsiveness in human tumour cells

Human tumour cells transfected with WT E6 and E7 had decreased protection against rhabdoviral infection (Figs 13a+b). Innate responses inhibit rhabdoviral infection of A549 cells however, proteins known to diminish type I IFN responsiveness, such as measles V enhance infection[284]. Transfection of A549 cells with WT E6E7 significantly increased infection relative to a control plasmid and the magnitude of increase was comparable to measles V (Figs 13a+b). HPV-associated head and neck cancer patient biopsies were infected *ex vivo* with MG1-GFP. Following infection fluorescence was confirmed microscopically (Fig 14) and viral titrations from biopsies were performed. All HPV-positive tumours were permissive to MG1 infection, with the HPV+ tumours displaying significantly enhanced viral replication (Fig 13c). Expression of E6 and E7 renders human tumour cells more permissive to MG1 replication.

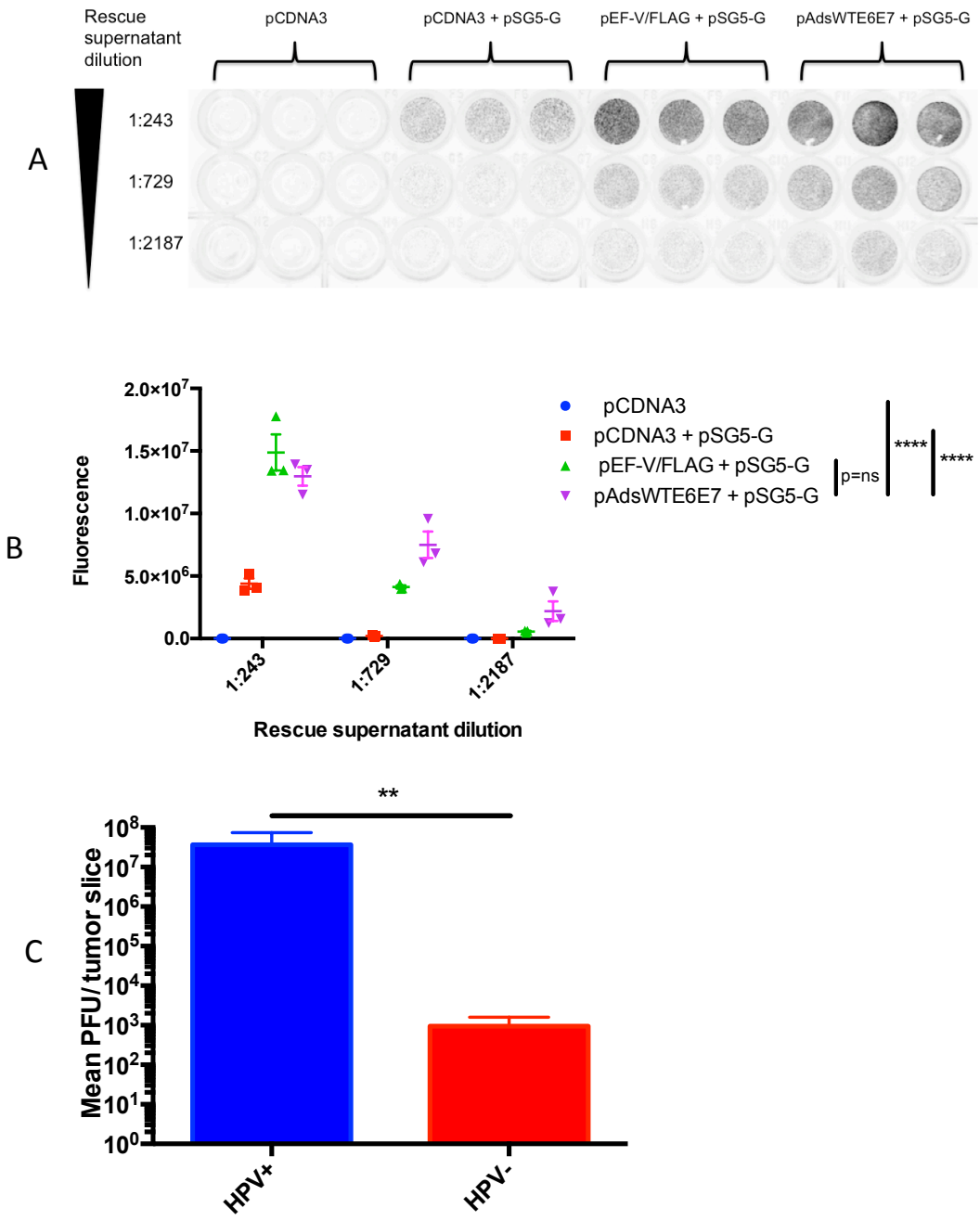


Figure 13. E6 and E7 facilitate MG1 replication in HPV-positive cancer. G deleted VSV assay performed on the human epithelial cancer line A549 following transfection with differing plasmids, including WT E6E7, reveals blunting of the innate immune response in the presence of these

HPV proteins in this human cell line (infection was detected by fluorescence from a GFP transgene and is depicted by black signal) (A). Fluorescence of wells was quantified using software and displayed graphically (mean and SEM displayed, comparison performed using 2 way ANOVA test, ****p≤0.0001) (B). Biopsies of patients suffering from HPV-positive (n=6) and negative (n=11) head and neck cancer were infected with MG1 Maraba *ex vivo* and viral titration assays were performed (mean and SEM displayed, comparison performed using Mann Whitney test, **p≤0.01) (C).

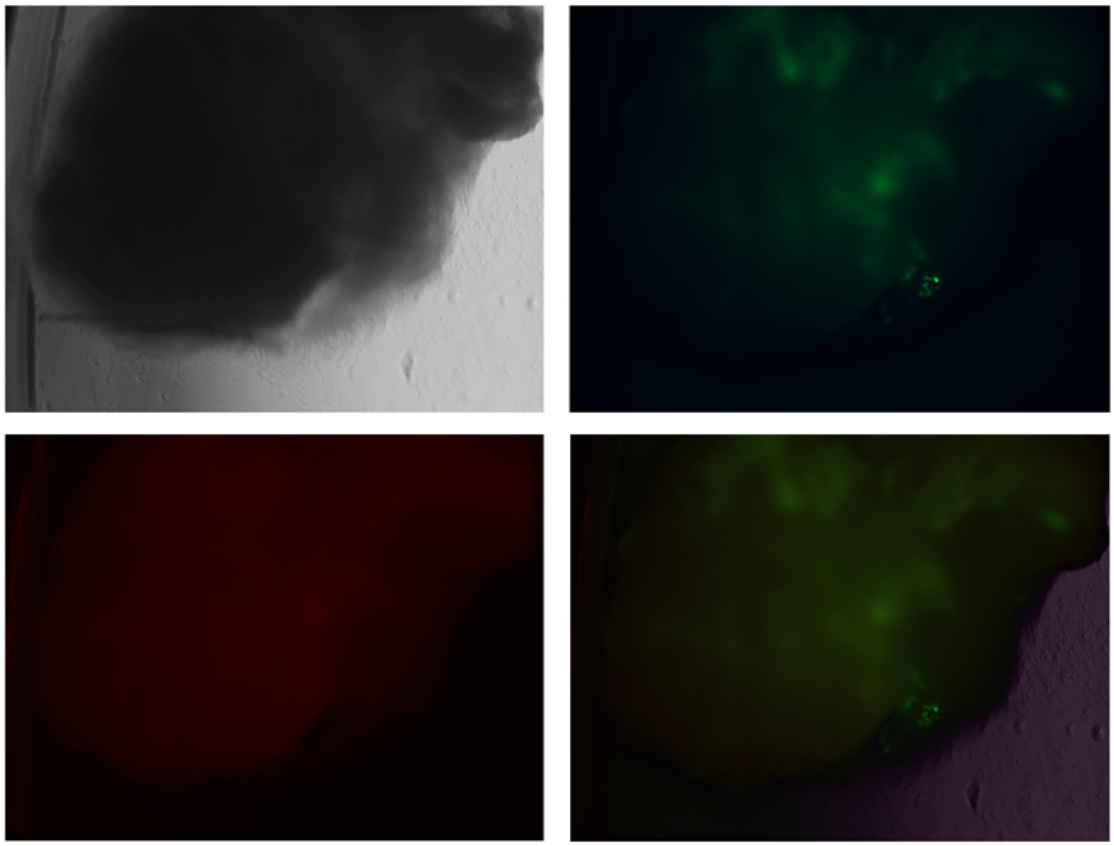


Figure 14. *Ex vivo* infection of an HPV+ head and neck tumour biopsy. Bright field, GFP (specific), RFP (background) and fluorescent overlay images from a representative HPV+ tumour biopsy following *ex vivo* infection with MG1-GFP, 4X magnification.

3.3. Discussion

Due to the intrinsically foreign nature of HPV-positive tumours, many therapeutic vaccine approaches have been applied in the clinic but with limited success as single agents against advanced disease. Here we describe a therapeutic approach in mice that induces complete and durable regression of the majority of advanced tumours with a mean volume of 300mm^3 at the start of treatment. The heterologous prime: boost regimen induces massive endogenous, specific CD8+ T cell responses with the potential to produce over fifty million E7-specific T cells per mouse. The ability of MG1 to acutely alter the transcriptional profile of the tumour microenvironment and preferentially replicate within HPV-positive patient biopsies justifies the rationale behind the design of this precision therapy and when combined with the vaccine's ability to generate such large numbers of T cells there is great promise for its future clinical application.

Mutations introduced to the therapeutic transgene neutralize the ability of the E6 and E7 HPV proteins to interact with and destabilize p53 and pRb. As Maraba is a rhabdovirus, it does not pose a risk of insertional mutagenesis due to the fact DNA is never manufactured in the virus' life cycle, which is entirely extra-nuclear[75]. The frequency of adenoviral integration into the host genome is low[301], however if cells transduced with Ad-E6E7 were to undergo an integration event with the E6E7 transgene, the resultant protein could not interfere with the functions of p53 or pRb. By including the full-length sequences of E6 and E7 from

HPV16 and 18 the vast majority of patients with HPV-associated cancer would be eligible for treatment with this vaccine with the potential to elicit responses against multiple potential epitopes. The mutant E6E7 transgene is devoid of the potential to destabilize p53 and pRb whilst maintaining an ability to induce antigen-specific immunity.

The dogma that the efficacy of an infectious disease vaccine is related to the ability of protective T cells being able to produce multiple cytokines is well established[302]. Multiple different populations of T cells, defined by their pattern of cytokine production, are induced following E6E7 vaccination, importantly all E7 specific, cytokine positive T cells are able to degranulate. Whilst Ad-E6E7 alone induces multifunctional T cells the numbers of these cells are vastly increased when MG1-E6E7 is used as a boost. More recently the value of multifunctional T cells has also been recognized in the realm of therapeutic cancer vaccines[303]. Boosting with MG1-E6E7 is able to generate significant numbers of antigen specific IL-2 positive CD8 T cells, such cells are a functionally important population for anti-cancer vaccine efficacy[304]. The ability of this oncolytic platform to generate multifunctional T cells further highlights its excellent profile as a therapeutic.

Treatment with this immunotherapeutic generates huge numbers of specific anti-tumour cytotoxic T cells and with the depletion of CD8+ T cells there is a loss of

efficacy. Intravenous administration of rhabdoviral vectors boost a primary immune response by optimally stimulating splenic central memory T cells in the absence of inhibitory effector T cells resulting in a massive proliferation of antigen specific T cells[282]. In patients the adoptive transfer of autologous tumour infiltrating lymphocytes following *ex vivo* expansion has the ability to be curative for metastatic cervical carcinoma with clinical outcome correlated to specific measures of anti-E6 and E7 immunoreactivity[51]. If a vaccine were to provoke sizeable specific immune responses then one would also expect a favourable clinical outcome, moreover a potent vaccine could be used to induce specific E6 and/or E7 responses in patients without these, thus enhancing prognosis. To the best of the authors' knowledge no studies have addressed the effect of number of cells adoptively transferred into TC1 bearing mice, however a positive correlation between numbers of cells transferred and tumour response does exist for treating established murine melanomas outlining the requirement for large T cell responses[305]. Therapeutic HPV vaccination strategies incorporating recombinant Listeria and peptide-based technologies are being extensively evaluated clinically due to their ability to generate specific CD8+ T cell immunity[306]; the responses generated by MG1-E6E7 are substantially greater in magnitude than those previously reported in the preclinical setting[307,308]. Recently other groups have been able to cure established TC1 tumours only when vaccines are administered with various other therapeutics leading them to speculate that vaccine induced endogenous immunity is only likely to be curative when part of complex

combination therapies[294,309]. Here we demonstrate that a straightforward vaccination regimen of two separate injections is able to generate extremely robust endogenous immunity and can completely ablate large murine tumours. Cured mice exhibit marked and durable persistence of specific CD8+ T cells with a relative expansion of central memory T cells over time, the lack of the latter is considered one potential cause for therapeutic cancer vaccination failure[303]. Whilst the prime: boost resulted in much more uniform regression of tumours, we were also able to demonstrate a somewhat remarkable response following treatment with a sham prime: MG1-E6E7; a single dose of systemic MG1-E6E7 was able to permanently cure a tumour comprising approximately 5% of the animal's body weight. We have shown in an advanced model of HPV-positive tumours that our MG1-E6E7 vaccination strategy is able to exert a curative effect.

Within 24 hours of the systemic administration of the oncolytic virus MG1-E6E7, the intra-tumoural immune transcriptional profile was significantly altered. Genes associated with antigen presentation and processing including Tapbp, Tap1, H2-K1 and B2m were up regulated following treatment with MG1-E6E7. Loss of antigen presentation is recognized as a significant cause of failure for cancer immunotherapy therefore therapeutic approaches to increase MHCI expression within the tumour, such as described here are highly desirable[310]. Alongside the induction of various genes associated with innate antiviral immune responses, increased expression of Cd28 and Il2ra was also of interest. Both CD28 and

IL2RA are receptor molecules with significant roles in the activity of T lymphocytes[311,312]; the exact mechanism by which MG1 is able to increase the expression of these genes and their respective roles within the tumour microenvironment remains to be determined. Interestingly Il1b was the only gene to have reduced intra-tumoural expression following MG1-E6E7 treatment. IL1 is frequently observed within tumours and secreted IL1β has been implicated in promotion of tumour invasiveness and systemic inflammatory syndromes associated with cancer such as cachexia[313]. Weight loss reminiscent of a cachexia type syndrome was observed in mice with progressing TC1 tumours so investigating the potential role of IL1β in this pathologic process is indicated. Maraba virus is able to acutely alter the expression of key immunologic genes within the tumour microenvironment thereby further enhancing its immunotherapeutic profile.

Multiple papilloma viruses are tumourigenic in many mammals, however viral infection is highly specific to the host species[314]. When E6 and E7 integrate into the genomes of human cells, as occurs in high-grade HPV malignancies cellular responses to type I IFNs are inhibited[293]. The data from the G-deleted VSV assay recapitulates this effect in a human epithelial cancer cell line. Primary HPV-positive head and neck tumour biopsies are highly permissive to MG1 infection when compared to HPV negative samples further emphasizing this phenomenon and predicting the sensitivity of such tumours to therapeutic oncolysis. The ability

of Maraba MG1 to exploit the cellular hallmarks of HPV-associated malignancy renders such tumours excellent targets for this novel therapeutic modality.

