IDENTIFYING NOVEL ENHANCERS OF THE ANTITUMOUR IMMUNE RESPONSE FOR CANCER IMMUNOTHERAPY

Oliver Varette

Supervisor: Dr. Jean-Simon Diallo

University of Ottawa

Abstract

Immunotherapy is a promising tool in the fight against cancer and aims to recruit patients own immune systems to seek out and destroy malignant cells. Options such as oncolytic viruses (OVs), autologous tumour vaccines and chimeric antigen receptors have shown clinical success to date, yet there remain significant hurdles to overcome. Here, we demonstrate a novel vaccine combining irrCell priming and infected cell boosting dramatically improves the tumour-specific CTL response against CT26 tumours and can be further enhanced using additional immunogenic factors (armed OVs, adjuvants). We also developed a novel fluorescence-based high-throughput screening platform to identify compounds that sensitize resistant solid tumours to killing by CAR-T cells, which ultimately revealed cardiac glycosides as putative tumour sensitizers. Overall, this thesis identifies several novel enhancers of the anticancer immune response, including a heterologous irr:ICV vaccine regimen and the potential ability to identify molecules to overcome resistance to CAR-T therapy.

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List of Abbreviations

aAPC- Artificial antigen presenting cell

ADCC- Antibody-dependent cellular cytotoxicity

ACT- Adoptive cell therapy

ACV- Autologous cancer vaccine

ACVS- Animal Care & Veterinary Services

ADJ- Adjuvant

AML- Acute myelogenous leukemia

APC- Antigen presenting cell

ATCC- American type culture collection

ATP- Adenosine triphosphate

bCG- Bacillus Calmette-Guerin adjuvant

CALR- Calreticulin chaperone protein

CAR- Chimeric antigen receptor

CEA- Carcinoembryonic antigen

CG- Cardiac glycoside

CFSE- Carboxyfluorescein succinimydyl ester

CTL- CD8+ cytotoxic T lymphocyte

CTX- Chemotherapy

DAMP- Danger associated molecular pattern

DARPin- Directed ankyrin repeat protein

DC- Dendritic cell

DMEM- Dulbecco's Modified Eagle's Medium

DMSO- Dimethyl sulfoxide

DNA- Deoxyribonucleic acid

d.p.i.- Days post-implantation/infection

FACS- Fluorescent assisted cell sorting

GFP- Green fluorescent protein

GMCSF- Granulocyte macrophage colony-stimulating factor

HCS- High Content Screening

HDACi- Histone deacetylase inhibitor

HMGB1- High-mobility group box 1

HTS- High-throughput screening

ICD- Immunogenic cell death

ICV- Infected cell vaccine

IFN- Interferon

IFNAR- Interferon alpha receptor

IFNγ- Interferon gamma

IL- Interleukin

i.p.- Intraperitoneal

irrCell- Irradiated tumour cell vaccine

ISG- Interferon stimulated gene

ITAM- Immunoreceptor tyrosine-based activation motif

ITIM- Immunoreceptor tyrosine-based inhibitory motif

i.v.- Intravenous

KIR- Killer cell immunoglobulin-like receptors

LPS- Lipopolysaccharide

MDA- Microtubule destabilizing agent

MDSC- Myeloid-derived suppressor cell

MHC- major histocompabtibility complex-1

MG1- Maraba-MG1 strain

NCR- Natural cytotoxicity receptors

NK cell- Natural killer cell

OMP- Outer membrane protein

ORV- Oncolytic rhabdovirus

OV- Oncolytic virus

PAMP- Pathogen associated molecular pattern

PBS- Phosphate buffered saline

p.c.- Peritoneal carinomatosis

PDL-1 – Programmed death ligand-1

PFA- Paraformaldehyde

PI-9 - Proteinase inhibitor-9

RFP- Red fluorescent protein

RIPK-1/3- receptor-interacting serine/threonine protein kinase

RPMI- Roswell Park Memorial Park Institute media

RNA- Ribonucleic acid

ROS- Reactive oxygen species

RTX- Radiotherapy

s.c.- Subcutaneous

TAA- Tumour-associated antigen

T_h1- CD4+ Th1 helper cell

TCR- T cell receptor

T_M- $CD45RO^+$ memory T cell

TME- Tumour microenvironment

TLR- Toll-like receptor

Treg- Regulatory T cell

TSA- Tumour-specific antigen

VSe- Viral sensitizer

VSV- Vesicular stomatitis virus

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1.0 Introduction

1.1 Cancer

1.1.1 *Hallmarks of cancer*

Cancer is a mosaic of related genetic diseases that are characterized by rapid and dysregulated cell division and the formation of malignant, often metastatic, neoplasms. There exist hundreds of types of cancers capable of affecting essentially all cell and tissue types . Despite this diversity, all forms of the diseases arise through the progressive accumulation of genetic mutations conferring a pre- malignant cell the abilities associated with tumour cells. This collection of shared phenotypes was coined "the Hallmarks of Cancer" by Hanahan and Weinberg in their landmark 2000 publication[1].

The seminal Hallmarks of Cancer report defines six characteristics acquired and shared by malignant cells, all of which can be tied back to an underlying genomic instability associated with pre-malignant precursor cells[2]. The original six hallmarks include an acquired ability to promote self-growth (e.g. overexpression of growth factor receptors such as HER2/neu[3]), resistance to growth-inhibitor signals, limitless replicative potential, evasion of apoptosis, ability to drive blood vessel generation (referred to as angiogenesis) and the capacity to metastasize and invade secondary, and sometimes distant tumour sites. These hallmarks serve not only as an organizing principle for the complex process human tumorigenesis, but also as opportunities for therapeutic intervention through a variety of platforms[1]. A follow up review in 2011 has proposed the addition of two supplemental hallmarks: a reprogramming of cellular energy metabolism pathways and evading detection by the host immune system[4], the latter representing a major factor in shaping the progression of cancer, and that also provides a compelling therapeutic rationale for the emerging field of cancer immunotherapeutics.

1.1.2 *Impact on Canadians*

The extension of the average humanlife span in recent decades is certainly a testament to incredible advances in basic scientific research and health care practices, yet, has simultaneously led to

widespread prevalence of cancer and other age-related illnesses among the ever-aging world population[5,6]. The impact of cancer on Canadian population is enormous, both in the context of human health and in terms of the financial strain placed on the health care system aiming to diagnose and treat the disease.

Based on statistics released by the Canadian Cancer Society, it is estimated that over 220 000 Canadians will be newly diagnosed with cancer in 2019 alone, with approximately 80 000 succumbing to the disease. More remarkably, cancer will prove to be the leading cause of death among Canadians, with 1 in 4 people dying from the disease, and 1 in 2 Canadians developing the disease at some point in their lifetime[7].

The impact of cancer on Canadians also extends far beyond the effect on the individual fighting the disease and their family, as the arms race against the disease on the part of researchers and health care providers alike is both costly and perpetual. A 2018 study by de Oliviera *et al*. estimated that expenses directly related to cancer and related patientcare totaled nearly $7.5 billion dollars in 2012 in Canada alone. More stunning is the fact that is more than double the $2.9 billion cost estimated for 2005[8]. This staggering imprint on the lives of Canadians, as well as the economic burden placed on the national healthcare system dramatically highlight the need for the development of novel and highly efficacious cancer therapy options.

1.1.3 *The pitfalls of traditional cancer therapies*

To this point in the history of cancer therapy, the most widely featured standards of care have included surgery, chemotherapy, and radiotherapy. Chemotherapy (CTX) involves involves the systemic administration of cytotoxic compounds to patients with the aim of killing rapidly dividing cells. The advent of chemotherapy traces back to anecdotal evidence from the First and Second World Wars showing that mustard gas exposure in soldiers correlated with a decreased leukocyte count.

Subsequent research into this phenomenon lead to the downstream development of early CTXs such as cyclophosphamide and chlorambucil, and eventually to subsequent modern chemotherapeutic agents[9,10]. The principle for CTX both old and new involves disruption of cellular metabolism and primarily aims to inhibit the growth of rapidly dividing cell populations. A major downfall of this approach is that it is by nature extremely toxic and will often result in severe off-target effects in rapidly dividing non-malignant cell populations (e.g. stomach mucosa, hair follicles). As a result, there are often an abundance of serious and unpleasant side effects associated with these treatment options.

Another enduring standard of care involves the use of surgical resection and/or radiotherapy (RTX). The physical removal of tumour tissue using surgical intervention has been practiced since at least the late 1890s[11]. Despite ongoing advances in surgical techniques and equipment over the decades, a major breakdown of this approach involves successfully removing all relevant malignant tissue, metastases and micrometastases[12,13,14,15]. If any tumorigenic cells remain following resection there exists a major risk for disease relapse, especially given the emerging body of research suggesting that surgery can actively contribute to an immunosuppressed, pro-tumorigenic environment in the host[16,17]. As a result, surgical techniques are often combined with additional therapeutic options such as RTX. This approach involves the use of targeted ionizing radiation in order to damage the DNA of rapidly dividing cells and was first explored as a therapeutic option in the late 1890s as well. Like CTX, radiotherapy is effective in its aim to kill rapidly dividing cells, but also produces significant off-target effects and is associated with a large range of patient side effects.

Overall, chemo and radiotherapies have been the long-standing front-line therapeutic options for cancer patients, yet both are associated with extreme off-site toxicity and lack crucial elements of cancer-selectivity. Furthermore, the aforementioned study by de Oliviera *et al.* indicated that the largest driver of cancer-related costs in Canada stem from the administration of these therapies[6]. Taken

together, it is strikingly evident that there exists a need for the development of more efficacious, selective and safe therapeutic options.

1.2 Cancer immunology and the rise of immunotherapy

1.2.1 *The immune response to tumours*

Among the first evidence highlighting the interplay between the human immune system and developing malignancies stems as a far back as the 1800s and the work of Dr. William Coley, who had performed intratumoural injections of live or inactivated bacteria with the hopes of stimulating the host immune system to combat tumours. Despite obvious safety concerns associated with clinical use of bacterial agents and the lack of thorough follow-up studies, there was documentation of significant regression of tumours following treatment. These landmark studies effectively laid the groundwork for genesis of cancer immunology and cancer immunotherapy strategies[18].

1.2.1.1 Innate immunity and natural killer (NK) cells

Like the immune responses generated against foreign pathogens and toxins, the anticancer immune responses can be divided into innate and adaptive pathways for their roles in cancer immunotherapy. The innate immune response is both rapid and largely non-specific in terms of antigen recognition and involves key cellular effectors such as Natural killer (NK) cells and immunostimulatory cytokines such as members of the interferon (IFN) and interleukin (IL) families. NK cells are often regarded as sentinels of the immune system and play an essential role in immunosurveillance of developing tumours and early pathogenic infections[19,20]. These cells play a crucial front-line role due to their ability to quickly recognize foreign/harmful motifs without prior exposure or sensitization, allowing rapid induction of effector functions upon recognition of targets and with minimal dependence on other immune cells.

NK cells maintain a range of activating and inhibitory receptors on their cell surface that are linked to downstream immunoreceptor tyrosine-based activation/inhibitory motifs (ITAM/ITIMs). These receptors allow NK cells to quickly discern "self" by detection of major histocompatibility complex-1 (MHC-I) molecules on target cells through interactions with killer cell immunoglobulin-like receptors (KIRs) and Ly49 in human and mice respectively. Contrarily, NK cells also feature a breadth of activating receptors such as natural cytotoxicity receptors (NCRs) and NGK2D, which quickly trigger NK cell-mediated cytotoxicity (for example through the release of perforin and granzyme or the release of apoptosis inducing) upon interaction with their cognate ligands commonly expressed on infected, malignant or otherwise distressed cells [21].

These cytotoxic immune cells also play a crucial role in facilitating antibody-dependent cellular cytotoxicity (ADCC), a process in which activated B cells from the immune system's adaptive compartment will produce antibodies against tumour or pathogenic antigens, coat them and target them for killing by NK cells. This represents a crucial interplay between the adaptive and innate immune responses against tumours and demonstrates the importance of recruiting both immune compartments in order to develop truly efficacious cancer immunotherapies[22].

1.2.1.2 Adaptive tumour immunity: the role of T cells

Tumour antigens have emerged as a lynchpin of cancer immunology and remain a key target for various forms of immunotherapeutic intervention. There exist numerous antigens associated with a wide range of tumour types, each serving as a potential target for destruction by immune effector cells such as cytotoxic T lymphocytes (CTLs). Antigens that appear solely in the context of tumours are referred to as tumour-specific antigens (TSAs), whereas antigens that exist on other cell types but are altered or overexpressed, for example, are designated as tumour-associated antigens (TAAs)[23]. Examples of such TAAs include the HER2/neu growth receptor overexpressed in several epithelial-derived cancers[24] or

mutated cancer-related protein targets such as members of the B-RAF serine-threonine kinase family featuring distinct missense mutations conserved in over 65% of melanomas and to a lesser extent in other forms of cancer as well[25].

The adaptive arm of the immune system represents a highly specialized and specific, counterpart to the innate component and the antitumour immune response. The adaptive immune responses against tumours involves the targeting of precise antigens by effectors such as the $CD4^+$ T helper cells and the vital cytotoxic $CD8^+$ CTL populations. The generation of immune responses encompassing these cells relies on the interaction of effectors and several other immune cells and mediators, for example specialized antigen presenting cells (APCs) such as dendritic cells (DCs), which represent a bridge between innate and adaptive immunity and contribute to the successful activation of antitumour immunity.

DCs are constantly sampling and processing antigens upon detection of infected, damaged or malignant cells and will undergo a process of maturation as they migrate to nearby lymph nodes and other lymph structures. The DCs will subsequently process and present antigenic peptide fragments via the MHC-I/II complexes on their cell surface and this presentation leads to clonal expansion of effector T cells which effectively recognize and respond to their cognate antigen. The process of antigen presentation and maturation of DCs is further promoted by factors including the recognition of danger- and/or pathogen-associated molecular patterns (DAMPS/PAMPs) by members of the innate immune system and the subsequent secretion of immunostimulatory factors that promote the generation of immune responses. An example is the potent IL-12 cytokine, which has been shown to promote the activity and maturation of both NK cells and DCs and is being explored as a method of improving several immunotherapy platforms currently being evaluated[26,27].

CTLs are characterized as CD3 and CD8 co-positive lymphocytes that play a vital role in the clearance of infected or transformed cells, and therefore represent a major target for therapeutic intervention aimed at recruiting the host immune system to combat cancer. The CTL response is highly antigen-specific and relies on the support of additional cellular components to proceed successfully. Naïve $CD8^+$ T cells reside in lymphoid tissue and become activated only upon recognition of their cognate antigen in the context of MHC-I molecules on a professional APC. This successful recognition and the concomitant provision of appropriate costimulatory protein interactions (e.g. CD40) and cytokine production (e.g. IL-2) results in clonal expansion of antigen-specific T cells capable of migrating to the site of infection or malignancy and exert their killing function through the release of cytotoxic enzymes such as perforin and granzyme, as well as through interaction of the Fas-Fas ligand cell death axis, both of which ultimately induce apoptosis, or programmed cell death, in the target cell. The generation of potent anticancer CTL responses and the accompanying breaking of immunological tolerance that can arise through tumour-associated immunological resistance mechanisms potentially leading to T-cell anergy (e.g. programmed death ligand-1 up-regulation in tumours) is a penultimate goal of cancer immunotherapy and carries the benefit of forming a reservoir of memory T cells specific for tumours, which can represent a life-long defense against relapse in patients[28].

1.2.2 *Cancer immune evasion and the tumour microenvironment*

Numerous studies have reported that a robust anticancer immune response and subsequent infiltration of tumours by immune cells correlates with improved disease prognosis and patient survival, as well as with a decreased incidence of disease relapse, for a number of highly relevant human cancers. Specifically, infiltration by CTL, $CD4^+$ Th1 helper cell (T_h1) and/or $CD45RO^+$ memory T cell (T_M) populations have been extensively linked to improved clinical outcomes for several cancers. Furthermore, various immunostimulatory proteins, such as chemoattractant CXCL9 and CXCL10, have

been shown to perpetuate tumour infiltration by immune effector cells[29]. Interferon gamma (IFNγ), for example, is a member of the type II interferon family and is a powerful immunostimulatory protein involved in the driving both the innate and adaptive arms of the immune system. Several studies have shown that in addition to facilitating the immune responses against pathogens, IFNγ can contribute strong direct anti-proliferative and pro-apoptotic effects on tumours, can enhance tumour immunogenicity and promote the recognition and infiltration of tumours by tumour-specific CTLs[30,31,32,33].

Despite the ability of the immune system to recognize and combat cancerous cells throughout their development, robust immune responses against tumours may be difficult to generate due to the ability of cancer cells to adapt to immune system selection pressure in order to evade detection through a process known as cancer immunoediting. Immunoediting features three key phases: elimination, equilibrium and escape. Elimination refers to the clearance of tumour cells by the immune system, yet a subset of cells may acquire additional mutations that confer the ability to evade detection by the host immune system and enter a phase of equilibrium in which there exists a balance between tumour cell elimination those cells evading detection. The escape phase involves the tumour eventually gaining the upper hand over the immune system and allow tumorigenesis to progress[34].

1.2.3 The aim of cancer immunotherapy

Tumours can only thrive if they can effectively evade surveillance by the immune system and/or subvert effector responses to facilitate their own growth. There remain several hurdles that hinder the generation of robust and efficacious anticancer immune responses, both naturally and through therapeutic intervention. First, malignant cells are self-derived and as a result the host immune system is frequently initially tolerant to relevant TAAs. Tumour cells are proficient at this process of immune escape through a vast range of additional mechanisms including downregulating immunogenic proteins

such as MHC molecules, increasing expression of inhibitory molecules (ex. programmed death-ligand 1) and recruitment of inhibitory immune cells (e.g. regulatory T cells) among many others[23].

Another barrier exists in the intricate interaction of tumour cells and the immune system in the context of what is known as the tumour microenvironment (TME). The tumour microenvironment is a complex collection of components including tumour and surrounding stromal cells, vascular networks, as well as several cells and factors from the host immune system. This complex biological milieu plays host to a range of both pro- and anti-tumorigenic factors and is the site of numerous crucial interactions between tumours and the immune effectors seeking to destroy them[35].

The discrepancy between the therapeutic benefits of a strong anticancer immune responses and the difficulty in inducing them naturally is the basis for the field of cancer immunotherapy, which ultimately seeks to re-awaken, improve, or induce new tumour-specific immune responses and aid in clearing a patient's malignant burden. There are several platforms being investigated to this end including therapeutic antibodies, oncolytic viruses, various therapeutic vaccination approaches and adoptive cell transfer therapies (ACT).

1.2.4 *Oncolytic viruses: multimodal biotherapies*

Oncolytic viruses (OVs) are a class of biotherapeutics that have emerged as a promising platform in the arsenal of cancer immunotherapy. These viral agents can be designed to specifically infect and destroy tumour cells (hence the term "oncolytic") while sparing non-cancerous tissue, thereby providing a heightened degree of cancer-specificity lacking in several standard of care therapies. This ability to specifically target cancerous cells can be achieved by exploiting the frequently abrogated antiviral response found in many tumours[36,37].

The type I interferon (IFNα/β) signalling axis is vital component of the cellular antiviral defense system. In response to viral infection, cells will produce and secrete IFNα/β, which bind to cognate

cellular receptors (IFNAR) and induce a downstream signalling cascade through the JAK-STAT pathway, culminating in the expression of a number of interferon-stimulated genes (ISGs) that possess a number of effector functions targeting essentially every stage of the viral life cycle[38,39]. Even though type I IFN pathway defects are common in cancer, tumour cells possess a high degree of genetic heterogeneity and display a spectrum of susceptibility to infection by OVs. This can result in viral replication in cells with partially intact antiviral pathways, making it challenging to achieve robust infection of malignancies with heterogeneous cell populations.

Biotherapies such as OVs are commonly referred to as "multimodal" by virtue of their ability to extend treatment benefits beyond the direct infection and physical destruction of malignant cells. An example of this therapeutic multiplexing is seen in the immunostimulatory milieu created in the TME following productive infection of tumours. Following infection and lysis of tumour cells, an array of immunostimulatory factors including critical DAMP/PAMPs, as well as immunostimulatory cytokines and chemokines are released and can significantly contribute to the perpetuation of the resulting anticancer immune response. This process of direct tumour cell destruction through infection releases a new repertoire of tumour antigens for sampling by the immune system that were previously inaccessible due to factors such as intracellular localization or altered/abrogated protein trafficking frequently found in cancerous cells.

Productive infection of malignancies by OVs has also been shown to promote shutdown of vascular networks supplying oxygen and nutrients the tumour bed, in addition to increasing the recruitment and activation of key immune effector cells such as macrophages, natural killer (NK) cells and tumour-specific T cells[40,41], while further aiding in the crucial process of breaking T cell tolerance towards TAAs originally derived from "self" antigen[42]. Overall, OV therapy represents a unique and powerful therapeutic tool that confers an element of cancer selectivity, as well contributing to

immunogenic cell death of tumours and perpetuate the induction of a systemic anticancer immune resposne[43].

1.2.4.1 Oncolytic rhabdoviruses: emergence of Maraba-MG1

A class of viruses that has shown pre-clinical and early clinical success as a cancer biotherapy platform includes members of the *Rhabdovirus* family. Members of this group feature relatively small negative-sense RNA genomes, distinctive bullet shaped capsids and include viruses such as rabies, vesicular stomatitis virus (a common livestock pathogen) and the more recently explored Maraba virus, the latter two representing the most appealing oncolytic rhabdoviruses being explored for cancer therapy to date. Rhabdoviruses are an attractive vector for OV therapy due to the relative ease of genetic manipulation associated with small RNA viral genomes (allowing for the introduction of transgenes or enhancing safety profiles) facilitated by reverse genetics techniques, as well as their relatively low seroprevalence in the human population. The fact that the rhabdovirus life cycle is exclusively supported in the cytoplasm alleviates concerns involving integration between viral and host genomes, and further bolsters their candidacy as key cancer therapy platforms.

