TARGETING TUMOUR ANTIGEN HETEROGENEITY WITH DUAL-SPECIFIC ADOPTIVE CELL TRANSFER.

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Abstract

Through the years, cancer therapies have progressed rapidly, pouring out novel treatments such as gene therapy, small molecule therapies and immunotherapy. One such immunotherapy, adoptive cell transfer (ACT), augmented through the addition of a chimeric antigen receptor (CAR), has proven success in treatment of hematological malignancies. Additionally, oncolytic viruses (OV) and OV-based (OVV) therapies, have shown promising results in both clinical and pre-clinical studies. In most instances, when applied as a monotherapy, the aforementioned treatment methods are incapable of inducing complete tumour remission. The Wan lab has developed an approach combining ACT with OVV therapies that dramatically increase therapeutic benefit resulting in complete regression of well-established solid tumours. Despite promising results, certain tumours can still escape this combination therapy through antigen loss resulting in antigen negative relapse (ANR). To further augment the therapy, the addition of a secondary receptor (CAR) provides the ACT multiple avenues of attack to prevent ANR. In this dissertation, we define culture conditions that promote strong expression of the CAR alongside confirmation of function in an *in vitro* setting. Following, it is demonstrated that OVV boosted dual-targeting T cells carry strong T cell activity by measure of cytokine release *in vivo*. Despite promising T cell activity data, dual-specific T cells are unable to improve tumour control and survival once relapse occurs. The failure to control relapse remains unclear however evidence points towards lack of T cell persistence, poor CAR function *in vivo* and a lack of endogenous T cell response leading to compounding effects that prevent dual-targeting T cells from preventing ANR. Although dual specific therapies have shown poor efficacy in preventing ANR, further study must be completed to identify areas of improvement – such as persistence, as the potential for success in using dual-targeting T cells coupled with OVVs still lies untapped.

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List of Abbreviations

ACK - Ammonium-Chloride-Potassium
ACT – Adoptive Cell Therapy
AICD – Activation Induced Cell Death
ALL – Acute Lymphoblastic Leukemia
ANR – Antigen Negative Relapse
APC – Antigen Presenting Cell
AREB – Animal Research Ethics Board
ATP – Adenosine Triphosphate
BiTE – Bispecific T-cell Engager
CAF – Central Animal Facility
CAsF – Cancer Associated Fibroblast
CAR – Chimeric Antigen Receptor
CD – Cluster of Differentiation
CRS – Cytokine Release Syndrome
CTLA-4 – Cytotoxicy T-Lymphocyte Associated Protein 4
DAMP – Damage Associated Molecular Pattern
DC – Dendritic Cell
DCT – Dopachrome Tautomerase
DMEM – Dubelco's Modified Eagle Medium
DPI – Days post injection
FACS – Fluorescence Assisted Cell Sorting
FBS – Fetal Bovine Serum
FDA – Food and Drug Administration
hBCMA – Human B Cell Maturation Antigen
HEV – High Endothelial Venule
HLA – Human Leukocyte Antigen
ICS – Intracellular Cytokine Staining
ID - Intradermal
IDO - Indoleamine 2,3-dioxygenase
IFN - Interferon
IL– Interleukin
IV – Intravenous
LB – Lysogeny Broth
MAGE-A3 – Melanoma Associated Antigen 3
MDSCs – Myeloid – Derived Suppressor Cells
mERK – Mutant ERK
MHC – Major Histocompatibility Complex
MMP – Matrix Metalloproteinase
mTOR – Mammalian Target of Rapamycin
NEAA – Non-Essential Amino Acids vii
NT – Non-transduced
NO – Nitric Oxide
OV – Oncolytic Virus
OVA - Ovalbumin
OVV – Oncolytic Viral Vaccine

PAMP – Pathogen Associated Molecular Pattern
PBS – Phosphate Buffered Saline
PD-1 – Programmed Cell Death Protein 1
PD-L1 – Programmed Death Ligand 1
PFU – Plaque Forming Unit
P/S – Penicillin / Streptomycin
RPMI – Rosalind Park Memorial Institute Medium
ScFv – Single Chain Variable Fragment
sf – Serum Free
TD - Transduced
TAA – Tumor Associated Antigen
TCM – Central Memory T-cell
TCR – T-cell Receptor
TEff – Effector T-cell
TGF-β – Transforming Growth Factor Beta
Th – T helper cell
TIL – Tumor Infiltrating Lymphocyte
TME – Tumor Microenvironment
TNF-α - Tumor Necrosis Factor Alpha
Treg – Regulatory T-cell
TSA – Tumor Specific Antigen
VSV – Vesicular Stomatitis Virus

Chapter 1

1. Introduction

1.1 Cancer

1.1.1 Cancer Biology

The disease cancer is described as "abnormal proliferation of the different kind of cells in the body", wherein hundreds of cancer types can drastically reduce the lifespan of an individual[1]. From an epidemiology perspective, cancer is one of the global leaders in causes of death, leading to millions of dollars being spent on a yearly basis in a race to find treatments[2].

Despite momentous levels of research, much about the disease remains a question, thus making it difficult to discover therapies that can treat a wide variety of cancer phenotypes. The inability to pinpoint specific targets for therapies lies within the highly mutative nature of cancer – attributed to the multiple changes seen in cell DNA sequences[3]. Each mutation arises due to selection for phenotypes that aid in aberrant cell growth, like Darwinian evolution, where these cells can now thrive and proliferate uncontrollably[1,3]. Mutations may also arise due to external factors such as radiation and chemical carcinogens, both a cause for DNA damage[1].

Across most cancer cell lines, genes that are responsible for cell growth or regulating cell growth are commonly found to be mutated thus leading to development of unnatural cell growth. Compounding mutations seen in proto-oncogenes and tumor suppressor genes will often lead to development of cancer[4]. Healthy genes such as *ras* aid in cell growth by encoding intracellular signal-transduction proteins[4]. A mutation seen here can cause overexpression of the downstream protein eventually leading to creation of proto-oncogenes and uncontrolled cell growth. At the same time, the tumor suppressor genes are responsible in acting as a safeguard to prevent

excessive cell proliferation to specifically prevent the creation of cancers[1,4]. A mutation seen here, such as in the gene p16, will allow for continuous progression of the cell cycle in phases where the cell cycle must be halted[5]. Other common mutations cause lack of apoptosis, and limited production of enzymes that are linked to DNA repair[4,6].

After mutations occur and tumours begin to form, multiple changes such as recruitment of anti-inflammatory cells and increase in factors that support growth can be seen in the surrounding area of tumour development. Cells in the surrounding area are modified to aid in tumour progression via reduction of function in cells that are responsible for protection as well as recruitment of cells that aid in growth[7]. Other modifications include inducing cell death in nearby effector cells, increasing blood flow and tissue repair, chronic inflammation, and recruitment of immunoregulatory cells[7]. As such, the tumour microenvironment is increasingly complex and an area that must be addressed to develop treatments that aim to reduce tumour load.

1.1.2 Standard & Experimental Cancer Treatments

From the initial findings of cancer, determining a form of treatment that massively improves cancer survival rates remains extremely difficult due to its mutative nature. Chemotherapy, the most well-known form of cancer treatment, saw initial usage as early as the 1930s[7]. Multiple iterations of chemotherapeutics were designed including the use of nitrogen mustard to treat lymphomas and folate antagonists for treatment of leukemia[8]. Modern day chemotherapeutics have shown to reduce tumour size and inhibit growth in multiple types of advanced cancers, including acute lymphoblastic leukemia, Hodgkin's and non-Hodgkin's lymphoma, small cell lung cancer, ovarian cancer, and choriocarcinoma[8]. Current efforts

involving chemotherapy are continuously aiming to improve on overall efficacy through multiple modes, such as targeting specific populations or by combining various therapeutics[8,9].

Other forms of traditional cancer treatment include both surgical removal of solid tumours and targeted radiation therapy. Surgical removal has been employed as treatment as early as the 1900s, as seen in numerous cases[10–12]. Surgery remains a staple for solid tumour treatment however, we have not seen major improvements to this therapy since the 1960s as survival rates plateaued[7]. The discovery of radiation therapy follows a similar trajectory to that of surgical removal. The discovery of radiation in 1895[7,13] led to the development of treatment strategies revolving around the use of radiation against solid tumours. Again, like surgical removal, radiotherapy improvement came to a standstill in the 1960s due to the lack of effectiveness for when the cancer had reached a metastatic state – leading to the need for improved or novel therapies that could induce complete remission long term.