To date outcomes from therapeutic vaccination of patients suffering from advanced HPV-positive tumours have been disappointing. In established cancers it has been proposed that modification of the tumour microenvironment by oncolytic viruses is one mechanism by which failure of vaccination monotherapy due to tumour associated immunosuppression may be avoided[315]. The data presented in this study reveal that this purpose designed and readily translatable immunotherapeutic for HPV can induce huge numbers of multifunctional, specific anti-tumour CD8+ T cells capable of durably eradicating advanced TC1 tumours in mice. When such an effect is combined with the oncolytic potential of MG1 Maraba in patients suffering from advanced HPV-positive tumours we are optimistic this preclinical promise can be realized as a much needed and potent new treatment option by exploiting the essential mechanistic hallmarks of HPV-associated neoplasms.

~CHAPTER IV~

COMBINING PEPTIDE VACCINATION WITH ONCOLYTIC MG1-E6E7 FOR THE TREATMENT OF HPV-ASSOCIATED CANCER

4.1. Introduction

Priming and expanding specific anti-tumour T cells within the patient are the primary tenets of therapeutic cancer vaccination and numerous approaches have been designed to enable this[58,315]. Synthetic long peptides (SLPs) are versatile vaccine platforms capable of inducing specific CD8+ and CD4+ T cell responses and have been evaluated in many pre-clinical and clinical settings[315]. Peptide vaccines offer a variety of potential advantages over other platforms including being easily and rapidly tailor made, cost effective to manufacture, straightforward to clinically administer and unlikely to induce anaphylaxis[316]. The advent of other successful immunotherapeutics has helped re-ignite interest in various vaccine platforms including SLPs with optimism surrounding the clinical combination of vaccines with other novel immunotherapeutics[58].

Expression of the transforming viral oncogenes E6 and E7 seemingly make HPV-associated cancers an attractive target for therapeutic vaccination[295]. Pre-clinically administration of such peptide-based vaccines induces specific CD8+ immunity against the viral antigens and has curative potential in syngeneic murine tumour models; this has been documented using a variety of approaches[308,317,318]. Against the early stage HPV-associated cancer, vulvar intraepithelial neoplasia, SLPs from E6 and E7 of HPV16 were able to induce specific anti-tumour immunity and resulted in clinical improvement in 12 of 20 women treated; complete responses were observed in nearly 50% of patients[319].

Whilst specific immune responses were again observed in patients with advanced HPV-associated cancer, SLP vaccination did not delay disease progression in this setting however, the treatments were well tolerated[320]. Vaccination with SLPs generates specific anti-tumour immunity in HPV-associated cancer but is only efficacious for early stage disease.

Maraba virus is a potent oncolytic virus and can be engineered to boost T cell responses against specific TAAs[75,281,282]. A customised oncolytic Maraba virus (MG1-E6E7) for the treatment of HPV-associated cancer has been manufactured. This virus contains a tetravalent transgene based on attenuated sequences of E6 and E7 from HPV16 and 18. When mice are primed with a replicate deficient adenovirus expressing the same attenuated transgene and boosted with MG1-E6E7, massive and specific CD8+ T-cell responses against an HPV16 E6 and an HPV16 E7 epitope have been detected; furthermore this regimen is able to cure mice bearing advanced HPV16 positive TC1 tumours. We have also demonstrated that HPV-associated head and neck tumour biopsies significantly enhance the replication of MG1 Maraba virus within the tumour compared to HPV negative samples as the presence of E6 and E7 decrease innate protection of tumour cells against rhabdoviral infection. Finally we have shown that MG1-E6E7 is able to rapidly alter the immune transcriptome within the microenvironment leading to increased expression of MHCI genes. The excellent oncolytic potential of MG1 Maraba and the impressive ability of MG1-

E6E7 to boost immune responses formed the rationale for combining this custom made biologic with SLP vaccination.

Combining SLP vaccination with an oncolytic virus for cancer treatment is reported here for the first time in the preclinical setting of HPV-associated cancer. By using a previously designed oncolytic vaccination strategy we are able to show the induction of immunity against multiple HPV-specific CD8+ T cell epitopes and this data facilitates the informed design of SLP vaccines. Moreover we reveal that SLP vaccination is able to induce specific CD8+ immunity against HPV epitopes and the magnitude of immune responses is increased when SLPs are combined with MG1-E6E7. Finally the efficacy of SLPs and MG1-E6E7 against large HPV16 positive murine neoplasms is demonstrated and as such forms the basis of a novel therapeutic approach for HPV-associated cancer.

4.2. Results

Maraba virus infects and kills HPV-positive tumour cells

The syngeneic C57BL/6 cell line TC1 expressing HPV16 E6 and E7 was grown in a monolayer and infected over range of MOIs for 48 hours with MG1-E6E7 and cells were subsequently fixed then stained with crystal violet (Fig 15a). MG1-E6E7 lethally infects TC1 cells *in vitro*. TC1 tumours isolated from mice 24 hours after treatment with intravenous MG1-E6E7 harbour oncolytic virus as

determined by standard plaque assay (Fig 15b). Maraba virus is cytotoxic to TC1 cells *in vitro* and infects TC1 tumours *in vivo*.

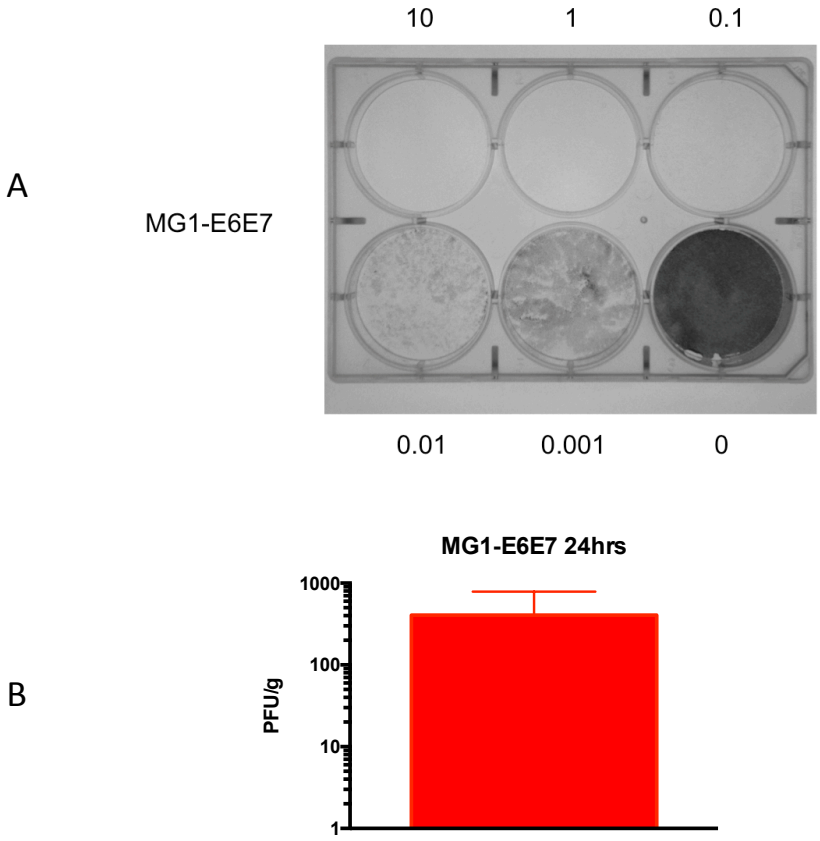


Figure 15. MG1-E6E7 virus lethally infects the murine HPV-positive cell line TC1 *in vitro* and is isolated *in vivo* from the tumour bed 24 hours after systemic administration. Monolayers of TC1 cells were cultured and infected with MG1-E6E7 then subsequently stained with crystal violet 48 hours after infection (MOI is indicated next to wells) (A). Mice bearing established subcutaneous TC1 tumours on their flank (n=4) received $1x10^9$ PFU MG1-E6E7 administered via their tail vein and tumours were harvested 24 hours after treatment for homogenisation and standard viral plaque assay (mean and SEM displayed) (B).

MG1-E6E7 induces specific immunity against multiple CD8+ epitopes

Epitope mapping was performed using an overlapping library of 15mer peptides from WT E6 and E7 sequences of HPV16 and 18. Splenocytes were isolated from mice primed with Ad-E6E7 intramuscularly and boosted with systemic intravenous MG1-E6E7 and were re-stimulated with peptides prior to intracellular staining for IFN-γ and surface staining for CD8+ and CD4+. The tetravalent transgene contained in these 2 viruses was able to induce CD8+ immune responses against many epitopes found in all 4 antigens of the sequence (i.e. HPV16 E6 and E7 as well as HPV18 E6 and E7) (Tables 5+6). Vaccination with Ad-E6E7: MG1-E6E7 is able to induce broad and marked immunity against multiple epitopes from E6 and E7 TAAs in two of the high-risk strains of HPV.

Table 5. Epitope mapping of the E6 antigens from HPV16 and HPV18. C57BL/6 mice (n=2) were primed with Ad-E6E7 and 9 days later were boosted with MG1-E6E7; 5 days after boosting mice were sacrificed and splenocytes were isolated. Intracellular staining of IFN-γ producing CD8+ T cells was performed after *ex vivo* re-stimulation of splenocytes with individual peptides from the overlapping 15mer peptide libraries of HPV16 and HPV18 E6. The magnitude of response to immunogenic peptides is summarised as follows: +=0.1%-1%, ++=1%-5%, +++=5%-20%, ++++=>20% of CD8+ T cells producing IFN-γ in response to the corresponding peptide. Peptides that were not synthesizable are denoted NS.

HPV16 peptides	**CD8+ IFNγ**	**HPV18 peptides**	**CD8+ IFNγ**
MHQKRTAMFQDPQER		MARFEDPTRRPYKLP	+
AMFQDPQERPRKLPQ	+	PTRRPYKLPDLCTEL	
QERPRKLPQLCTELQ	+	KLPDLCTELNTSLQD	+
LPQLCTELQTTIHDI	+	TELNTSLQDIEITCV	NS
ELQTTIHDIILECVY		LQDIEITCVYCKTVL	NS
HDIILECVYCKQQLL		TCVYCKTVLELTEVF	NS
CVYCKQQLLRREVYD		TVLELTEVFEFAFKD	NS
QLLRREVYDFAFRDL	+	EVFEFAFKDLFVVYR	+
VYDFAFRDLCIVYRD	+	FKDLFVVYRDSIPHA	
RDLCIVYRDGNPYAV		VYRDSIPHAACHKCI	+
YRDGNPYAVCDKCLK		PHAACHKCIDFYSRI	
YAVCDKCLKFYSKIS		KCIDFYSRIRELRHY	
CLKFYSKISEYRHYC		SRIRELRHYSDSVYG	
KISEYRHYCYSVYGT		RHYSDSVYGDTLEKL	
HYCYSVYGTTLEQQY	+	VYGDTLEKLTNTGLY	
YGTTLEQQYNKPLCD		EKLTNTGLYNLLIRC	+
QQYNKPLCDLLIRCI		GLYNLLIRCLRCQKP	
LCDLLIRCINCQKPL		IRCLRCQKPLNPAEK	
RCINCQKPLCPEEKQ		QKPLNPAEKLRHLNE	
KPLCPEEKQRHLDKK		AEKLRHLNEKRRFHN	
EKQRHLDKKQRFHNI	+	LNEKRRFHNIAGHYR	+
DKKQRFHNIRGRWTG		FHNIAGHYRGQCHSC	
HNIRGRWTGRCMSCC		HYRGQCHSCCNRARQ	+++
WTGRCMSCCRSSRTR		HSCCNRARQERLQRR	++
SCCRSSRTRRETQL		ARQERLQRRRETQV	+

Table 6. Epitope mapping of the E7 antigens from HPV16 and HPV18. C57BL/6 mice (n=2) were primed with Ad-E6E7 and 9 days later were boosted with MG1-E6E7; 5 days after boosting mice were sacrificed and splenocytes were isolated. Intracellular staining of IFN-γ producing CD8+ T cells was performed after *ex vivo* re-stimulation of splenocytes with individual peptides from the overlapping 15mer peptide libraries of HPV16 and HPV18 E7. The magnitude of response to immunogenic peptides is summarised as follows: +=0.1%-1%, ++=1%-5%, +++=5%-20%, ++++=>20% of CD8+ T cells producing IFN-γ in response to the corresponding peptide. Peptides that were not synthesizable are denoted NS.

HPV16 peptides	**CD8+ IFNγ**	**HPV18 peptides**	**CD8+ IFNγ**
MHGDTPTLHEYMLDL		MHGPKATLQDIVLHL	
TLHEYMLDLQPETTD		TLQDIVLHLEPQNEI	+
LDLQPETTDLYCYEQ		LHLEPQNEIPVDLLC	+
TTDLYCYEQLNDSSE		NEIPVDLLCHEQLSD	++
YEQLNDSSEEEDEID	NS	LLCHEQLSDSEEEND	++++
SSEEEDEIDGPAGQA	++	LSDSEEENDEIDGVN	++++
EIDGPAGQAEPDRAH	+++	ENDEIDGVNHQHLPA	+
GQAEPDRAHYNIVTF	++++	GVNHQHLPARRAEPQ	++
RAHYNIVTFCCKCDS	++++	LPARRAEPQRHTMLC	+++
VTFCCKCDSTLRLCV	++	EPQRHTMLCMCCKCE	++++
CDSTLRLCVQSTHVD		MLCMCCKCEARIKLV	++
LCVQSTHVDIRTLED	+	KCEARIKLVVESSAD	++
HVDIRTLEDLLMGTL		KLVVESSADDLRAFQ	++++
LEDLLMGTLGIVCPI		SADDLRAFQQLFLNT	++
GTLGIVCPICSQKP	+	AFQQLFLNTLSFVCP	NS
		LNTLSFVCPWCASQQ	NS

Informed design of synthetic long peptides

Dominant CD8+ epitopes from each of the four arms of the tetravalent transgene were used as a centre point to design 29mer SLP vaccines and these were custom manufactured; the minimal HPV16 E6 and E7 epitopes as used to re-stimulate PBMCs in chapter II were confirmed to be immunodominant by epitope mapping and were used for HPV16 SLP construction. 15mer peptides were

selected from HPV18E6 and HPV18E7 based on immunodominance and were used to design HPV18 SLPs (Fig 16). The four SLP vaccines namely 16E6, 16E7, 18E6 and 18E7 were designed to enable assessment of immunogenicity, and for the HPV16 vaccines, anti-tumour efficacy in a preclinical murine setting.