A promising OV-based strategy involves the use of Maraba virus, which was isolated in 1984 from Brazilian sand fleas[44], and was found to be a potent inducer of cell death in human tumours. It was observed using a panel of various *in vitro* human tumour lines that the Maraba virus displayed a robust cytolytic capacity paired with desirable levels of virus progeny production and burst size, in comparison to other rhabdoviruses including the initially explored and closely related vesicular stomatitis virus (VSV). It was further reported that the introduction of mutations in the M (L123W) and G (Q242R) proteins (resulting in the Maraba-MG1 nomenclature) could attenuate the virus in such a way that cancer-selectivity was enhanced with no impairment to virus-induced cytotoxicity, thereby greatly enhancing the therapeutic index of this platform[45]. Similarly, several studies have reported the

enhancement of VSV tumour-selectivity through engineering of mutations into methionine 51 of the viral M protein, thereby interfering with the viral ability to dampen cellular interferon production (VSV-Δ51) and hampering the ability of the cell to engage the antiviral mechanisms necessary to stave off infection.

1.2.5 *Viral sensitizer technology*

A major hurdle to OV therapy is the genetic heterogeneity of malignant cells, both between different cancer types and even between individual malignant cells within the same tumour. This often results in tumour cells exhibiting a spectrum of susceptibility to viral infection that can hamper the multimodal therapeutic effects that are so valuable in the OV platform. Viral sensitizers (VSes) are a class of chemical compounds that modulate aspects of the cellular antiviral response and greatly improve the spread and/or replication of OVs in resistant tumour cells in a cancer-specific manner[46].

A diverse collection of small molecules that have been observed to contribute these viral sensitizing effects include several novel small molecules identified through exquisite high-throughput screening (HTS) assays in addition to an intriguing pool of previously approved pharmaceuticals that have been shown by our research group to possess these desirable bioactive properties. Among the latter are several approved pharmaceuticals previously investigated for cancer therapy including members of the histone deacetylase inhibitor family (e.g. MS-275 and another example), which are known to epigenetically modify the DNA of tumour cells, as well as established microtubule destabilizing agents (MDAs). In addition to directly improving infection of tumours, several VSe compounds such as the MDA colchicine have been shown potentiate the killing of uninfected bystander tumour cells when used in combination with oncolytic rhabdovirus[47], a term appropriately referred to as the "bystander killing effect".

In addition to repurposing established pharmaceuticals and chemotherapies, the collection of VSes shown to potentiate OV therapy includes a number of compounds that are novel in their use for cancer therapy, including the hallmark VSe-1 compound discovered and shown by Diallo *et al.* to synergize with the attenuated vesicular stomatitis virus VSV- Δ51 and dramatically enhance the infection and killing of several previously resistant tumour cell lines *in vitro* and *in vivo*. More recently, drug compounds that have previously been explored in contexts unrelated to cancer and/or immunotherapy have been demonstrated to contribute the desirable immunomodulatory and pro-viral effects seen with previous generations of VSe technology. Among these are members of the fumaric acid esters (such as dimethyl fumarate) being followed for the treatment of psoriasis and multiple sclerosis, and compounds based on the transition metal vanadium (e.g. sodium orthovanadate) explored for its use as an insulin-mimetic for the treatment of diabetes in humans.

While MG-1 and oncolytic viruses in general can be effective as monotherapies, it is recognized that combination approaches are critical to achieve maximum anticancer effects. For instance, several studies have demonstrated the benefits of using MG1 as part of a virus-based cancer vaccine strategy. Groups from the McMaster Immunology Research Center have demonstrated that VSV-Δ51 and MG1 alone were unable to generate a strong response to virally encoded antigens, owing to the relatively strong immunostimulatory profile of the viral components, to which the host is naïve. However, in some models, using oncolytic rhabdovirus as a boosting vector following priming with a heterologous viral vector (such as adenovirus) when both encode the same TAA can direct the immune response towards the common TAA and lead to rapid induction of a robust and potent tumour-specific T cell response[48,49]. These heterologous prime-boost strategies can be further improved through the incorporation of factors such as immune checkpoint inhibitors and through combination with sensitizing compounds such as HDACis among others[50,51].

1.2.5.1. Vanadium-based VSes

An interesting class of VSes being exploring in recent years include molecules based on the transition metal vanadium, which occurs naturally in human tissues in relatively small amounts. This unique element exists in numerous oxidation states which can change based on factors such as cellular pH levels. Vanadium-based molecules have been explored in the treatment of type 2 diabetes due to their ability to regulate glycemia and contribute insulin-like effects, a function mediated through restoring signal transduction via the insulin receptor and by increasing glucose uptake via the GLUT4 receptor[52]. Furthermore, these compounds are known to act as pan-phosphatase inhibitors owing to their structural resemblance to phosphate, and have been explored to a limited extent in cancer therapy owing to their ability to induce reactive oxygen species (ROS) [53].

A more novel application for the use of vanadium-based compounds was recently uncovered by our lab and involved the ability to be combined with OV therapy for improved therapeutic benefits. This "pharmacoviral" approach was shown to not only enhance the infection of formerly resistant tumours by OVs *in vitro* and *ex vivo*, but also to enhance antitumour efficacy in several syngeneic murine tumour models, even those otherwise refractory to treatment with either monotherapy. In addition to simply enhancing the degree of tumour infection in virotherapy, the combination of vanadium-based VSes and VSV-Δ51 was observed to promote the transition of type I antiviral interferon responses towards a more pro-inflammatory IFN type II response. This type of response involves a shift in the secretory profile of infected cells towards cytokines such as IFN-β and IL-6, which promote immune stimulation and can contribute to robust antitumour immune responses. Furthermore, the combination of vanadium and virus was able to induce the production of leukocyte-attracting chemokines like CXCL9, an extremely potent T cell chemoattractant, and this effect was not observed when tumour cells were treated with either virus or vanadium alone[54].

1.2.6 Cancer vaccine platforms: whole and infected tumour cell vaccines

The advent of vaccines for humans can undeniably be regarded as one of the most pivotal advancements in the history of medicine and biomedical research. The study of immunizing humans against future pathogenic infection dates back as far as the fifteenth century, and really came to fruition with the seminal work of Edward Jenner in 1798 in his quest to use a relatively innocuous cowpox virus to protect inoculated subjects from subsequent exposure to the far more pathogenic smallpox virus[55].

The concept of vaccine booster doses was later shown to improve the generation, perpetuation and longevity of immune responses in response to antigen exposure, and thereby improving the efficacy of several vaccines aimed at eradicating or preventing crucial human disease such as polio, measles and hepatitis among others[56–58]. The purpose of immunological boosting for vaccination centers on the long held dogma that successive exposure to antigens can promote more rapid and robust immune responses, and that this enhanced immunological efficiency of the antigen-specific response replenishes the pool of effectors capable of preventing infection by relevant pathogens or toxins.

Like the approaches used for infectious diseases, cancer vaccination can educate the immune system to key disease-associated antigens for the purposes of inducing adaptive immune effector and memory responses. Therapeutic cancer vaccination has been explored in a number of contexts including the use of OVs encoding TAAs, DC-based vaccines, synthetic tumour peptides and using autologous tumour cells to produce personalized therapies[49,59,60,61]. Autologous cancer cell vaccines (ACVs) are comprised of whole tumour cells rendered more immunogenic through several processes, for example γ-irradiation, and are used to educate the immune system to the identity of targets for both humoral and cell-mediated anticancer immune responses. The use of ACV as a treatment platform carries several benefits including exposing the immune system to a complete repertoire of putative TAAs and concurrent presentation to $CD4^+$ and $CD8^+$ T cells. In addition, autologous vaccines inherently contain

unique mutated antigens that are patient/cell line-specific, thus conferring ACVs the ability to induce a personalized, broad anticancer response capable of fighting tumour growth[62].

Despite decades of research into the benefits of using ACVs as a cancer vaccine platform, clinical success to date can be described as moderate and variable. Cancer immunotherapies have been explored in the treatment of colorectal cancers for over a decade and strategies featuring ACVs have shown signs of promise in several phase II/III trials, and are generally well tolerated by patients[63]. However, patient responses in trials are inconsistent, with strong responses often resulting in equilibrium between tumour and immune system and stable neoplastic lesions[64]. One strategy that has been explored to improve ACV-induced immune responses is the use of adjuvants. Studies by Hanna *et al*. have shown that combining a colon tumour-based ACV with the bacterial adjuvant bacillus Calmette-Guérin (bCG) significantly improved clinical performance[65]. Compounds capable of inducing immunogenic cell death (ICD) can also be considered as putative enhancers of ACV-induce immunity. For example, anthracycline compounds such as mitoxantrone have been shown to be excellent inducers of ICD, which is considered a critical driving factor of ACV immunogenicity[66].

Infected cell vaccines (ICVs) are yet another strategy aimed towards improving the immunogenicity and efficacy of autologous vaccines. ICVs are prepared in the same fashion as ACVs but are irradiated and subsequently infected with OV, in hopes of harnessing the immunogenic properties of viral infection to enhance vaccine performance *in vivo*. An additional benefit to this method is administration of live virus in the vaccine regimen, which can contribute to therapeutic efficacy through virus spread, tumour oncolysis, transgene expression, and by acting as a powerful adjuvant. A study by Lemay *et al.* has shown that ICVs prepared from resistant melanoma cells had a significant protective capacity in murine tumour challenge models when prepared with VSV-Δ51, and even more so when the virus was armed with granulocyte macrophage colony-stimulating factor (GM-

CSF)[42]. Similarly, Alkayal *et al.* have demonstrated that ICVs prepared using colon carcinoma cells infected with MG1 expressing IL-12 showed success in pre-clinical models of murine peritoneal carcinomatosis[67]. Results like these give credence to the fact that ACVs are a powerful therapeutic vaccine platform and have the potential to be enhanced through multiple strategies aimed at improving vaccine immunogenicity.

1.2.7 Chimeric antigen receptor-T cell therapy

Adoptive cell therapy (ACT) is a branch of cancer immunotherapy aimed at re-targeting a patient's own T cells towards relevant TAAs and amplifying these cells *ex vivo* prior to reinfusion into the patient. The use of chimeric antigen receptors (CARs) has emerged as a powerful method of conferring artificial immune effector specificity for selected antigens and doing so in a manner that does not rely on TCR/MHC interaction. All generations of CARs are composed of a specific antigen-binding domain fused to key intracellular signalling domains such as CD3ζ, CD28 and 4-1BB[68]. This results in immune cells with an artificial specificity that capable of initiating the necessary signalling pathways following antigen-receptor interaction in order to carry out effector functions, such as killing of tumour cells.

The production and implementation of CAR-T cells for adoptive cell therapy requires careful selection of antigenic targets in order to maintain crucial elements of cancer-selectivity. The design of the antigen-binding domain of the CAR is achieved through several approaches including the use of single-chain variable fragments derived from monoclonal antibodies and through designed ankyrin repeat protein (DARPin) sequences. Ankyrin repeat proteins are a class of binding proteins that are highly prevalent in the human genome, and through screening of diverse libraries of sequences, can be identified and used as highly specific targeting molecules against virtually any target antigen of interest[69]. Ongoing advances in the generation and selection of these proteins sequences has led to

enhanced binding affinities between novel DARPins and their target proteins. For example, DARPins screened specifically for the HER2 tumour antigen have been reported with binding affinities in the picomolar range, compared to previous generation that were roughly 3000-fold less potent at binding their cognate proteins[70]. CAR-expressing T cells are typically generated by harvesting a patient's own T cells from blood, transducing the cells with a relevant genetic construct encoding the CAR (using viral vectors such as a lentiviruses) and then expanding the cell culture to a high level *ex vivo* prior to administration to a lymphodepleted patient[68].

There are several therapeutic benefits associated with the use of CAR-T cells for cancer immunotherapy. Due to the artificial specificity of T cells expressing the CAR complex, antigen recognition and the subsequent initiation of immune cell effector function (e.g., release of granzyme B or perforin) are executed in a manner that is MHC-independent. This is especially beneficial for cancer therapy as many tumours have been shown to downregulate MHC at the cell surface as a means of evading immune detection during tumorigenesis and immunoediting[71]. Furthermore, CAR-T cell constructs are able to recognize a diverse range of cell surface antigens including protein, carbohydrate and glycolipid-based molecules[72]. From a clinical standpoint, the ability to produce a high number of TAA-specific CTLs in a relatively short timeframe is a major benefit as well[73].

Despite significant therapeutic upside, an immunosuppressive tumour microenvironment and inherent cellular resistance to T cell cytolytic activity remain significant hurdles to the clinical success of CAR-T cell therapies. Tumours and their microenvironment are able to supress CAR-T cell-mediated effector function through an array of mechanisms including the production of immune-dampening cytokines (e.g. IL-4, TGFβ), inhibitory ligands such as PD-L1 and the recruitment of immunosuppressive regulatory T cells and myeloid-derived suppressor cells[68]. In addition, malignant cells can subvert the induction of apoptosis by CTLs, as many tumours harbour signalling defects in key

apoptotic pathways or express anti-apoptotic proteins. Several cancers have further been show to produce cellular proteins that directly inhibit CTL effector function, such as intracellular serpin proteinase inhibitor 9 (PI-9), a direct inhibitor of granzyme B function, and cellular FLICE-inhibitory protein, which has been shown to inhibit apoptosis stemming release of perforin/granzyme B or death receptor binding by CTLs[74,75].

1.3 Experimental Rationale and Aims

Resistance to cancer immunotherapy is a hurdle that needs to be overcome to maximize the potential therapeutic benefit of these promising therapeutic modalities. As previously demonstrated using viral sensitizers like vanadium in the context of OV therapy, small molecules can be harnessed to support immunotherapy approaches by targeting fundamental resistance mechanisms. Similarly, adjuvants are frequently used in the context of vaccines to amplify immune responses and downstream therapeutic effects. For this project we rationalized that these compounds possess direct beneficial immunomodulatory properties when combined with virotherapy. We further rationalize that accessory vaccine factors (such as viral sensitizing compounds and adjuvants) could improve the immunogenicity and *in vivo* performance of our autologous cancer vaccines, specifically, in the context of our heterologous prime/boost vaccination regimen. Furthermore, we hypothesize that our proposed high-throughput screening platform will allow us to identify chemical enhancers of CTL killing function that ideally can be implanted to further improve the efficacy of our optimized autologous cancer cell vaccine.

AIM 1: ***Preparing the optimal autologous cancer cell vaccine***

Autologous cancer cell vaccines are a powerful tool for cancer immunotherapy but have not shown overt clinical success under investigations to date. Work by our group and by close collaborators has shown that autologous whole cell vaccines manufactured solely of tumour cells irradiated to the point of growth arrest can generate a high level of protection from subsequent tumour challenge in

syngeneic murine models of colon carcinoma, colon fibrosarcoma and melanoma. Further, the addition of OV to produce an ICV has been shown to be increase the potency of the vaccine-induced anticancer immune response and enhance therapeutic outcomes in several tumour models that were initially refractory to OV therapy or autologous vaccination alone. We are proposing to investigate the potential of VSes to improve autologous vaccine immunogenicity and as a result, *in vivo* efficacy in our murine tumour models. We hypothesize that through the addition of VSes such as the tyrosine phosphatase inhibitor orthovanadate, ICV efficacy will be improved due to the immunostimulatory nature of a productive viral infection and the ability of the incorporated small molecules to directly modulate the immune system. In addition, we propose to explore the use of adjuvants and other alterations to our current vaccination regimen to enhance autologous vaccine performance for cancer immunotherapy.

AIM 2*: Identifying novel enhancers of cytotoxic (CAR)-T cell effector function*

The penultimate goal of cancer immunotherapy is to induce a potent tumour-specific CTL response, though this can be inconsequential if tumours remain resistant to killing by the CTL effector response generated therapeutic intervention. We propose to design and employ a novel high-throughput screening assay to identify compounds capable of enhancing CAR-T cell-mediated killing of resistant tumour cells. Additionally, we anticipate that the successful identification of candidate compounds from a small FDA-approved library will serve as a proof-of-concept and set the groundwork for larger, future screens aimed at identifying more and possibly novel compounds of interest.

1.4 Significance

Immunotherapy approaches such as autologous tumour cell vaccination have been explored to treat cancer for years yet have failed to produce major positive clinical outcomes to date. We are proposing to attempt to identify novel enhancers of the autologous-vaccine-induced anticancer immune response by optimizing the preparation and administration of a CT26.WT-based autologous vaccine regimen, and by further identifying chemical factors capable of potentiating CTL-mediated killing of

tumour cells. We are also aiming to demonstrate that viral sensitizers (e.g., orthovanadate) and biological adjuvants can be combined with a vaccination regimen to generate more efficacious vaccine strategies to treat not only colorectal cancers, but to improve disease outcomes for a wide range of human cancers that are resistant or refractory to existing therapies. Furthermore, the identification of drugs that specifically enhance CTL-mediate killing of malignancies could have a tremendous impact on many immunotherapeutics for cancer and other human diseases, as the ultimate aim of these therapies is to induce robust CTL responses capable of effectively eliminating cells expressing disease-associated antigen.

2.0 Methods

2.1 Cancer cell lines and viral vectors

CT26.wild-type (WT), CT26.LacZ (both murine colon carcinoma) and Vero (African green monkey) cell lines was obtained from the American Type Culture Collection (ATCC, Manassas, VA). These cells were maintained in HyClone Dulbecco's Modified Eagle's Media (DMEM; GE Healthcare, Mississauga, ON, CA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO). A549.WT (human lung adenocarcinoma), LOX-IMVI (human melanoma) and OVCA-3 (human ovary carcinoma) cells were a generous gift from the laboratory of Dr. Johnathan Bramson (McMaster Immunology Research Center, Hamilton, ON). These cell lines were maintained in HyClone Roswell Park Memorial Institute Media (RPMI; GE Healthcare) supplemented with 10% FBS (Sigma-Aldrich). All cell lines were also maintained at 37°C and 5% CO_2 and were routinely monitored for mycoplasma contamination using Hoescht staining/microscopy and eMyco VALiD Mycoplasma PCR detection kits (iNtRON Biotechnology, Sangdaewon-Dong, South Korea).

Viruses (VSVΔ51-GFP, Maraba-MG1, MG1-GFP, MG1-IL-12 and MG1-IL-12/IL-18) were propagated in our laboratory through the amplification of seed stocks in Vero cells. Viral titers were

subsequently assessed using plaque assay as described in published methodology[76]. The Maraba-MG1 virus platform is protected under the intellectual property of Turnstone Biologics (New York, NY).

2.2 Animals

Six-week-old female Balb/c mice were purchased from Charles River Laboratories (Wilmington, MA) and were housed in pathogen-free biosafety conditions under the supervision and care of the University of Ottawa Animal Care and Veterinary Services (ACVS). All animal studies were carried out in compliance with Canadian Council on Animal Care guidelines and were approved by the University of Ottawa Animal Research Ethics Board.

2.3 Irradiated (irrCell) and infected cell vaccine (ICV) preparation

Cell line were cultured in vitro, harvested in suspension, and washed twice with Phosphate Buffered Saline (PBS; Corning, Manassas, VA) prior to re-suspension at a density of 5e7cells/mL in serum-free DMEM. The cell suspensions were subsequently irradiated with γ-irradiation for a total dose of 60 Gray (Gy). Infected cell vaccines (ICVs) were then prepared by subsequently infecting irradiated cell mixtures at a multiplicity of infection (MOI) of 10 using various Maraba constructs for two hours under constant rotation in a 37°C, 5% CO_2 incubator. Irradiated cell vaccines were prepared similarly while foregoing the infection step.

2.4 in vivo murine tumour models

For prophylactic tumour models, irrCell and ICVs were prepared using CT26.WT or CT26.LacZ cells as described in ***Methods 2.3***. Mice were vaccinated with either vaccine alone, or a heterologous combination of both, via intraperitoneal (i.p.) injection at a concentration of 5e7 cells/mL in 100 μL. For vaccine regimens featuring either homologous or heterologous priming and boosting, doses were given seven days apart via i.p. injection. Tumour challenges were administered seven days following the boosting dose in the form of 5e5 live CT26.WT (or LacZ) suspended in 100 μL of PBS (Corning) via

subcutaneous (s.c.) injection on the right flank of the animal. Tumour outgrowth volume and survival were then monitored with measurements three times weekly minimum with a pre-stablished humane endpoint of 1625 mm^3 (calculated as length x $width^2$) adhered to as the endpoint of the study.

An aggressive therapeutic model was established and investigated by first implanting 5e5 tumour cells s.c. in 100 μL and beginning a vaccination regimen once the tumours had reach a palpable starting volume of approximately 5mm x 5mm. At this point, two doses of either irrCell or ICV were administered i.p., seven days apart as described for the prophylactic model. Again, tumour sizes exceeding 1625mm^3 signaled a human endpoint for tumour outgrowth and murine survival.

A model of peritoneal carcinomatosis (P.C.) was also used to investigate the efficacy of various autologous vaccine preparations. CT26.WT cells were implanted i.p. at density of 1e5 cells to establish a murine model of peritoneal carcinomatosis. Treatment regimens were started three days post-implantation and featured either homologous doses of either irrCell or ICV preparations, or a heterologous combination of both were administered six bi-weekly doses of vaccine as described for previous murine models. The endpoints of these studies included visible abdominal distension and respiratory disease brought on by peritoneal disease, as assessed by our highly trained veterinary technicians and ACVS staff.

Adjuvants used in this study were a proprietary microbial outer membrane protein-based adjuvant generously provided by our close collaborators at Biodextris (Montreal, QC, Canada).

2.5 Chemical compounds

The primary vanadium-based compound explored in this study was sodium orthovanadate (Na_3VO_4, Sigma-Aldrich, cat: 450243), which was prepared by dissolving the drug powder in distilled water. The solution was subsequently pH-adjusted to 10, boiled until translucent to ensure thorough

dissolution and then cooled to room temperature. These steps were required to ensure the establishment of stable monomeric vanadate[77] and stock solutions were stored at -20°C and thawed as necessary for *in vitro* and *in vivo* studies. Cell viability assays were performed using AlamarBlue Cell Viability Reagent (ThermoFisher Scientific, Waltham, MA) diluted 1:10 in the experimental well and read for fluorescence 24- or 48-hours post-infection with virus or post-incubation with drug. The Prestwick chemical library (Prestwick Chemical, Strasbourg-Illkirch, France) used for pilot screening features 1280 FDA approved compounds and was daughtered out to library subsets in house. Our screening stock used a base stock of 100 μM drug compounds in 10% DMSO diluted to the appropriate concentration for screening using our suite of robotic liquid handlers.