Each traditional form of therapy has shown success to varying degrees when pitted against a diverse set of cancers. Prominent examples of this include a greater than 90% 10 year survival rate in thyroid, testis and prostate cancer, while other forms of cancer such as esophageal, pancreatic and lung cancer possess 5 year survival rates that remain lower than 20%[7,14]. As such, an immense amount of effort and resources are funneled into to the discovery of novel forms of therapeutics to further improve long term survival of this disease.

More recent discoveries have seen the use of experimental therapies like nanoparticles, genetic modifications and immunotherapeutics[7]. Uses of nanoparticles and gene therapies (sometimes through nanoparticles) have shown to be targeted and more specific to sites of cancer development, due to their ability to only affect targeted tissue[7]. Typically, nanoparticles are capable of binding to selective moieties (e.g., folate receptor) followed by release of therapeutic

agents such as chemotherapeutic drugs[7,15]. Other methods of therapeutic interventions involving nanoparticles include: photodynamic therapy, where reactive oxygen se are produced, hyperthermia, where tumours are heated to render them susceptible to traditional therapy and gene therapies to remove dysfunctional cells or induce control in cancer cells[7].

The use of immunotherapy is another relatively new field wherein cells or biomolecules involved in the natural immune response, can be harnessed, and enhanced to induce anticancer functions. The first ground-breaking use of an immunotherapy came after the discovery of programmed death ligand 1 (PD-L1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)[16]. In short, these molecules are typically responsible for preventing excessive activation and killing of effector cells in the system, such as T cells, to prevent unnecessary tissue damage in times of infection of inflammation. Cancers can use this mechanism in a negative fashion where the immune response is evaded by upregulating these checkpoint molecules. To combat this, antibodies that block these molecules are employed to prevent cancer cells from activating these pathways, resulting in correction of immune effector function. Other forms of immunotherapy that have been recently developed and are undergoing study include the adoptive transfer of T cells (ACT), oncolytic viruses (OVs) and genetic engineering approaches to further augment these novel immunotherapies.

1.2 The Immune System and Cancer

1.2.1 The Immune System – an Overview

The human immune system is a complex machine, made up of multiple sets of cells, tissues and biomolecules that work together to prevent infections, eliminate pathogens and deal with injuries. Diving deeper, the immune system can be broken down into the innate and

adaptive system, where the former is responsible for prevention and immediate response to infection while the latter is involved with a delayed and long-term response. Within the adaptive response, a wide variety of cell phenotypes exist with a specific set of functions that are carried out for removal of pathogens and other adverse cellular bodies. The adaptive response contains both humoral immunity and cell mediated immunity wherein the primary focus of the humoral immunity is to elicit immediate and long-term immunity through B cells and antibodies[17]. T-lymphocytes and cytokines are the primary factors involved within cell-mediated immunity where adaptive function is executed via induction of apoptosis, release of cytotoxic materials or recruitment of phagocytic cells to the site of infection for the clearance of a pathogen[18,19].

T-lymphocytes are generally divided into two categories when considering pro-inflammatory cell subtypes; the $CD4^+$ "helper" T cells and $CD8^+$ "killer" T cells[18,20]. T-helper cells (Th) carry out effector function through recruitment of secondary cells to the site of infection, such as cytotoxic T cells, or through release of cytokines which may lead to further downstream cell recruitment or direct acting function, dependant on the type of infection/injury[20]. Killer T cells function through direct cytotoxic activity where apoptosis is activated via release of cytotoxic granules or expression of death ligands such as FasL[20]. Both Th and killer T cells recognize targets through the T cell receptor (TCR), a unique receptor that is generated upstream of T cell formation. TCR formation is carried out via RAG1 and RAG2, proteins that are responsible for gene re-arrangement which promotes the wide array TCR specificities that make up for the limitless number of peptides found in nature[18,20]. In the context of CD8 T cells, binding to a target cell requires the TCR to recognize a molecule found on all cells within the body, the major-histocompatibility complex (MHCI), producing the TCR-MHC complex. The main function of MHCI is to present intracellular peptides to surveying immune

cells for recognition to potentially initiate an immune response during presentation of a danger signal[21]. The human form of MHC, human leukocyte antigens (HLA), are highly polymorphic and each individual will contain a specialized HLA factor which affects the composition of the MHC. This uniqueness leads to the development of TCRs that will only recognize one phenotype of HLA[21]. Development of HLA provides the immune system a mechanism to recognize foreign or pernicious intracellular bodies for elimination via T-lymphocytes.

1.2.2 The Immune Response to Cancer

Inherent features of the immune system such as MHC recognition and danger signals allow for the initiation of protective mechanisms against cancerous cells. Inflammation induced by cancer is a major draw of immune cells to the site of a tumour – thus promoting an immune response via innate and adaptive function[22]. Additionally, inflammation will give rise to genomic instability and epigenetic modification leading to creation novel antigens, prompting the immune system to act with $CD8^+$ activation following recognition to mutant antigens[23,24]. The $CD8^+$ T cell response is the primary method of action in eliminating malignancy.

In many cases, this initial response is not mediated by interaction of TCR and MHC localized to the tumour/cancer cells[25] but rather through presentation of mutant antigens via professional antigen presenting cells (APC) to T cells[26]. APCs such as the dendritic cell (DC), will phagocytose a tumour associated antigen (TAA), break it down and present tumour associated peptides to naïve immune cells within secondary lymphoid organs[25]. Activation of naive $CD8^+$ T cells pushes differentiation into effectors cell[27], which may now elicit cytotoxic activity against the cancerous cells in hopes that the cancer is eliminated.

The expectation remains that the T cells may remove all intracellular forms of dangers such as malignant growths seen during cancer however this is not always the case. In solid tumours, the effector cells may traffic to the site of the tumour however in many instances, the immune system fails to prevent development of disease[7]. A lack of response is evoked through numerous mechanisms found innately within the immune system alongside mechanisms that exist inherently within the tumour. In particular, the tumour can react to the immune response in a manner that may reduce immune system function or via direct modifications in tumour microenvironment or antigen landscape, rendering the immune response ineffective.

1.2.3 Cancer Response to the Immune System – Immune System Modification

The tumour microenvironment (TME) is a complex combination of cells and factors that aid in the progression of tumour growth and immune system evasion, leading to poor disease prognosis[7]. Initially, the tumour can harness growth factors, such as EGFR[7] to push for activation and rapid proliferation. Additionally, the tumour may use other growth factors to accelerate intraepithelial proliferation, breakdown of the basement membrane, intravasation, extravasation, dissemination and angiogenesis[7]. Tumour cells may also harness immune system factors for regulation, like the cytokine TGF-β, an immunosuppressive cyotkine[28].

Alongside promoting growth and the use of regulatory cytokines, the TME has other mechanisms that reduce anti-tumour immune response. Both regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSC) are cells that are typically involved with prevention of autoimmunity and excessive immune responses. The TME exploits these cells in a manner to prevent subsequent reactions and response against TAAs[7,29]. Tregs typically function through both direct and indirect mechanisms wherein they can inhibit $CD8^+$ T cells by competition for consumption of cytokines needed for T cell proliferation and differentiation or through

production of cytokines seen in anti-inflammatory responses (such as IL-10)[7,30]. Similarly, the MDSC population interacts with effectors through both a direct and indirect manner[31,32]. Some examples include the metabolism of L-arginine, an essential molecule involved in T cell proliferation, production of reactive oxygen species, induction of Tregs, and the metabolism a tryptophan, another essential amino acid involved in T cell proliferation[7,33,34]. Other factors such as PD-L1 and CTLA-4 may also be involved in T cell suppression – leading to a massively decreased immune response against TAAs.