Peptide design

HPV16 E6 minimal epitope: VYDFAFRDL

SLP: YCKQQLLRREVYDFAFRDLCIVYRDGNPY

HPV16 E7 minimal epitope: RAHYNIVTF

SLP: DGPAGQAEPDRAHYNIVTFCCKCDSTLRL

HPV18 E6 overlapping epitopes: HYRGQCHSCCNRARQ/
HSCCNRARQERLQRR

SLP: NIAGHYRGQCHSCCNRARQERLQRRRETQ

HPV18 E7 epitope: KLVVESSADDLRAFQ

SLP: CKCEARIKLVVESSADDLRAFQQLFLNTL

Figure 16. Schema for rational HPV SLP vaccine design. Epitope mapping data was used to confirm the immunodominance of previously characterised 16E6 and 16E7 CD8+ peptides and these minimal epitopes were the centre point of 29mer SLP vaccines with additional E6 and E7

sequences flanking these minimal epitopes. Epitope mapping revealed 2 contiguous 15mer peptides to be immunodominant in the 18E6 antigen and they formed the centre point of the 18E6 SLP. A single immunodominant peptide was used as the centre point for the 18E7 SLP.

MG1-E6E7 enhances the immunogenicity of SLP vaccination

MG1-E6E7 was able to boost immune responses to HPV peptide vaccination. SLPs were administered to mice in combination with an agonistic murine anti-CD40 monoclonal antibody and polyIC as an adjuvant and given intraperitoneally. Groups of 5 C57BL/6 mice were vaccinated with either a single dose of the 4 SLPs, 2 doses of SLPs 8 days apart or SLPs boosted after 8 days with intravenous MG1-E6E7. ICS of CD8+ T cells was performed after re-stimulation with the short peptides (used for the design of SLPs) on PBMCs from blood samples taken 5 days after boosting (Figs 17a-d). Immune responses were elicited following vaccination with the 16E6, 16E7 and 18E7 peptides. No specific response against the 18E6 SLP was detected; epitope mapping of HPV18 E6 following Ad-E6E7: MG1-E6E7 vaccination was repeated and a separate vaccination experiment with 18E6 SLP: MG1-E6E7 was performed (data not shown) confirming lack of immunogenicity of the 18E6 SLP vaccine. An instability index for each of the SLPs was calculated as previously described[285]. For the 3 other SLP vaccines immune responses were detectable after vaccination with SLPs alone. However, when SLPs were boosted by MG1-E6E7, significantly higher frequencies of both single positive IFN-γ as well as double positive IFN-γ and TNF-α producing CD8+ T cells were induced against 16E6, 16E7 and 18E6

compared to both single and double dosing of SLPs (Figs 17e-h). MG1-E6E7 significantly increases the magnitude of specific CD8+ immune responses generated by SLP vaccination.

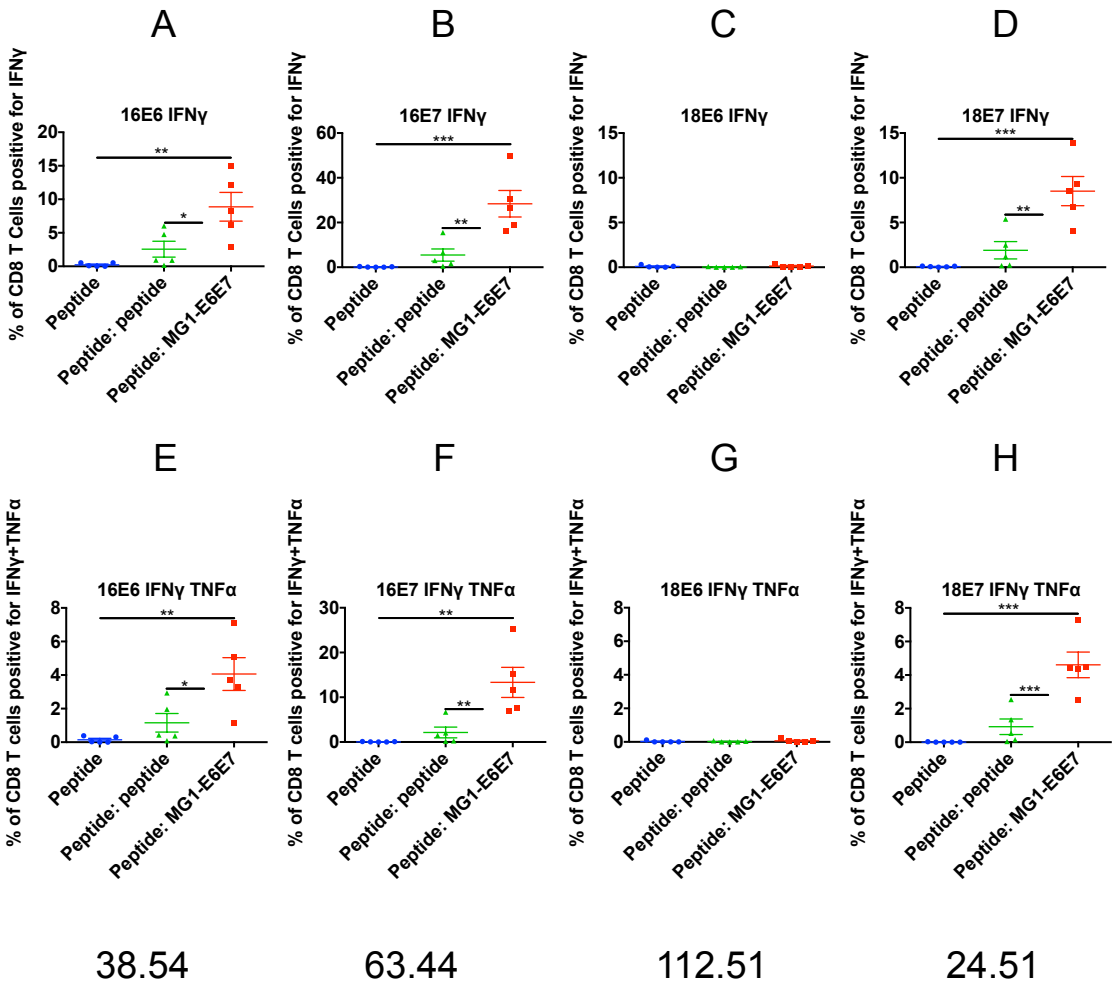


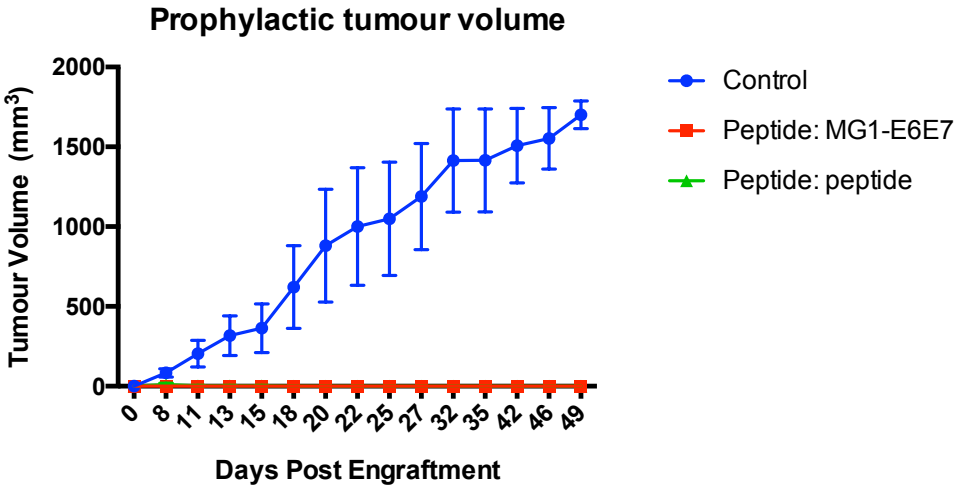
Figure 17. SLP vaccination induces specific CD8+ T Cell immunity. Tumour free C57BL/6 mice were vaccinated with a single dose of custom designed SLPs (n=5), 2 doses of SLPs (n=5) or a single dose of SLPs followed by MG1-E6E7 (n=5) to assess the induction of specific immune responses against the peptide epitopes used to design the SLPs as indicated by IFN-γ and TNF-α production from CD8+ T cells. Peripheral blood was collected 5 days after boosting. Frequencies

of single (IFN-γ) cytokine positive CD8+ T cells specific for 16E6 (A), 16E7 (B), 18E6 (C) and 18E7 (D) are shown at the top of the panel. Frequencies of double (IFN-γ + TNF-α) cytokine positive CD8+ T cells specific for 16E6 (E), 16E7 (F), 18E6 (G) and 18E7 (H) are shown in the middle of the panel (mean and SEM displayed, comparisons performed using ANOVA, *p≤0.05, **p≤0.01, ***p≤0.001). Corresponding peptide instability scores, displayed at the bottom of the panel, were calculated for each SLP with a higher score correlated with increased instability of peptide sequences.

SLP vaccination induces sterilising immunity against HPV-positive cancer

Following vaccination with 2 doses of the SLP vaccines (peptide: peptide) or SLP vaccination boosted by MG1-E6E7 (peptide: MG1-E6E7) mice were challenged with $1x10^6$ of TC1 cells (murine HPV16 positive cell line) subcutaneously. Both vaccination protocols were completely protective compared to a control group of untreated mice that all succumbed to tumour related end point (Fig 18). Both peptide: peptide and peptide: MG1-E6E7 displayed prophylactic activity in a syngeneic HPV mouse tumour model.

A



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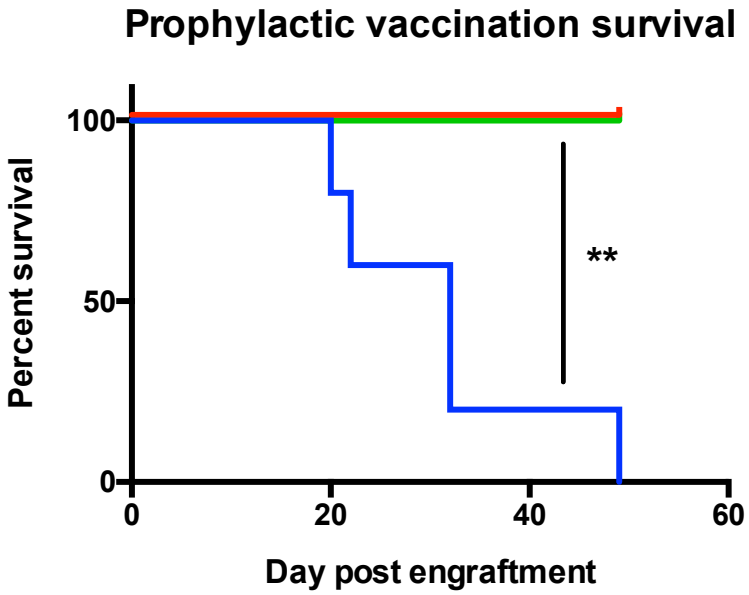


Figure 18. SLP vaccinated mice have complete sterilising immunity when challenged with HPV16 positive TC1 cells. Untreated mice (n=5), mice treated with 2 doses of SLP (n=5) and mice treated with SLP followed by MG1-E6E7 were challenged with $1x10^6$ TC1 cells subcutaneously 7

days after boosting. Mean tumour volumes with SEM are displayed (A). Survival times are displayed using a Kaplan Meier curve (B) (log rank test used to compare survival times, **p≤0.01).

SLP vaccination and MG1-E6E7 induce specific immunity in mice with advanced HPV-positive tumours

Mice bearing advanced subcutaneous TC1 tumours (mean volume of approximately 300mm^3) were treated with either 2 doses of 16E6 and 16E7 SLPs (peptide: peptide), 2 doses of MG1-E6E7 (MG1-E6E7: MG1-E6E7) or one dose of 16E6 and 16E7 SLPs followed by MG1-E6E7 (peptide: MG1-E6E7) with the 2 separate treatments being administered 8 days apart, a control group of untreated mice was also included. No detectable immune responses were observed in untreated mice. Responses against the immunodominant 16E7 epitope were documented in all groups of treated mice; the frequencies of single positive (IFN-γ) and double positive (IFN-γ + TNF-α) CD8+ T cells were significantly higher for the 16E7 epitope in mice treated with peptide: MG1-E6E7 compared to all other groups of mice (Figs 19b+d). Specific single and double positive CD8+ T cells against the 16E6 epitope were detected in both peptide: peptide groups and peptide: MG1-E6E7 mice, however no response was detected in mice treated MG1-E6E7: MG1-E6E7 for 16E6 (Figs 19a+c). Substantial immune responses were induced by SLP vaccination in tumour bearing mice and by combining SLP vaccination with MG1-E6E7 the size of

response against the immunodominant HPV16 E7 epitope in C57BL/6 mice was significantly increased.

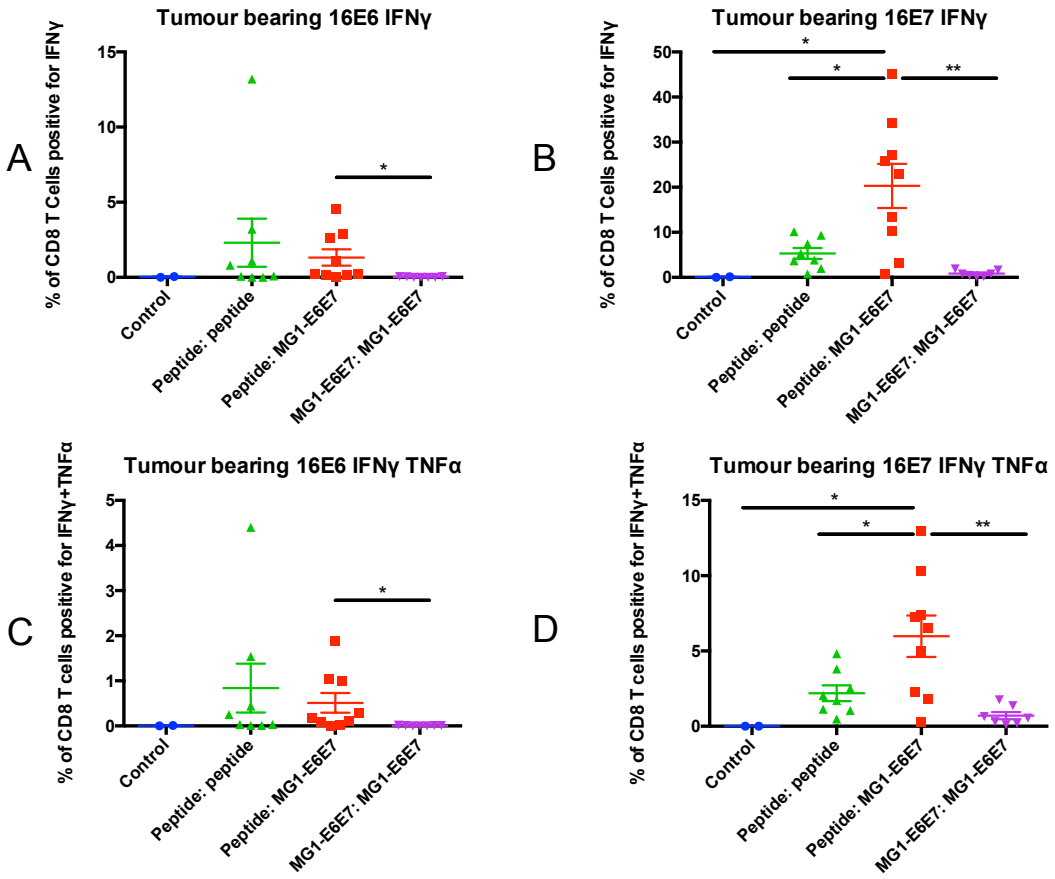


Figure 19. SLP vaccination induces dual cytokine positive CD8+ T cells in tumour bearing mice. Peripheral blood was collected from C57BL/6 mice with established HPV16 positive TC1 tumours to assess for the induction of specific immune responses against the HPV16 peptide epitopes used to design the SLPs as indicated by IFN-γ and TNF-α production from CD8+ T cells. PBMCs were isolated from mice vaccinated with 2 doses of HPV16 SLPs 8 days apart (n=8), 2 doses of MG1-E6E7 8 days apart (n=7) or a single dose of HPV16 SLPs followed 8 days later by MG1-E6E7 (n=9); an untreated control group (n=2) was also included. Blood was collected 5 days post boost. Frequencies of single (IFN-γ) cytokine positive T cells specific for 16E6 (A) and 16E7 (B)

are shown at the top of the panel. Frequencies of double (IFN-γ + TNF-α) cytokine positive T cells specific for 16E6 (C) and 16E7 (D) are shown at the bottom of the panel (mean and SEM displayed, comparisons performed using ANOVA, *p≤0.05, **p≤0.01).