2.6 in vivo CTL assay and flow cytometric preparation and analysis

The *in vivo* CTL assay featured in this study was largely adapted from previous work completed by Tzelepis *et al.* for the purposes of investigating vaccine responses against infections disease pathogens[78]. In short, naïve mice were sacrificed for splenocyte harvest and these splenocytes were subsequently labelled with either a high or low concentration of carboxyfluorescein succinimydyl ester (CFSE; ThermoFisher Scientific, Waltham, MA), 5μM or 0.5μM, respectively. The CFSE-high ($CFSE^{Hi}$) population of naïve splenocytes is subsequently peptide-loaded with exogenous tumour gp70 tumour peptide (H2kd-restricted, SPSYVYHQF; MBL International, Woburn, MA), whereas the CFSE-low ($CFSE^{Lo}$) population was left unloaded. An equal proportion and number of $CFSE^{Hi}$ and $CFSE^{Lo}$ populations were combined and 2e7 total splenocytes were administered intravenously (i.v.) to previously vaccinated mice. Eighteen hours later, vaccinated mice were also sacrificed and splenocytes were harvested from individual subjects as previously described. These mouse splenocytes were subsequently used for additional immune analysis including intracellular staining assays (***Methods 2.7***). Flow cytometry was used to assess the relative elimination of the tumour antigen loaded, $CFSE^{Hi}$

population compared to control groups, and this was used as a surrogate interrogation of CTL responses generated because of vaccine treatment.

2.7 Ex-vivo peptide stimulation and intracellular staining

As mentioned in ***Methods 2.6*** *ex vivo* splenocytes from vaccinated mice were harvested and isolated for further intracellular staining. These cells were restimulated with H2kd-restricted peptides at a concentration of 5uM to assess responses against the gp70 tumour antigen (MBL International) and the VSV N protein (VSVn, MPYLIDFGL; New England Biolabs, Ipswich, MA), which is cross-reactive with the Maraba-MG1 N viral protein, in order to assess the antiviral response generated through our vaccination protocols. Peptide stimulation proceeded for sixteen hours overnight in a 37°C, 5% CO_2 incubator. After eleven hours of stimulation, GolgiPlug protein transport inhibitor containing brefeldin A (Becton Dickinson (BD), Franklin Lakes, NJ) was added to the experimental conditions for the final five hours of incubation. Cells were subsequently washed and treated with Fixable Viability Dye eFluor 780 (eBioscience, San Diego, CA) and treated with purified rat anti-mouse CD16/CD32 Mouse Fc Block (BD). The splenocytes were then stained for extracellular markers such as CD3, CD4, CD8 using antibodies purchased from BD Bioscience. Intracellular staining for IFNγ was carried out following fixing/permeabilization using the Cytofix/Cytoperm kit (BD) and following the provided manufacturer's protocol. Flow cytometric experiments were carried out with the assistance of the University of Ottawa Flow Cytometry & Virometry Department using the BD LSRFortessa.

2.8 Chimeric antigen receptor (CAR)-T cell expansion and culture

A human HER2-specific CAR-T cell construct (generously provided by the Bramson laboratory) was the primary immune cellular modality used for designing our high-throughput screening assay and implementing our initial pilot screen. This construct is chimeric in design and features a G3 (H10-2-G3) DARPin described by Zahnd *et al.*, fused to a CAR scaffold of the human CD8a extracellular hinge, as

well as transmembrane and intracellular domains from human CD28 and the intracellular domain from human CD3ζ (**Figure 3.10a**). The construct was the result of stable lentiviral transduction of human peripheral blood mononuclear cells as describe in the thesis of Daniela Tantalo[79].

The expansion of the human HER2-CAR construct relies on the use of artificial antigen presenting cells (aAPCs), in this case K64-41BBL again provided by the Bramson laboratory. K64-41BBL saws were thawed in RPMI containing 5% human AB serum (Sigma-Aldrich), 10% HEPES buffer (Corning), 1x Penicillin-Streptomycin Solution (Corning) and 100 U/mL recombinant human IL-2 (h1l-2; Peprotech, Rocky Hill, NJ), hereafter referred to as T-Media. T-media was filter sterilized prior to use in culture of any immune cells. The cells were allowed to expand for a minimum of three days without allowing cell numbers to exceed 5e5 cells/mL, as this can cause a buildup of cellular metabolic byproducts that can hinder further expansion.

After sufficient expansion, cultured aAPCs were irradiated for a total dose of 100 Gy in order to induce cellular senescence, exogenously loaded with 0.5μg/mL of both anti-CD3 (OKT3, eBioscience) and anti-CD28 (Clone 9.3, eBioscience) and then cocultured with newly thawed HER2-CAR-T cells in a ratio of 2:1 (T cell:stimulatory cell) in T-Media further supplemented with 10ng/mL of recombinant human IL-7 (Peprotech). Cell density and corresponding viability were assessed every 48 hours to avoid exceeding 5e5 cells/mL

2.9 High-throughput (HTP) assay optimization

The plating of all adherent tumour and control cells for experimental use was done in 96-well format and performed using a sterilized Biotek MicroFlo select dispenser to ensure accuracy and minimize sources of variance during screen optimization. In order to more easily visualize and interrogate target tumour cells using our ArrayScan High-Content Screening Microscope, A549, OVCA-3 and LOX-IMVI cell lines were stably transduced to express green fluorescent protein (GFP) using a

lentiviral expression system. Following puromycin selection, GFP positive cell lines were sorted into low, medium and high expressors using fluorescent-accelerated cell sorting (FACS). These individual populations were subsequently grown out in cell culture using complete RPMI as previously described and were placed into liquid nitrogen storage for further investigation. Regardless of cell type or plating density, cells were always plated in 100 μL of complete RPMI.

An additional step in the preparation of CAR-T cells for screening involved labelling with a cell surface stain that would not impact their viability or functionality. To this end, Far Red CellTrace was used and was applied following successful co-culture/expansion and immediately prior to incubation with the target tumour lines. Staining protocols were carried out in precise accordance with the manufacturer recommendations. Addition of CAR-T cells to target cells was again carried out using a MicroFlo Select dispenser and in a final ratio of 1:1 complete RPMI:T-Media.

2.10 Pilot FDA-approved compound HTP screen and analysis

Target A549-GFP^{Hi} cells were plated at a density of 20 000 cells/well (100 μL) within the middle ten columns of a 96-well plate, while columns 1 and 12 were seeded with alternating populations of Target A549-GFP^{Hi} and LOX-IMVI-GFP^{Hi} (HER2^{+}, sensitive to killing) to serve as negative and positive controls, respectively.

All drug dilutions and treatments of target cells were performed using a robotic Biotek Precision Liquid Handler. The Prestwick Chemical Library was periodically thawed, mixed via shaking and spun down prior to use in screening studies. The liquid handler was programmed to mix the library plates and their contained compounds, aspirate 45μL of drug and dispense 15μL to the corresponding well of a 96 well assay plate, each analyzed in triplicate. Positive and negative control cell lines in the outer columns of the plates were treated manually with 1% DMSO (final well concentration for assay plates following

treatment) immediately after addition of library compounds. Assay plates were treated and incubated for 2 hours at 37°C, 5% CO_2.

Meanwhile, HER2-CAR-T cells were thawed and amplified five days prior to screening as described in ***Methods 2.9***. During the drug incubation, the CAR-T cells were stained with CellTrace as previously described and each set of triplicate assay plates was removed from incubation and 35μL containing 20 000 stained CAR-T cells were added to each assay well exactly two hours post-treatment, again using the MicroFlo Select dispenser. The target-effector cocultures were incubated for a total of 24 hours post-drug treatment prior to being fixed with 1% paraformaldehyde (PFA). Assay plates were then stored at 4°C for three days before being scanned and measured using the ArrayScan High Content Screening Microscope.

Top hits from the pilot screen were those wells which featured the lowest measured levels of residual GFP signal, an indication of loss of target cells through T cell or drug induced cytotoxicity. In order to select compounds that appeared to enhance the performance of the T cells and were not simply cytotoxic in nature, top candidates were re-sorted by Far Red signal, exemplifying wells were library compounds that facilitated killing of target cells without exerting harmful effects on the T cells themselves. We also had the luxury of performing a previous screening assay with the A549 cell line and Prestwick Chemical library and had some pre-existing data regarding cytotoxicity levels or various drugs in the library. We were therefore able to reference this data while performing our post-screen analysis.

3.0 Results

AIM #1: Enhancing autologous tumour vaccine strategies

3.1 *CT26 colon carcinoma as a murine model for cancer immunotherapy*

The colon tumour #26 wildtype (CT26.WT) is a highly explored murine model of colon carcinoma syngeneic to the Balb/c background and represents an intriguing platform for the investigation of immunotherapy platforms for several reasons. It is a line that has been assessed considerably in the field of cancer therapy and it's most highly expressed gene is a mutated tumour antigen referred to as gp70[80], originally derived from the murine leukemia virus envelope protein, and is commonly regarded as a prototypical biomarker for murine tumour burden in various murine tumour models[81]. Another benefit associated with this cell line is access to the CT26-LacZ subclone, which we have explored extensively and have demonstrated to be dramatically more susceptible to OV infection and oncoviral therapy than the wildtype counterpart[82] (**Figure 3.1a**), thus serving as a surrogate for enhanced viral infection (e.g. to mimic the effect of a VSe compound) in vaccine studies. Lastly, CT26.WT has been extensively characterized in terms of its genomic, transcriptomic and immunomic properties and was found to be largely similar to aggressive, undifferentiated, refractory human carcinomas of similar origin[80].

Preliminary studies using this cell line have revealed that the CT26.WT is a highly murine tumour model, both in the context of both subcutaneous flank model and intraperitoneal (i.p.) peritoneal carcinomatosis (P.C.) models. The median 50% survival was found to be approximately 25 days post implantation (d.p.i.) for a s.c. flank tumour challenge model and 20 d.p.i. for the P.C. challenge (**Figure 3.1b, d**). It was also observed that tumour outgrowth was rapid in the s.c. tumour model, reaching an average volume of 1000 mm^3 within 19 days of tumour implantation (**Figure 3.1c**). Both models represent intriguing platforms to investigate our developing cancer immunotherapy platforms as they are

clearly aggressive, rapidly growing models and have several published reports have indicating a clear resistance of to various immunotherapies including autologous vaccines and virotherapy[42,83].

3.2 *CT26-based irradiated cell vaccines demonstrate a remarkably high protective capacity*

Initially our studies focused on a simple prophylactic, homologous prime-boost vaccine regimen with vaccine preparations and administrations executed as described in **Figure 3.2a**. Irradiated cell (**irrCT26**) and infected cell (**CT26-ICV**) vaccines were prepared as described in ***Methods 2.3*** using either the CT26.WT cell line, which is known to resist infection and monotherapy approaches involving oncolytic rhabdovirus including VSVΔ51 or Maraba-MG1, or the relatively more infectable CT26.LacZ

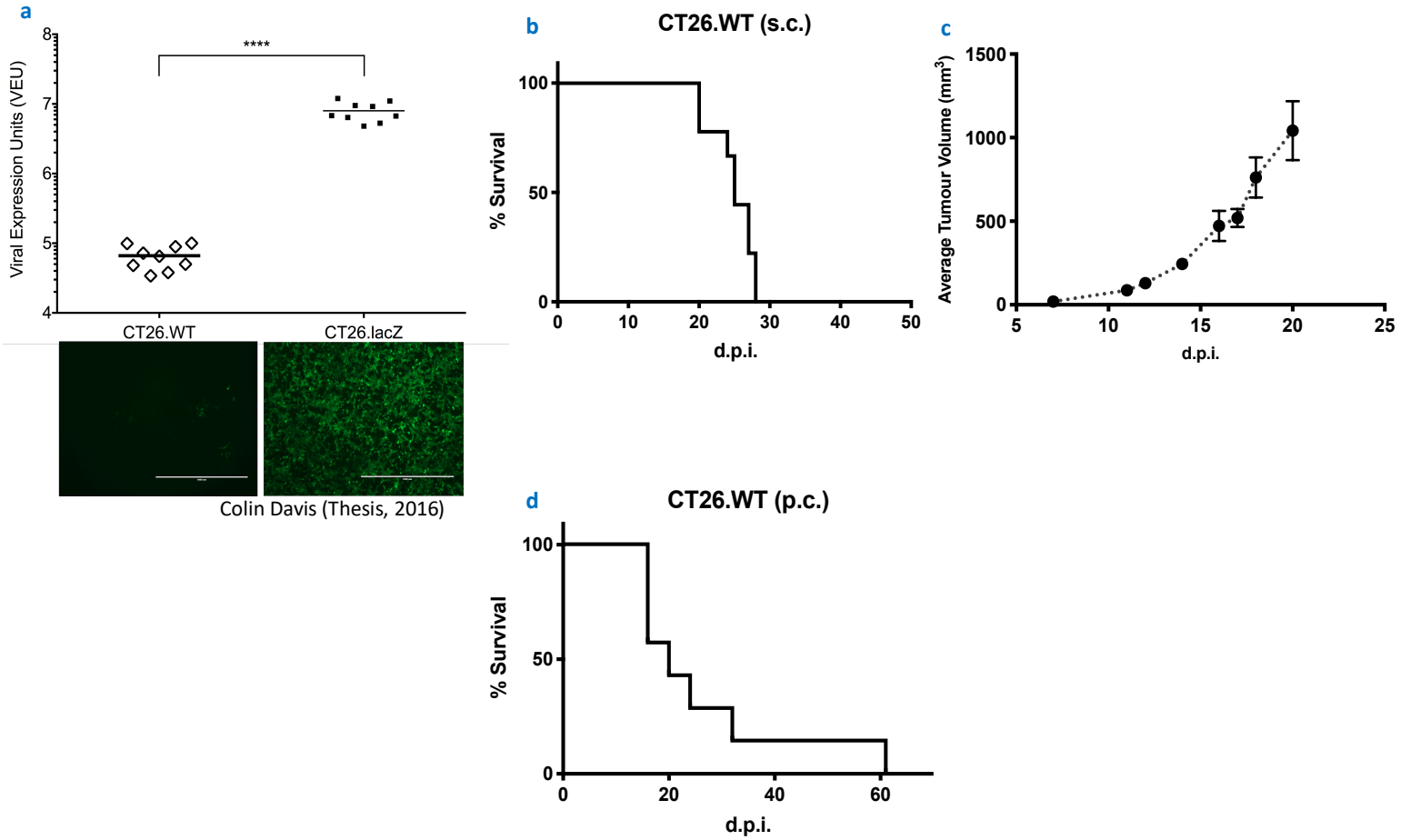


Figure 3.1 CT26 murine colon carcinoma as a model for cancer immunotherapy. **a) Previous work by group member Colin Davis demonstrates the striking difference in infection of CT26.WT and CT26.LacZ[82]. In this study, confluent monolayers of CT26 cells were infected with VSVΔ51 (MOI:0.01). Images shown were captured 24 hours post-infection whereas viral titers in viral expression units (VEUs) as determined by luciferase high-throughput tittering as described in Garcia *et al.*[84]**

(** p<0.0001, unpaired two-tailed student's t-test) b) 5e5 CT26.WT cells were implanted s.c. on the right flank of 6-week old Balb/c mice. Tumour measurements for outgrowth are shown in c) and it was determined that median survival occurred approximately 26 after implantation. (n=10). 5e5 CT26.WT cells were implanted i.p. in order to establish a murine model of peritoneal carcinomatosis (p.c.). Median survival was found to be approximately 21 d.p.i. (N=10) as shown in d).**

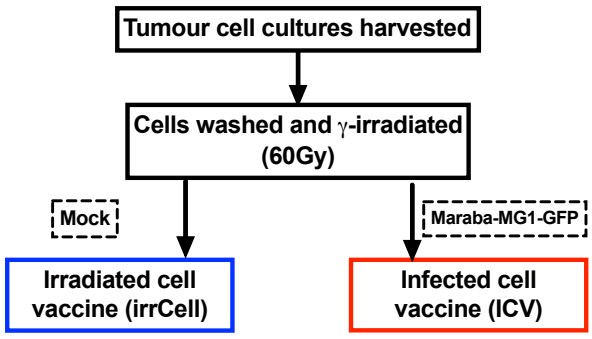
subclone. Our initial predictions regarding the outcomes of these pilot studies were largely predicated on the belief that a higher infection rate in the context of ICV would represent a more immunogenic vaccine platform, and subsequently a heightened protective capacity associated with CT26-ICV compared to irrCT26, even more so when using LacZ compared to wildtype (**Figure 3.2b**). In addition, the CT26.LacZ murine model is generally more responsive to pre-clinical interventions including OV therapy, and therefore it was hypothesized that these cells would represent a less aggressive tumour challenge compared to the WT counterpart (**Figure 3.2b**).

Surprisingly, it was observed that two doses of either irrCT26.WT or irrCT26.LacZ was able to confer nearly 90% protection from subsequent s.c. tumour challenges on the right flank of the animals. Interestingly, this was robustly higher than observed for ICVs prepared with either of the CT26 cell lines, which conferredapproximately 50% protection from challenge (**Figure 3.2c**). It is also important to note that this protective capacity extended beyond the cell line used specifically for vaccine preparation, as evidenced by the ability of CT26.WT-based vaccines to protect from subsequent CT26.LacZ challenge, and vice versa.

A follow up study confirmed this inherent protective capacity associated with CT26 tumour-based vaccines and it was found to be independent of the dose of γ-irradiation used for vaccine preparation. It was previously established that doses of 30, 45 and 60 Gy were sufficient to induce the necessary cellular senescence for a safe autologous tumour vaccine incapable of outgrowth once administered. However, decreasing the dose of γ-irradiation below the 60 Gy used extensively for our

vaccine studies did not appear to hinder the protective capacity of either cell line upon a s.c. flank challenge with the more refractory and aggressive CT26.WT cell line (**Figure 3.2d**).

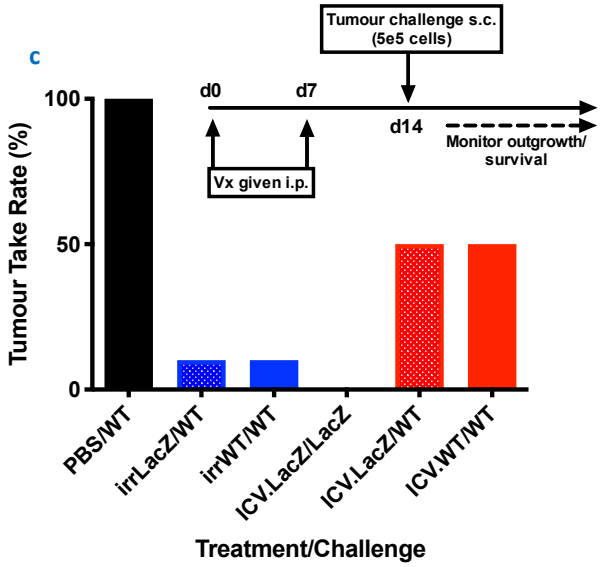
a



b

Treatment/Challenge	Predicted Tumour Outgrowth
PBS/CT26.WT	+++
irrCT26.WT/CT26.WT	+++
irrCT26.LacZ/CT26.WT	++
ICV.LacZ/CT26.LacZ	-/+
ICV.LacZ/CT26.WT	+/++
ICV.WT/CT26.WT	++/+++

c



d

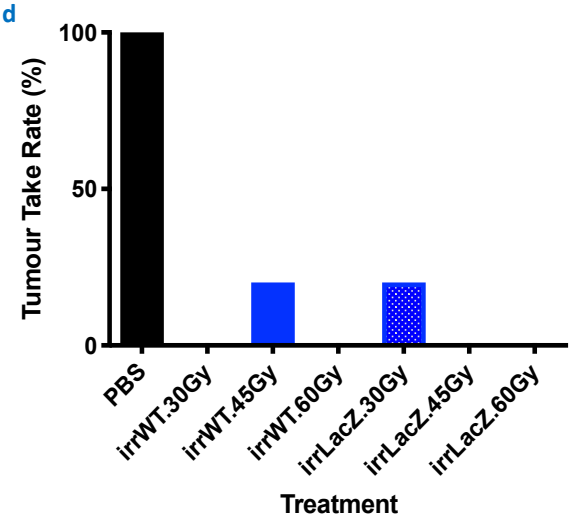


Figure 3.2 CT26-based irradiated cell vaccines demonstrate a remarkably high protective capacity. **a) The preparation of irradiated CT26 (irrCT26) and infected cell vaccines (ICV.WT/LacZ) both involved harvesting monolayers of cultured tumour cells and irradiating them for 60G. ICV preparation involved the subsequent infection of irradiated cells with Maraba-MG1-GFP at MOI:10 for 2 hours under constant rotation in an incubator. irrCT26 vaccines were mock infected during this time. b) Predictions for pilot study with "+" denoting expected tumour outgrowth a "-" the possibility of**

none. Highest likelihood of outgrowth predicted (other than PBS) for uninfected CT26.WT with a challenge of refractory WT cells (irrCT26.WT/CT26.WT). Lowest predicted for ICV.LacZ/CT26.LacZ due to higher infectability and a less aggressive tumour challenge. c) Prophylactic tumour challenge experiment showing the percent of mice in each group with palpable flank tumours as of day 21 post-implantation (tumour take rate, n=10/group) Treatment represents the two-dose regimen given while Challenge denotes the cell line used for s.c. tumour challenge. d) Prophylactic challenge experiment following same protocol as c) except all treatments are two doses of cells irradiated for various doses and all groups were challenged with the more aggressive CT26.WT s.c. on the right flank.

3.3 *Homologous vaccination with irrCT26 and ICV-CT26 lacks therapeutic efficacy in CT26.WT flank model*

A therapeutic CT26.WT syngeneic tumour model was established by implanting 5e5 tumour cells s.c. on the right flank of six-week-old female Balb/c mice. Tumour outgrowth was monitored over the following days and therapeutic vaccination regimens only commenced upon average tumour volume reaching a palpable volume of 5 mm x 5 mm. The experimental design as well as survival data for this pilot therapeutic experiment are shown in **Figure 3.3a**. It was observed that neither homologous dosing of irrCT26.WT or CT26.WT-ICV were capable of contributing significant extension to median survival compared to PBS control groups (median survival for all three groups fall around 28-32 days post-implantation). Furthermore, it was observed that neither vaccine platform when administered in successive, homologous doses was able to significantly delay tumour outgrowth as observed through three times weekly tumour volume measurements (**Figure 3.3b**).