1.2.4 Cancer Response to the Immune System – Genetic Modifications

The ability for malignant cells to adapt to the immune system extends beyond modification of the surrounding microenvironment to include genetic modifications that aid in evasion of the immune system[35]. In a process known as immunoediting, malignant cells can modify their genetic code on two main fronts – the modification of recognizing factors such as MHC and the deletion of immune system targets[36]. As mentioned, expression of MHC allows for immune detection and verification of existing healthy cells throughout the system[36,37]. To avoid the detection of unregulated proliferation and growth, tumours undergo MHC downregulation leading to evasion from surveying APCs and activated T cells. Cancerous cells can then go undetected until they reach a latter stage whereby the effects on the body are irreversible[36,38]. Looking upstream, it has also been noted in literature that tumour cells may modify antigen processing machinery such as proteosome subunits or transporter associated proteins (TAP), reducing overall peptide presentation to surrounding APCs[38].

Deletion of an immune target or selection of an antigen negative variant is typically not observed until an initial immune response is formed and is more commonly seen after the use of anticancer therapeutics such as immunotherapies[7,37]. Loss of antigen target occurs following the

immune system targeting of one highly immunogenic epitope whereafter the tumour will downregulate the expression of the target antigen[31] and begin to promote an antigen negative variant to overcome the immune system. In the context of therapy, the growth of antigen negative variants typically follows initial remission generating antigen negative relapse (ANR) where the previously successful therapy may no longer remain efficacious[7]. As such, the target selection carried out by the immune system and antigen selection for immunotherapies is crucial to ensure prevention of ANR.

1.3 Cancer Immunotherapy

1.3.1 Immune Checkpoint Inhibitors

The first major advancement in the field of cancer immunology came with the discovery of the immune checkpoint, such as CTLA-4 and PD-1. Initiation of T cell response requires activation through TCR-MHC interaction, co-stimulation via CD28 and growth cytokines[39]. CD28, the cognate receptor for CD80 and CD86, regulates T cell immunity in preventing unwanted anti-self responses while promoting anti-pathogenic responses during infection[40]. During TCR-MHC binding, lack of CD28 stimulation causes anergy, where a T cell may become unresponsive to further stimulation[41]. The receptor CTLA-4 is expressed following T cell stimulation and is upregulated during prolonged immune response[41]. CTLA-4 is a direct competitor to CD28 in that they share the same ligands and binding of CTLA-4 and CD28 blocks T cell activation resulting in anergy[41,42]. Further study indicates that CTLA-4 may play a role in counteracting kinase signals induced by TCR and CD28 binding, further inducing the anergic response[44].

In the context of cancer, the TME creates chronic inflammation, thus leading to prolonged T cell responses and T cell exhaustion[42]. T cell exhaustion is known to promote

upregulation of inhibitory receptors such as CTLA-4, causing early retirement of T cells [41,42]. TME recruitment of Tregs may encourage further interaction of the CTLA-4 receptor reducing T cell effectiveness[42]. Following the discovery of CTLA-4 the use of blocking antibodies was achieved when Dr. James Allison[43] and colleagues found that usage of the CTLA-4 antibody increases the therapeutic window[44]. This discovery pushed for clinical trials in which it can be shown that CTLA-4 is efficacious for treatment of a myriad of cancers [43–45].

PD-1 is the other receptor that is emerging as a promising target for immune checkpoint therapy. In healthy T cells, PD-1 binds to the PD-1 ligand (PD-L1) during times of prolonged immune response and inflammation[40]. Like CTLA-4, the PD-1 receptor regulates T cell responses following activation to safeguard against overactive T cells[40,46]. Binding of the PD-1 receptor induces apoptosis of T cells halting the immune response[40,46]. The TME will abuse this mechanism through recruitment of regulatory cells such as the Treg, TAM or APC, all of which can produce PD-L1, resulting in excessive T cell apoptosis during response to the tumour[47]. To date, many PD-1 and PD-L1 blocking antibodies have been produced and approved by the American FDA for therapy in a wide variety of cancers[46]. The discovery of PD-1 blockade and CTLA-4 blockade has opened the door to novel immunotherapies by encouraging the study of using the immune system as a weapon against cancer.

1.3.2 Adoptive Cell Therapy

Adoptive cell therapy (ACT) is an additional form of immunotherapy that has been in the spotlight for its potential in treating liquid cancers[48]. ACT is the process whereby tumour-specific T cells are generated from patient extracts of tumour-infiltrating lymphocytes (TILs) or peripheral blood mononuclear cells (PBMCs), which are then cultured *ex vivo*, and subsequently reinfused back into the patient[49]. Despite the success of ACT seen in preclinical and clinical

models[54], the culturing of efficacious tumour antigen specific T cells has proved to be difficult because of lower frequencies of $CD8^+$ tumour specific T cells. To overcome this issue, extensive and precise *in vitro* protocols have been developed to enrich both quantity and quality of tumour specific T cells. Additionally, to further increase the viability of ACT, autologous T cells have genetically modified to express antigen specific T cell receptors (TCRt) or chimeric antigen receptors (CAR)[49,50]. In particular, the CAR has shown to be efficacious in treating relapsed liquid cancers, such as B-cell acute lymphoblastic leukemia and is now currently available as treatment in multiple countries[51].

Prior ACT research studied the use of terminally differentiated T cells, known as effector cells, due to their ability to elicit cytotoxic activity during the immune response[52]. The use of effector cells would provide immediate destruction of tumour cells, thus leading to reduction of tumour load and improving disease prognosis. Unfortunately, effector cells are unable to provide persistent, long term responses to tumours and in many cases have resulted in tumour relapse[52]. To combat the short-term nature of the effector T cell, using early progenitor T cells or T cells with self renewal activity allows the cells to persist longer after transfer into the patient while giving rise to a large effector population with a strong recall response[52]. T cell phenotypes such as stem cell memory (T_{scm}) and central memory (T_{cm}) have proven to provide more efficacious tumour protection against both infection and malignancy when compared to their effector counterparts[52,53]. Clinical studies are currently investigating whether using T cells with a self renewing, expansion ability is capable of entering the modern pharmaceutical market[7].

In the modern day pharmaceutical market, adoptive cell therapy has shown curative potential over a multitude of clinical trials ranging from solid melanomas to liquid leukemias. In trials targeting the B cell lymphoma target CD19, the cohorts receiving the CAR ACT therapy

showcased complete response rates >60%[54–56]. Despite success in clinical studies, ACT does come with downfalls. As aforementioned, ACT may fall short in terms of long-term persistence leading to relapse and an inability to repeat treatment[57–59]. Relapse is not solely the fault of the ACT and may arise due to inherent features of malignant cells however, by further improving on ACT we may prevent relapse. Furthermore, it can be shown that ACT fails to produce strong clinical results in solid malignancies. It is seen in a review article published by Dr. Jessica Wagner, that numerous ACT based clinical studies have been carried out against multiple antigens and solid malignancies[60]. In a vast majority of the studies, clinical efficacy remains non-significant or statistically significant but inferior when comparing to results of clinical trials against liquid cancers. The issues found may arise from the TME, homing issues, and T cell fitness[60].

Alongside inefficiencies and relapse seen within ACT therapies, it also shown that ACT may result in therapeutic toxicities. Toxicity may arise from on-target, off-tumour tissue damage[61,62] where reactions to infusion range from fever, rash, seizures and diarrhea[66]. More adverse effects include cytokine release syndrome (CRS) and neurotoxicity[63]. CRS occurs following transfer due to the highly activated nature of the transferred cells, where abnormal levels of cytokines are produced[61,62]. CRS is characterized by fevers, rigors, hypotension, hypoxia and vasodilation[64]. Severity of symptoms following CRS varies and is not attributed to a single cytokine however, the onset of CRS must be closely monitored as it has been shown that CRS may lead to death if left untreated[65].

The upcoming treatment of ACT has shown to be promising as a monotherapy however, the issues mentioned remain to be a hurdle when it comes to fully implementing the therapy in a

clinical setting. As such, it was proposed that the further augmentation such as genetic manipulation or combination of therapies be instated to further enhance ACT.

1.3.3 Chimeric Antigen Receptors and Transduced T Cell Receptors

To augment ACT, an above-mentioned method involves genetically engineering the T cell to express a novel receptor to improve therapeutic efficacy. The use of the CAR or TCRt further enhances adoptive cell therapy to solve multiple issues that arise before and during the use of ACT. The TCRt mimics the natural TCR in that the machinery for activation of a T cell remains the same (α and β chains linked to an intracellular signalling domain)[66] providing an increase in tumour specific T cells during therapy (Figure 1.1). More importantly, these TCRt T cells are engineered to avoid potential toxicities[67].