Custom designed vaccination causes complete and durable regression of large TC1 tumours

Mice with large TC1 tumours had prolonged survival when treated with either peptide: peptide, MG1-E6E7: MG1-E6E7 or peptide: MG1-E6E7. Whilst MG1-E6E7 alone only delayed tumour progression in all but 1 mouse that had a complete response, 80% of peptide: peptide and 80% peptide: MG1-E6E7 treated mice had complete tumour regressions (Figs 20a-d). Cure rates of 60% and 50% were achieved for mice treated with peptide: MG1-E6E7 and peptide: peptide respectively (Fig 20e). Mice that were cured of disease were re-challenged with $2.5x10^6$ TC1 cells subcutaneously 104 days after boosting and all cured animals exhibited complete sterilising immunity whilst all treatment naïve animals succumbed to tumour progression (Fig 20f). Two doses of custom designed SLP vaccine or 1 dose of SLP followed by oncolytic MG1-E6E7 resulted in frequent, durable and complete responses when administered to mice with bulky neoplastic disease.

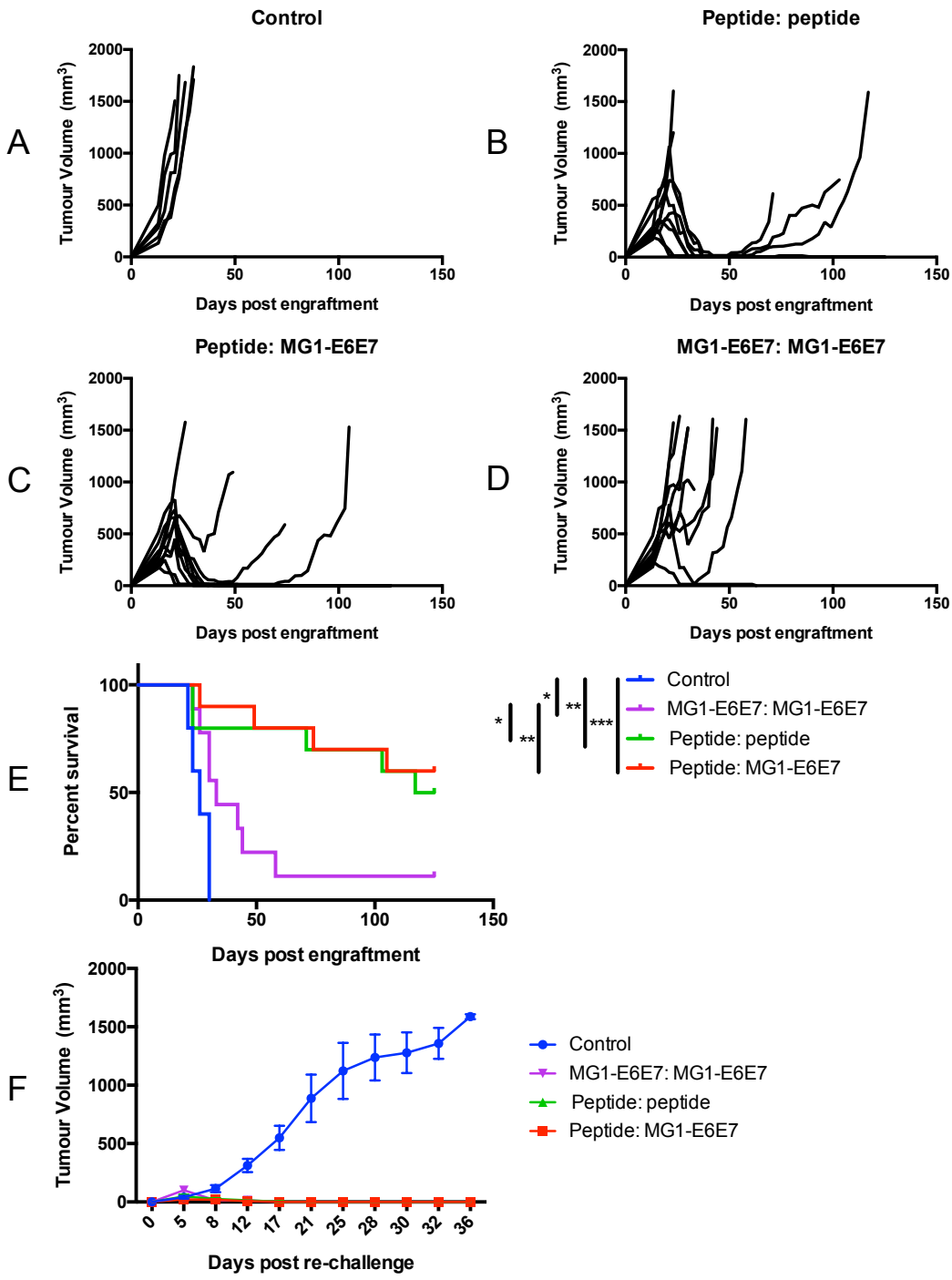


Figure 20. SLP vaccination results in complete regression of advanced TC1 tumours in mice. C57BL/6 mice with established TC1 tumours were vaccinated with 2 doses of HPV16 SLPs 8 days apart (n=10), 2 doses of MG1-E6E7 8 days apart (n=9) or a single dose of SLPs followed 8

days later by MG1-E6E7 (n=10) and a control group (n=5) of untreated mice was also included. Mice were primed 14 days after engraftment when mean tumour volume was approximately 300mm^3 and were boosted 22 days after engraftment. Individual tumour plots for the 4 different groups are displayed (A-D) and survival times are displayed using a Kaplan Meier curve (E) (log rank test used to compare survival times, *p≤0.05, **p≤0.01, ***p≤0.001)). Mice that were cured of disease (MG1-E6E7: MG1-E6E7 n=1, peptide: peptide n=5, peptide: MG1-E6E7 n=6) were re-challenged with 2.5x10^6 TC1 cells on the flank contralateral to the original tumour 104 days after boosting and a control group (n=5) of untreated mice was also challenged (F) (combined mean tumour volumes and SEM displayed).

4.3. Discussion

Currently therapeutic vaccination for advanced HPV-associated tumours is not clinically efficacious despite the recent progress of cancer immunotherapy. The data presented here demonstrate the rational and successful design of HPV specific SLP vaccines in a preclinical model of advanced HPV-associated cancer utilising the custom made oncolytic virus MG1-E6E7. Furthermore by combining SLP vaccination with an oncolytic virus, that is capable of altering the tumour microenvironment, we were able enhance the immunogenicity of SLP vaccinations and for the first time have shown this novel multimodal therapy is capable of eradicating large solid tumours.

Oncolytic viruses have a variety of anti-neoplastic effects and as such are versatile cancer therapeutics. MG1 Maraba was identified as a promising and selective candidate OV based on potent killing of numerous murine and human

tumour cell lines[75], the data here demonstrates the custom manufactured MG1-E6E7's *in vitro* killing activity in the TC1 cell line over a variety MOIs. Viable virus was isolated from established subcutaneous TC1 flank tumours 24 hours after administration via the tail vein. Intravenous dosing of OVs allows treatment of multicentric tumours with a single injection that practically is easier to administer than intra-tumoural delivery[266]. Once within the tumour bed OVs have various mechanisms by which they are cytotoxic to cancer cells including inducing apoptotic cell death, immunogenic cell death, immune cell activation, anti-angiogenesis and resultant tumour ischemia[54,55]. MG1-E6E7 is cytotoxic to a syngeneic murine HPV-positive cell line and systemic administration of this OV results in delivery to tumour sites distal to the injection.

Multivalent vaccines capable of inducing responses against a variety of epitopes are considered superior to vaccines invoking responses against single antigenic targets, as antigenic drift is less likely to occur with the former[321]. We have previously documented the generation of huge responses against a single E7 epitope using the Ad-E6E7: MG1-E6E7 oncolytic vaccination strategy; the data presented here complements this by revealing the broad diversity of responses invoked by this regimen. A number of other groups have published studies revealing the generation of specific anti-tumour immunity against various multi-epitope vaccines based HPV E6 and/or E7 utilising differing vaccine platforms and vectors[322–328], MG1-E6E7 is the first OV encoding a custom designed and

attenuated multivalent HPV transgene. Vaccination with Ad-E6E7 prime: MG1-E6E7 boost induced significant and specific immune responses against multiple epitopes encompassing all 4 antigens of the tetravalent transgene.

Epitope mapping data provided immunodominant epitopes and facilitated the informed design of HPV SLP vaccines in a preclinical setting. Specific immune responses against the 16E6, 16E7 and 18E7 SLPs were demonstrated via intracellular staining of CD8+ T cells from vaccinated mice. For the 3 effective SLPs, 2 doses of SLP 8 days apart resulted in a non-significant increase in the number of antigen specific CD8+ T cells relative to a single dose; when SLP vaccination was boosted with MG1-E6E7 the magnitude of responses for both single- and dual-cytokine positive T cells were significantly higher for all responding vaccinees. Mice vaccinated with either 2 doses of SLP or SLP combined with MG1-E6E7 had complete sterilising immunity when challenged with the TC1 HPV16 tumour model, thus clearly demonstrating the functional ability of the vaccine-invoked immune response. Induction of high numbers of dual functional IFN-γ+ TNF-α+ CD8+ T cells has been shown to be of benefit in preclinical therapeutic vaccination of HPV-associated cancer[329]. By rationally designing SLP vaccines functional HPV specific CD8+ T cells were generated, moreover the novel combination of SLPs with the bespoke oncolytic virus MG1-E6E7 was able to significantly enhance the magnitude of responses detected in immune competent mice.

Factors limiting the immunogenicity of anti-cancer vaccines have clear ramifications for the efficacy of these therapeutic agents. In the context of the virally encoded E6E7 transgene, overt immune responses were provoked against all 4 of the candidate epitope regions used to design the SLPs. Repeated testing failed to document any specific immunity following 18E6 SLP vaccination. The 18E6 peptide had an extremely high instability index as calculated by a widely cited algorithm based on dipeptide composition thus predicting instability of 18E6[285]. Of late there has been much interest around prediction of MHCI peptide binding affinities, the ability to estimate this *in silico* and the repercussions these processes have on vaccine design and efficacy for cancer immunotherapy[330,331]. The data presented here highlight the need to also consider the form in which immunogenic peptides are delivered *in vivo* as the physical and pharmacokinetic properties of SLPs can significantly affect their immunogenicity prior to antigen processing[332].

Intelligent design of HPV SLPs enabled eradication of advanced bulky TC1 tumours and for the first time preclinical activity of a novel SLP: MG1-E6E7 combination therapy was demonstrated. Treatment with MG1-E6E7 alone also delayed tumour progression compared to untreated control mice, however the therapeutic profile of MG1-E6E7 was enhanced when an SLP prime was employed exploiting MG1 Maraba's profile as a potent booster of CD8+ immunity[282]. Proposed mechanisms of activity for MG1-E6E7 include induction of

specific anti-cancer immunity, direct oncolysis of neoplastic cells as well as a previously demonstrated ability to acutely alter the transcriptome of the immune microenvironment. Furthermore human HPV-positive tumour biopsies greatly enhanced MG1 replication relative to tissue matched HPV negative tumour samples highlighting the oncolytic potential within this group of neoplasms. Although a few other studies have also demonstrated preclinical anti-cancer activity of OVs in HPV cancer, further research into the potential benefit of this versatile class of biologics for this indication is justified[298,333,334]. Whilst the eradication of large subcutaneous tumours by SLP vaccines in immunocompetent mice is impressive, other groups have published similar data utilising alternative peptide vaccine regimens demonstrating obvious efficacy in the TC1 model; this has also been observed in early clinical disease[308,317–319]. However, there has been no documented anti-tumour activity in clinically advanced HPV-associated cancer[320]. When SLP vaccination was combined with MG1-E6E7 in the therapeutic setting this regimen induced significantly higher frequencies of single- and dual-cytokine positive CD8+ T cells against the immunodominant 16E7 epitope relative to 2 doses of SLP vaccination and this therapeutic vaccination regime was also effective in ablating established solid HPV-positive tumours. Rejection of an aggressive re-challenge dose of TC1 cells emphasised the durability of the tumour responses observed. Logical vaccination strategies utilising MG1-E6E7 resulted in complete tumour responses in mice with advanced HPV-positive cancer.

Oncolytic viruses are relatively new anti-cancer agents available for use by oncologists and due to their diverse mechanisms of actions many of these viruses are currently undergoing assessment for clinical activity[262]. Unfortunately the early promise of SLP vaccination has not given rise to success in the treatment of advanced HPV-associated cancer and as such new combination therapies require investigation. For the first time we demonstrate the preclinical efficacy of combining SLP vaccines with the custom made OV, MG1-E6E7 as an alternate approach to SLP vaccination. As MG1-E6E7 is able to exert a variety of anti-cancer effects complimentary to those of standard vaccines clinical assessment of SLP: MG1-E6E7 is warranted. Moreover, as SLP vaccines have well demonstrated safety and immunogenicity, such a combinatorial therapy is readily translatable to the human clinic where novel immunologic approaches to treat advanced HPV-associated cancers are in great demand.

~CHAPTER V~

ONCOLYTIC VIRAL IMMUNOTHERAPY FOR CARCINOMA OF THE PROSTATE

5.1. Introduction

In 2012 it was estimated that prostate cancer had the second highest incidence rate for male non-skin tumours across the world accounting for approximately 1,100,000 cases as well as claiming over a quarter of a million lives in that same year[9]. Mortality rates in some developed countries are decreasing and this is thought to be from a combination of improved screening via PSA (prostate-specific antigen) testing, earlier intervention and advancement of more definitive treatment strategies[335,336]. Despite improvement in overall mortality rates, one study in 2014 found that over the previous 20 years there had been no survival improvement in patients presenting with *de novo* metastatic disease[337]. Recently there have been slightly more positive results regarding advanced forms of prostate carcinoma with cabazitaxel, abiraterone and sipuleucel-T all being licensed for CRPC (castrate resistant prostate cancer) since 2004[338]; a large study has also demonstrated the benefit of docetaxel for metastatic disease[339]. High-risk prostate cancer continues to pose a significant threat to male health and notwithstanding the recent progress of some systemic therapies new treatments for advanced disease are urgently required.