3.4 *A novel heterologous prime-boost vaccination regimen improves immune responses against gp70 tumour antigen*

Following the suboptimal *in vivo* performance of both homologous prime-boost regimens in the CT26.WT therapeutic model, we elected to undertake a deeper analysis of the immune responses generated through vaccination, and whether the combination of autologous CT26.WT vaccine vectors in a heterologous prime-boost framework could ultimately enhance the immunogenicity of the vaccine regimen.To this end, naïve Balb/c female mice were used for analysis in a prophylactic vaccine experiment where vaccines were administered i.p. seven days apart as in previous vaccine studies, and the experimental readout would involve quantifying the relative level of CTL responses generated

against the highly disease-relevant gp70 antigen, as well as the proportion of CTLs specific for gp70 or viral antigen (VSVn) that are also positive for the potent immunostimulatory cytokine IFNγ.

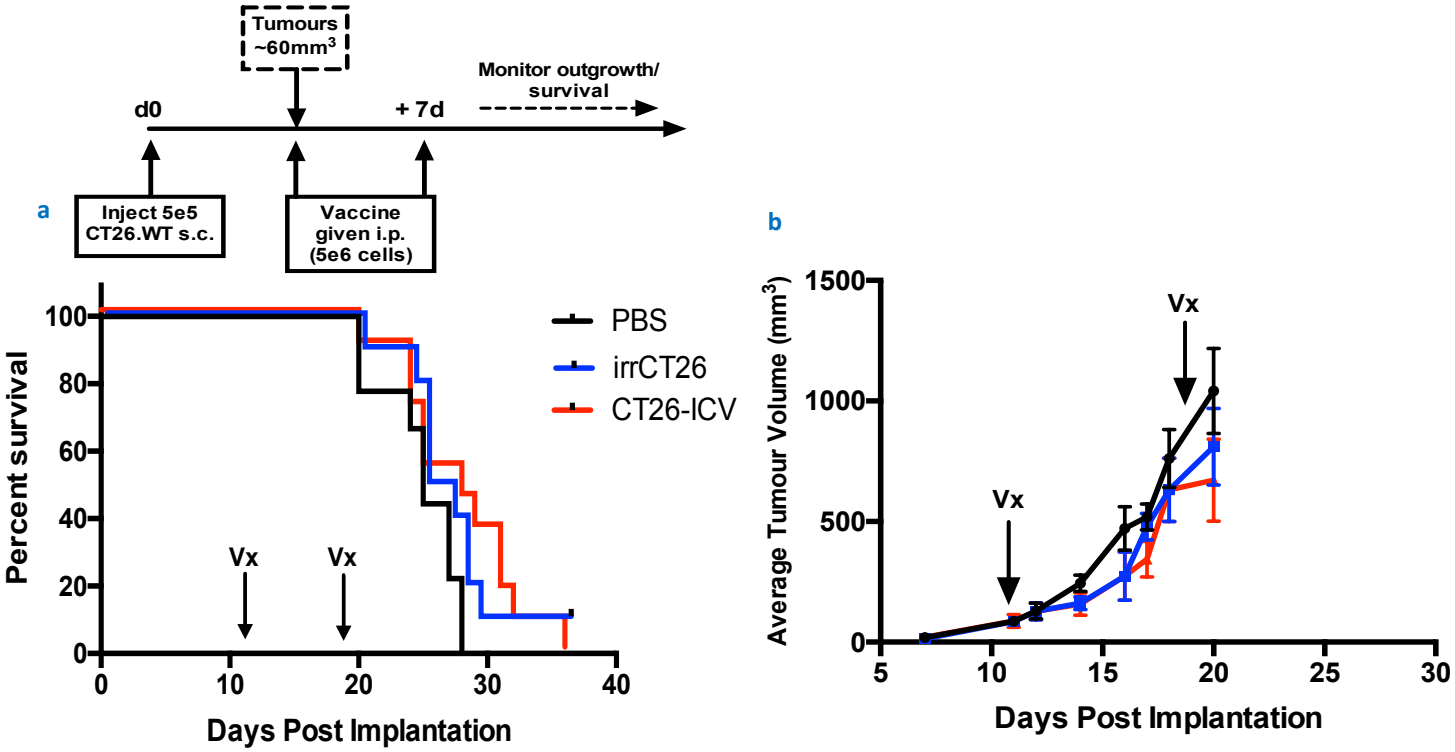


Figure 3.3 Homologous vaccination with irrCT26 and ICV-CT26 lacks therapeutic efficacy in CT26.WT flank model. **A therapeutic CT26.WT syngeneic flank model was established by implanting 5e5 tumour cells s.c. on the right flank. Vaccine regimens were only administered upon most tumours in treatment groups reaching a palpable size of 5mm x 5mm, and vaccine priming and boosting doses are denoted by the black arrows. All vaccines and controls were administered in 100 µL via i.p. injection. a) Survival data for initial exploration of CT26.WT therapeutic flank model with the corresponding tumour measurements shown in b). Tumour measurements were taken three times weekly and average tumour volume lines terminate at the first endpoint per group (1625mm^3, n=10/group).**

The experimental workflow for these combined *in vivo* CTL assay and intracellular staining (ICS) experiments are described in ***Methods 2.7*** and visualized in **Figure 3.4a**. Following the preparation, staining, peptide loading and injection of the mixed population of naive CFSE^{Hi} and CFSE^{Lo} splenocytes to vaccinated mice, spleens from treated mice were harvested and prepared for flow cytometry and ICS protocols. **Figure 3.4b** depicts a typical readout for CFSE staining as measure by flow cytometry. In untreated (PBS) groups, there exists a nearly exact 50% split between the CFSE^{Lo} and CFSE^{Hi} peaks, which also serves as an indicator of proper preparation and administration of the naïve

splenocytes. The primary readout for the in vivo CTL assay is percent (%) cytotoxicity, which is determined by analyzing the elimination of the $CFSE^{Hi}$, gp70-loaded peak and thus serves as a surrogate indicator of the CTL-response generated against CT26 tumours as a result of vaccination (for example, as observed in the lower right panel of **Figure 3.4b**).

It was observed that two homologous doses of irrCT26.WT (which had previously shown a robust protective capacity in prophylactic studies, **Figure 3.2**) was consistently able to induce an approximate 20% elimination of $CFSE^{Hi}$ gp70-loaded splenocytes, whereas the recorded immune response generated through homologous ICV dose was surprisingly close to undetectable. Even more striking was the observed CTL response generated using a novel heterologous prime-boost approach combining an irradiated cell prime and ICV boost, specifically. Using this strategy, we were able to detect an average CTL response of approximately 40%, with two instances of almost complete elimination of the gp70-loaded splenocyte subset (**Figure 3.4c)**. Furthermore, it was observed that this heterologous strategy dramatically increases the proportion of gp70-specific CTLs that are copositive for IFNγ following restimulation and ICS, and that this enhancement in IFNγ staining correlates well with improved CTL response as a result of vaccination **(Figure 3.4d,e)**.

Interestingly, this effect was not observed when ICV is used to prime an irradiated cell vaccine boost, possibly highlighting a unique priming capacity associated with irrCT26.WT and/or a more relevant boosting capacity inherent to ICVs. It is also important to note that in addition to the largest CTL and IFN responses detected against gp70, the irrCT26:ICV regimen also generated the highest proportion of IFN^{+} CTLs specific for the VSV N viral peptide (**Figure 3.4c,d)**. This relatively large antiviral immune response does not appear to detract from the generation of antitumour responses in this context, yet this division of immune focus between tumour and viral antigens is an important component to consider while moving forward with ICV preclinical and clinical models.

3.5 *Exploring heterologous prime-boost vaccine strategies in the context of therapeutic murine tumour models and the addition of armed OV vectors*

Following the observed enhancement of gp70-specific CTL responses by the novel irrCT26:ICV heterologous regimen in a prophylactic setting (**Figure 3.4**), we elected to investigate this phenomenon in the context of therapeutic tumour models. In a therapeutic flank model established similarly to those outlined **Figure 3.3**, it was observed that an irrCT26.WT prime followed by an ICV-CT26 boost was able to extend survival moderately, yet significantly, when compared to homologous doses of either autologous vaccine vector. This extension of survival in mice treated with the heterologous prime-boost regimen was, however, not especially robust and represents an opportunity to further potentiate the therapeutic response to vaccination through additional immunostimulatory factors (**Figure 3.5a**).

To further interrogate the therapeutic potential of this proposed vaccination process, we used a syngeneic murine model of peritoneal carcinomatosis (P.C.), like those employed in reports published by Alkayyal *et al.*, using CT26.WT to implement an aggressive i.p. model. The model was established by implanting 1e5 CT26.WT cells in the peritoneal cavity of six-week-old Balb/c females and beginning treatment following three days of tumour outgrowth. It was also decided to include a group of ICV vectors prepared with Maraba-MG1-eGFP (as was used with previous ICV experiments to this point), as well as Maraba-MG1 vectors expressing the powerful immunostimulatory cytokine IL-12, which has been shown in previous publications to drive vaccine efficacy through enhanced recruitment and activation of NK cells and other key immune players[67]. For the P.C. model, homologous doses of irrCT26 and various ICVs preparations were administered i.p. as previously outlined, but instead dosing frequency was increased to three times weekly for a total of six doses, in keeping with previous models of P.C. explored by collaborators and previous published reports. The “heterologous” treatment group in **Figure 3.5b** refers to a single prime and single boost regimen (totaling two injections) using irrCT26 and ICV-CT26, respectively. Additionally, this regimen was administered as two doses spaced seven

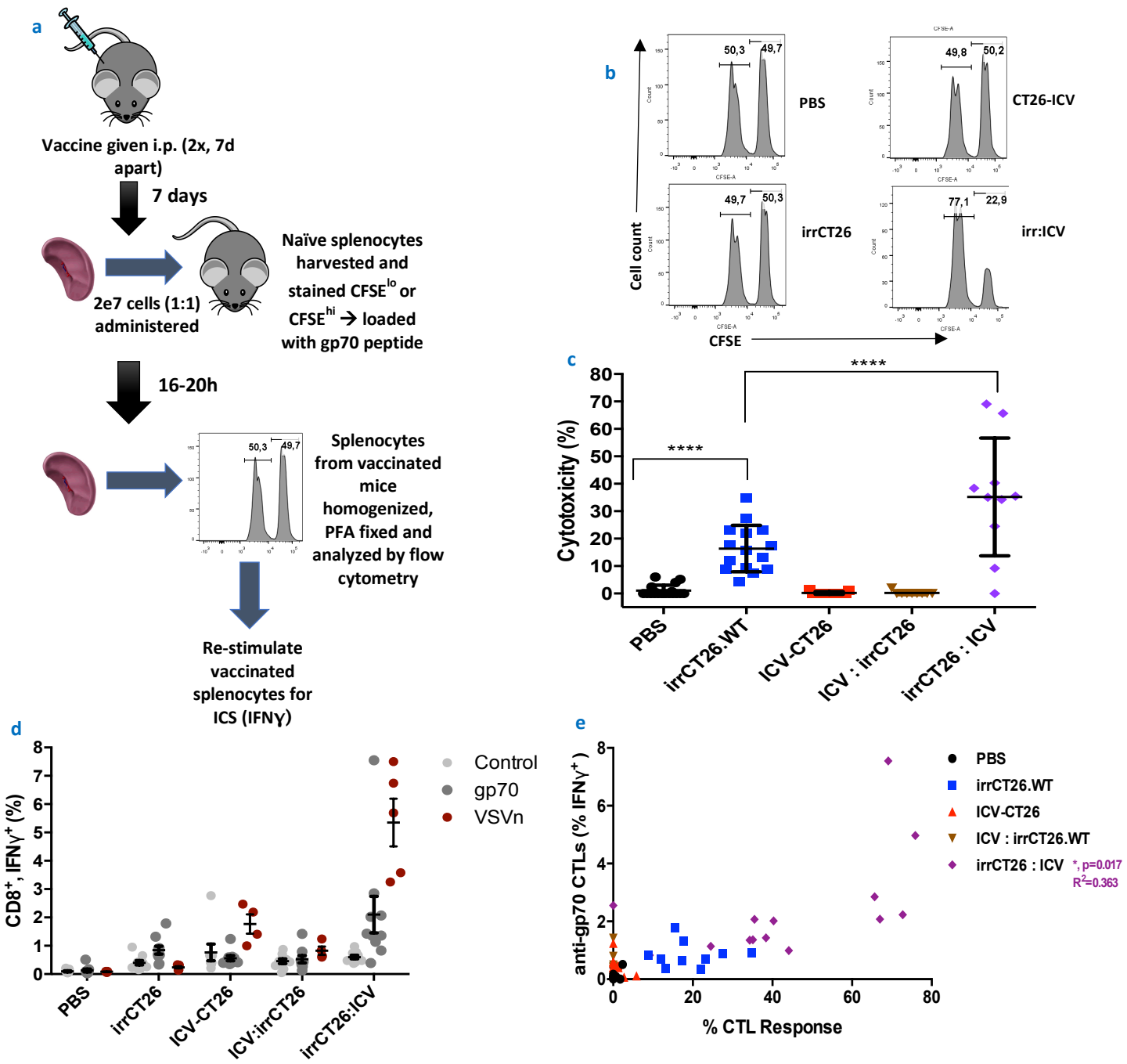


Figure 3.4 A novel heterologous prime-boost vaccination regimen improves immune responses against gp70 tumour antigen. a) Experimental workflow for prophylactic studies using *in vivo* CTL assay and intracellular staining for IFN responses against gp70 and/or viral peptide. Essentially, naïve splenocytes are prepared and stained with either a high (5μM) or low (0.5μM) concentration of CFSE, with the former being exogenously loaded with gp70 tumour peptide and both populations being injected intravenously to treated mice in a 1:1 ratio. Elimination of the CFSE high population (as seen in the bottom right panel of b)) indicates a tumour-specific CTL response and this value is given as % cytotoxicity after normalization to the control group. b) Flow cytometry analysis showing the presence of $CFSE^{Lo}$ and $CFSE^{Hi}$ peaks, and their subsequent changes in relative proportion caused by vaccine-driven, gp70-specific CTL responses. c) *in vivo* CTL assay results for various combinations of homologous and heterologous prime-boost vaccine regimens in a prophylactic

setting. Vaccines were administered i.p. seven days apart as with previous prophylactic studies, and vaccine responses quantified seven days post-boost (n=10/group, **** p<0.001). d) Flow cytometric data showing the proportion of CTLs copositive for IFN staining following stimulation with gp70, VSVn viral peptide or control (n=10/group). A correlation analysis between CTL response (% cytotoxicity) and IFN staining (% positive) is shown in e).

days apart between prime and boost. This schedule was chosen to assess the performance of our novel heterologous tumour-based vector strategy between different tumour models and also to determine how this regimen directly compares to the six-dose treatment schedule used by collaborators exploring the P.C. model of cancer.

Despite generating the highest observed anti-gp70 CTL responses in a prophylactic tumour model (**Figure 3.4**), it was evident that the heterologous prime-boost strategy using irradiated cells followed by ICV boost failed to contribute any striking extension of murine survival in the therapeutic tumour flank model (**Figure 3.5a**) or in the CT26.WT P.C. model (**Figure 3.5b**). Even at the relatively low seeding density of 1e5 cells used for the P.C. model, it is clear that this model is aggressive, with a median survival of approximately 20 days for untreated mice. There were several surprising takeaways from this study, all of which highlight the potential for crucial future research questions. First, this is one of the first instances where homologous vaccination with irrCT26.WT produced a noticeable therapeutic effect, despite consistently producing moderate gp70-specific CTL responses in previous prophylactic studies (**Figure 3.4c**). In addition, it was striking to see such a low therapeutic response associated with the heterologous prime-boost strategy in the p.c. model, given the consistently robust values typicalltypically y obtained for *in vivo* CTL studies. It was also interesting to observe the extent to which incorporating the IL-12-producing Maraba-MG1 vector was able to improve ICV performance *in vivo* compared to ICV prepared with the standard MG1-eGFP, with six homologous doses driving a survival rate of nearly 90 percent in mice with established peritoneal disease (**Figure 3.5b**).

It was important to also determine if any differences exist in *in vivo* CTL assay results obtained from naïve vs. tumour bearing mice, as well the impact of repeated vaccine dosing, as these factors may

contribute to the unexpected findings presented in the pilot p.c. model study. An *in vivo* CTL/ICS flow assay was performed by establishing p.c. disease as previously described and then treating with either two doses of heterologous vaccine or six doses of homologous preparation, with immune analysis taking place on day 17 post-implantation. For this study we also elected to include a heterologous treatment group including ICV boosted with irradiated cells to investigate if this inverse strategy has added benefits in a therapeutic, tumour-established setting. Again, it was observed that homologous dosing with irrCT26.WT was able to generate a substantial CTL response against gp70, as was the heterologous strategy using irrCT26 to prime ICV.

Furthermore, it was evident that ICV:irrCT26, as well as homologous doses of ICV, were unable to promote a significant CTL response against gp70 (**Figure 3.5c**). Even more striking were the effects observed for vaccine preparations made using the IL-12 expressing virus. Despite the strong therapeutic efficacy of CT26.WT-ICV-IL12 in the p.c. model (**Figure 3.5b**), repeated doses of this vector were not able to promote an increase in the CTL response against gp70 (**Figure 3.5c**) or improve the proportion of these cells co-positive for IFN staining as detected through ICS and subsequent flow cytometry (**Figure 3.5d**). It was also observed during this analysis that there was an apparent shifting of the CTL response generated through irr:ICV treatment from one that was primarily targeted towards viral proteins, to one more skewed towards gp70 by virtue of including the IL-12 transgene.

3.6 *Immunogenic adjuvants in combination with heterologous prime-boost autologous vaccines drive strong antitumour immune responses*

Following observations regarding the immunogenic potential of combining irradiated cell and infected cell vectors in a heterologous prime-boost cancer vaccine system, we elected to investigate the potential of biological adjuvants to further enhance the anticancer immune responses generated through therapeutic interventions. A secondary, yet equally important, experimental question centers on the comparison between the use of Maraba-MG1 (which was used for all previous vaccine experiments) and

VSVΔ51 for preparation of the ICV components. Despite the large genomic similaritiesbetween these two commonly used oncolytic rhabdoviruses, they have been shown to possess varying biological properties *in vitro* and *in vivo* when being utilized as OV biotherapies. It is therefore vital to directly compare which would provide the optimal viral component for ICV therapy as well as combination with irradiated cell priming and other accessory immunogenic factors.

To this end we again used of a CT26.WT therapeutic flank model and designed a workflow through which two simultaneous experimental questions could be explored. The focus of the first component was quantifying the immune response stemming from vaccination (with or without adjuvant administered via two potential routes) while the second featured the same protocol and treatment groups but concentrated on survival and tumour progression/outgrowth. In both cases, treatments regimens were only initiated after the majority of the animals displayed palpable tumours of approximately 5mm x 5mm and were subject to regrouping in order to have all groups starting at a similar average tumour volume prior to therapeutic intervention.

The adjuvant used in the study was administered at a dose of 15 μg/mouse either admixed with the vaccine itself and given i.p. or via intranasal (i.n.) injection immediately before vaccination. This dosage was determined both through early optimization studies in combination with irradiated cell vaccines and through direct consideration from the vaccine developer. Early studies investigating the combination of adjuvant with homologous irradiated CT26.WT vaccination demonstrated a modest enhancement of gp70-specific CTL responses when mice were given a 30 μg dose concurrently with the initial priming dose of vaccine. Furthermore, increasing doses of adjuvant alone was able to drive an increased abundance of neutrophil and macrophage migration to the i.p. cavity in tumour-na.ve animals when assayed by a peritoneal lavage and subsequent flow cytometric analysis (**Figure 3.6a**).

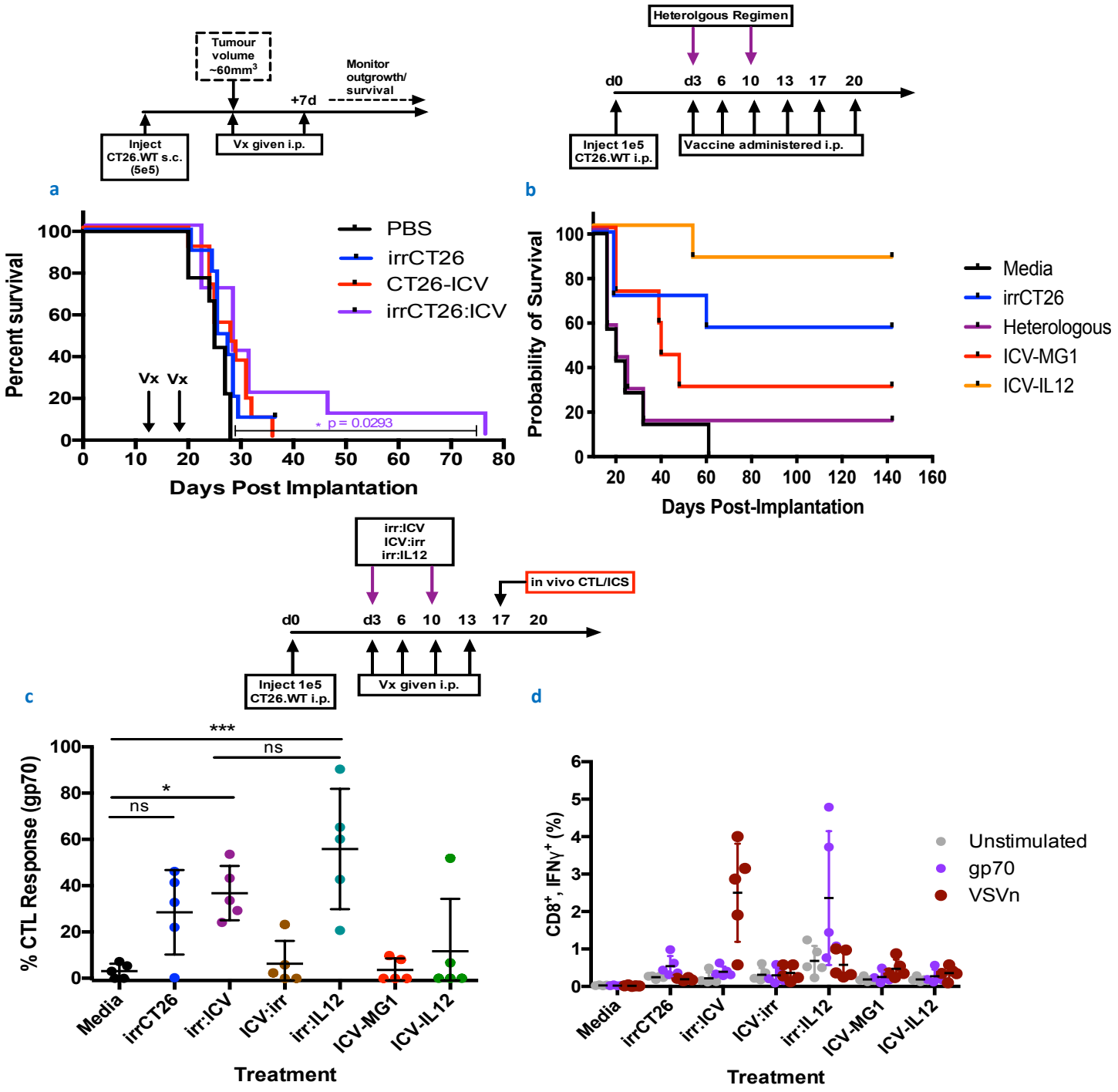


Figure 3.5 Exploring heterologous prime-boost vaccine strategies in the context of therapeutic murine tumour models and the addition of armed OV vectors. a) Survival results for a therapeutic s.c. flank CT26.WT experiment aimed at investigating the potential of a heterologous prime-boost vaccination strategy (irradiated CT26 followed by ICV-CT26, prepared using Maraba-MG1-eGFP). It was observed that only this heterologous treatment cycle was able to produce a significant extension of survival (p=0.0293, Logrank test for trend). b) A CT26.WT peritoneal carcinomatosis model was established by implanting tumour cells in the i.p. cavity of six-week-old Balb/c female mice and following the treatment schedule as displayed. c) *in vivo* CTL data generated in mice bearing CT26.WT p.c. disease. It was observed that only treatments involving the irr:ICV regimen (irr:ICV, irr:ICV-IL12) were able to significantly improve gp70-specific CTL responses (one-way ANOVA p<0.0001, *p<0.05, ***p<0.001, Holm-Sidak multiple comparisons test). d) Analyzed flow

data for ICS analysis of $CD8^+$, IFN^+ co-positivity following stimulation with gp70 or viral VSVn peptide. This data was generated simultaneously along with data for c).