Like the TCRt, the CAR is an antigen recognizing receptor that is embedded into T cells but will act as replacement for the T cell receptor. The CAR typically consists of a variable region of an antibody, linked to the CD3 intracellular signalling domain of a TCR alongside a co-stimulatory molecule (Figure 1.1)[68]. T cells will then be activated through the CAR once encountering the target antigen. Engineering CAR T cells can drastically increase the quantity of antigen specific T cells, improving the potential of ACT by reducing the need longer culture times and providing larger initial quantities of tumour specific lymphocytes[69]. More significantly, the addition of a CAR allows for targeting of full surface protein complexes in an MHC independent manner removing the need for peptide presentation from the suppressed immune system. As previously mentioned, CAR therapies have seen success against many liquid cancer forms[70] and have been FDA approved to treat such cancers. Like in the monotherapy of ACT, it is common to observe that patients who have initial remission of cancer eventually give rise to relapsed tumours, many in the form of ANR. Additionally, CAR therapy has shown little

success when treating solid tumours due to the intensely suppressive microenvironment, and it is common for CAR T cells to be unable to home to the site of the tumour or for CAR T cells to become exhausted post-transplant[71,72]. As with other monotherapies, we must look to augment CAR therapy, particularly in solid tumours, to maintain complete remission in a manner that avoids or prevents loss of antigen.

Another point for discussion in the development of successful exogenous receptor therapies lies within selection of a target antigen. Potential target antigens found in cancer cells can be categorized based on origin. Self-antigens or differentiation antigens exist simultaneously in healthy tissue and in cancerous tissue and may be overexpressed, making it a potential target for the immune system[73]. Cancers may also propagate through viral infection, such as HPV leading to cervical cancer[74], where the cancerous cells have propensity to express viral antigens, producing a potential target[75]. An additional, and attractive form of target antigen is the neoantigen, where proteins have undergone numerous mutations marking it a non-self antigen and producing an epitope target for elimination[76]. Differentiation such as self-antigens make an attractive target due to high frequency and conservation of expression across multiple patients however, T cell responses to a differentiation antigen may lead to on-target off-tumour toxicities because of corresponding protein being expressed in healthy tissue[77]. Neoantigens and viral antigens carry the benefit of not being expressed on healthy, somatic tissue, and targeting T cells are not subject to central and peripheral tolerance[76] making it a much safer alternative to differentiation antigens. Despite their safety, targeting neoantigens can be complicated due to the need for the immune system to generate novel naïve T cells in sufficient numbers[76]. As such, multiple points must be considered when selecting a target for use in genetically modified ACT to enact a successful treatment regimen.

1.3.4 Oncolytic Viruses

Virotherapy is a concept that dates to the early 1900s, wherein doctors observed that cancer patients who recently suffered a viral infection had shown improvement regarding the cancer[78]. Virotherapy, the direct use of viruses in cancer treatment, originated from this, and is now being studied as a method to resolve cancer when observing their lytic capabilities alongside potent endogenous immune stimulation induced through the release of molecules such as interferon and tumour antigens[78]. Virotherapy in the context of cancer is also viewed as a safe method of therapy due to the propensity of the virus to divide in the rapidly dividing tumour, reducing off-target toxicity[79]. The viruses utilized in therapies have tropism for replication in cancer cells due to the rapid proliferation state of cancer as well as their dampened immune response. Finally, oncolytic viruses may also be genetically modified to remove viral genes that allow for immune evasion, to prevent replication in healthy cells for safety purposes[79].

Clinically, the use of OVs as a treatment against cancer dates to the 1940s where Pack et al. utilized an attenuated virus against melanoma, leading to a partial response[80]. Following, multiple experiments have shown partial success of the OV in patients with a variety of malignancies, leading to further study[81,82] and the eventual approval of multiple OV therapies by drug administrations in various countries[79,82]. Typically, therapeutic response across multiple studies indicates poor success when aiming for complete tumour remission[79] but the main payoff from these studies lies within the discovery of the large anti-tumour response elicited by the OV. The immunogenic cell death reaction triggered by OVs induces a strong inflammatory response and thus multiple different immune cells are recruited to the site of inflammation, despite the TME, allowing for APC antigen presentation and subsequent T lymphocyte activation[83]. Further destruction initiated by the OV leads to release of antigens – inducing a stronger immune

response against TAAs after inflammation[83]. This reaction can be described as turning a "cold" tumour "hot", wherein the primary immune response can be triggered following application of the OV[84].

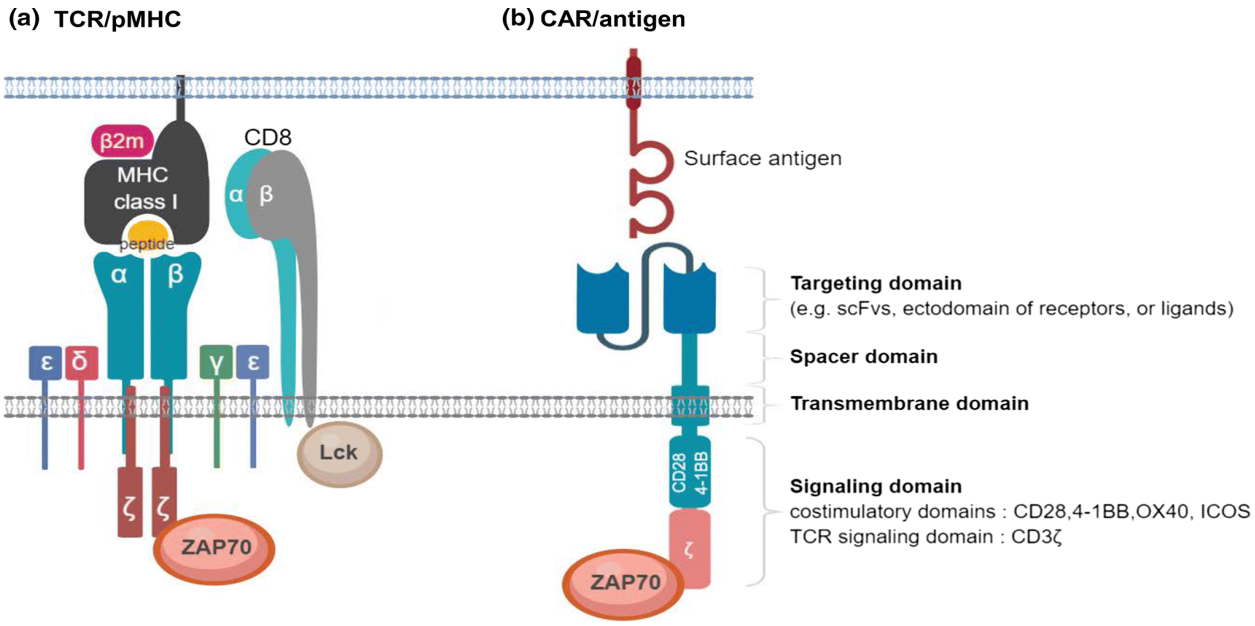


Figure 1.1: Visual representation of the structure of the (a) TCR and (b) CAR and their interactions with their targets. The structure of the TCR differs heavily from the CAR as the recognition domain will bind to a MHC-peptide complex whereas the CAR is capable of recognition for full length proteins, independent of MHC. The CAR is typically broken up into the ScFV, antigen recognition domain, and the constant region which consists of stimulatory and co-stimulatory molecules to propagate a T cell response. Development of the CAR lead to the creation of multiple CAR generation where additionally co-stimulatory regions were added to further promote activation signals after binding of target antigen. Taken from: Lee YH., Kim, C.H. Evolution of chimeric antigen receptor (CAR) T cell therapy: current status and future perspectives.[100]

1.3.5 Oncolytic Viral Vaccines

Consequent ingenuity led to the development of genetically modified oncolytic viruses that express tumour associated antigens (TAA) to drive expansion of previously existing TAA specific endogenous T cells[85]. By encoding the TAA, the oncolytic virus can induce an immunological response known as "boosting" where endogenous T cells that are specific for the TAA begin to divide and expand eliciting a more robust response to cancer cells[85,86.] The term coined oncolytic viral vaccines (OVV), is used to describe viruses that specifically target tumour

cells while inciting an antigen specific immune response. If the initial pool of TAA specific T cells does not exist, OVVs are capable of priming, wherein the OVV can establish a primary response to the tumour antigen to produce a pool of T cells that are ready to be boosted for a robust secondary response[86]. The combination of these effects induces stronger therapeutic outcomes due to the impact seen through the endogenous cell response[87].