Over the last decade a variety of immunotherapeutic approaches have been assessed clinically for prostate cancer. Of particular note trials using autologous cell vaccines (sipuleucel-T) and poxvirus (rilimogene galvacirepvec) based therapies (targeting the prostatic acid phosphatase (PAP) antigen and prostate-

specific antigen (PSA) respectively) have both led to increased median survival times in patients with CRPC[64,340]. These vaccine-based studies alongside evidence of clinical efficacy of CTLA4 blockade via the monoclonal antibody ipilimumab[341] have all fuelled interest in the role of immunotherapy for the treatment of advanced prostate cancer.

Despite the recent progress made treating prostatic carcinoma with immunotherapy a significant opportunity remains to optimise these therapeutics by developing novel and combinatorial approaches[342]. Oncolytic viruses offer a new option for the treatment of prostate cancer. Numerous oncolytic viral strategies utilising VSV, adenovirus, vaccinia and HSV have been investigated at the preclinical level emphasising the potential of these biologics for this indication[232,343–346]. MG1 Maraba virus is a versatile OV that is able to exert direct oncolytic anti-neoplastic effects as well having the capacity to express various transgenes including tumour associated antigens that induce marked and specific anti-tumour T cell immunity[75,281]. A combinatorial approach employing viral oncolysis as part of a robust heterologous prime: boost platform for a relevant prostatic tumour associated antigen represents an unexplored avenue for prostate cancer therapy.

TRAMP-C2 is a transplantable prostatic cancer cell line isolated from a transgenic C57BL/6 male mouse that specifically expressed the proto-oncogene

SV40 large T antigen in prostatic epithelium under the control of the rat probasin promoter[347]. The TRAMP-C2 line is able to form tumours in syngeneic C57BL/6 mice and TRAMP-C2 have favourably lost expression of SV40 large T antigen (in this case this antigen was required for malignant transformation but not for the maintenance of this phenotype) whilst maintaining other features consistent with the lineage of prostatic malignancy[347]. Two candidate TAAs identified in human prostatic carcinoma are six-transmembrane antigen of the prostate (STEAP) and Wilms' Tumour Protein (WT1) with expression of both increased in malignant prostatic tissue[348,349]. Both STEAP and WT1 are reportedly expressed in TRAMP-C2 cells making this a murine tumour model with potential for investigating induction of an anti-neoplastic immune response against these two relevant antigens[350,351]. WT1 is a transcription factor that plays important roles in cellular differentiation and growth with expression in normal tissues limited to kidneys, reproductive organs and mesothelium; in the setting of cancer WT1 is overexpressed in many malignancies including various leukemic and solid neoplasms including prostatic carcinoma[352]. In normal tissue convincing data exists for the presence of STEAP protein within the prostate; mRNA expression has been variably reported in a variety of other tissues including the kidneys, testes, bladder and lungs[349,353,354]. STEAP's role in normal homeostasis is somewhat unclear, but it is believed to function as transmembrane ion channel and is required for intercellular adhesions and communication[354]. Tumours derived from multiple organs including prostate, bladder, lung and kidney

overexpress STEAP[354,355]. *In vivo* studies of mice with TRAMP-C2 tumours facilitate preclinical evaluation for targeting relevant overexpressed prostate cancer antigens in an immune competent setting.

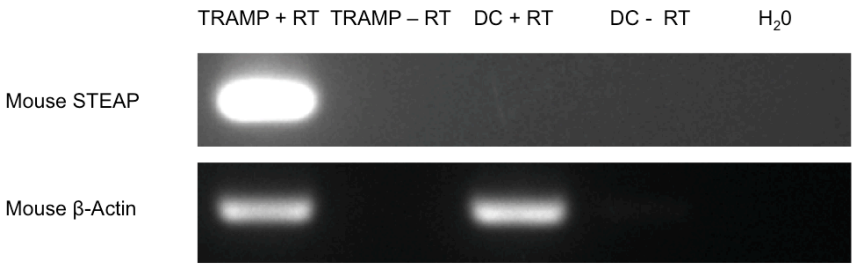
We hypothesised that by engineering MG1 Maraba virus to express prostatic TAAs we would be able to break tolerance against these self-antigens leading to therapeutic efficacy in an advanced model of prostate cancer in immune-competent mice. MG1 Maraba is able to exploit type I IFN defects of TRAMP-C2 cells and infect TRAMP-C2 tumours leading to transient reductions in tumour volumes. When MG1 Maraba is engineered to express human STEAP (MG1-STEAP) sizeable and specific CD8+ T cell immunity is induced against multiple STEAP epitopes leading to specific inflammation in the prostate of tumour free mice demonstrating the significant potency of this vaccine platform in breaching tolerance. A significant enhancement in the survival of male mice bearing large TRAMP-C2 tumours alongside a marked influx of tumour infiltrating lymphocytes (TIL) was demonstrated following treatment with oncolytic MG1-STEAP vaccination. Collectively the data presented here introduce a novel treatment approach for prostate cancer supporting clinical assessment of this strategy for this highly prevalent disease.

5.2. Results

TRAMP-C2 expresses relevant prostatic TAAs

TRAMP-C2, a syngeneic C57BL/6 murine model of prostate cancer was screened for the expression of TAAs applicable to human disease. RT-PCR performed on whole RNA isolated from TRAMP-C2 cells demonstrated expression of murine STEAP and murine WT1 (Figs 21a+22a), the products amplified were cloned and then submitted for DNA sequencing thus confirming their identity. Immunofluorescence was subsequently performed on fixed TRAMP-C2 cells and revealed cytoplasmic and membranous positivity for STEAP and nuclear staining of WT1 (Figs 21b+22b). TRAMP-C2 cells express both STEAP and WT1 antigens.

A



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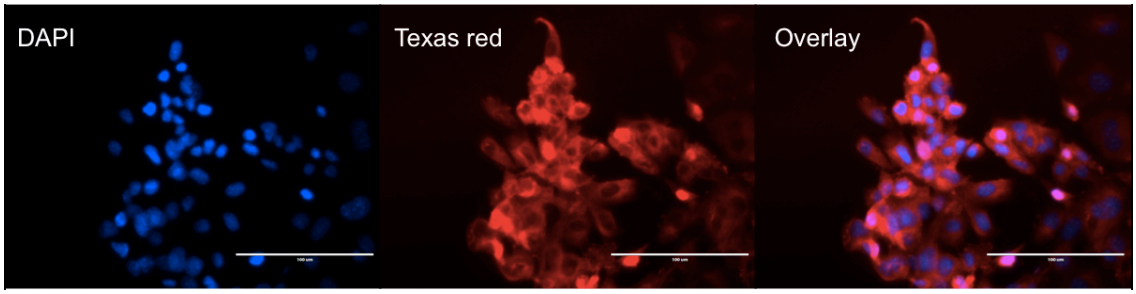
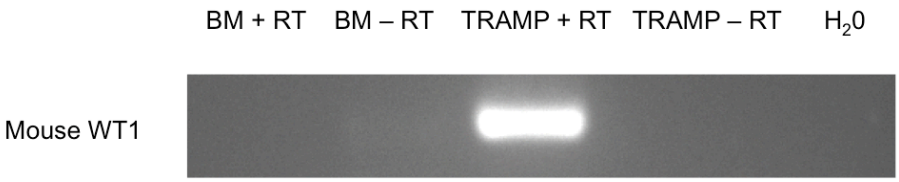


Figure 21. TRAMP-C2 cells express the TAA STEAP. RT-PCR confirms expression of STEAP in TRAMP-C2 cells. RNA was extracted from TRAMP-C2 cells and murine dendritic cells (DC) as a negative control; standard RT-PCR was performed to amplify cDNA (reactions were also performed without reverse transcriptase to confirm subsequent products were not amplified from genomic DNA contamination). Primers for murine STEAP, murine β actin and a reaction containing water in the absence of template were used to complete standard PCR and products were run out on agarose gel and subsequently imaged to confirm gene expression (A). The STEAP band was dissected out, cloned and sequenced. Immunofluorescence of TRAMP-C2 cells was performed using a polyclonal STEAP antibody and a fluorescent-conjugated secondary antibody and nuclei were stained with DAPI. Images were captured on the DAPI and Texas Red channels and a composite overlay image was made (B) (white bar represents 100μm).

A



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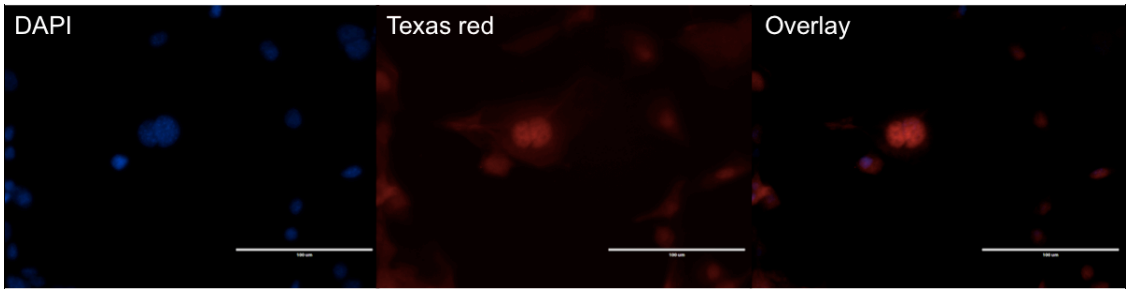


Figure 22. TRAMP-C2 cells express the TAA WT1. RT-PCR confirms expression of WT1 in TRAMP-C2 cells. RNA was extracted from TRAMP-C2 cells and murine bone marrow (BM) as a negative control; standard RT-PCR was performed to amplify cDNA (reactions were also performed without reverse transcriptase to confirm subsequent products were not amplified from genomic DNA contamination). Primers for murine WT1 and a reaction containing water in the absence of template were used to complete standard PCR and products were run out on agarose gel and subsequently imaged to confirm gene expression (A). The WT1 band was dissected out, cloned and sequenced. Immunofluorescence of TRAMP-C2 cells was performed using a polyclonal WT1 antibody and a fluorescent-conjugated secondary antibody and nuclei were stained with DAPI. Images were captured on the DAPI and Texas Red channels and a composite overlay image was made (B).

Maraba virus lethally infects TRAMP-C2 cells and TRAMP-C2 are poorly protected by type I IFN

TRAMP-C2 cells were screened both *in vitro* and *ex vivo* for potential susceptibility to Maraba viral oncolysis. TRAMP-C2 cells in a monolayer were infected with MG1-GFP over a range of MOIs and cells were killed by 48hrs at all MOIs as assessed by crystal violet viability staining (Fig 23a). Pre-treatment of TRAMP-C2 cells with murine IFN- β led to only partial protection from rhabdoviral infection compared to the IFN responsive L929 cell line (Fig 23b). Subcutaneous TRAMP-C2 tumours were harvested from mice, infected with MG1-GFP and maintained in tissue culture conditions; infection of tumour cores was demonstrated by fluorescence (Fig 23c) and confirmed by MG1 immunohistochemistry (Fig 23d). Mice bearing advanced subcutaneous TRAMP-C2 tumours (mean tumour volume of 250mm^3 at first treatment) had 3 doses of MG1-GFP administered IV and tumour volumes were monitored. Treatment with the direct oncolytic regimen led to temporary decrease in tumour size but did not significantly enhance overall survival (Figs 23e+f). TRAMP-C2 cells are susceptible to infection by Maraba virus both *in vitro* and *ex vivo*; TRAMP-C2 cells are only mildly protected by type I IFN and direct oncolytic treatment of mice bearing advanced TRAMP-C2 tumours results in a transient reduction in tumour volumes.

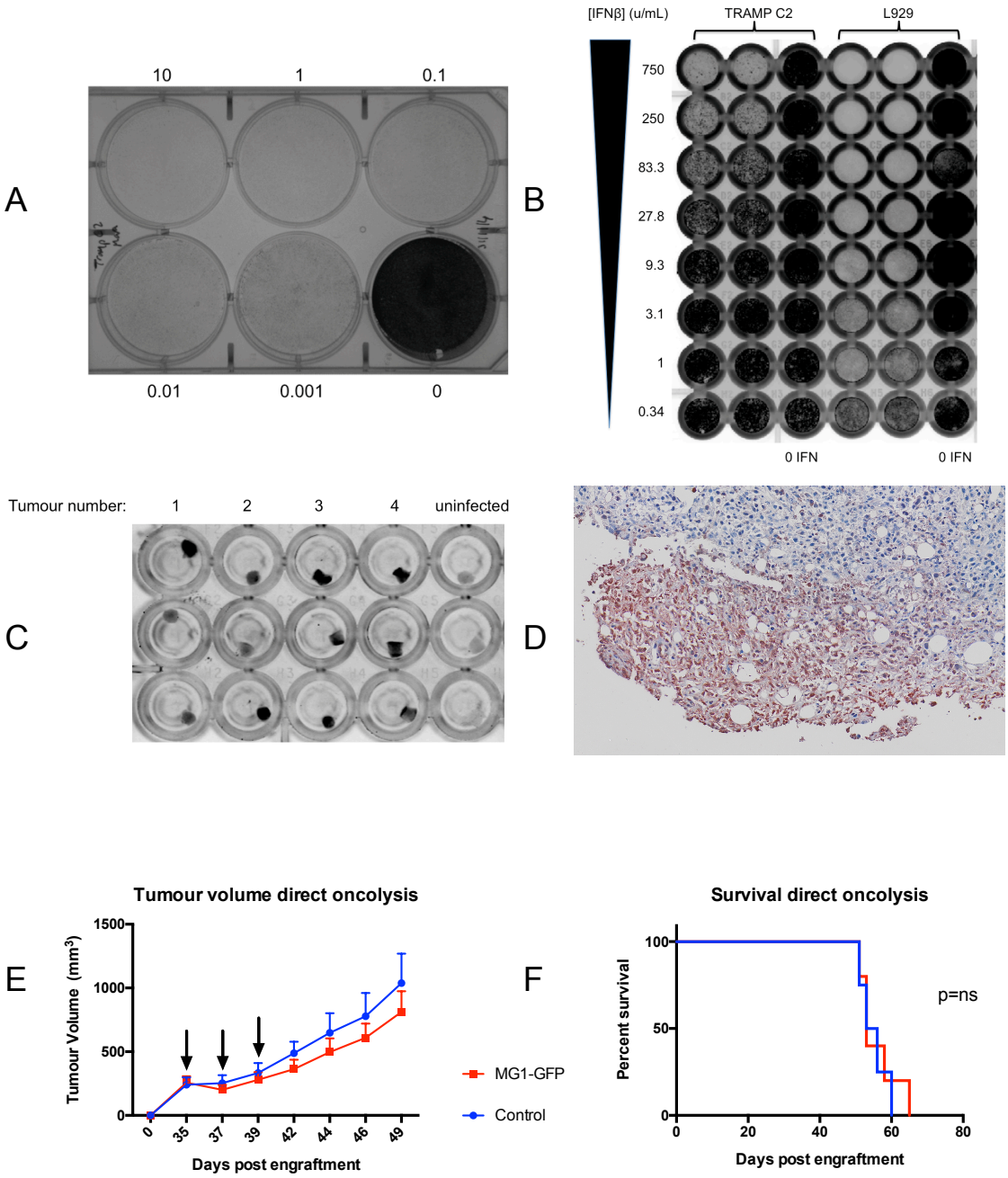


Figure 23. Direct viral oncolysis of TRAMP-C2 tumours by MG1-Maraba. Monolayers of TRAMP-C2 cells were cultured and infected with MG1-GFP then subsequently stained with crystal violet 48 hours after infection (A) (MOI is indicated next to wells). Monolayers of TRAMP-C2 and the type I IFN responsive control L929 cells were pre-treated with murine IFN-β (excluding 0 IFN

control columns) prior to infection with a WT VSV expressing GFP and infection was demonstrated by black signal indicative of fluorescence (B). TRAMP-C2 tumours were harvested from mice (n=4), processed into uniform cores, maintained in tissue culture and infected with MG1-GFP *ex vivo*; infection was demonstrated by fluorescence as depicted by black signal (C) and confirmed in 1 core immunohistochemically (D) (IHC captured using a slide scanner at 20x magnification). Treatment of male mice with established TRAMP-C2 tumours with 3 intravenous doses of MG1-GFP (n=5) results in transient decrease in tumour volumes (mean and SEM displayed) (E), but does not significantly prolong survival over untreated control mice (n=4) (log rank test used to compare survival times) (F).