Follow up adjuvant-vaccine studies sought to answer two fundamental questions contributing to the preparation of the optimal autologous vaccine regimen: 1) Does the route of adjuvant administration effect the generation of downstream anti-tumour and/or anti-viral immune responses following vaccination, and 2) do either Maraba-MG1 or VSV-Δ51 possess any inherent therapeutic advantages when used as viral vectors for the preparation of ICVs in homologous or heterologous vaccine regimens? It is evident based on immune analysis data that both MG1 and VSV are able to elicit similar, relatively robust gp70-specific CTL responses in mice bearing established CT26.WT flank tumours, and that i.p. coadministration of adjuvant in the context of an irrCT26:ICV-MG1 vaccine failed to improve immune responses against gp70. Homologous doses of irrCT26.WT in combination with adjuvant also failed to produce detectable CTL response as was observed in previous tumour-naïve animal models. Most striking of all however was the extremely potent gp70-specific CTL responses generated through heterologous prime boosting (irrCT26:ICV-VSVΔ51) when combined with a distal i.n. administration of adjuvant. These were the highest aggregate *in vivo* CTL assay percent cytotoxicity values observed to date (approximately 70%) and were significantly higher than observed for either heterologous regimen alone or in combination with i.p. adjuvant (**Figure 3.6b**).

The biological salience of i.n. adjuvant administration was observed in the corresponding ICS data as well, as it was observed to contribute to a robust population of $CD8^+$, $IFN\gamma^+$ cells following gp70 stimulation. This was also concomitant with an enhanced antiviral immune response as was observed for other heterologous regimens with and without i.p. adjuvant, yet the i.n. adjuvant was the only condition in which a noticeable increase in gp70-specific increases were observed in tandem (**Figure 3.6c**). Another interesting finding of this

pilot adjuvant study was that despite a range of biological differences in the immune compartments of animals receiving vaccine with or without adjuvant, no treatments were able to generate a level of therapeutic efficacy sufficient to significantly emerge as an optimal, or even discernable, therapeutic candidate in terms of delaying tumour outgrowth (**Figure 3.6d**) or extending survival.

3.7 *Vanadium-based VSes show biological activity in combination with OVs and ICVs in vitro and in vivo*

To this point, the pursuit of a highly optimized autologous vaccine strategy has centered on developing a novel, combined irradiated cell and ICV heterologous vaccine platform and on pursuing further enhancement of efficacy through the incorporation of adjuvants. The combination of VSe compounds and ICV therapy represents another intriguing avenue to pursue in the aim of driving overall vaccine therapeutic performance. Previous and current work by our group has continued to highlight the broad range of powerful biological activities associated with these small molecules and has highlighted them as immunomodulatory factors that could potentially potentiate the activity of our autologous vaccine strategy.

Previous work by members of our research group has exquisitely demonstrated the powerful immunomodulatory capacity of vanadium-based phosphatase inhibitors (i.e., meta- and orthovanadate) when used in combination with OV therapy for the treatment of resistant tumour models[85]. For example, the combination of VSVΔ51 and metavanadate in resistant human cancer cell lines has been shown to enhance the expression of highly immunostimulatory genes such as IFNB, IL-6 and tumour necrosis factor-α (TNFα) among others (**Figure 3.7b**). In addition to this is a strong upregulation of CXCL9, which is a powerful T cell chemoattractant, and in combination with increased viral infection (**Figure 3.7a**), could provide a means of improving the efficacy and performance of our ICV treatment platforms.

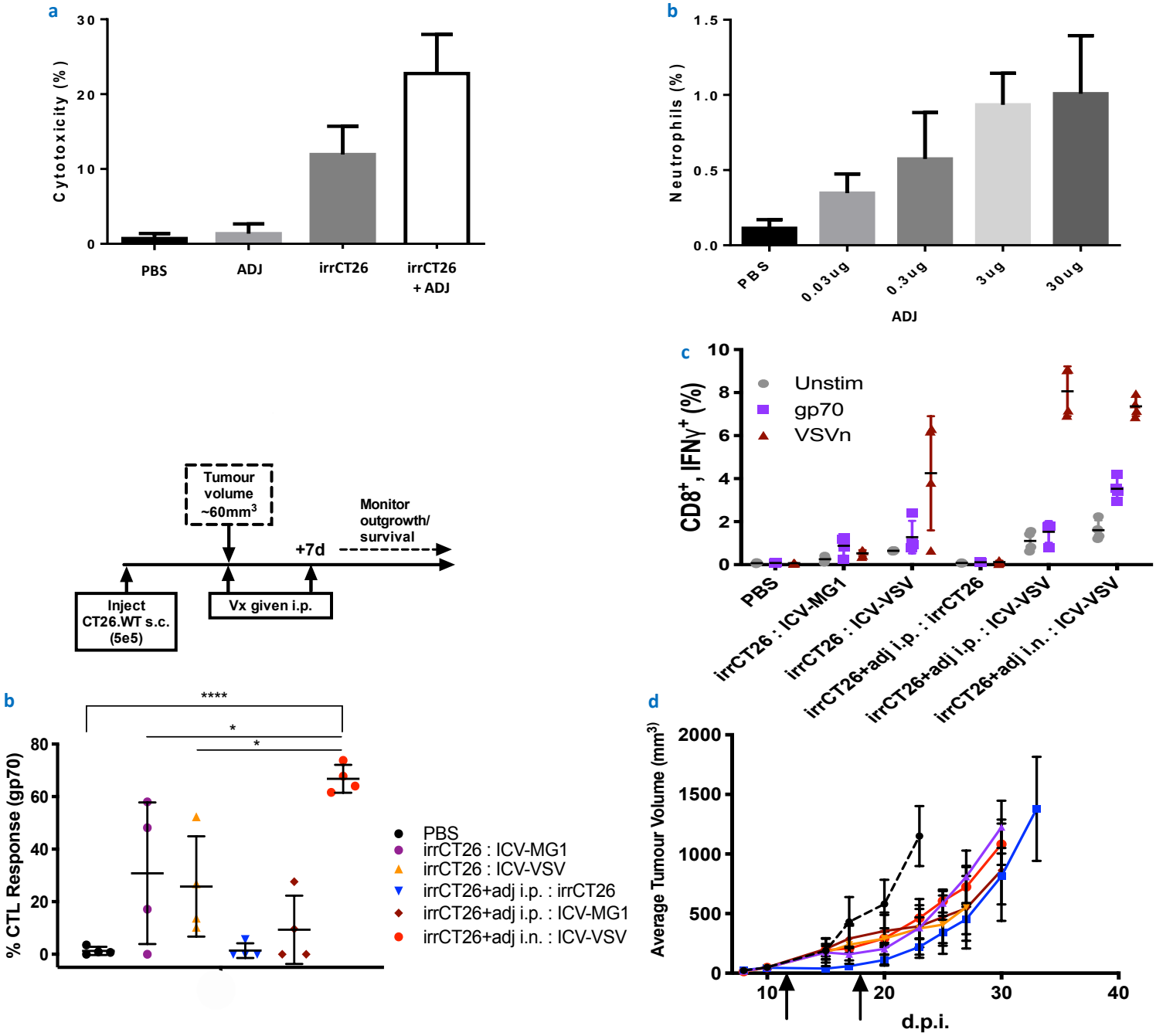


Figure 3.6 Immunogenic adjuvants in combination with heterologous prime-boost autologous vaccines drive strong antitumour immune responses. **a) Initial investigation of our chosen adjuvant shows the propensity of the adjuvant to improve gp70 CTL responses when combined with two doses of irrCT26 vaccine, and that increasing doses of adjuvant can lead to increased number of neutrophils and macrophages in the i.p. cavity as detected by flow cytometry following peritoneal lavage b) *in vivo* CTL data for CT26.WT therapeutic flank models were set us as previously described and were used to investigate the differences in immune responses generated using ICVs made with either Maraba-MG1-eGFP (ICV-MG1) or VSVΔ51 (ICV-VSV) and whether or not the inclusion of a known immunogenic adjuvant can improved antitumour responses following vaccination. Adjuvant was administered at a dose of 15 µg/animal either i.p. admixed with the vaccine preparation or administered intranasally (i.n.) immediately prior to i.p. vaccination (n=5/group, One-**

way ANOVA <0.0001, ****p<0.0001, *p<0.05, Holm-Sidak multiple comparisons test). c) Flow cytometry data showing the relative proportions of $CD8^+$, IFN^+ cells following treatment groups from b). d) Average tumour outgrowth volume corresponding to treatments from b), n=5 per group for survival/outgrowth experiments.

The CT26.WT cell line when cultured in monolayer is robustly resistant to infection as seen in both **Figure 3.7a** and in various published studies. Work by our research group has demonstrated the ability of several classes of small molecules, including several based-on a vanadium backbone (e.g., orthovanadate and metavanadate), to enhance both the infection and multimodal killing of various resistant cancer lines. **Figure 3.7a** shows the ability of a dose of as little as 17 μM orthovanadate to increase the expression of virus-driven fluorescent signal 24 hours post-infection. In addition, doses of 11 μM and above were able to potentiate the killing of CT26.WT 24- and 48-hours post infection when combined with OV (**Figure 3.7a**).

The combination of vanadate and virotherapy represents an appealing therapeutic possibility in the context of ICVs as well. Initial investigation of this idea showed nearly undetectable enhancement of ICV-driven CTL responses against gp70 when orthovanadate was directly admixed with vaccine preparations to a concentration of 100 μM and administered to tumour-naive mice in a two-dose regimen. Interestingly as well, it appeared to hinder the previously robust CTL responses generated by successive doses of irradiated CT26.WT (**Figure 3.7c**). Despite the poor performance observed in CTL assays, incorporation of 100 μM orthovanadate with Maraba-MG1 directly in the ICV preparation appears to modestly improve the protection of naive mice against subsequent CT26.WT flank challenge (**Figure 3.7d**), as well as delay tumour outgrowth (**Figure 3.7e**).

AIM #2: Identifying novel enhancers for CAR-T cell therapy of resistant solid tumours

The second aim of our research project targeted towards identifying novel enhancers of the anticancer immune response involves attempting to uncover existing or novel pharmaceuticals that can selectively enhance the cytotoxic activity of CAR-T cells on tumour

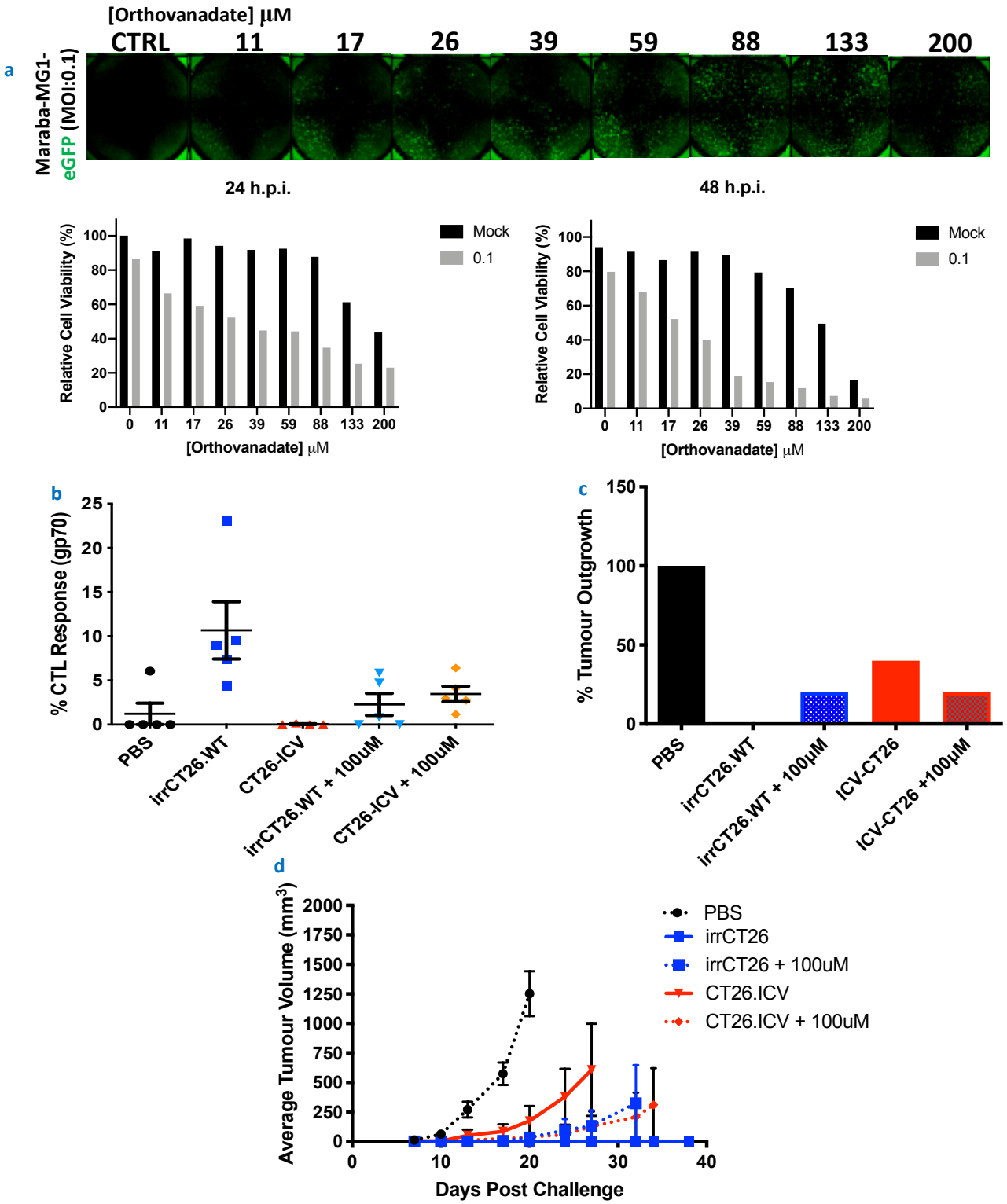


Figure 3.7 Vanadium-based VSes show biological activity in combination with OVs and ICVs in vitro and in vivo. a) **fluorescent microscopy images showing the enhancement of Maraba-MG1-eGFP infection (MOI:0.1) of resistant monolayers of CT26.WT cells by sodium orthovanadate in a dose dependent fashion. Images were obtained 24 hours post-infection. Below is cell viability data showing the potentiation of CT26.WT infection by MG1-eGFP in combination with orthovanadate at both 24 and 48 hours post infection, as detected by AlamarBlue Cell Viability Dye. b) *in vivo* CTL data generated in tumour-naïve mice exploring the addition of orthovanadate to irradiated CT26.WT and CT26-ICV**

preparations with Maraba-MG1. c) Rates of tumour outgrowth for mice vaccinated with homologous doses of autologous with or without VSe. d) Tumour outgrowth measurements accompanying c).

cells that were previously resistant to killing. Solid tumours have been reported to resist killing by CAR-T cells through a variety of cellular mechanisms despite expressing relatively high levels of target tumour antigen specifically recognized by the CAR-T construct. Our study into this phenomenon centered on the use of an A549 human lung adenocarcinoma cell line that had previously been shown by collaborators to robustly resist killing by a HER2- specific CAR-T cells construct despite relatively high surface expression of HER2[86].

Our aim using this framework is to identify drugs, either through uncovering a new therapeutic indication or through the discovery of compounds that are truly novel, capable of overcoming this cellular resistance of CAR-T cell therapy and potentiating the therapeutic potential of this exciting immunotherapy for solid tumours.

Figure 3.8 GFP-transduced human lung carcinoma cells can be stably fixed and quantified using High Content Microscopy

In our search for small molecules that specifically enhance the cytotoxic capacity of CAR-T cells, we elected to base our screening platform on the human A549 lung adenocarcinoma line. This tumour line has been explored by our group for several other screening experiments in combination with our Prestwick chemical library of FDA-approved small molecules, and as a result we have the benefit of a surplus of data from previous screening experiments for analysis. This cell line has been specifically explored by collaborators at the McMaster University Immunology Research Center and has been observed to resist killing by CAR-T cells despite relatively robust expression of the target HER2 surface antigen (**Figure 3.9b**). These factors in tandem highlight A549s as an ideal target cell line to interrogate putative tumour sensitizing compounds using our existing high-throughput screening (HTS) tools to generate a novel screening platform for this aim.

To capitalize on our access to a suite of powerful HTS tools, we elected to explore the design of a fluorescence based HTS assay using our ArrayScan High Content Microscope to simultaneously photograph individual assay wells and quantify the average fluorescent intensity emitted from the cultured cells therein. To this end, A549 cells were stably transduced with a GFP-encoding lentivirus for 7 days under puromycin selection. The resulting cells were visually confirmed to express GFP and were seeded in culture and subsequently, broad GFP expression was further confirmed using flow cytometry and flow assisted cell sorting (FACS, **Figure 3.8a**, leftmost panel). The GFP^{+} cell population was further sorted using FACS into three cell population based on average GFP signal intensity: GFP^{Lo} (bottom 33% of expressers), GFP^{Mid} (middle 33%) and GFP^{Hi} (upper 33%) and these cell populations were subsequently grown out in culture and frozen in liquid nitrogen for further use and analysis.

The three GFP^{+} subpopulations were plated at increasing cell densities in replicate in 96-well format and plates were read for average GFP intensity/well 24 hours post-seeding. It was initially observed that only the A549-GFP^{Hi} population provided discernable differences based on varying cell density and was therefore selected as the target cell line moving forward. The A549-GFP^{Hi} line generates reliable and detectable differences in GFP signal intensity relative to cell seeding density, particularly up to the point of 20 000 cells/well. The use of GFP signal as a screen readout is also strengthened by the observably tight linear association between cell density and the measured GFP intensity, for example an increase from a GFP intensity value of approximately 50 for 10 000 cells/well increasing to approximately 90 for 20 000 cells/well (**Figure 3.8a**).

Lastly, it was observed that fixation of the assay plates with 1% paraformaldehyde (PFA) was sufficient to stably fix GFP signal at a relatively steady level for up to 7 days post-fixation. Furthermore, this was true even when the fixative was left directly in the well without removal or performing any subsequent PBS washes (**Figure 3.8b**). This is a major logistical benefit for the execution of a screen

featuring a large volume of assay plates that must be quantified at specific time intervals, while also minimizing the number of handling steps required for our complex screening platform design.

3.9 *A549-GFP^{Hi} cell death induced through various agents is detectable using ArrayScan GFP analysis*

An important follow-up question for optimizing the proposed CAR-T cell HTS assay is ensuring that losses in cell viability in the population of A549 target cell and the associated loss of GFP signal can be reliably detected using the ArrayScan fluorescence protocol following PFA fixation. A combination of various known cytotoxic agents and oncolytic VSVΔ51 was used to treat A549-GFP^{Hi} cells in culture (20 000 cells/well), in a dose-dependent manner, and were subsequently fixed (with or without subsequent PBS washes) or subjected to AlamarBlue based cell viability analysis as described previously.

Mitoxantrone is anthracenedione chemotherapy agent known to inhibit type II topoisomerase and prevent DNA synthesis/repair through nucleotide base pair intercalation. It has been explored as direct therapeutic agent for the clinical treatment of several cancers including prostate cancer and acute lymphoblastic leukemia with moderate anticancer activity reported in both cases[87–89]. It was observed that decreases in A549-GFP^{Hi} cell viability driven through increasing doses of mitoxantrone could be detected through AlamarBlue assay as well as corresponding loses in GFP signal after fixation with 1% PFA for 5 minutes (**Figure 3.9a**, bottom and top panel, respectively).

In addition to chemotherapy drugs such as mitoxantrone, similar results were obtained when testing additional known inducers of cell death including oncolytic VSVΔ51 and hydrogen peroxide as the anticancer agents, suggesting the assay is robust in detecting cell death induced by chemotherapeutic, reactive oxygen species, and biological agents (**Figure 3.9b, c**).