The use of non-OV vaccines for purpose of therapy against malignancy is not a novel concept as two different vaccines, Sipulecel-T and HSPPC-96, have been approved by the American FDA for use in therapy[86]. Despite the approval of both vaccines for use in therapy against prostate cancer and glioblastoma, it was found that both vaccines have limited efficacies in improving disease-specific outcomes such as disease response and time to progression[86,88]. Poor efficacy seen in non-OV vaccines led to further study in an attempt to produce a vaccine like treatment against malignancy. Through previous experiments involving the non-OV vaccines and OVs monotherapies, it was rationalized that the use of a replicating peptide vaccine (OVV) may lead to improved efficacies[89]. Current clinical trials for OVVs remain in stages 1/2 and are evaluating the Adenovirus:Maraba-virus prime-boost strategy in patients with MAGE-A3 positive solid tumours[90,91].

1.4 Combination Therapies

1.4.1 Immune Checkpoint Inhibitors

Therapies with only one avenue of attack (monotherapies) are commonly used a last resort against malignancies such as advanced/metastatic melanoma[92] thus, monotherapies may fall short of complete remission[92] due to the more advanced nature of the cancer. Immune checkpoint blockade (ICB) therapy is one such therapy as it remains susceptible to relapse following initial remission, as seen during treatment of non-small cell lung carcinoma and

melanoma[93,94]. To combat immune escape following ICB therapy or classical therapies, combination therapies were developed to further the advance of immunotherapies. Combining the use of different therapies has shown to improve efficacy due to the creation of synergy[95–97]. Most commonly, it has been shown that combining ICB with therapies such as conventional chemotherapy or the newly approved CD19 CAR therapies generates a potent anti-cancer effect[98,99]. Although ICB therapy aims to rescue the endogenous immune response through reduction of the oppressive TME, ICB combined with ACT would also improve transferred T cell function[99]. Variations of combination therapies are being tested on both a preclinical and clinical level to prevent relapse in the context of immunotherapy.

1.4.2 Adoptive Cell Therapy and Oncolytic Viral Vaccines

The primary focus of the Wan lab is the use of T_{cm} ACT in combination with OVV therapy to produce a more robust immunotherapy against solid tumours. To potentiate the use of T cell phenotypes such as T_{cm} in ACT, the addition of the OVV can provide benefits that are uncharacteristically seen with ACT alone[100]. Particularly, the OVV can provide a more precise response to the site of infection due to the inflammatory nature of the infection[49]. Most importantly, the OVV can promote cross-presentation of the OVV cancer transgene to induce a boosting response regarding the T_{cm} cells[49].

This combination can provide the immune system with direct access to the cancer antigen via the OVV, allowing for professional antigen presenting cells (APC) to present the TAA in the lymph nodes, where T_{cm} cells will most likely reside after transfer[100]. Upon stimulation independently from the tumour, the transferred cells will proliferate and expand *in vivo* while differentiating to an effector-like phenotype, to carry out effector function against the target[49].

By combining ACT and OVV therapies, the limitations found in ACT of poor persistence and low T cell quantity are overcome, for a more complete form of therapy [49,100,101].

It has been seen in multiple *in vivo* murine models that the combination of T_{cm} cells and OVVs can lead to complete remission of tumours with induced neoantigens for the purpose of study. In a published *in vivo* study[100], treating the B16gp33 melanoma model expressing the genetically engineered gp33 epitope derived from lymphocytic choriomeningitis virus (LCMV), with transgenic T cells containing a gp33 specific TCR (P14 T cells) and OVV engineered to express gp33 (VSV-gp33), improves the therapeutic response compared to treatment with ACT or OVV alone (Figure 1.2). This data can be replicated when observing the CMS5 fibrosarcoma model in immunodeficient mice, where the tumour has mutated to express an induced neoantigen in mERK[87].

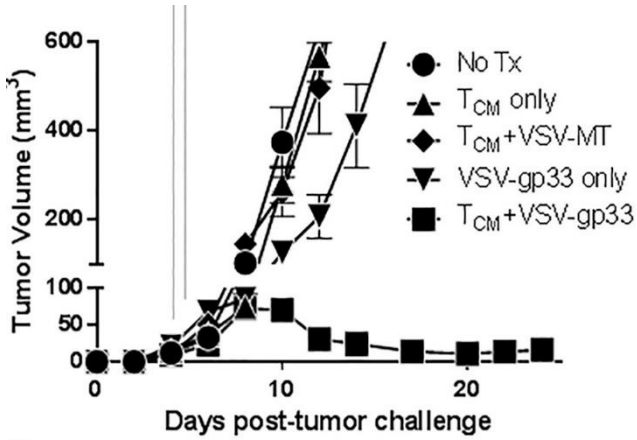


Figure 1.2: Adoptive cell therapy of tumour specific T_{cm} coupled with boosting VSV improves therapeutic benefit in the CMS5 tumour model. $1x10^5$ P14 memory cultured T cells were transferred into mice bearing the B16gp33 tumour model expressing the on day 0 followed by infusion of $2x10^8$ PFU VSV-gp33 on day 1. Other groups received Tcm alone, VSV-gp33 alone and non-specific VSV couple with T_{cm} therapy Tumour measurements were taken every 3 days until the mice reached endpoint or until the study was complete[100].

1.4.3 Antigen Negative Relapse in Combination Therapies

As mentioned, many currently available and successful immunotherapies result in relapse and during second administration of the initially successful therapy, there is a failure to reduce tumour load[99,100]. In many instances, this relapse is strongly related to loss of the target antigen

(ANR). To combat ANR, we must first understand its underlying mechanisms. A common form of ANR originates due to the innate rapid mutation ability of a tumour[102]. Further to this, therapeutic pressure can create tumour stress, leading to genomic instability and allowing the tumour to shed the target antigen thus abrogating single target monotherapies[103].

Acquired resistance to immunotherapies may also arise due to the heterogenous landscape of antigens found within a tumour[104,105]. By targeting one antigen through a monotherapy, we are capable of treating and totally eliminating the tumour cells exhibiting this specific antigen. By eliminating these tumour cells, natural selection will give rise to a population of tumour cells that inherently do not express the target antigen. Eventually, the antigen negative populations begin expanding exponentially and produce a large antigen negative population that is immune to consequent applications of the same immunotherapy[103,104].

Former students in the Wan lab have exhibited this phenomenon in not only monotherapy, but with combination therapy utilizing both T_{cm} cells and OVV that target one single antigen. In Figure 1.3, the combination of memory culture P14 T cells combined with VSV-gp33, is pitted against B16-gp33. Figure 1.3A demonstrates that the combination of the two therapies results in tumour remission followed by relapse. Figure 1.3B then reveals that initial B16gp33 did indeed carry the target antigen (gp33) by verification through PCR while the relapsed form did not carry the target antigen gene. To prevent relapse, we must then look to further augment the T_{cm}, OVV therapy to maintain complete remission in a manner that avoids or prevents loss of antigen.

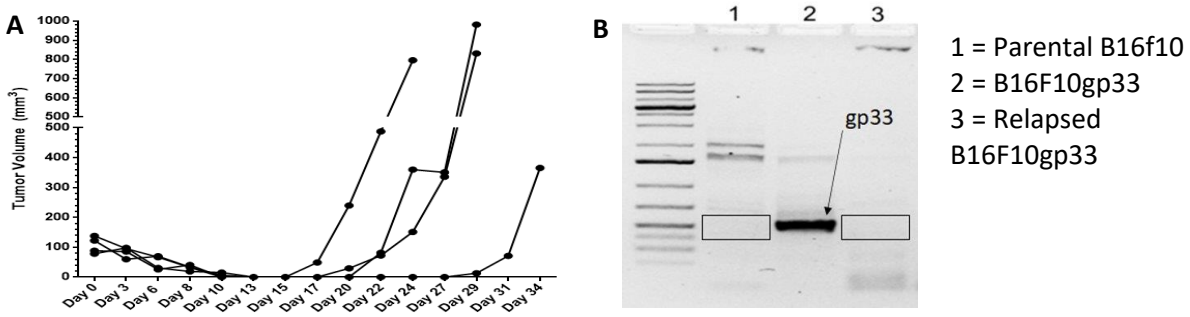


Figure 1.3: Adoptive transfer of tumour associated antigen targeted memory T-cells followed by boosting with a tumour-associated antigen expressing oncolytic virus results in initial tumour remission followed by antigen negative relapse. A – P14 memory cultured T-cells were transferred to mice bearing B16-gp33 tumours on day 0 followed by boosting with gp33-expressing VSV on day 1. Tumour measurements were taken every 3 days until animals reached endpoint, where each line represents one single mouse. **B** – PCR was used to amplify the gp33 gene from 1 – parental B16F10 cells which do not express gp33, 2 – B16F10 cells expressing gp33 and 3 – B16F10 cells isolated from relapsed tumours. Data presented in this figure was generated by Boris Simovic.