Oncolytic vaccination with prostatic xenoantigens induce CD8+ immunity

Two pairs of replication deficient adenoviruses and MG1-Maraba viruses were manufactured with the first set containing a human STEAP transgene and the other human WT1. For initial screening 2 groups of tumour-free female C57BL/6 mice were vaccinated with either WT1 or STEAP vectors. ICS was performed on PBMCs re-stimulated with STEAP or WT1 peptides from blood samples obtained 13 days after adenoviral priming and 6 days after the first dose of MG1. Only minor specific CD8+ T cell immunity was observed post MG1 boost following re-stimulation with one of the WT1 peptides in WT1 immunised mice (Figs 24a+b). Specific CD8+ T cell responses were documented against all 4 STEAP peptides in STEAP vaccinated mice with the largest magnitude observed for the human specific epitope (h327) (Fig 24c+d). Tumour-free male C57BL/6 mice were subsequently vaccinated with the STEAP vectors alongside a separate group of control-vaccinated mice; only STEAP vaccinated mice had specific immunity and

the largest responses were again directed against the h327 epitope, no responses were observed against the conserved 5-13 epitope in male mice (Fig 24e+f). Specific T cell immune responses can be generated by immunising mice against relevant prostate cancer xenoantigens in the context of an oncolytic vaccination regimen.

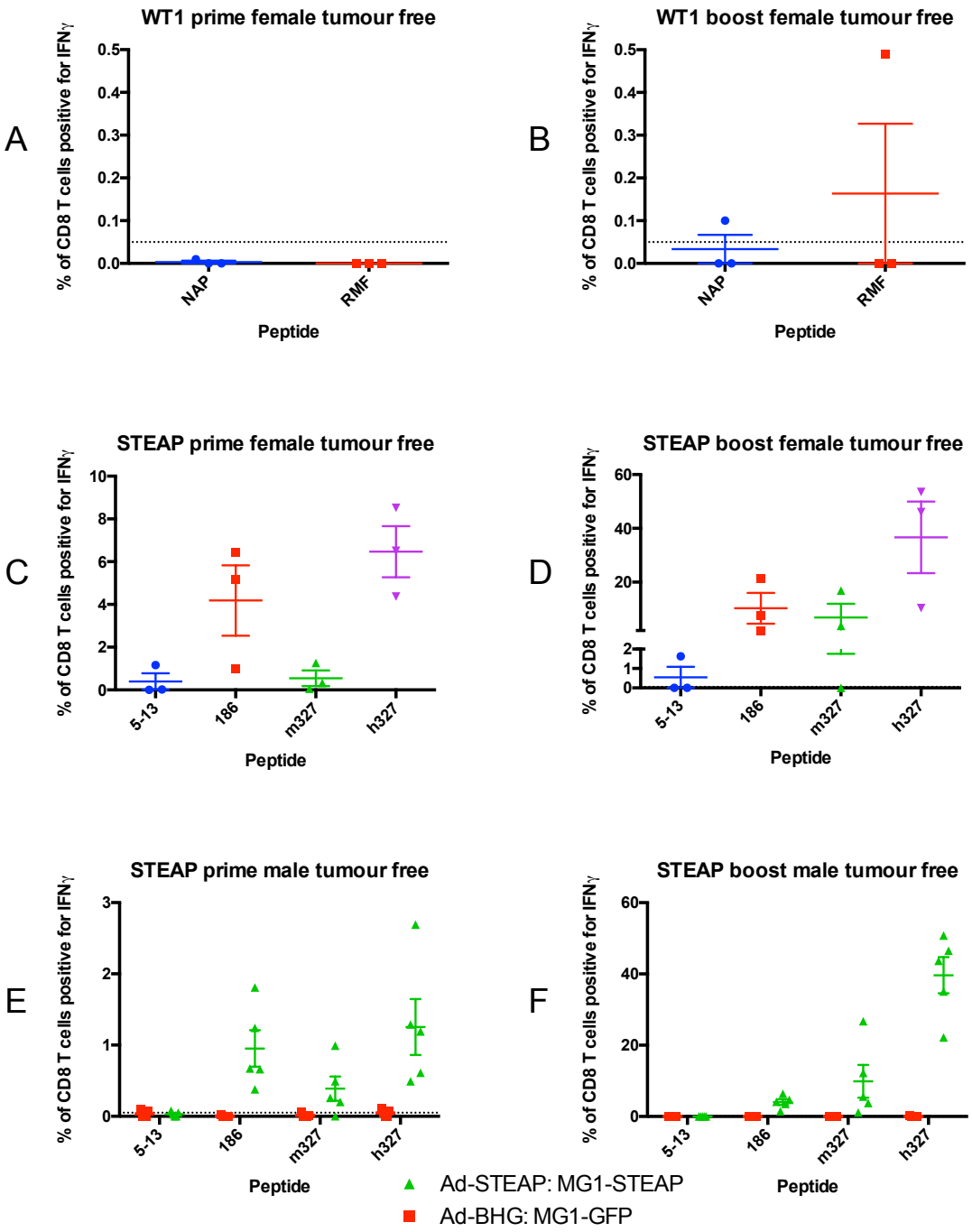
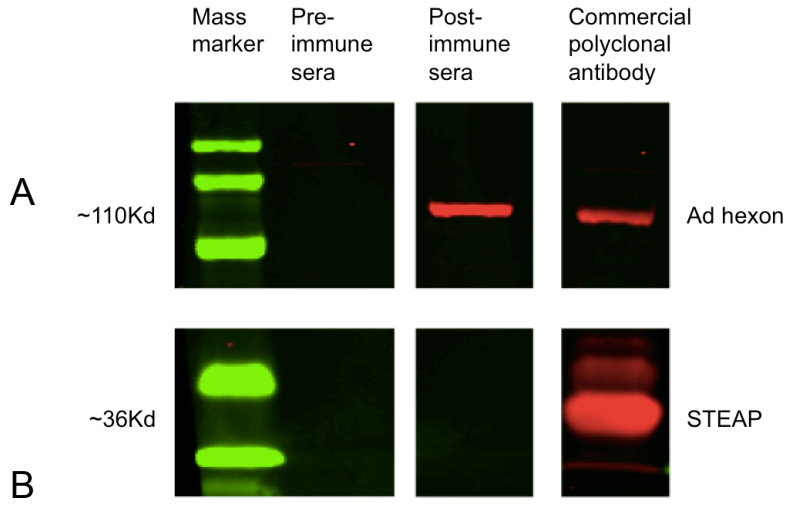


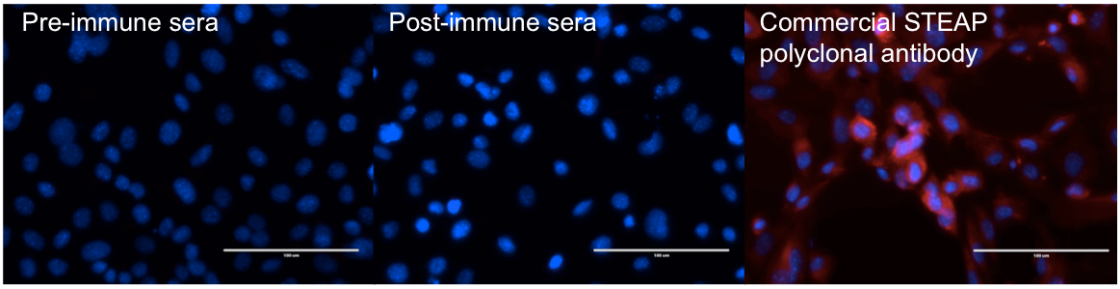
Figure 24. Oncolytic vaccination induces specific CD8+ T cells against multiple transgene epitopes. Tumour free female C57BL/6 mice were treated with oncolytic vaccination against WT1 (n=3) (Figs 4a+b) and STEAP (n=3) (Figs 4c+d) to assess for the induction of specific immune responses against known WT1 and STEAP peptide epitopes as indicated by IFNγ production

from CD8+ T cells collected after the prime and boost (mean and SEM displayed). Tumour free male mice were treated with oncolytic vaccination against STEAP (n=5) as well as a control group of mice treated with Ad-BHG: MG1-GFP (n=5) and ICS was performed after prime (Fig 4e) and boost (Fig 4f) (mean and SEM displayed).

Oncolytic vaccination does not result in humoural immunity against STEAP

As STEAP is a surface associated antigen, sera were collected and pooled from 5 mice for analyses 15 days after boosting in mice vaccinated against STEAP. Sera were used to probe protein lysates of HEK293s infected with Ad-STEAP; post-vaccinated mouse sera revealed seroconversion against the adenoviral hexon protein but no antibodies were detected against STEAP, commercial antibodies confirmed the presence of both proteins in the lysate (Fig 25a). Post-immune sera was also used to probe fixed TRAMP-C2 cells for immunofluorescence; STEAP was not detectable using either pre- or post-immune sera (Fig 25b). A functional assay for assessment of antibody-dependent complement mediated cytotoxicity revealed no evidence of cell killing in post-vaccinated sera in the STEAP positive cell line TRAMP-C2 (Fig 25c). Evidence of antibody generation against the adenoviral priming vector was documented but seroconversion against the STEAP transgene was not observed in any of three separate assays.





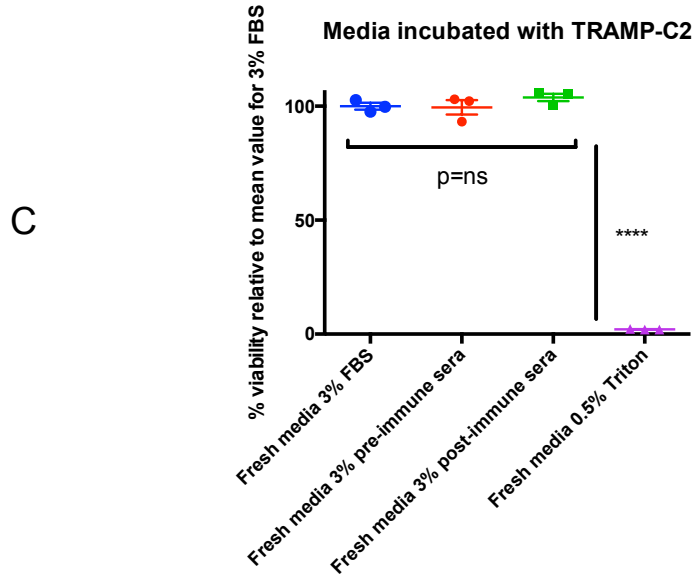


Figure 25. Oncolytic vaccination does not result in humoural immunity against the STEAP transgene. Lysates of HEK293 cells infected with Ad-STEAP were subjected to western blot and

probed with pre- and post-vaccination sera from mice treated with the oncolytic STEAP platform; the adenoviral hexon protein was detected using post-vaccination sera but STEAP was not observed, commercial antibodies demonstrated the presence of both proteins within the lysate (A). STEAP was not detected using immunofluorescence when TRAMP-C2 cells were probed with either pre- or post-immune sera; STEAP expression was previously confirmed in TRAMP-C2 cells using a commercially available antibody (white bar represents 100μm) (B). Antibody-dependent complement mediated cytotoxicity of TRAMP-C2 cells using immune sera was not detectable following STEAP vaccination in a functional *in vitro* assay (C) (mean and SEM displayed, comparison performed using ANOVA test, ****p≤0.0001).

Oncolytic STEAP vaccination induces target organ inflammation

As STEAP is an auto-antigen and oncolytic STEAP vaccination induces large CD8+ T cell responses tumour free male mice were vaccinated with the STEAP vectors and organs were harvested 6 weeks post MG1 for assessment of immunopathology. Mice treated with Ad-BHG: MG1-GFP and untreated mice were included as controls. No gross pathology was identified in any mouse. Histologic examination of H&E sections revealed no evidence of architectural change representative of primary pathology in the testes, lungs, bladder or kidneys of any mouse. No pathology was identified in the genito-urinary tract of the untreated and Ad-BHG: MG1-GFP mice. All 4 STEAP vaccinated mice had histologic changes within the prostatic tissue ranging from mild polymorphic cellular infiltrate within the glandular lumen to severe pleocellular infiltrate with accompanying epithelial dysplasia and epithelial hypertrophy consistent with prostatitis (Fig 26); prominent lymphoid follicles were noted within the bladder

wall of the mouse with the most marked prostatic changes and this was consistent with local reactive lymphoid hyperplasia secondary to prostatitis as no other abnormalities were identified within the remainder of the genito-urinary tract. Oncolytic STEAP vaccination is able to break immune tolerance resulting in prostatitis.

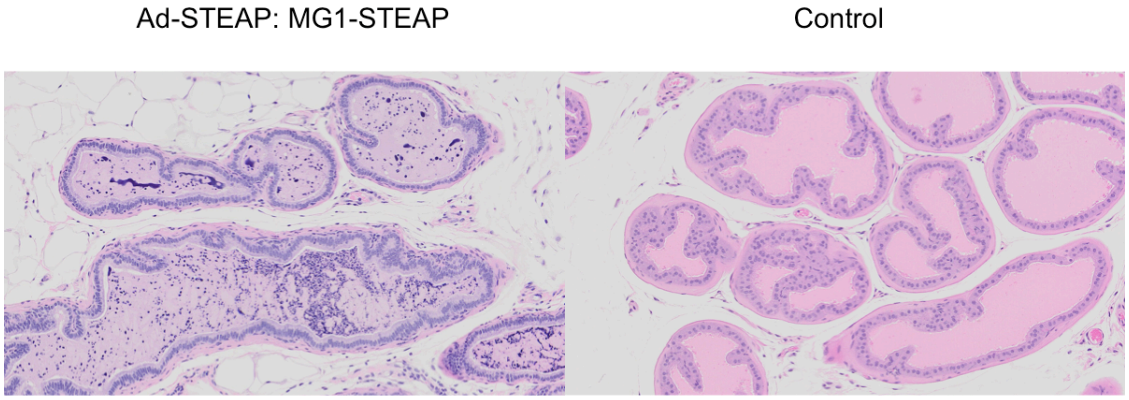


Figure 26. STEAP vaccination induces prostatitis. Prostatic tissue was harvested 6 weeks after MG1-STEAP boost was administered to male mice and sections were stained with haematoxylin and eosin. Prostatic tissue was also collected from age matched non-vaccinated control mice (images captured using a slide scanner at 20x magnification).