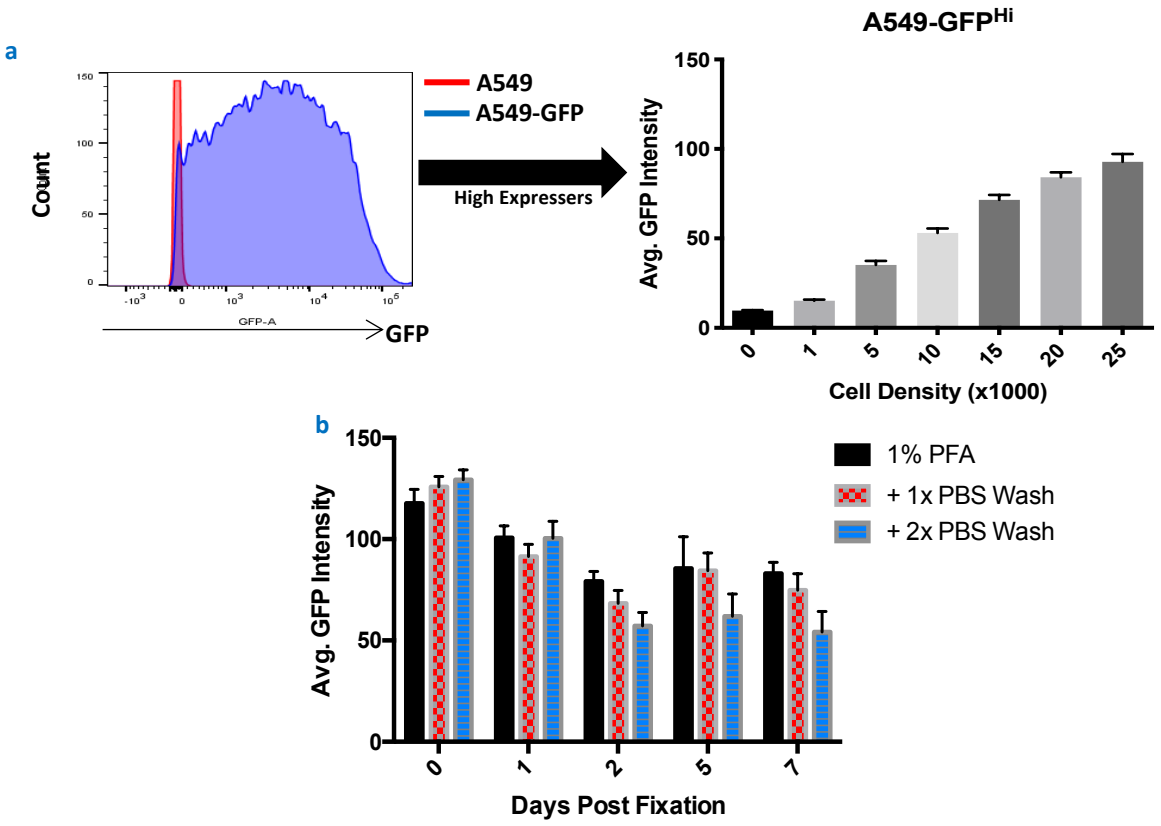


Figure 3.8 GFP-transduced human lung carcinoma cells can be stably detected, fixed and quantified using High Content Microscopy. **a) Flow cytometry plot showing the stable and broad expression of GFP from A549 human lung carcinoma cells. High expressors were sorted using FACS and this population was observed to generate significant and quantifiable differences based on differential seeding densities as measured by ArrayScan High Content Screening Microscope. Each line in the line central panel represents a replicate (n=8) and data is summarized in the right-most bar graph. b) A549-GFP^{Hi} cells were seeded at a density of 20 000/well and were subsequently fixed with 1% PFA for 5 minutes, with or without subsequent PBS wash(es). The read out is average GFP intensity as measured by ArrayScan.**

3.10 *Optimization of CAR-T cell and target cell co-culture ratios for novel dual-fluorescence HTS assay*

Following confirmation that A549-GFP^{Hi} cell death induced through various means could be reliably detected using ArrayScan assay protocols, subsequent optimization of our novel fluorescence-based HTS system focused on confirming proper effector to target ratios (E:T; CAR-T cell:A549-GFP^{Hi}) to use in order to ensure adequate interrogation of putative enhancers of CAR-T cell-mediated killing, as well as confirming that GFP signal loss through immune cell driven killing of target cells can be detected 24 hours following co-culture with and without fixation.

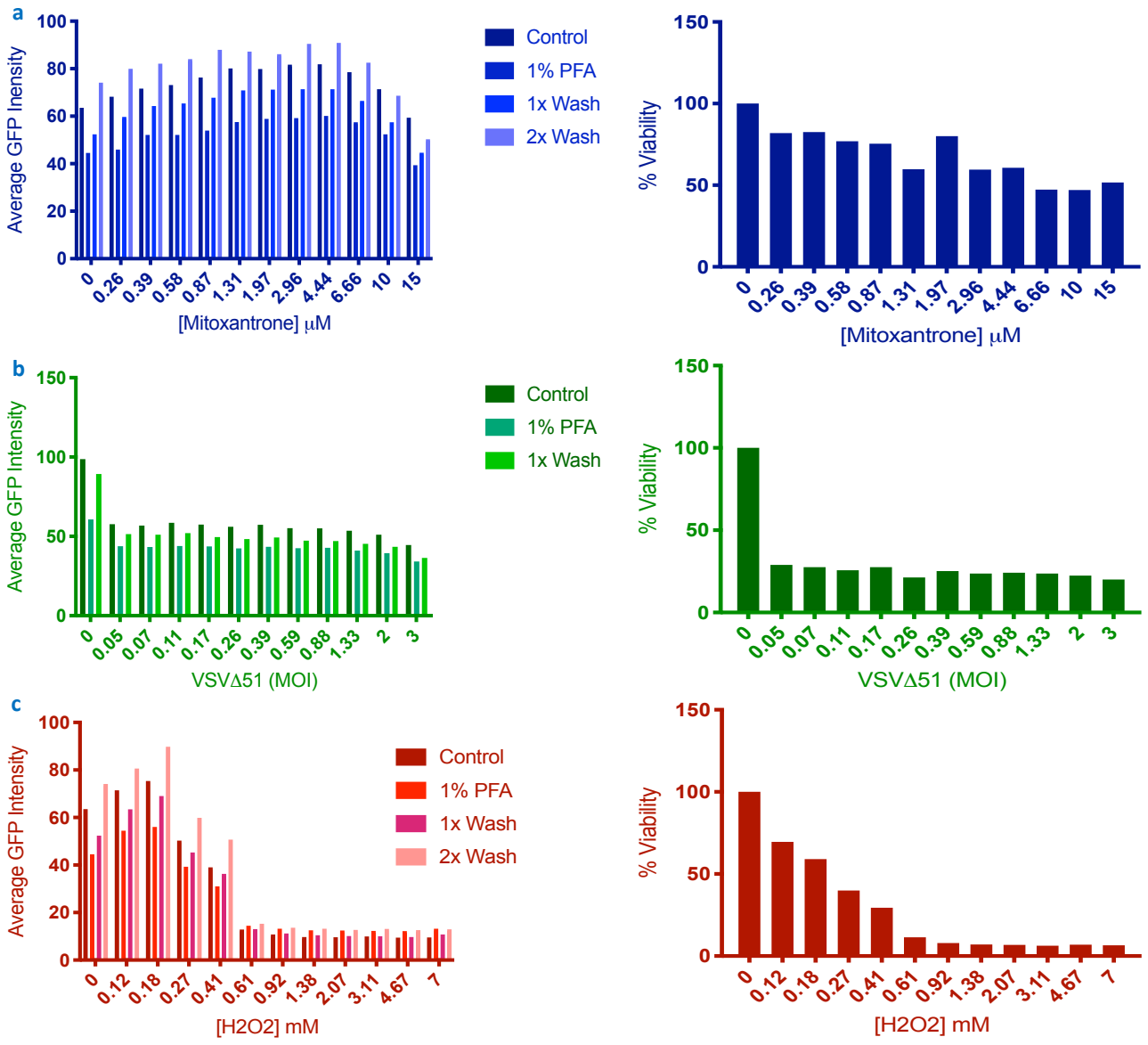


Figure 3.9 A549-GFP^{Hi} cell death is detectable using ArrayScan GFP analysis. **A549-GFP^{Hi} cells were plated at a density of 20 000 cells/well in 96 well format and were subsequently treated with serial dilutions of known cytotoxic drugs or oncolytic virus. A subset of assay replicates were fixed +/- PBS washes and read for GFP signal intensity using the ArrayScan, while a second set was used to quantify relative cell viability using AlamarBlue Metabolic Dye. a) Dose-response GFP intensity reads (top) and cell viability data (bottom) for target cells treated with mitoxantrone HCL at increasing concentrations. Reads obtained 24 hours post-treatment. b) Dose-response GFP intensity reads (top) and cell viability data (bottom) for target cells treated with VSVΔ51 at increasing MOIs. Reads obtained 48 hours post-infection. c) Dose-response GFP intensity reads (top) and cell viability data (bottom) for target cells treated with hydrogen peroxide (H_2O_2) at increasing concentrations. Reads obtained 24 hours post-treatment.**

Early stages of planning for our HTS study were carried out in close collaboration with the group of Dr. Johnathan Bramson at the McMaster University Immunology Research Center, who also provided several key materials and protocols for the optimization of the assay. Previous work by the Bramson laboratory has demonstrated that despite a relatively robust surface expression of the HER2 epidermal growth factor receptor (as well as NKG2D NK cell ligands), A549 cells remain strongly resistant to killing by human CAR-T cell constructs specific for HER2 (**Figure 3.10a**). The human HER2-specific CAR-T cell construct generously gifted by the Bramson laboratory features a directed ankyrin repeat (DARPin) sequence specific for the tumour antigen fused to a scaffold encompassing the extracellular hinge of CD8a, intracellular domains of human CD28 and intracellular domain of human CD3ζ, which are is crucial for the downstream transduction of signaling necessary for the induction of immune responses following the recognition of cognate antigen by the extracellular portion of the receptor[86] (**Figure 3.10a**).

Two additional GFP-expressing cell lines were also used for the purposes of determining an optimized E:T ratio. LOX-IMVI-GFP cells are a human melanoma cell line that does not express detectable levels of the HER2 surface antigen and therefore serves as a useful negative control. The OVCA3 cell line on the other hand is a human ovarian adenocarcinoma line known to express HER2 at a robust level and has been shown by the Bramson lab to remain sensitive to killing by CAR-T cells, unlike the A549 lung cancer line, and was therefore used as a positive control for optimization[90]. In both cases, we had received cell lines that were previously transduced to stably express GFP, and detection of this signal was confirmed using ArrayScan as previously performed for the A549-GFP^{Hi} cell line. It was observed that both LOX-IMVI-GFP and OVCA-3iv-GFP cells could be reliably detected using GFP signal intensity and that this signal increased proportionally to the number of cells initial seeded (**Figure 3.10b**).

In order to determine an optimal E:T for screening, human HER2-specific CAR-T cells were thawed and cultured using the aAPC system previously described. Concurrently, LOXIMVI-GFP ($HER2^{-}$), OVCA-3iv-GFP ($HER2^{+}$, sensitive) and A549-GFP^{Hi} ($HER2^{+}$, resistant) cells were seeded at a density of 20 000 cells/well in 96 well format. The CAR-T cells were counted, quantified for relative cellular viability, and subsequently subsets of CAR-Ts were either labelled with CellTrace Far Red dye (Thermo Scientific) or mock labelled prior to addition to target cells for co-culture. The rationale for this was to allow us to further take advantage of the ArrayScan High Content Screening capabilities by using a dual fluorescence-based system, in which target tumour cells are labelled with GFP and effector CAR-T cells are labelled in FarRed. This increases the value of our screening platform as it allows the simultaneous assessment of both cell populations in co-culture following exposure to small molecules or other immune modulators.

As expected, the LOX-IMVI cell line remained impervious to killing by the HER2-CART cell construct at all tested E:T ratios, both with and without the addition of CellTrace labelling, as detected by ArrayScan quantification of GFP signal intensity. The OVCA-3iv cell line in stark contrast immediately saw decreases in GFP signal at E:Ts as low as 0.5:1, and a nearly 50% reduction in total signal at a ratio of 1:1. It is also interesting to note that the CellTrace label appears to have no impact on the biological ability of the CAR-T cell to recognize target antigen and exert downstream effector function (**Figure 3.10c**), and that the quantification of FarRed RFP signal appears to also rise and fall proportionally with the number of T cells assayed (**Figure 3.10d**).

In the case of the A549-GFP^{Hi} cell line, no discernable GFP signal loss was detected until E:T ratios eclipsed 2:1. This finding suggests that an optimal screening ratio would be 1:1 (E:T) due to the fact A549s resist killing at this level, whereas a non-resistant cell line such as OVCA-3iv are clearly susceptible. Furthermore, the loss of A549-based GFP signal at higher E:T ratios (both with and without

T cell labelling) confirms the expression and availability of HER2 for the CAR-T cells to target, therefore cell cytotoxicity is only observed when saturating the co-culture with high E:T ratios (**Figure 3.10c**). Representative visualizations obtained through ArrayScan quantification of E:Ts of 1:1 for each cell line used for optimization are shown in **Figure 3.10e**.

3.11 *Proposed pilot HTS assay for enhancers of HER2-specific CAR-T cell-mediated killing of resistant target cells*

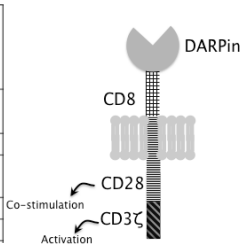
The overall proposed screening plan for interrogating the Prestwick chemical library, which features nearly 1280 small molecules approved for various clinical indications by the FDA, is outlined in Figure 3.11. The A549-GFP^{Hi} target tumour cell line will be plated using high precision Biotek Microflo liquid dispenser at a density of 20 000 cells/well (in 100µL of complete RPMI) and incubated overnight at 37°C and 5% CO_2, with each set library compounds being screened and read in quadruplicate. In addition, twelve wells of OVCA-3iv cells are included on the periphery of each assay plate each to serve as a positive control. Seven days prior, human HER2 specific CAR-T cells are thawed and co-cultured using the aAPC culture system previously described.

The following day, Prestwick Chemical library plates were thawed at room temperature while protected from light, shaken, and spun at 1500 rpm for 3 minutes to settle drugs to bottom of the plates. Biotek Precision liquid handlers will be employed to perform drug dilution and transfer of drug from library plates to assay plates in quadruplicate. During the two-hour drug pre-treatment, HER2-specific CAR-T cells were cultured and isolated prior to staining with CellTrace Far Red tracking dye. Exactly two hours following addition of library drugs, stained CAR-T cells were added to each plate at a density of 20 000 cells/well (E:T=1) in a volume of 35µL using the Biotek Microflo reagent dispenser Assay plates were then replaced in the incubator overnight and were subsequently spun down and fixed using 1% PFA exactly 18 hours post-treatment. Fixed assay plates were protected from light and stored for 3 days at 4°C prior to reading using the ArrayScan and our novel dual fluorescence protocol.

a

	NKG2D Ligands	HER2	Sensitivity to Killing – hchNKG2Dζ T cells	Sensitivity to Killing – hHER2-CAR T cells
A549	5251.9	1953.5	+	+
MCF-7	26.2	57.3	+++	N/A
MDA-MB-231	2243	2306	++	+++
NCI-H460	847	1168	+++	N/A
T47D	13510	8258	+++++	+++++

(Modified from D. Tantalo, Thesis, 2015)



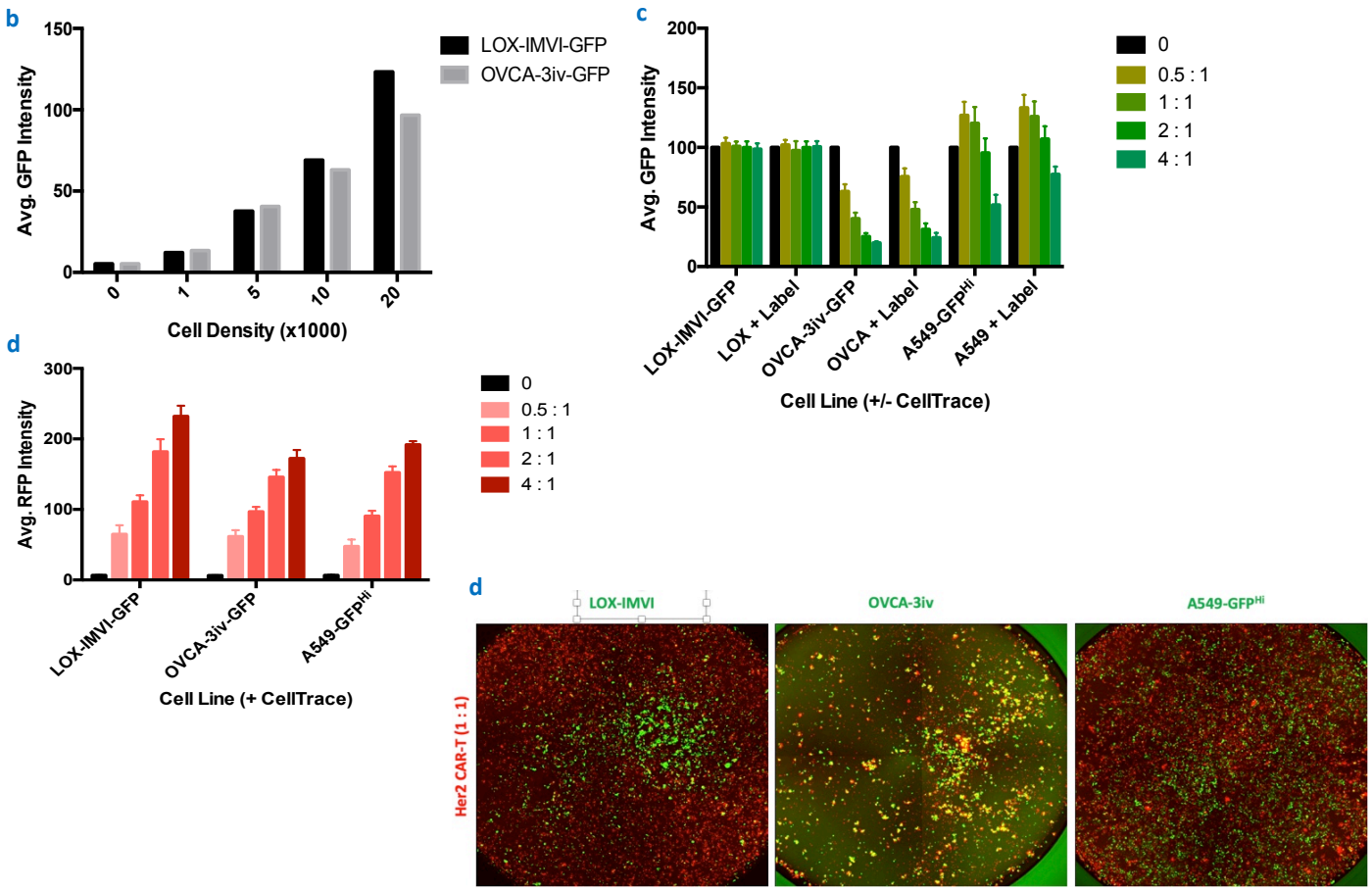


Figure 3.10 Optimization of CAR-T cell and target cell co-culture ratios for novel dual-fluorescence HTS assay. **a) Table showing the relative expression of two relevant CAR-T cell targets (NKG2D stress ligand and HER2) by various cancer cells. Red asterisks highlight the A549 human lung adenocarcinoma selected as a target line for the proposed novel HTS assay due to relatively high expression of HER2 (and NKG2D ligand) and a striking inherent resistance to killing by CAR-T cells in both cases. A schematic of the HER2-specific human CAR-T cell construct used for the study is also included. Data for this panel was adapted from the thesis of Daniela Tantalo, 2014. b) ArrayScan GFP signal intensity quantification for various seeding densities of LOX-IMVI-GFP (HER2- human melanoma) and OVCA-3iv-GFP (HER2+, sensitive, human ovarian adenocarcinoma) cell lines used as negative and positive controls, respectively, for screen optimization. c) Average GFP intensity values measured by ArrayScan for various co-culture ratios of HER2-specific CAR-T cell to control or A549 cell lines (E:T) 24 hours following co-culture and fixation with 1% PFA. "Label" refers to the staining of CAR-T cells with CellTrace Far Red dye immediately prior to co-culture. FarRed fluorescence values obtained through ArrayScan quantification simultaneously with GFP measurement for various E:T ratios is shown in d). e) Representative images of co-cultures of HER2 CAR-T cell and optimization cell lines 24 hours following culture captured by ArrayScan (E:T = 1). Cancer lines are labelled in green, effector cells red and areas of yellow overlap denote immune interaction between effector and target (in case of OVCA-3iv-GFP).**

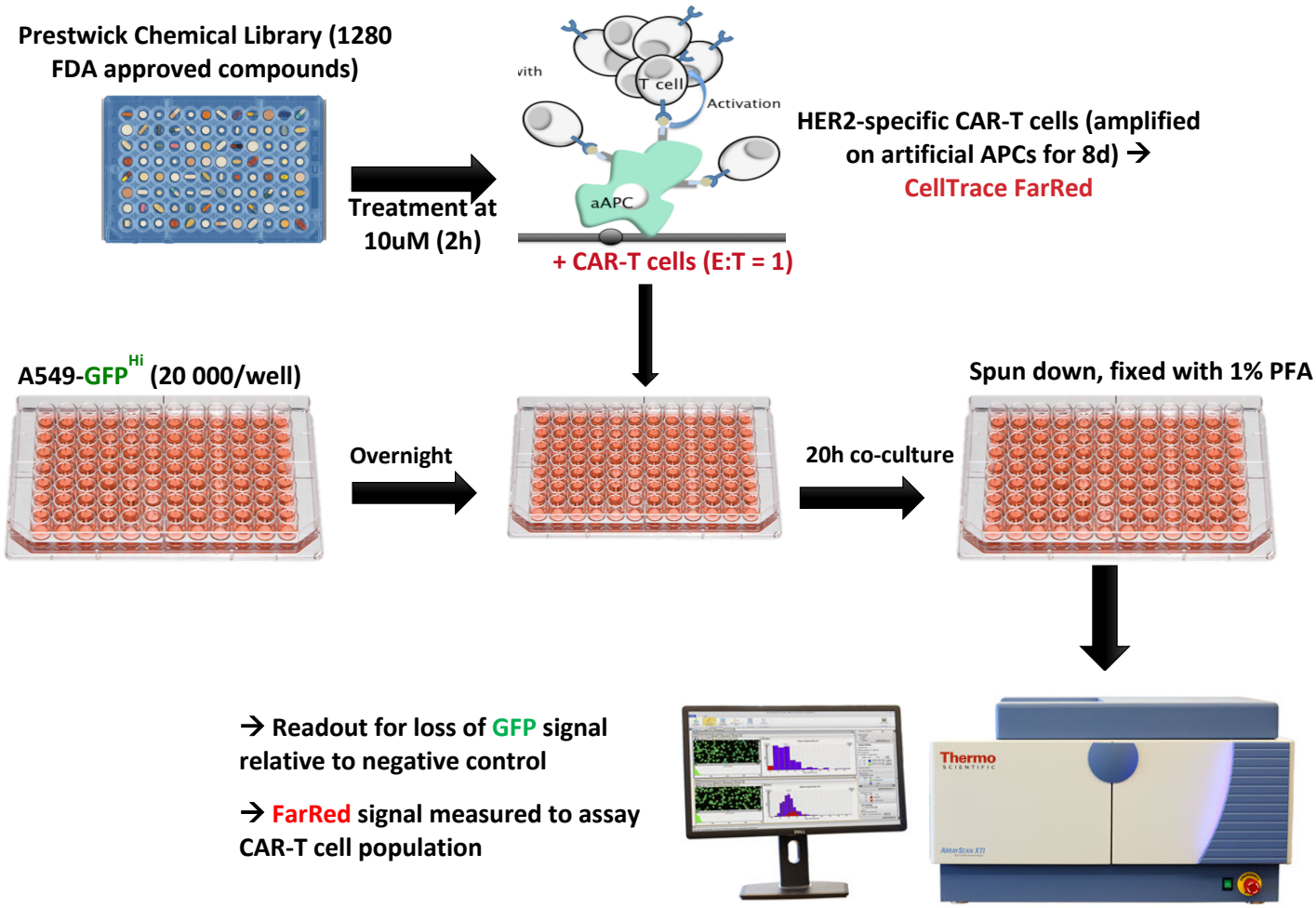


Figure 3.11 Proposed pilot HTS assay for enhancers of HER2-specific CAR-T cell-mediated killing of resistant target cells.
Schematic overview of the proposed HTS assay using the highly optimized dual fluorescence based ArrayScan platform for interrogating the Prestwick Chemical Library of FDA approved small molecule compounds.