1.5 Dual-Specific T cells

1.5.1 Combatting Antigen Negative Relapse

Numerous strategies are in development to combat antigen negative relapse to further improve the use of immunotherapies. Methods employed to reduce ANR typically stem from a genetic engineering perspective where the endogenous functionality of the TCR can be harnessed with exogenous molecular therapies or through genetically engineering of a secondary receptor for ACT[105]. As mentioned, a common strategy involves the re-stimulation and rescue of a pre-existing immune response in the face of the TME, such as using anti CTLA-4 or anti-PD-1 antibodies[106]. A combination of ACT targeting one antigen and the rescue of tumour specific endogenous T cells may lead to prevention of antigen negative relapse.

The Bi-specific T cell engager (BiTE) and Mutual antibody T cell engager (MATE) are forms of immunotherapy that utilize small chemical molecules to enact a stronger internal response against target antigens[107]. The BiTE and the MATE operate in a similar manner which exploits a receptor on the operating T cell and links it to a TAA, for improved responses as the interactions operate without the need for co-stimulation and typical MHC-peptide presentation[108]. BiTE therapy utilizes the natural TCR physiology where the double-sided antibody binds to the CD3 stimulation domain of the TCR – bypassing the need for classical T cell activation[108,109]. MATE therapy (or zipCAR[110]) requires genetic engineering of the T cells to create T cells that express the Fc region and stimulation domain of a CAR, with a leucine zipper[110], while lacking the single chain variable fragment (scFv). Transfusion of a molecule like the BiTE, where one region binds to the Fc portion while the other end is specific to a TAA, creates a bond utilizing the leucine zipper to connect the Fc and scFv regions to enhance ACT[111], bypassing the need for co-stimulation and MHC presentation[110]. Both modes of therapy are amenable to adapt to ANR as target specificity is derived from the free-floating molecule rather than the TCR or CAR. By infusing a patient with BiTEs targeting multiple TAAs or by infusion of a secondary BiTE at a later time point, specific to a secondary TAA, T cells have the ability to act against multiple antigens in the hopes of preventing relapse.

The idea of targeting multiple antigens during immunotherapy is not novel as ANR has been a prominent cause for relapse in cancer therapeutics. Another potential solution to ANR are dual-specific T cells where T cells specific for one antigen are genetically engineered to express a secondary receptor to attack tumours from a secondary angle[112]. Targeting a secondary antigen with dual-specific T cells has shown to reduce tumour escape and relapse in both pre-clinical and clinical settings against liquid malignancies[113,114]. Furthermore, the addition of the secondary

receptor can lead to better anti-tumour efficacy[111], highlighting the potential of dual-targeting T cells to enhance the outcome during immunotherapies.

1.5.2 The Triple Threat Combo: CAR Therapy, TCR Therapy and OVV Therapy to Combat Antigen Negative Relapse

We have shown in the Wan lab that the combination of T_{cm} ACT and OVVs have the potential to elicit extremely powerful anti-tumour responses leading to initial regression of a solid tumour (Figure 1.2). Simultaneously, this successful therapy has also shown to be unsuccessful in preventing ANR bringing into question the reproducibility of the combination therapy (Figure 1.3). As such, we look to strengthen the ACT + OVV platform to circumvent ANR. Multiple methods have been proposed, such as adding additional therapeutics (such as ICB), modifying OVV vectors or the addition of small molecules (MS-275) that have shown efficacy in enhancing immune response. Again, dual-targeting T cells, produced through the addition of a CAR, have shown success in prevention of tumour relapse[113,114] and thus, it was proposed that by augmenting our ACT with a secondary receptor, we may further improve our current therapeutic platform beyond its current capabilities.

1.5.3 The Creation of Dual-specific T cells and Their Use

Clinically, TILs and PBMCs harbour previously established antigen specific TCRs that provide an avenue of attack against malignancies. In the context of the Wan lab ACT, T cells originate from transgenically modified mice with TCRs specific to a pre-established neoantigen. As such, T cells specific for one tumour antigen are readily available – pushing for the need of genetic modifications to provide a secondary receptor. The addition of a CAR is carried out most

commonly through retroviral vectors[115]. Herein, the creation of the dual-specific T cell for this project is carried out with a retrovirus encoding a CAR targeting the human B cell maturation antigen (hBCMA), allowing for TCR activation via two TAAs. Experimentally, we have now created the tool to further augment T_{cm} ACT + OVV in an attempt to curb ANR. As the dual-specific therapy remains similar to typical ACT, preparation of T cells and other therapeutic materials remains similar with minute modifications to the therapeutic platform.

1.6 Rationale and Hypothesis

ACT and OVV combined therapies have shown capacity for remission (Figure 1.2 and 1.3) and it is thought that by targeting antigen negative tumour variants we can curb the tumour antigen loss response. We hypothesize that engineering tumour antigen specific T cells (P14 in this case) to express a secondary receptor such as a CAR with specificity to a secondary tumour antigen, we will provide transferred T cells the tools to target multiple tumour antigens and prevent tumour antigen-escape (Figure 1.4). For this study, the secondary CAR target, human B cell maturation antigen (hBCMA), will be expressed alongside the initial target antigen in the tumour to artificially represent the heterogenous nature of the solid tumour. Through targeting multiple antigens, the ‘dual-specific’ T cells will be boosted through their natural TCR via exogenous administration of OVV while executing cytotoxic activity simultaneously against TCR antigen and hBCMA. Under ideal conditions, T cells will proliferate and traffic to the site of the tumour to carry out anti-tumour activity. The initial treatment of ACT + OVV will lead to tumour remission and resulting selective pressures will be mitigated due to elimination of TCR antigen negative clones through the secondary CAR receptor.

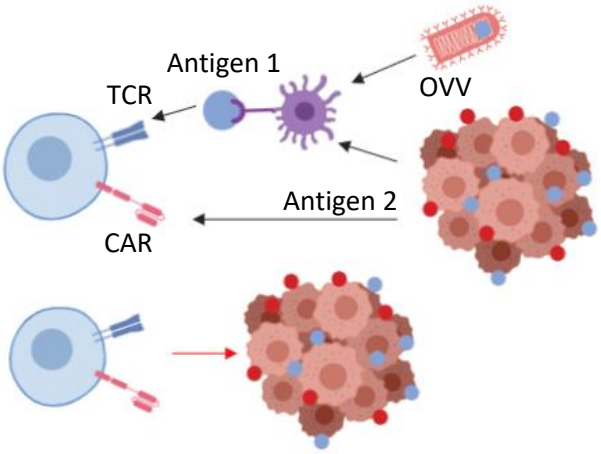


Figure 1.4: Representation of dual specific T cells during antigen presentation and tumour attack. Dual-specific T cells can be stimulated via TCR with antigen stemming from the tumour and/or OVV as well as through the CAR from tumour antigen expression. During tumour attack, the T cell can home to the tumour and elicit cytolytic effect through either TCR or CAR.

This dissertation will describe the progress pathed during the two years of my study beginning with the *in vitro* development of the T cell therapy. The *in vitro* development will detail the issues involved with generating genetically engineered T cells that express the transgene at high efficiency while maintaining cell quantity and quality, in terms of cell numbers, cell viability and maintenance of a memory phenotype. Following the production of dual-specific T cells, *in vitro* characterization is outlined, providing evidence that indeed, these dual-specific T cells function as intended in both proliferative capacity and killing capacity. Both proliferation assays and modified killing assays are utilized to establish the capacity of the T cells to function through both TCR and CAR. Ensuing work observes the capacity of the dual-targeting T cells to function *in vivo* during ACT + OVV combination therapies. Initially, we determine the capacity for T cells to expand, differentiate and function through markers such as IFNγ and TNFα in comparison to classical Wan lab ACT + OVV protocol. Finally, we observed therapeutic efficacy of this therapy against tumour bearing mice to determine the ability of the T cells in preventing ANR.