Oncolytic STEAP vaccination prolongs survival in mice bearing advanced TRAMP-C2 tumours

Male mice were engrafted subcutaneously with TRAMP-C2 and upon reaching a mean volume of approximately 250mm^3 were treated with various oncolytic vaccination regimens. Groups of mice received Ad-BHG: MG1-GFP, Ad-WT1: MG1-WT1, Ad-STEAP: MG1-STEAP and an untreated group of mice were

included as controls. Immune analysis by ICS of PBMCs re-stimulated with the relevant pooled murine peptides was undertaken after adenoviral priming and again after MG1 boosting. Compelling and specific CD8+ immunity was only noted in TRAMP-C2 bearing mice following vaccination with STEAP vectors in samples re-stimulated with the STEAP peptides (Fig 27a). Vaccination with the STEAP vectors resulted in a significant survival advantage over all the other groups of mice (Figs 27b+c). Whilst neither treatment with the Ad-BHG: MG1-GFP or the WT1 vectors improved survival significantly transient tumour responses were observed in both of these groups following systemic administration of MG1 Maraba (Figs 27b+c). Maraba virus has oncolytic activity against an established murine prostatic carcinoma and oncolytic vaccination against STEAP induces compelling CD8+ immunity and significantly prolongs survival in this model.

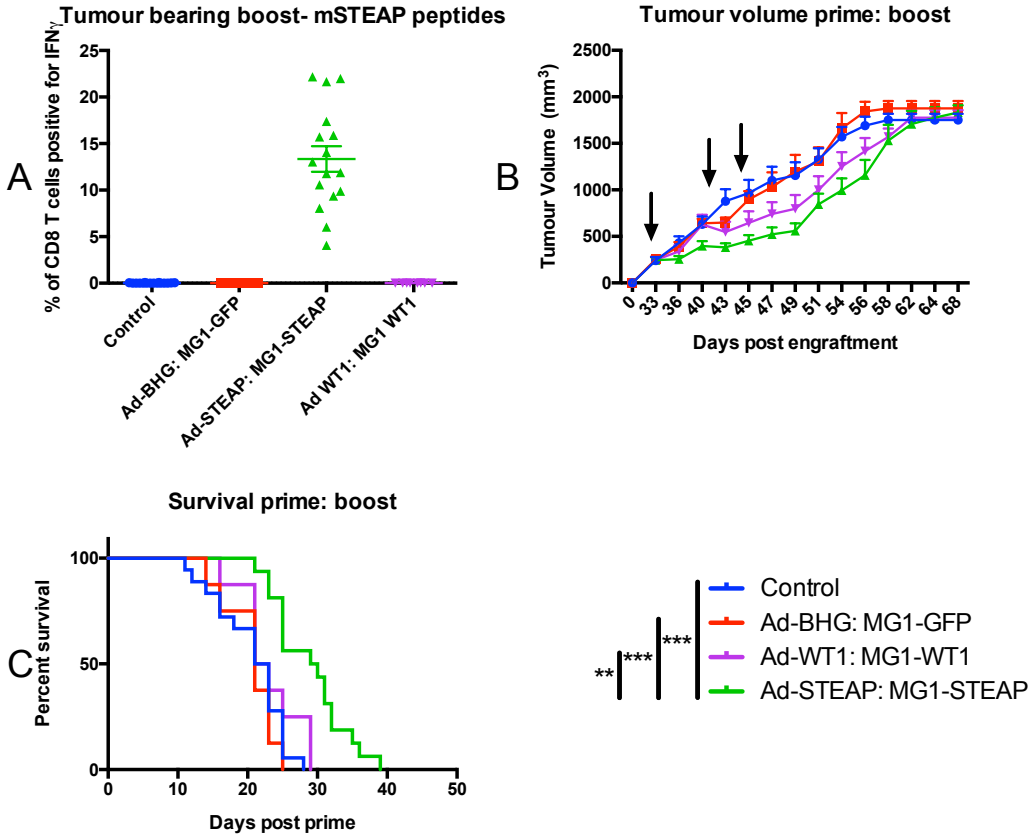


Figure 27. STEAP vaccination significantly prolongs survival in an aggressive murine model of prostate cancer. Male mice with established TRAMP-C2 tumours were treated with Ad-BHG: MG1-GFP (n=8), Ad-STEAP: MG1-STEAP (n=16) or Ad-WT1: MG1-WT1 (n=8) and compared to a control group of untreated mice (n=18) (data combined from 2 experiments). ICS on peripheral blood cells following re-stimulation with pooled murine STEAP peptides was performed at the time of peak boost responses (A) (mean and SEM displayed). Tumour volumes are plotted for each group from a single experiment (B) (arrows denote timings of Ad and MG1 treatments, mean and SEM displayed) and collective median survival times were calculated from the initiation of treatment (C) (log rank test used to compare survival times, *p≤0.05, ***p≤0.001).

Oncolytic STEAP vaccination induces intra-tumoural lymphocytic infiltrate

TRAMP-C2 tumours were harvested from mice vaccinated against STEAP 6 days after receiving the first dose of MG1-STEAP (n=6), untreated control tumours (n=4) from age-matched mice were collected concurrently and both sets were stained for CD3 and CD8a cell surface antigens. A marked CD3+ (Fig 28) and CD8a+ (Fig 29) inflammatory cell infiltrate consistent with an influx of TILs was noted following STEAP vaccination relative to a sparse population of such cells in untreated controls. Oncolytic STEAP vaccination leads to significant intra-tumoural lymphocytic recruitment thus transforming the microenvironment of immunologically quiescent tumours.

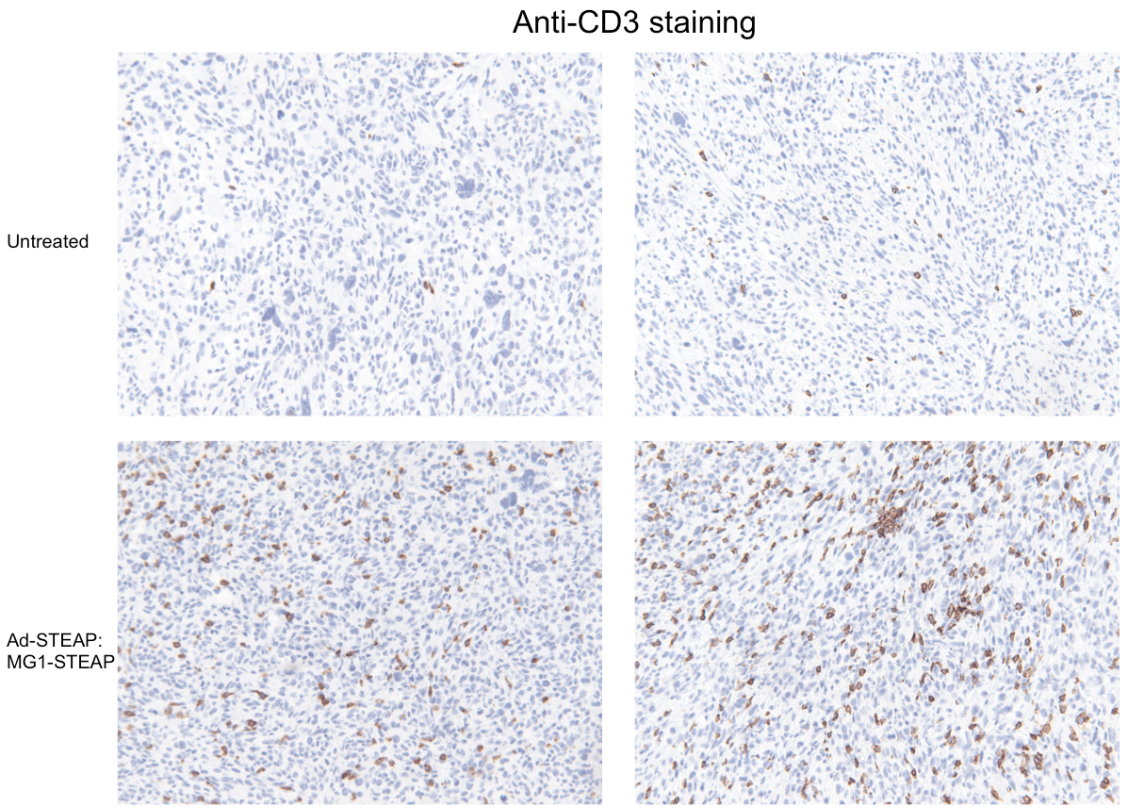


Figure 28. STEAP vaccination induces intra-tumoural CD3+ infiltration. TRAMP-C2 tumours were harvested from mice 6 days following the first dose MG1-STEAP and stained immunohistochemically for the CD3 cell surface antigen, nuclei were counterstained with haematoxylin, tumours were harvested at the same time point from untreated mice (20x magnification).

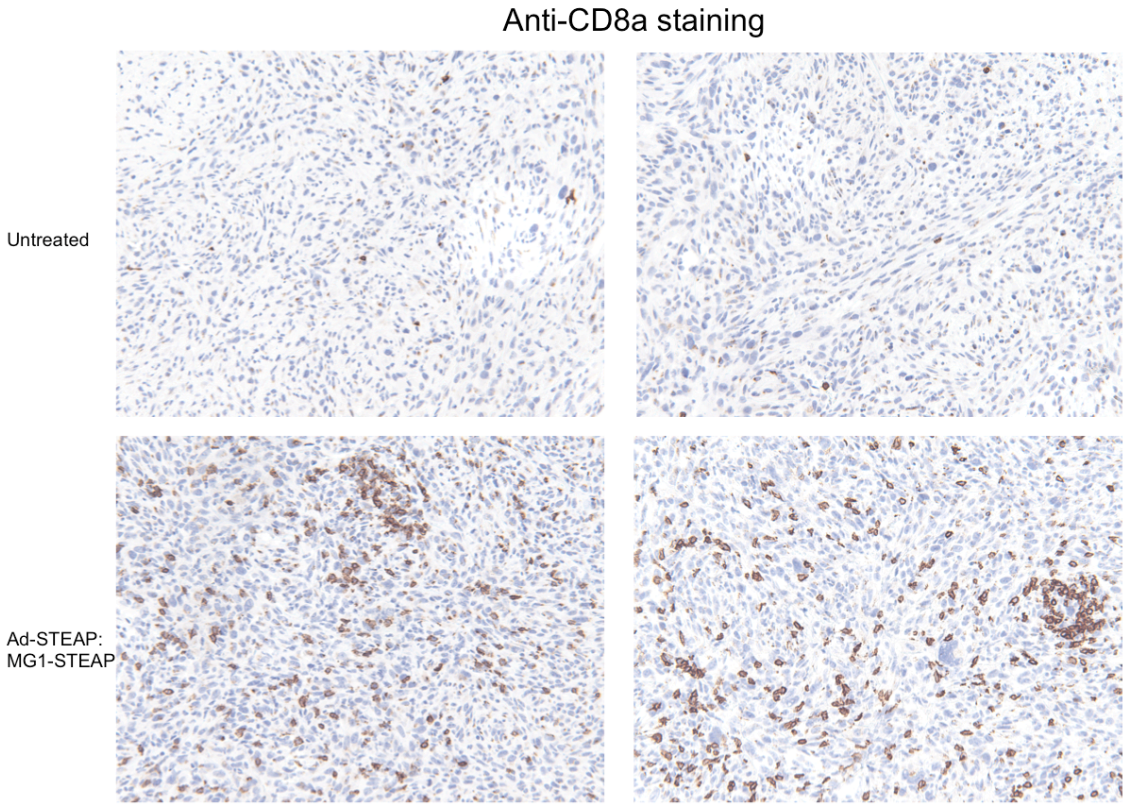


Figure 29. STEAP vaccination induces intra-tumoural CD8a+ infiltration. TRAMP-C2 tumours were harvested from mice 6 days following the first dose MG1-STEAP and stained immunohistochemically for the CD8a cell surface antigen, nuclei were counterstained with haematoxylin, tumours were harvested at the same time point from untreated mice (20x magnification).

5.3. Discussion

Heterologous prime: boost vaccination strategies are a relatively new therapeutic option for men suffering from advanced prostate cancer. PROSTVAC is the best characterised example and uses a vaccinia prime and fowlpox boost encoding

prostate-specific antigen (PSA) resulting in targeted immunity against PSA and a significant enhancement in overall survival[356]. Whilst such results are promising there exists much room for improvement as treatment with PROSTVAC alone does not enhance progression free survival and the majority of patients succumb to disease within 5 years of treatment[64]. Here for the first time we report the use an oncolytic virus as part of a heterologous prime: boost vaccination strategy in the setting of prostate cancer. The ability of this multi-faceted novel combination therapy to induce marked and specific anti-tumour immunity and significantly enhance survival in an immunocompetent murine model of advanced prostatic carcinoma supports the future evaluation of this approach in the clinical setting.

Incorporating an OV into a vaccination strategy broadens the anti-neoplastic mechanisms of the treatment described over conventional prime: boost platforms. Infection of tumours by OVs can result in a pro-inflammatory change to the tumour microenvironment[55] resulting in a tumour that is more likely to respond to immunotherapy[357]. Our data reveal that TRAMP-C2 cells have defective type I interferon responses and are susceptible to infection both in monolayer cultures but also in a heterogeneous *ex vivo* setting from advanced neoplasms. Whilst the treatment of bulky neoplasms with a direct oncolytic regimen did not extend survival times a repeatable transient tumour response was observed after systemic administration of MG1 Maraba. Defects in IFN responses have been demonstrated in number of primary human prostatic cancers predicting

susceptibility to oncolysis by MG1[358]. Recent observations have revealed that many tumour suppressor genes are key players in the type I IFN pathways[109]. PTEN is a tumour suppressor that is frequently mutated in prostatic cancer[359] and this mutation enhances oncolytic apoptosis induced by another rhabdovirus, namely VSV by exploiting IFN defects in a murine model of prostate cancer[360]. In the clinical setting we anticipate that the oncolytic activity of MG1 Maraba will be complimentary to its ability to induce marked immunity to relevant prostatic TAAs.

By engineering MG1 Maraba to express a human xenoantigen we were able to break tolerance against the murine form of the protein as demonstrated by responses against multiple CD8+ epitopes. Initial screening revealed that vaccination against STEAP resulted in much greater CD8+ immune responses than WT1, therefore STEAP was selected as a candidate antigen for further investigation. By encoding the entire antigen of interest we were to induce responses against various conserved, human and murine epitopes *in vivo*. Predicting immunogenic epitopes *in silico* is becoming more popular however; such approaches rely on complex bioinformatic procedures and as yet are not universally reliable[361]. Utilising a vaccine platform that encodes an entire antigen negates the need for such bioinformatic approaches and offers potential therapeutic value to all individuals irrespective of MHC haplotype.