3.12 *Identification of putative CAR-T cell enhancers from pilot HTS study*

A simplified experimental goal of the proposed screen is shown in **Figure 3.12a** and visualizes the pursuit of small molecules that contribute to converting the intact monolayer of resistant A549-GFP to sparse cell populated with significant loss of GFP, as is observed when CAR-T cells were incubated with the OVCA-3-GFP positive control line in the presence of library vehicle (1% DMSO). The stark contrast between positive and negative control lines observed in **Figure 3.12a** also lends credence to the reliability and validity of the employed screening protocol, as the CAR-T cells used were clearly functional and able to kill the sensitive OVCA-3-GFP cell line.

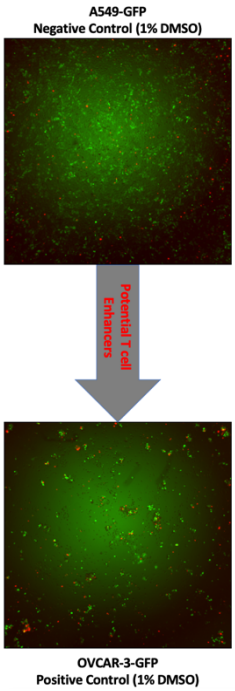
A list of the top putative tumour sensitizing compounds identified through a pilot Prestwick Library screen is presented in **Figure 3.12b**. The initial list was generated by averaging the GFP signal intensity values for quadruplicate wells and subsequently normalizing this value against the average of the negative control for each set of assay plates. This provides a measure of the relative reduction in GFP intensity and is denoted, along with relative statistical significance values, as "relative GFP signal". A similar measure was conducted for the Far Red (RFP) signal observed for each well. The change in RFP signal is a parameter that facilitates the assessment of relative drug toxicity towards the CAR-T cells specifically. This is crucial as the ultimate goal of the screen is to identify compounds that synergize with CAR-T cells and help to overcome previous cellular resistance, and not simply drugs that are toxic to both tumour and T cell components. Furthermore, we have the benefit of having previously screened this library at this concentration with the A549 cell line. As a result, a secondary sorting parameter on top potential screening hits was employed based on their known relative toxicities in A549s.

One of the most striking trends evident in the list of potential top candidate drugs (**Figure 3.12b**) is the relatively high enrichment of compounds from the cardiac glycoside family, a large family of naturally derived compounds sharing a common steroidal structural motif. Cardiac glycosides are known to actively bind and inhibit cellular Na^+/K^+-ATPase and have been a long-standing frontline therapeutic for the treatment of congestive heart failure and atrial arrythmias[91].

4.0 Discussion

Oncolytic virotherapy, autologous tumour cell vaccines and CAR-T cell platforms represent three of the most rapidly progressing and clinically promising immunotherapy strategies being explored to eradicate cancer and alleviate the resulting burden on global healthcare. It is becoming increasingly evident in these circles all three aforementioned platforms possess key therapeutic benefits hampering

a



b

Drug ID	Relative Target Signal (GFP)	P-value (ΔGFP vs. – control)	Relative Effector Signal (Far Red)	P-value (ΔFR vs. – control)	Mechanism/ Approved Use	Relative Cell Viability (AlamarBlue, previous screens)
Digoxin	47.22	3.73E-03	92.72	2.99E-02	Cardiac glycoside	117.49
Lanatoside C	67.93	1.50859E-10	85.92	6.67E-02	Cardiac glycoside	108.19
Digitoxigenin	46.37	3.25E-04	101.30	5.97E-01	Cardiac glycoside	107.89
Proscillaridin A	45.48	2.26E-05	92.76	2.76E-01	Cardiac glycoside	104.24
GBR 12909 dihydrochloride	86.24	3.91E-02	92.36	4.31E-02	Dopamine reuptake inhibitor	100.55
Digoxigenin	61.30	1.19E-04	69.09	3.83E-03	Hapten/non-isotopic label	98.75
Dequalinium Dihydrchloride	82.28	2.00E-06	100.90	1.38E-01	Antibacterial	77.13
Pyrimethamine	74.82	7.04E-02	96.85	5.51E-02	Antiprotozoal	75.77
Alexidine Dihydrochloride	33.43	4.09E-04	80.45	2.00E-03	Antibacterial	65.69
Ciclopirox ethanolamine	84.50	3.44E-02	83.39	6.31E-02	Antifungal	64.41
Thioguanosine	72.15	6.10E-04	98.60	1.22E-01	Nucleoside analog	63.19
Gemcitabine	79.50	2.85E-03	103.73	3.50E-01	Anticancer	34.07
Amethopterin	70.97	5.13E-04	85.55	1.48E-05	Dehydrofolate reductase inhibitor	27.08

Sort 1- Relative Target Signal

Sort 2- Relative cytotoxicity

Figure 3.12 Identification of putative CAR-T cell enhancers from pilot HTS study. **a) Representative images showing negative (A549-GFP + vehicle) and positive (OVCA-3-GFP + vehicle) co-cultures with human HER2-specific CAR-T cells (+ label). The idea of the screen is to identify library drug compounds that enhance CAR-T cell-mediated killing of target cells. b) List of putative CAR-T cell enhancers identified through pilot HTS assay. Compounds were first sorted from largest to smallest decrease in measured GFP signal intensity by ArrayScan, and were sorted through a secondary parameter based on known cytotoxicity of library drugs based on previous screening of the Prestwick library with A549s at the assay concentration of 10μM. This should order putative hits based on their ability to increase killing of the target cell population through T cell related mechanisms as opposed to pure drug-induced cytotoxicity.**

them from achieving status as truly broad and reliable frontline cancer therapies. OVs have long stood as one of the most promising advances in immunotherapy and even feature one FDA approved therapy currently available for clinical use (a modified herpes simplex virus dubbed T-VEC). Major hurdles involved in OV treatment can include the massive degree of tumour heterogeneity associated with the cell populations comprising a tumour mass[92,93].

Specifically, there exists widespread variability in the antiviral response and other immune-related pathways, leading to dramatic disparity in the susceptibility of different tumours (both within the same type of disease and between types of tumours) to infection. Two methods employed to overcome this are the combination of OV with VSe compounds known to modulate key aspects of cellular immunity to virus, as well as to employ *ex vivo* infection of tumour cells at high multiplicities of infection in order to ensure robust infection of potentially resistant tumour cells and to harness the immunostimulatory nature of the viral infection for the vaccine[46,94]. Despite advances in these areas, the clinical success of autologous tumour vaccines can largely be described as underwhelming and largely inconsistent to date[95–97]. It is a primary aim of this study to explore the combination of each of these therapies, both with each other and with additional immunogenic factors (e.g., VSes or adjuvants) to improve vaccine-mediated therapeutic efficacy in formerly resistant tumour models.

Our second aim primarily focused on uncovering previously unidentified enhancers of cancer immunotherapy, through improving the poor performance of CAR-T cell therapies for solid tumours. The advent of CARs and associated adoptive cell therapies undoubtedly represent two powerful breakthroughs in cancer immunotherapy yet and have shown remarkably therapeutic potential for the treatment of leukemias and other blood-based cancers. In fact, despite emerging to the forefront of immunotherapy circles relatively recently, several approved CAR-T cell therapies are currently approved and commercially available, including Kymriah and Yescarta, both of which are approved for

the clinical treatment of human cancers including acute lymphoblastic leukemia and various B-cell lymphomas, respectively[98,99].

Despite this promising outlook for blood-based cancers, CAR-T cell therapy has shown a distinct lack of clinical efficacy is in the treatment of solid, non-hematological malignancies. Putative reasoning for this may include the ability of CAR-Ts to successfully infiltrate and survive in solid tumours, and to ultimately be able to properly execute their effector functions[100]. This therefore presents an opportunity to attempt to intervene and overcome to this apparent resistance of solid tumours, while potentially opening the door for the use of powerful CAR-T therapies for a broader range of clinical indications. To this end, we developed a novel high throughput screening platform featuring a dual fluorescence-based readout to identify exist novel indications of previously approved pharmaceuticals that could enhance the performance of CART cell therapy, and potentially enhance anticancer immune responses generated through various means of cancer immunotherapy.

Our preliminary exploration of CT26 murine colon carcinoma as a refractory model for immunotherapy using an autologous tumour vaccine platform demonstrated a striking protective capacity associated with two homologous doses of γ-irradiated cells prophylactically to tumour challenge (**Figure 3.1, 3.2**). There exists a growing body of evidence highlighting the biochemical changes occurring in tumour cells following radiotherapy (RT) such as through direct doses γ-irradiation, and that these effects extend beyond the induction of apoptosis or cellular senescence and contribute to the immunogenicity of the tumour cell through downstream immunomodulatory effects[101,102]. Interestingly, our murine studies also revealed that treatment with irradiated CT26 (irrCT26), wildtype or LacZ subclone, outperformed infected cell vaccines (ICVs) prepared from the same cell line (**Figure 3.2**). This degree of inherent immunogenicity and associated immune protection has yet to be reported for this cell line and advanced pre-clinical and burgeoning clinical studies have

failed to demonstrate a level of consistent and robust efficacy in similar autologous vaccine studies[42,65,85].

One of the most intriguing discoveries unearthed through our pursuit of a highly optimized autologous CT26 vaccine protocols was the powerful immunostimulatory effect produced through using a heterologous regimen using irradiated cell priming and a boosting ICV vector. It was observed that a priming dose of irrCT26 with an ICV-CT26 boost induced a dramatic enhancement of the CTL response specific for the gp70 tumour antigen, the most highly expressed gene in the tumour line and a common surrogate marker for the immune response against CT26 (**Figure 3.4**)[80]. Even more interesting is the fact that this effect is only maintained when irrCT26 is used to prime and not vice versa. Furthermore, additional exploration of the autologous irrCT26:ICV-CT26 regimen at its core demonstrated even further enhancement of the anticancer immune response by means of including additional immunogenic factors such as OVs armed with immunostimulatory cytokines (**Figure 3.5**), combination with potent adjuvants (**Figure 3.6**) and admixing with novel viral sensitizer technology (**Figure 3.7**). Despite the promising immune response generated through this platform, a significant hurdle remains the translation of potent CTL responses *ex vivo* to meaningful therapeutic responses *in vivo*, something that has proven evasive to this point in our studies.

4.1 *Irradiated CT26 cells induce potent T cell responses, yet fail to exert significant therapeutic effects against tumours in vivo*

It was immediately evident through the early phases of the CT26.WT pilot studies that there existed a striking protective capacity associated with irradiated CT26 (irrCTW6) when administered prophylactically as a two-dose homologous prime-boost regimen. It was also evident that this protective capacity could be induced with as little as 30 Gy of γ-irradiation, was stable across increasing doses of irradiation during vaccine preparation and occurred regardless of whether the wildtype or LacZ CT26 subclones were used as the primary cell line (**Figure 3.2d**).

The primary goal of RT for human cancers is to induce widespread, largely nonspecific damage to key cellular biomolecules including lipids and genomic DNA, resulting in irreparable damage to the cell. This subsequently initiates a range of adaptive stress responses and ultimately either entering a permanent state of growth arrest known as cellular senescence or engaging highly regulated cell death pathways, the most common of which being apoptosis. The primary mechanism for this in solid tumours involves the induction of intrinsic apoptotic pathways following mitotic catastrophe, which is a failure of tumour cells to successfully undergo mitosis following extensive DNA damage and an inability to induce functional repair pathways.

There has been progressive elucidation of the immunomodulatory effects associated with the induction of cell death pathways, as well as how these cellular changes can contribute towards the efficacy of autologous tumour vaccines including an irradiated cell component. The immunostimulatory and cellular responses accompanying irradiation are defined as either "on- or off-target" depending on whether the immunomodulatory change stems from the tumour itself or from non-malignant components of the tumour stroma or microenvironment. Furthermore, it is believed that RT in the context of tumour cell vaccines directly impacts the three "cardinal aspects" underlying a clinically relevant immune response to tumours following therapeutic intervention: antigenicity, adjuvanticity and the promotion of a permissive immunological microenvironment[101].

Irradiation is known to enhance the antigenicity and adjuvanticity of tumour vaccines through several mechanisms including the increased expression of cell surface MHC I molecules and immunostimulatory calreticulin (CALR) chaperone proteins. In addition to the upregulation and/or elevated expression of surface antigens, RT and γ-irradiated tumour cell vaccines specifically have been shown to promote NK cell activity (e.g., upregulation of activating receptors), as well antigen presentation through DCs through the induced upregulation of important costimulatory receptors

such as CD40 and CD80.

A growing body of research focuses on the investigation of cell death associated with lethal RT doses and this phenomenon has been found to be highly immunogenic owing largely to the release of many endogenous molecules with adjuvant-like properties, including vital DAMPs such as CALR, cellular energy carriers such as adenosine triphosphate (ATP), high mobility group box 1 (HMGB1) proteins, release of IFNγ and the downstream induction of chemoattractant proteins such as CXCL9/10[104,105]. In totality, it is evident that there are numerous immunological benefits associated with γ-irradiation that extend beyond the compulsory step of inducing the growth arrested phenotype necessary to ascertain a level of therapeutic safety for autologous tumour vaccines.

Despite the extensive body of literature on the biological alterations resulting from γ-irradiation, it remained striking to observe the prominent potential of homologous irrCT26 vaccine to outperform its ICV counterpart (ICV-CT26) in prophylactic tumour challenge studies. An early rationale for ICV-based vaccination underscores harnessing the immunostimulatory nature of a productive viral infection to enhance the vaccine immunogenicity to improve the antitumour immune responses generated through vaccination. As previously mentioned, infection of tumours by OVs has been shown to promote a range of multi-modal factors that contribute to an immunogenic tumour microenvironment conducive to the generation of downstream immune responses against relevant tumour antigens.

A major focus in OV research centers on the propensity of these biologics to induce local inflammation and immune stimulation at the tumour site, as well as “waking up” the tumour microenvironment (TME). In addition to the induction of a “cytokine storm” encompassing several key immunostimulatory cytokines such as IL-6 and IFNγ, and to stimulating the activity of innate effectors such as NK cells, it has become evident that OVs alleviate impeding or regulatory aspects of the TME, such as the relative abundance of suppressive regulatory T cells (Tregs) or myeloid derived suppressor

cells (MDSCs)[106]. Furthermore, the lytic nature of tumour infection by rhabdoviruses such as Maraba-MG1 and VSVΔ51 releases a pool of tumour antigens for immune sampling, which may have been previously unavailable for recognition by immune effector cells, and in essence serve as a method of *in situ* vaccination against relative tumour antigens[107].

The observed protection from tumour challenge conferred by irrCT26 vaccination was unanticipated in terms of both our initial experimental rationale and based on previous publications investigating similar vaccine platforms. A 2012 study published by Lemay *et al.* had previously explored the use of CT26.WT-based autologous vaccine and its propensity to be improved by using armed OVs for ICV preparation. This paper elegantly demonstrated the antitumour activity of direct OV therapy towards aggressive tumours such as CT26 and that these effects were largely dependent on robust recruitment of the T cell compartment of the immune system. For example, it was reported that the strong antitumour effects associated with VSVΔ51 therapy in tumour-bearing mice was largely compromised in athymic mice, and that splenocyte transfer from mice that were cured of their disease was sufficient to confer protection to naive mice later challenged with the same disease[42].

A critical contrast between this study and our own pilot study was the reported degree of protection obtained for homologous irrCT26 and ICV-CT26 treatments. The Lemay *et al*. publication reports nearly complete protection from tumour challenge with a two-dose ICV-CT26 regimen, whereas our study showed values closer to 50% protection. More intriguing however, is the nearly 100% protection observed for irrCT26.WT in this platform, whereas irrCT26 alone conferred little to no protection in this previously published report. A possible reason for these stark differences in the context of *in vivo* efficacy could simply be the initial point of therapeutic intervention and the volume and/or composition of the tumour at this time. It is becoming increasingly evident in immunotherapy research circles, both in the laboratory and in clinical settings, that the initial size and structure of tumours at the

time of therapy, as well as the degree of vascularization, remain vital determinants for subsequent clinical response and overall therapeutic efficacy associated with immunotherapies such as the OV platform.

Another potential explanation for the differences in performance observed for our irrCT26.WT vaccines and those in previous studies could be the initial source and phenotype of the cell lines, as well as the culture conditions used to propagate both cells and virus, as these may all influence subsequent data obtained through *in vitro* and *in vivo* experiments. Work by previous group members have extensively explored the genomic and phenotypic differences not only between wild-type CT26 (CT26.WT), LacZ subclone (CT26.LacZ), and a wide range of slightly differing subclones as well[82]. It was evident that the genetically similar, yet distinct subclones displayed heterogenous phenotypes in the context of several key biological properties including the robustness of the antiviral response and resulting susceptibility to OV infection both *in vitro* and *in vivo*, and even more fundamental cellular properties such as the rate of cell division. It therefore stands to reason that different subclones used to establish experimental assays or systems could potentially display differently immunological profiles when used for autologous vaccine preparation, be it through differential baseline immunogenicity of the tumour cells, the pool of antigens most salient or readily available for immune sampling or the susceptibility to infection of the tumour cells by live OV for ICV therapy[82].

It is also important to note that the majority of the early CT26 autologous infected cell vaccine experiments used frequently in the Lemay *et al.* publication[42] were prepared using VSVΔ51, in contrast to the Maraba-MG1 backbone used throughout our early vaccine studies. Despite a direct comparison of the immune responses resulting from CT26-based ICVs prepared with MG1 versus VSVΔ51 and minimal observed differences as a result (**Figure 3.6c,d**), it still remains a potential explanation for the contrasting results obtained in the two studies.

A powerful takeaway from the Lemay *et al.* ICV landmark ICV paper, regardless of the difference in findings, is the dramatic increase in autologous vaccine performance when ICV preparations featured OVs expressing granulocyte-macrophage colony-stimulating factor (GMCSF), a powerful glycoprotein cytokine that drives the production of new leukocytes, as well as promoting the migration and function of mature immune cells such as neutrophils. In the context of cancer therapy, it was exquisitely demonstrated that VSVΔ51-GMSCF and ICVs prepared with this armed vector promoted pro-immunogenic factors such as the rapid activation of dendritic cells and the strong infiltration of challenge tumours by activated and functional T and NK cells because of vaccination. The combination of these factors and the powerful immunostimulatory nature of the ICV itself contributed to significant therapeutic efficacy *in vivo* for mice treated both prophylactically and therapeutically[42].

These findings clearly emphasize the importance of rapid and early immune activation and the robust infiltration of tumours by activated T cell and NK cells as key drivers of autologous tumour vaccine efficacy in a clinical, albeit murine, context. It is therefore clear that the design of these vaccine platforms, the selection of accompanying immunogenic factors, and the regimes used for treatment should all aim at the generation of robust immune responses capable of migrating to and infiltrating, the tumour burden of a living system[42].

4.2 A novel heterologous prime-boost vaccine improves immune responses against target tumours

One of the most intriguing discoveries to stem from our optimization of an efficacious autologous vaccine platform was the striking potential of our novel heterologous prime-boost platform, specifically an irrCell prime followed by ICV boost, to robustly elevate tumour-specific CTL responses compared to either vaccine given alone. It is an emerging sense in cancer vaccine research that the use of multiple boosting doses may be key in overcoming the many immunosuppressive mechanisms engaged by tumours and the surrounding microenvironment. The therapeutic benefits associated with

repeated administration of autologous vaccine boosters was something we explored specifically in the context of ICVs in the murine CT26 colon carcinoma model (**Figure 3.5**). In this context, it was observed that recurrent dosing with ICV-CT26 at the tumour site contributed a high degree of therapeutic efficacy compared to the two-dose heterologous vaccine regimen (**Figure 3.5**), even though the latter has consistently demonstrated the highest levels of immune response in response to vaccination in both naïve and tumour bearing mice in previous studies (**Figures 3.4, 3.5, 3.6**).

The Auer group has dedicated extensive efforts to the exploration of multi-boost ICV strategies in the highly aggressive murine peritoneal carcinomatosis (P.C.) model, specifically attempting the assess the impact of vaccination on various compartments of the immune system with an emphasis on the induction of antitumour NK cell responses. A 2017 study published by Alkayyal *et al.* showcased impressive therapeutic efficacy associated with multiple doses of ICV produced with a Maraba-MG1 vector expressing IL-12. They were further able to demonstrate that armed OVs such as these can reliably induce high levels of the encoded gene product in ICV preparations and improve the performance of the autologous tumour vaccines used. The incorporation of an IL-12-encoding OV was also demonstrated to enhance the vaccine-induced immune response including the migratory capacity and activation of NK cells and the ability of these cells to aide in tumour rejection and survival in murine tumour challenge studies[86,87].