Chapter 2

2. Materials and Methods

2.1 Mice and source of T cells

C57BL/6 mice were purchased from Charles River Laboratories and housed in a specific pathogen–free room in the McMaster University Central Animal Facility. T cells are harvested from the P14 transgenic mouse strain that has been genetically engineered on the C57BL/6 background. $CD8^+$ T cells have been engineered to recognize the LCMV epitope, gp33, via TCR and express the Thy1.1 T cell marker for analytic purposes.

2.2 Retrovirus

The retrovirus construct was designed by Rebecca Burchett, a graduate student in the Bramson Lab. The construct of the 1067 retrovirus plasmid contains a second-generation CAR with the hBCMA targeting domain linked to murine CD3ζ and CD28 costimulatory molecule. The construct contains green fluorescent protein (GFP), to be used as a transduction marker.

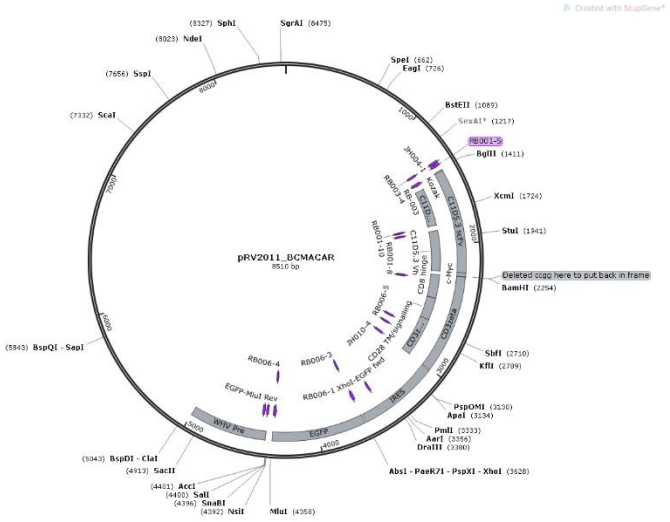


Figure 2.1: Plasmid map of the retrovirus construct containing the hBCMA CAR.

2.3 Retrovirus production

The production of retroviruses follows a protocol borrowed from Rebecca Burchett of the Bramson lab. To summarize, the virus production is completed using the PLAT-E packaging cell line[21]. Cells are plated at $5x10^6$ cells/mL 1 day prior to transfection in a T-75 tissue culture flask. PLAT-E cells are cultured in DMEM supplemented with inactivated FBS (10% final), 1mM

HEPES, 0.2mM L-glutamine, 89μL/mL normocin, 8.9μg/mL blasticidin and 0.89μg/mL puromycin. After one day of culture, the media is changed during transfection to remove blasticidin and puromycin while adding 10μg of the retroviral construct with 10μg of pCL-Eco packaging plasmid and 45μL of Lipofectamine 2000. The cells are incubated overnight and maintained by one media change 24 hours after transfection. The following day, the virus can be harvested through concentration with an Amicon Ultra 100K centrifugal filter. Retrovirus is then frozen at -80˚C following production.

2.4 Generation of hBCMA expressing cell lines

To carry out efficacy studies both *in vitro* and *in vivo*, parental B16 cells (B16F10) and cells that have shown ANR properties after treatment (B16gp33) are transduced via lentivirus encoding for hBCMA and puromycin resistance. The lentiviral plasmid was kindly provided by Rebecca Burchett from the Bramson lab. To produce lentivirus, $3x10^6$ 293TM cells are plated with DMEM supplemented with inactivated FBS (10% final), 1mM HEPES, 0.2mM L-glutamine and 89μL/mL normocin in a 10cm tissue culture dish and incubated at 37˚C overnight. The following day, transfection is carried out where lentiviral plasmid (20μg), envelope plasmid (3μg), rev packaging plasmid (1 μg), gag/pol packaging plasmid (10 μg) and lipofectamine 2000 (2μL) in optiMEM is added directly to 293TM plates and incubated overnight. 12 hours after transfection, media is changed and after a subsequent 24 hours, lentivirus can be harvested for transduction.

As aforementioned, the B16gp33 cell line has shown initial tumour remission with subsequent ANR. In a 6 well plate, the cells are cultured overnight at $2x10^5$ cells per well in MEM-Earles media supplemented with 1x non-essential amino acids, 1x MEM vitamins, 0.2mM L-glutamine, inactivated FBS (10%), 0.05 mM β-mercaptoethanol and 1mM HEPES. During this time, fresh lentivirus is prepared for use and can be used once B16 cells reach 70% confluency

the following day. The lentivirus is harvested, filtered, and added directly to each well at varying volumes (0, 1, 10, 50, 100, 500μL). The following day, 1 μg/mL puromycin is added to each well to begin selection for hBCMA expressing cell lines. Media is changed every 48-72 hours to remove dead cells and wells are monitored for single cell colonies. Once single cell colonies are achieved, monoclonal selection is carried out whereby one single colony of hBCMA expressing cells are grown up and then frozen down for use in experiments. Expression of hBCMA is confirmed through flow cytometry.

2.5 T cell culture and transduction

Splenocytes are harvested and processed immediately where cells are plated in a 24 well plate at 3 million cells/well in 1mL of culture media. The T cell culture protocol uses RPMI with FBS (10% final), 1mM HEPES, 0.2mM L-glutamine, 100μg/mL pen/strep, 0.05 mM β-mercaptoethanol, 1x non-essential amino acids (NEAA), 1 mM sodium pyruvate along with **10 ng/mL IL-7, 0.001 ng/ml IL-2, 100ng/mL CD3e and 100ng/mL CD28e**. 24 hours after harvest, cells are spun down and ~0.5mL of media is removed while retrovirus is added. The retrovirus solution contains 1.0μL/mL Lipofectamine 2000 and 0.8μL polybrene (1mg/mL stock) as well as ~25μL thawed retrovirus (optimal viral volume should be titrated/titered prior to transduction). The T cells are spinfected in the centrifuge at 2000xg for 1.5 hours at 37˚C followed by a 3-4 hour incubation period at 37˚C. 0.5mL RPMI with supplemented cytokine (no stimulation) is added and the cells are left to incubate overnight. On the next day and every subsequent 2 days, cells are scaled up to maintain a cell concentration of $2x10^6$ cells/mL with the respective media and cytokine supplements. Assessment of transduction and phenotype is carried out via flow cytometry with CD8-APC (1:200), CD44-PE (1:200), CD62L-APC-CY7 (1:200), IgG-BV421 (1:200) as well as live/dead staining with BV510 (1:200) as appropriate.

2.6 Flow based killing assay

Briefly, target cell lines (B16F10, B16-gp33, B16-hBCMA, B16-gp33-hBCMA) are cultured prior to start of the assay. Target cells are then labelled with CFSE to differentiate between target cells and effectors cells during the assay. As T cells express a GFP marker for transduction, T cells are further stained for expression of CD8 to differentiate target and effector cells. Following CFSE staining, target cells are plated in a 96 round bottom well plate at $1.5x10^5$ cells/well. Effectors cells (cultured T cells) are added to the 96 well plate at predetermined ratios (1:0.5, 1:1, 1:4) and are left to incubate for 12 hours. Following incubation, cells are stained with fixable viability dye and ran on flow cytometry to determine levels of killing. After normalization using the basal levels of target cell death, levels of cell death can be plotted and compared across ratios and cell lines.

2.7 Flow based proliferation assay

The methodology of the proliferation assay is similar to the killing assay in that target cells and effector cells are co-cultured for a period of time and the results are then analyzed through flow cytometry. In short, target cells (B16F10, B16-gp33, and B16-hBCMA) are cultured in a 12 well plate prior to co-culture to ensure sufficient target cells. On the next day, transduced (TD) and non-transduced (NT) T_{cm} cells are stained with violet proliferation dye 450 (V450) and are added to the 12 well plates at a predetermined ratio (1:1) in triplicate. The co-culture is left for 72 hours and monitored to feed cells when needed. After the 3-day period, the T cells can be harvested from the 12 well plate, stained for viability and analyzed via flow cytometer to determine number of cell divisions based on the number of visible V450 peaks.