Vaccinating tumour free mice against STEAP resulted in target organ inflammation whilst sparing other tissues providing evidence of safety and specificity in the preclinical setting. Normally murine STEAP expression is highest in prostatic tissue with low level expression reported in the kidneys and testes of male mice[353]. Consistent with this we observed the induction of prostatitis following oncolytic STEAP vaccination however; no gross or microscopic pathology was noted at six weeks post vaccination in the kidneys, testes, lungs or urinary bladder. Prostatitis following STEAP vaccination highlights the potency of our oncolytic platform, as indicated by a functional breach in immune tolerance. We have also observed a similar phenomenon in mice vaccinated with the same strategy against DCT (TRP-2), an antigen involved in the process of melanogenesis, developing vitiligo[281]. STEAP is over-expressed in various human malignancies including but not limited to prostatic, lung, renal and urinary bladder cancers[355,362–364]. Oncolytic STEAP vaccination results in the generation of specific immunity leading to prostatitis and this finding along with the absence of any other observed pathology emphasises the potential utility of directing cancer immunotherapies capable of inducing significant CD8+ immunity against this antigen.

In advanced and bulky TRAMP-C2 tumours oncolytic vaccination against STEAP significantly delayed tumour progression. Other groups have employed adenoviral STEAP primes followed by boosting with either DCs pulsed with

tumour cell lysates[365] or a modified vaccinia (Ankara) virus[366] and were able to demonstrate therapeutic efficacy in the TRAMP-C1 prostate model once tumours became palpable. In this study TRAMP-C2 tumours were treated at a more advanced and challenging stage when mean tumour volume reached 250mm^3 and we documented larger peripheral blood immune responses by ICS than those obtained in alternative STEAP based prime: boost platforms[365,366]. Boosting with MG1-STEAP compares favourably with other heterologous prime: boost vaccines targeting STEAP as not only is MG1 oncolytic but it also generates immune responses of a greater magnitude than previously described approaches.

Oncolytic vaccination against STEAP resulted in the generation of STEAP specific CD8+ T cell immunity whilst anti-STEAP antibodies in mouse sera were not detected. The exclusion of CD8+ T cells from the tumour microenvironment (TME) is considered a roadblock to successful immunotherapy in many solid tumours including prostatic cancer[367]; marked and widespread CD8+ infiltration of TRAMP-C2 tumours following oncolytic STEAP vaccination supports the ability of our therapeutic strategy to overcome this checkpoint. Low levels of MHCI expression by TRAMP-C2 cells act as a mechanism for evasion of immune attack by cytotoxic T cells in this model[368], thus the induction and intra-tumoural infiltration of large numbers of specific anti-tumour CD8+ T cells would not be anticipated to be curative for TRAMP-C2 tumours. Native STEAP is primarily

expressed within plasma membranes therefore generation of humoural immunity may also exert an anti-neoplastic effect[354,364]. Combining oncolytic STEAP vaccination with immunotherapeutic modalities capable of targeting membranous STEAP, such as monoclonal antibodies or CAR T cells, to enhance the efficacy of oncolytic vaccination against TRAMP-C2 tumours is worthy of future investigation.

Current clinical and preclinical data supports the value of vaccine-based approaches for the treatment of prostate cancer but combinatorial therapy and further optimisation of such therapeutics are required to improve their efficacy[342]. This study reports the first time a replicating oncolytic virus has been used as part of a prime: boost regimen with a relevant prostatic antigen. MG1-STEAP is able to boost substantial and specific CD8+ T cell immune responses, convert an immunologically “cold” TME to a “hot” (active) TME as well as exerting direct oncolytic activity against bulky murine prostatic tumours. Oncolytic STEAP vaccination represents a novel medical avenue worthy of clinical appraisal in the ongoing quest to develop safe and effective therapies for aggressive prostate cancer.

~CHAPTER VI~

SUMMARY AND CONCLUSIONS

This body of data summarises the work performed throughout my doctoral studies. The preclinical data compiled here describes novel, efficacious and readily translatable therapeutic approaches for two highly prevalent and frequently deadly groups of malignancies namely HPV-associated cancer and carcinoma of the prostate.

6.1. Summary of findings

By engineering MG1 Maraba to express TAAs relevant to human epithelial tumours we are able to combine a highly potent vaccine platform capable of boosting large numbers of tumour-specific CD8+ T cells with a multifunctional oncolytic virus. Developing technologies that induce robust effector and memory T cell responses is of paramount importance for the success of therapeutic cancer vaccination[369]. Of late there has been a great resurgence of interest in therapeutic cancer vaccines however, various barriers remain prior to their clinical establishment for the treatment of advanced malignancies[58]. Arguably one of the largest hurdles impeding the success of therapeutic vaccines is the immunosuppressive effect of the tumour microenvironment[315,369]. Oncolytic viruses have a unique functional ability to infect the tumour bed and thus convert a suppressive tumour niche to that of a pro-inflammatory environment via the liberation of TAAs, danger signals and immunostimulatory cytokines[55,370]. In the preclinical setting of advanced HPV-associated and prostatic cancer this strategy significantly extends survival times thus paving the way for future clinical trials.

Recognition of TAAs by activated T cells has been fundamental for the success and great amount of recent interest in cancer immunotherapy; this specific interaction has been pharmacologically targeted using a variety of approaches including checkpoint blockade, adoptive cellular therapies and vaccination[371]. Taken together this thesis demonstrates the potency of systemically administered MG1 Maraba to boost significant numbers of specific anti-tumour CD8+ T cells against foreign viral antigens in the case of HPV, and a tolerized self antigen in the case of STEAP, moreover the versatility of MG1 as a boost is also revealed as for the first time a novel combination of MG1 Maraba with SLP vaccination is also unveiled. In both of the models used within this thesis significant peripheral T cell immunity was accompanied by evidence of acute intra-tumoural T cell activation (Fig 12) or a marked influx of T cells in the TME (Figs 28+29), highlighting the ability of oncolytic viral immunotherapy to induce specific anti-tumour T cells capable of exerting an effect within the target tumour tissue. Whilst the induction of specific anti-cancer T cells using a vaccination approach is not new, the magnitude of responses that are documented here compare favourably with therapeutic vaccination strategies currently undergoing clinical appraisal for HPV-associated cancer[307,308] as well as pre-clinical prime: boost approaches designed to incite anti-STEAP immunity[365,366]. In the context of the prime: boost, direct infection of splenic follicular B cells by systemically administered MG1 Maraba facilitates antigen transfer to DCs in the follicular region of the spleen; in the case of a primed immune response TCM cells specific for the TAA against

which they were primed reside in these follicles, however effector T cells are absent from this niche[282]. This key functional and anatomic feature affords MG1 Maraba (encoding the TAA) the privilege of directly boosting TAA specific TCM cells (via follicular DCs that are unimpeded by T effector cells) resulting in a huge expansion of specific anti-tumour CD8+ T cells[282]. Intravenous MG1-E6E7 and MG1-STEAP are capable of inducing marked and specific anti-tumour immunity in preclinical HPV and prostatic cancer models and are therefore excellent candidates for development as therapeutic cancer vaccines.

For the successful outgrowth of a tumour a complex evolutionary myriad of changes take place within the TME and intrinsic to this pathologic process is the acquisition of many mechanisms by which cancer is able to evade immune destruction. *In vitro* MG1 Maraba was able to lethally infect both TC1 and TRAMP-C2 cells. Treatment of immunocompetent mice bearing advanced neoplasms with MG1 Maraba alone was infrequently curative. Whilst in terms of overall survival the effect of MG1 Maraba treatment was modest, significant changes to the immune composition of established TC1 and TRAMP-C2 tumours were documented. Loss of MHCI as well as the absence of TILs within the TME are two established negative prognostic indicators in a variety of human epithelial neoplasms[372]. Pre-clinically MG1 Maraba is able to increase the expression of MHCI and the TIL population, as shown in this thesis, and as such carries obvious translational potential for cancer immunotherapy alongside therapeutic

vaccine development. A variety of other approaches including chemotherapy, radiation therapy, adoptive cellular therapy and checkpoint blockade are being investigated for their ability to reverse tumour-associated immunosuppression[372]. Therapeutic utilisation of an OV to reverse such suppression following selective infection of the tumour bed seems logical as the recognition of microbes, including viruses, by the immune system results in an inflammatory response, the instigation of adaptive immunity and is an essential physiologic function of complex life forms[373]. MG1 Maraba is able to infect and immunologically transform the TME of HPV-associated cancer and carcinoma of the prostate, thus offering a novel multi-faceted mechanism of anti-tumour activity.

6.2. Limitations and future directions

Data from murine cancer models form the bulk of this thesis and undoubtedly the contribution of the laboratory mouse to the advancement of cancer research should not be underestimated. There are however, inherent limitations that prevent the direct translation of mouse-based research into the hospital, hence the need for stratified and stringent clinical appraisal of novel therapeutics. Relatively few tumour cell lines; spontaneous immunogenicity of transplantable models; rapid growth rate curtailing long term studies; genetic homogeneity specifically with relation to haplotype; limited spontaneous models and controlled environments that are incomparable to that of humans have all been described as potentially misrepresentative of human neoplastic pathology and

pharmacologic development[374,375]. One possible alternative to murine models is the use of pet dogs harbouring spontaneous malignancies for the study of novel cancer immunotherapeutics, this approach side steps many of the previously cited issues and is of potential benefit to both human and veterinary species[16,374]. The predictive value of using a mouse model of a tumour induced by a human cancer-causing virus, as is the case in HPV, also raises certain concerns regarding the biological relevance of this scenario. Species specific papilloma viruses result in neoplastic disease not only in people but also within cattle and dogs for example by specific bovine and canine papilloma viruses respectively[314]. It follows that the retroviral-mediated transduction of a murine cell line with the human viral proteins E6 and E7 from HPV16, as is the case for the TC1 cell line[376], is not the most aetiologically sound representation of HPV-associated carcinogenesis. Whilst the findings unveiled in this thesis allow for cautious optimism in the quest for new treatments of epithelial cancer, ultimately the success of any novel therapeutic approach will be judged by safety and efficacy profiles gleaned from trials within the cancer clinic.

Beyond the larger question of whether MG1 Maraba can be successfully applied to treat human epithelial tumours several lines of preclinical enquiry remain. In TRAMP-C2 tumours the largest immune responses and only significant survival advantages were seen following vaccination against STEAP, nonetheless, there were hints of immunologic activity following vaccination against WT1. As only 2

previously reported epitopes from WT1 were assessed, purchasing an overlapping peptide library may help uncover any other potential immunogenic epitopes within the WT1 sequence. Given the expression profile of this antigen within various tumours this may allow assessment of oncolytic WT1 vaccination in other suitable tumour models. In a similar vein the assessment of oncolytic STEAP vaccination in other models is also indicated to attempt to broaden the potential indications for this vaccine target. Oncolytic viral immunotherapy in mice with TC1 tumours was able to cure the vast majority of mice with advanced disease, therefore, assessing combinatorial strategies to enhance survival may prove challenging in this model due to the large numbers of mice required to power a significant survival advantage, however, we were unable to cure any of the TRAMP-C2 tumours. Two subsequent questions arise following this statement: why was the therapy not curative in the prostatic model and how can this approach be improved? Lack of MHCI expression, loss of target antigen (STEAP) expression and lack of a sustained T cell infiltrate within the TME coinciding with tumour growth are all plausible explanations for eventual disease progression in TRAMP-C2 and all are worthy of investigation. In terms of improving therapeutics the combination of vaccination with anti-STEAP antibody based approaches may address the first issue raised, however, if antigenic loss occurs then this would suggest that targeting STEAP alone may be problematic. Further evaluation of the TME at different time points as well as potential TIL profiling, may also shed light on reasons for treatment failure and how one would

attempt to rectify this. Finally the expression data from the NanoString® experiment in TC1 tumours would be bolstered by immunohistochemically comparing MHCI, CD28 and IL2RA in untreated tumours to tumours from mice treated with MG1-E6E7. Whilst the intrinsic limitations and remaining questions carry scientific intrigue, clinical trials assessing the use oncolytic viral immunotherapy for epithelial cancer do not hinge on the answers to such investigations and indeed such specifics may not be entirely relevant to the setting of human disease.

6.3. Concluding remarks

In summary the following points encapsulate the main findings of this thesis:

1. Ad-E6E7: MG1-E6E7 vectors encoding for an attenuated transgene based on HPV16 and 18 E6 and E7 induce massive and specific CD8+ T cell immunity capable of eradicating very large HPV+ TC1 tumours.

2. Ad-E6E7: MG1-E6E7 induces specific immunity against multiple CD8+ epitopes across the 4 arms of the encoded tetravalent transgene. These epitopes can be used to rationally design SLP vaccines capable of being used in concert with MG1-E6E7 resulting in durable cures of established TC1 tumours in an aggressive re-challenge model.

3. Ad-STEAP: MG1-STEAP provokes immunity against multiple CD8+ epitopes of this auto-antigen resulting in a functional breach of tolerance against the end target organ manifesting as prostatitis. When applied to an aggressive and bulky TRAMP-C2 model of prostatic carcinoma this treatment significantly extends survival times.

4. MG1 Maraba virus is able to infect HPV-associated cancer and prostatic carcinoma. In the setting of established murine tumours MG1 Maraba acutely increases the intra-tumoural expression of MHCI in the TC1 model and leads to a dramatic influx of CD3+ and CD8a+ cells in TRAMP-C2 tumours thus altering the immunosuppressive TMEs of these carcinoma models.

New clinical approaches to treat aggressive epithelial tumours are required to improve the outlook for numerous cancer patients. Currently no clinical trials are assessing the possible benefit of specifically targeting HPV-associated cancer with an oncolytic virus[262]. The use of therapeutic vaccinations for advanced prostatic carcinoma has unearthed the potential of this treatment strategy[342] and a variety of oncolytic viruses are at different stages of clinical evaluation for prostate tumours[377]. Using an oncolytic virus in the context of a potent vaccination platform tailored for prostate cancer is described for the first time in chapter V. Collectively the data compiled in chapters III, IV and V of this thesis

provide clear pre-clinical evidence that the novel approach of using custom designed oncolytic Maraba virotherapy is worthy of clinical appraisal for HPV-associated cancer and carcinoma of the prostate.

Despite the remarkable recent progress of cancer immunotherapy, advanced solid malignancies are continuing to prove challenging to cure[283]. Currently Ad-MAGEA3: MG1-MAGEA3 is undergoing clinical evaluation for a variety of solid neoplasms as a standalone therapeutic (NCT02285816) and also in combination with the checkpoint inhibitor pembrolizumab (NCT02879760). Data presented herein forms the major part of a pre-clinical body of evidence used to secure funding for future clinical trials of the MG1 Maraba platform to treat advanced HPV-associated cancer and carcinoma of the prostate. Well-designed clinical trials assessing the safety and efficacy of novel therapeutics are a cornerstone in the ongoing progress of modern medicine. It is the author's sincere hope that the subsequent clinical studies based around this thesis are able to help turn the tide and assist in improving the prognoses of people suffering from these commonly encountered epithelial malignancies.

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