Establishing this heterologous, autologous tumour vaccine combining the potential of both irradiated tumour cell priming and ICV boosting is a concept that is truly novel and has not been explored thus far in the realm of cancer immunotherapy research. As with all emerging therapies, a deeper elucidation of the biological mechanisms underpinning the capacity of the irrCT26:ICV-CT26 regimen to induce such robust T cell responses is required in order to maximize the therapeutic potential of this system.

A group of our close collaborators at the McMaster Immunology Research Center has extensively explored the use of oncolytic rhabdoviruses (ORVs) for cancer vaccine-based approaches. Through this, they have demonstrated that the use of ORVs encoding tumour associated antigens, for example the human dopachrome tautomerase (hDCT) highly relevant to human melanoma, can drive a robust antigen-specific T cell response. Remarkably, it was further reported that the use of an adenovirus-Ag vector to prime the ORV-Ag boost dramatically enhanced the magnitude of the immune response launched against the encoded antigen, and that a dramatic retargeting of the immune system had taken place as the response against the highly immunostimulatory viral vectors had been hampered in both instances, while the subsequent response against the tumour was robustly enhanced. This Ad:ORV vaccine regimen has shown extremely promising results in several models of murine cancer including melanoma and glioblastoma, and has even been shown to possess a desirable safety profile in non-human primates and is currently being investigated in several human clinical trials for cancer. As mentioned previously, irradiated CT26 cells have a strong capacity to prime anti-tumour immune responses and there clearly exists some biological effect associated with these cells having undergone the process of irradiation-induced senescence that is powerful in terms of establishing the early stages of the immune response against the tumour.

It is evident that the highly immunostimulatory ICV is optimally employed particularly in a boosting role following irrCell priming. Intriguing findings of our early vaccine studies included not only the striking ability of heterologous combinations of tumour cell-based vaccines to enhance the resulting anticancer immune responses, but the fact that this effect only occurs when the ICV is used for boosting and not vice-versa. This is conceptually in line with previous studies using the VSVΔ51 and MG1 OVs for cancer vaccine studies that have demonstrated a specific power associated with these viral vectors used in the boosting phase[88].

We initially posited that one potential reason the homologous ICV regimen failed to elicit a significant immune response against gp70 or outperform irrCT26 treatment in murine tumour models could be the vaccine-generated immune response being shifted from one that is tumour-specific toward the highly immunostimulatory live OV present in the ICV. However, when we directly compared the T cell responses generated against viral antigen versus gp70 we observed a surprisingly robust CTL responses against the viral N peptides resulting from irrCT26:ICV-CT26 vaccination (**Figure 3.5, 3.6**). Interestingly, this was also frequently observed concomitantly with the highest observed gp70-specific CTL responses.

This could simply be a result of the timing of our immunological analysis related to dosing in the vaccine regimen. For example, the large antiviral immune response could be explained by the fact that the immune system is seeing highly immunostimulatory virus for the first time approximately seven days before analysis, and it can therefore be expected that the response against the virus may be around then peak in response to novel pathogen exposure. It is also possible that the irrCell:ICV regimen as a whole is highly immunostimulatory and is causing elevated $CD8^+$ $IFN\gamma^+$ T cell responses across multiple target antigens (gp70 and viral N peptide included) due to a potential elevation of baseline immune reactivity following heterologous vaccination. It would be interesting to assess whether this can contribute to an enhancing the breadth of the immune vaccine-induced immune response as well as its ultimate magnitude, and perhaps expand the pool of tumour antigens recognized and sought out by induced effector cells.

Based on findings such as these it is clear that another critical follow in our exploration of new heterologous tumour vaccine strategies should involve further interrogate the irrCT26:ICV-CT26 prime-boost regimen in order to confirm an optimized dosing schedule that would combine the potent effects of irrCT26 priming and the benefits stemming from repeated ICV boosting. For this to be successful it

would be crucial to address additional parameters such as the timing between irrCell prime and ICV boost, the number and timing of additional boosting doses, as well as an optimized methodology for selecting and incorporating accessory immunostimulatory factors such as adjuvant or VSes.

A major limitation of our investigation to this point has been our examination of the immune response against tumours solely in the context of the gp70 tumour antigen. Although this antigen is known to be highly expressed and is often regarded as a prototypical surrogate marker for immune response against CT26 tumours[89], it is possible that ICV treatment may shift or expand immune system focus towards a variant set of antigens that are perhaps more salient to the immune system in the context of a productive viral infection. Also, the pool of tumour antigens accessible to the immune system for sampling could be drastically different between irrCT26 and ICV-CT26 vaccination. The irrCell compartment likely promotes immune sampling of antigens primarily on the cell surface while ICV features a high proportion of lytic cells and potential access to a pool of intracellular tumour antigens that may have been previously unavailable to immune monitoring. A major benefit associated with autologous vaccinations is their ability to serve as pan-antigen vaccines, and as a result it will be essential going forward to interrogate a more global immune response against a broad pool of relevant tumour antigens.

4.3 *Exploring the use of accessory immunogenic factors to further enhance autologous tumour vaccine performance*

It is evident from the success of a combined vaccine approach featuring both irradiated tumour cells and ICV that differential preparations of autologous vaccines possess strong immunostimulatory properties best exploited through administration at specific points in the treatment timeline. The immunogenic properties of live OV in the ICV preparations appears to exert a potent adjuvant-like effect when administered in the boosting phase of our heterologous prime-boost regimen. To further push this envelope in hopes of producing an optimal tumour vaccine protocol, we elected to explore the

incorporation of additional immunogenic factors such as VSes and known biological adjuvants such as the proprietary bacterial outer membrane adjuvant used in the study.

The use of biologically active adjuvants, such as those derived from bacteria, have long been employed to improve the performance of vaccines for a variety of indications. Following the observed therapeutic potential of the irrCT26:ICV tumour vaccine platform, we elected to undertake partnerships allowing us to explore the use of powerful commercial adjuvants such as the proprietary bacterial outer membrane adjuvant generously provided by Biodextris Therapeutics. This adjuvant is a preparation incorporating outer membrane proteins (OMPs) from one bacterial strain covalently linked to lipopolysaccharide (LPS) molecules from an unrelated bacterial strain, and these highly immunogenic PAMP stimulate the immune system through a variety of mechanisms largely mediated through recognition of these conserved patterns by the toll-like receptors (TLRs) prevalently expressed by cells of the innate immune system.

The adjuvant is prepared to a nanoparticle consistency and has been demonstrated to facilitate its immunogenic properties primarily through stimulation of TLR-2 by the bacterial OMP fractions and through interaction of LPS isolates with TLR-4. The downstream effect of these interactions culminates in several pro-immunogenic outcomes including increasing the production of pro-inflammatory IFNγ and IL-2 by immune cells, as well as increasing serum antibody levels against relevant vaccine antigen compared to groups without adjuvant. This provides an opportunity to combine adjuvants with our autologous irrCell and viral-based ICV vaccine vectors, the latter of which is known to exhibit similar pro-inflammatory effects through engagement of TLR-3/7 by viral RNA molecules. This therefore presents the prospect of producing a vaccine capable of engaging a broad range of innate PAMP receptors through the combinations of highly immunogenic bacterial and viral molecules in the context of a pan-antigen autologous tumour vaccine.

This adjuvant of interest has been explored extensively in the context of several vaccine applications and is primarily administered through the intranasal delivery route due to a potent capacity to engage mucosal immunity. Early experiments with this adjuvant exhibited that doses as low as 0.3 μg administered i.p. at the site of vaccine administration was sufficient to noticeably increase the relative abundance of both residential and inflammatory macrophages, as well as neutrophils, as detected by peritoneal lavage and subsequent flow cytometry analysis. In addition, 30 μg of adjuvant administered alongside irrCT26 vaccine was both well tolerated by mice and led to a robust enhancement in the gp70-specific CTL response when interrogated using *in vivo* CTL assay protocol.

These findings, coupled with an abundance of literature highlighting the potential of similar adjuvants to improve *in vivo* vaccine performance represent a clear rationale for the pursuit of similar therapeutic combinations in the context of a tumour-based therapeutic cancer vaccine[90]. However, it was still surprising to observe the striking levels measured in our animal studies when adjuvant ("+adj") was co-administered intranasally (i.n.) at the time of i.p. vaccine administration. When administered i.n. at both the time of priming and boosting, therapeutic groups involving the adjuvant demonstrated remarkably strong CTL responses against gp70, generating the highest values observed to date in any previous studies, at close to 80% specific elimination of loaded splenocytes by tumour-specific T cells in vaccinated animals.

There is evidently a biologically salient mechanism underlying the ability of i.n. adjuvant to boost the distally administered tumour vaccine to such an extent, as it is clear that administration directly at the site of vaccination is incapable of inducing T cell responses to the same robust levels. Though these studies are preliminary, the combination of i.n. adj and i.p. irrCell:ICV clearly represents an important research focus moving forward in an aim to both understand and further exploit the

mechanisms underlying the observed potentiation of T cell responses, as well as to continue optimizing this system in terms of correct timing and dosing of the regimen.

In addition to the encouraging, yet preliminary, results obtained through combinations of heterologous irrCell:ICV platform and these kinds of biological adjuvants, we also explored the incorporation of additional immunogenic components for ICV preparation such as a OV vectors encoding therapeutic transgenes, as well as previously characterized immunogenic VSe compounds that have been shown to potentiate OV-driven therapy in a variety of contexts.

Through close collaboration with the Auer group, we had access to a wealth of knowledge concerning the use of OVs expressing the highly immunostimulatory IL-12 cytokine, as well as the highly aggressive peritoneal carcinomatosis (P.C.) murine model itself. In the context of p.c., a six-dose, twice-weekly homologous regimen adapted from Auer lab vaccine studies, as well as a two-dose heterologous irrCell:ICV group for comparison, were used for therapeutic intervention. Our exploration of this model revealed that repeated dosing with ICV preparations containing IL-12-armed OVs was able to contribute an extension of median survival in tumour-bearing mice, despite a relatively innocuous gp70-specific CTL response detected through concurrent *in vivo* CTL assays. It was also observed that a two-dose heterologous prime-boost regimen induced robust CTL responses and that the addition of the IL-12 component appeared to potentiate the generation of CTL response against the tumour (**Figure 3.5**).

Despite the relatively high gp70-specific CTL response recorded following heterologous prime-boost vaccination, these effects evidently did not efficiently translate to therapeutic efficacy in the p.c. tumour model. This hurdle between *ex vivo* immune analyses and the successful translation to *in vivo* efficacy should surely represent a major focus of research moving forward in order to identify the roadblocks hindering the vaccine-generated CTL responses from exerting their therapeutic influence in a

profound way at the tumour site. Whether it is necessary to gauge vaccine-induced CTL responses against other tumour antigens aside from gp70 as a marker of vaccine therapeutic success, or if there are immunological blockades disrupting immune cell migration or function at tumour sites is a key question that should be explored to pursue to production of highly efficacious autologous vaccine therapies.

We also investigated the capacity of VSe compounds to enhance the therapeutic potential of autologous vaccines, primarily in the context of the ICV boosting component. These small molecules have been demonstrated by our group to impact various aspects of the innate immune system and overall, to potentiate the infection and anti-tumour effects associated with OV therapy[91]. We chose to investigate the potential combination of sodium orthovanadate with oncolytic rhabdovirus therapy due to the published immunostimulatory effects associated with this pharmacoviral combination approach, which extend beyond the robust enhancement of resistant tumour infection, including the CT26.WT murine model. Among these immunostimulatory effects are the upregulation of powerful immunomodulatory cytokines such as IFNγ and the potent T cell chemoattractant CXCL9. Despite this combination of ICV and VSes being a primary experimental focus at the genesis of this project, we unfortunately did not spend an extensive amount of time exploring this avenue to both the extensive time commitments shared between vaccine studies and the creation of our CAR-T cell screening platform..

In our pilot experiment featuring VSes as part of the vaccination protocol, it was encouraging to observe that admixing of as little as 100 μM sodium orthovanadate directly in ICV preparations at the time of infection was capable of subtly enhancing the associated biological activity and that this certainly warrants further investigation moving forward. In particular, the ICV and VSe combination was able to elevate a perpetually nonexistent gp70-specific CTL response following ICV treatment to a detectable level of approximately 3-5%. More noticeably, there appears to exist a slight survival benefit

to animals receiving ICV and VST compared to ICV alone. This suggests that some underlying biological effects are being triggered through the inclusion of VSe compounds, and if gp70 is a true surrogate for the antitumour immune response, this slight gp70 response was able to translate some degree of therapeutic benefit for reasons that are not yet elucidated (**Figure 3.7**).

Regardless, the combination of VSe and autologous tumour vaccine deserves to be further explored moving forward and is currently being pursued by members of our research group. It would also be extremely intriguing to assess a final, highly optimized autologous vaccine platform involving a heterologous irrCell:ICV approach, the potential addition of an armed OV vector combined with VSes for the boosting phase, as well as i.n. bacterial adjuvant to further drive a highly optimized and efficacious vaccine approach. To this end, future steps are required moving forward to continue optimizing each component, the appropriate dosing and timing of each, as well as the most therapeutically beneficially combination of vaccine vectors therein.

4.4 Development of a novel high-throughput screening assay for the identification of chemical enhancers of CAR-T cell activity: cardiac glycosides emerge as a promising family

Adoptive cell therapy and in particular, chimeric antigen receptor (CAR)-T cell therapy, is certainly one of the most exciting and promising avenues in cancer immunotherapy being investigated currently. These engineered T cell have shown tremendous success in human trials for the treatment of blood cancers such as acute myelogenous leukemia (AML), yet their success in the treatment of solid tumours is far less impressive and remains a major hurdle in the field[115–117]. A reason for this is the vast array of mechanism used by solid malignancies to evade or dampen the host immune response against the tumour[118,119].

To overcome this, we developed a novel high-throughput screening platform using the wealth of screening robotics and ArrayScan High Content Screening Microscope at our disposal. This approach

featured a dual fluorescence-based readout allowing for simultaneous assessment of drug effects on both target tumour cells (GFP) and CAR-T effector cells (Far Red) following treatment with library compounds and subsequent co-culture of these cell populations. We were able to generate a reliable assay readout which was used to interrogate the Prestwick Chemical Library of FDA approved pharmaceuticals, by which we were able to identify and generate a list of putative T cell enhancers capable of potentially enhancing CAR-T cell efficacy and/or sensitizing resistant tumours. It was observed that there was a conspicuous enrichment of cardiac glycoside (CG) drug family members as being able to putatively enhance T cell mediated killing of formerly resistant A549 human lung cancer cells.

For the purposes of a pilot screen, we elected to assay the Prestwick Chemical Library of nearly 1250 FDA-approved pharmaceuticals previously indicated for a vast range of therapeutic conditions. A major benefit of this library is that the featured compounds are all approved by regulatory agencies for existing clinical indications and therefore there exists published research to reference when attempting to elucidate pathways and mechanisms of interest, as well as a much easier path to clinical translation should any promising tumour sensitizing compounds emerge from the screen. Additionally, we have employed this library in previous screens for various research applications, and as such we have access to existing data regarding activity of this library in the A549 cell line, of particular interest being cytotoxicity data previously obtained for the library at similar concentrations to our CAR-T cell screens. This allowed us to strategically sort putative hits based both on their ability to dissipate target tumour cell GFP signal when combined with HER2-specific CAR T cells, but also by their known relative cytotoxicity alone in the context of A549 cells. This facilitates the identification compounds that ideally potentiate CAR-T cell activity as opposed to simply being cytotoxic directly to the tumour cells. This sorting strategy is outlined in **Methods 2.10**.

It was strikingly evident that there was a strong enrichment of members of the cardiac glycoside (CG) drug family among the top capable of inducing significant reduction in target GFP signal with relatively moderate reductions in CAR-T RFP signal. CGs are a class of organic compounds commonly sharing a steroid-based structure linked to both glycoside molecule as well as several other functional groups aimed at influencing the activity of the compounds. It has been widely reported throughout their time as approved human therapies that the CGs influence specific aspects of cellular Na^+/K^+-ATPase and have largely been used in the context of treating congestive heart failure and other cardiac arrhythmias. These drugs are known to produce several off target effects and to possess relatively narrow therapeutic windows when used for the treatment of cardiac disorders, but more recently are emerging as putative compounds for driving a slew of anticancer properties, including being potent inducers of immunogenic cell death[91].

Emerging bodies of evidence show that several of the top candidate molecules identified in our HTS assay, including digoxin, digitoxin and others, can induce strong antiproliferative effects in the context of human lung and prostate cancers. Beyond these antiproliferative capacities, the CGs have also been reported to act as strong inducers of various cell death pathways. Compounds such as digoxin, oleandrin and lanatoside C among others are known inducers of both intrinsic and extrinsic cellular apoptosis, mediated largely through BAK/BAX activation and upregulation of the FAS receptor/ligand pathway, respectively, while other have been shown to act as strong inducers of other cell death modalities such as the autophagy pathway[120].

Recent research focusing on the therapeutic deployment of CG drugs has also highlighted the ability of these to serve as potent inducers of immunogenic cell death (ICD), which combined with the direct cytotoxic effects of compounds allows the release of several crucial DAMPs such as ATP and HMGB1, which may further improve immune surveillance and immune responses against the tumour.

This biologically powerful scenario of ICD carries major benefits for the purposes of cancer therapy, as essentially all therapies capable of contributing to this outcome can serve as drivers of immune responses against tumours in the capacity of a sort of pseudo-vaccine.

The mechanisms underlying the ability of CGs to drive ICD remain somewhat unclear, though a growing body of research suggests that the CG interaction with NA^{+}/K^{+}-ATPase drives changes in intracellular Ca^{2+} homeostasis. This resulting alteration in cellular Ca^{2+} concentration drives several cell signaling pathways resulting in pro-immunogenic outcomes such as increased calreticulin expression at the tumour cell surface and a possible nudging of the cell towards an autophagy fate, facilitating the release of key DAMPs. Further understanding of the mechanisms driving this emerging field of cancer therapeutics is certainly required and is currently underway to pursue the use of CGs and other known ICD inducers to further promote efficacious and reliable cancer therapies[120–122].

The identification of CGs through our screen presents an intriguing crossroads in the context of this project, and cancer therapy in general, because compounds such as these may hold the potential to not only contribute direct antitumour effects as a result of therapy but could also promote/enhance the resulting anticancer immune response stemming from therapies such as OV therapy, autologous tumour cell vaccination and CAR-T cell therapies among others. Essentially, all immunotherapies aiming to generate potent T cell responses could benefit from a putative combination with compounds that enhance the induction of these responses. It may even be interesting to investigate the ability of these compounds to enhance the powerful irrCell:ICV vaccine platform we have been developing throughout this project.

Before this occurs however, it is of crucial importance to validate the results from the screen to confirm then results obtained through our pilot experiment. Although we did not have a chance to actively pursue the validation of our CAR-T cell screen, the fact that such a robust enrichment of drugs

from the same family (CGs) appeared clustered in our sorting, as well as the fact this enrichment is supported by known literature focused on the use of CGs for cancer therapy, allows us to speculate that there was a high degree of accuracy and validity associated with our screening assay.

5.0 Conclusions

In conclusion, this study aimed to identify powerful and potentially novel enhancers of the antitumour immune responses generated through cancer immunotherapy approaches. Our primary aim involved the pursuit of a highly optimized and efficacious autologous tumour vaccine regimen. This therapeutic strategy encompasses several desirable benefits including their use as pan-antigen vaccines, delivering a broad pool of relevant tumour antigens for immune sampling, yet have failed to demonstrate consistent levels of clinical success to date.

We were able to demonstrate a striking capacity associated with irradiated CT26 cells (irrCT26) to protect mice from subsequent tumour challenges, and interestingly, to outperform CT26 infected cell vaccines (ICVs) in terms of prophylactic tumour challenge models and in generating $CD8^+$ $IFN\gamma^+$ cytotoxic T cell (CTL) responses against the highly expressed gp70 tumour antigen. Most striking was our discovery of a newly reported heterologous tumour cell vaccine strategy specifically incorporating an irrCT26 prime and an ICV-CT26 boost to greatly enhance the vaccine-induced CTL response in treated animals.

Our goal of generating a highly optimized vaccine regimen subsequently focused centrally on this novel irrCell:ICV platform through which we investigated the potential of accessory immunostimulatory factors such as OVs armed with therapeutic transgenes, bacterial adjuvants and viral sensitizers (VSes). We were able to show that treatment with ICV-CT26 prepared with an OV expressing the powerful IL-12 cytokine improved CTL responses against the tumour compared to homologous ICV groups and heterologous groups prepared with empty OV in murine tumour models.

Interestingly, the highest observed CTL responses stemming from autologous tumour cell vaccination were generated through a combination of intraperitoneal irrCell:ICV treatment and concomitant intranasal administration of adjuvant.

Our second experimental aim centered on improving the performance of CAR-T cell therapy for solid tumours, as a resistance to treatment by solid tumour represents a longstanding and critical hurdle for this rapidly growing therapeutic avenue. To this end, we developed a novel fluorescence based HTS platform capable of simultaneously interrogating signals from both target tumour cell population and the co-cultured CAR-T cells. Using this system and the Prestwick Chemical Library of FDA approved pharmaceuticals, we identified an enrichment of cardiac glycoside drugs as putative sensitizers of resistant human lung carcinoma cells.

Future work should undoubtedly involve further exploration of the irrCell:ICV platform in order to fully optimize the therapeutic potential of this novel vaccine regimen. Major points to focus on for this aim should certainly include optimizing critical parameters such as the dosing schedule required to drive therapeutic translation, including elucidating the optimal number of each irrCell priming doses and ICV boosters, as well as the timing between them. Furthermore, the role of VSes and their ability to putatively enhance the biological activity of ICV treatment was unfortunately underexplored in our study yet remains a highly intriguing avenue thanks in part to the extensive body of knowledge highlighting the ability of these compounds to enhance OV therapy in a number of contexts. Also, further investigation the CAR-T cell enhancement aim should involve a involve validation of the cardiac glycosides identified through our pilot screen to combine with CAR-T cell and overcome solid tumour resistance. Our screening platform can also be used to potentially interrogate other chemical libraries, including a small molecule library as used in the pilot VSe screen performed by Diallo *et al*. In all, the work in this thesis identifies novel enhancers of the antitumour immune response that can potentially be

further developed to produce highly efficacious cancer immunotherapy options moving forward and attempt to render cancer a disease of human history.

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7.0 Curriculum Vitae

Please contact author for an up-to-date academic CV and with any questions relating to the writing of this thesis and the work therein.