2.8 Memory T cell re-stimulation assay

After culture, T_{cm} cells are harvested and prepped for co-culture with antigen presenting cells (APCs). For antigen presentation we are using the immortalized murine dendritic cell line (DC2.4). The DC2.4 cells are pulsed with 1ug/mL gp33 for 1 hour at 37˚C where they are now

ready to be used for stimulation of P14 T cells. P14 T cells are co-cultured with peptide pulsed DC2.4s overnight at a ratio of 1:1. The next day, the T cells are harvested for use in a flow based killing assay with target cells. Simultaneously, flow cytometry is completed to ensure effector phenotype of the T cells. After a subsequent 6 – 8 hours, the killing assay is complete and the levels of cell death in target cells are measured to determine effector cell efficacy.

2.9 *In vivo* work

Briefly, approximately $7.5x10^5$ tumour cells are injected into the C57BL/6 mice 7-10 days prior to treatment. On day -1, transduced and non-transduced T_{cm} cells are injected into mice with $1x10^6$ cells per mouse. On day 0, T cells are boosted via gp33 expressing VSV at $5x10^8$ pfu. Results include tumour size measurements every 2-3 days and collection of blood once a week to examine levels of T cell activity (IFNγ and TNFa) through flow cytometry. Extraction of tumour sample is completed through physical harvest of tumour mass collected into tubes. Following physical break up of tumour, they are then digested and processed with 25mg Collagenase IV, 10mg DNase and 50mL of cRPMI and allowed to mix for 30 minutes at 37˚C for 30 minutes. Cells are strained and then can be kept for flow cytometry analysis or subsequent culture for future purposes.

Chapter 3

3. Results

3.1 *In Vitro* Development of Dual-specific T cells for ACT + OVV Therapy

3.1.1 Traditional Wan Lab T_{cm} Culture yields poor transduction efficiency following retroviral infection

The traditional ACT culture protocol observed in clinical procedures utilizes IL-2 to initialize expansion of T cells[116] for transfer. Clinically, IL-2 culture short-term is used to prevent

terminal differentiation – improving therapeutic outcomes during ACT[117]. Despite literature highlighting the ability to culture memory-like T cells using IL-2, the Wan Lab has shown that culturing with IL-2 will often lead to a large proportion of T cells differentiating into an effector phenotype. Thus, a new protocol for T cell culture was developed to induce T_{cm} cells for the purpose of harnessing the full potential of the OVV + ACT boosting therapy. Classical Wan lab ACT culture contains IL-15, IL-21 and rapamycin in replacement for IL-2, alongside splenocyte stimulation with peptide instead of the standard CD3e and CD28e antibodies[118]. Indeed, this protocol allows for proportions of central memory T cells to reach up to 80%, generating therapeutic effects in solid tumour treatment when combined with OVV therapy, in pre-clinical studies (Figure S1). This protocol is consistently repeatable in P14, VSV-gp33 therapy against B16gp33 model where strong tumour remission is observed (Figure 1.2)[100]. To maintain *in vivo* therapeutic success, we look to carry out T cell transduction in T cells of the T_{cm} phenotype.

To produce dual-specific T cells, splenocytes are cultured with a retrovirus encoding for the hBCMA CAR, 1 day after splenocyte harvest. On day 6 after splenocyte harvest, T cell phenotype, viability, yield and transduction efficiency are examined where phenotype is defined by CD62L and CD44 expression and transduction efficiency is measured through the transduction marker, GFP during flow cytometry. Dual expression of CD62L and CD44 are representative of murine cells with a T_{cm} phenotype[119]. As described, culture with IL-15, IL-21 and rapamycin yield T cells with a T_{cm} phenotype (Figure S1). Importantly, it is observed that transduction efficiency under these culture conditions yield a transduction efficiency of <10% whereas cells of an effector phenotype observed transduction efficiencies up to 80% (Figure 3.1B). It is commonly found that minimizing T cell differentiation for ACT culture can decrease levels of transgene expression, such as the CAR, when compared to cells cultured in IL-2

alone[120,121]. To overcome this issue, we must then modify the procedure to generate T_{cm} cells such that transduction efficiency is improved to create dual-targeting T cells. To do so, we created multiple variations of our standard T_{cm} protocol and tested them on P14 splenocytes while transducing with the hBCMA CAR retrovirus to measure levels of transgene expression.

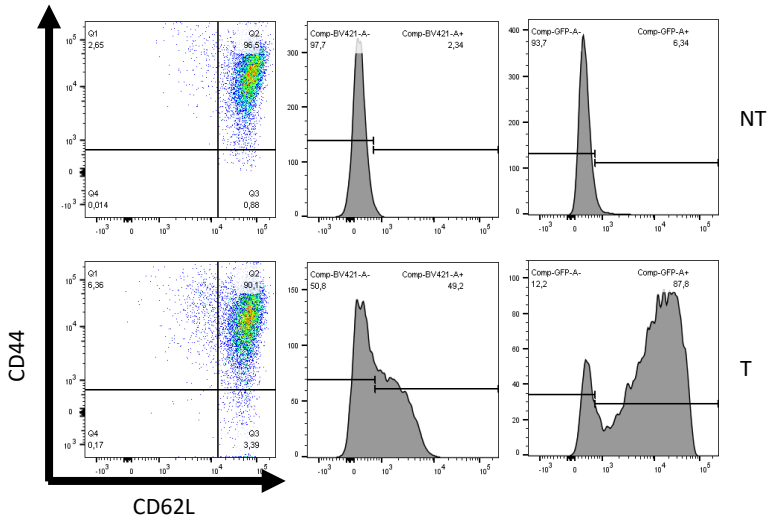
3.1.2 Transduction efficiency is improved significantly while maintain a central memory phenotype

To determine ideal culture conditions for dual-specific T cell therapy, 4 metrics must be met. Maintenance of T_{cm} phenotype, improved transduction efficiency, maintenance of T cell health, and maintained or improved T cell yield. T_{cm} phenotype conditions achieved during culture described in the Wan lab T_{cm} protocol will serve as a benchmark for all T cell cultures (Figure S1) while transduction efficiencies achieved during IL-2 T cell culture may act as the comparable for successful transduction. Therefore, we must look to improve on transduction efficiencies that are seen within IL-15, IL-21 and rapamycin T cell cultures while striving to achieve transduction efficiencies comparator to IL-2 culture.

Multiple variations of the IL-15, IL-21, rapamycin cultures were tested and measured for their ability to alter transduction and maintain the central memory phenotype as each cytokine provides differential properties that affect T cell culture. Additionally, we cultured cells using IL-2 as a positive control and another addition to our memory culture panel, IL-7, was added due to its ability to induce large proportion of T_{cm} during culture while granting high levels of transduction[122]. Furthermore, we compared epitope stimulation (as described in the Wan lab protocol) against CD3e and CD28e antibodies where it was found that peptide stimulation

massively reduced transduction efficiency (Figure S2). Among different culture conditions, it is observed that all conditions, apart from IL-2 culture, maintain comparable levels of the desired phenotype when compared Wan lab memory culture conditions (Figure 3.1B).

Observation of GFP expression reveals rapamycin as a major inhibitor of transduction, as all conditions utilizing rapamycin show extremely low levels of GFP expression (Figure 3.1C). Other conditions enhance transduction when compared to the Wan lab culture conditions (Figure 3.1C). Importantly, we note that the IL-7 culture conditions retain comparable levels of T_{cm} distribution while considerably increasing GFP expression (Figure 3.1C). Herein, we focused on IL-7 as a culture cytokine due to its ability to enhance T cell transduction.


CD44
CD62L
Q1
2,65
Q2
96,5
Q4
0,014
Q3
0,88
Comp-BV421-A-
97,7
Comp-BV421-A+
2,34
Comp-GFP-A-
93,7
Comp-GFP-A+
6,34
NT
Q1
5,36
Q2
90,1
Q4
0,17
Q3
3,39
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Comp-GFP-A-
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Comp-GFP-A+
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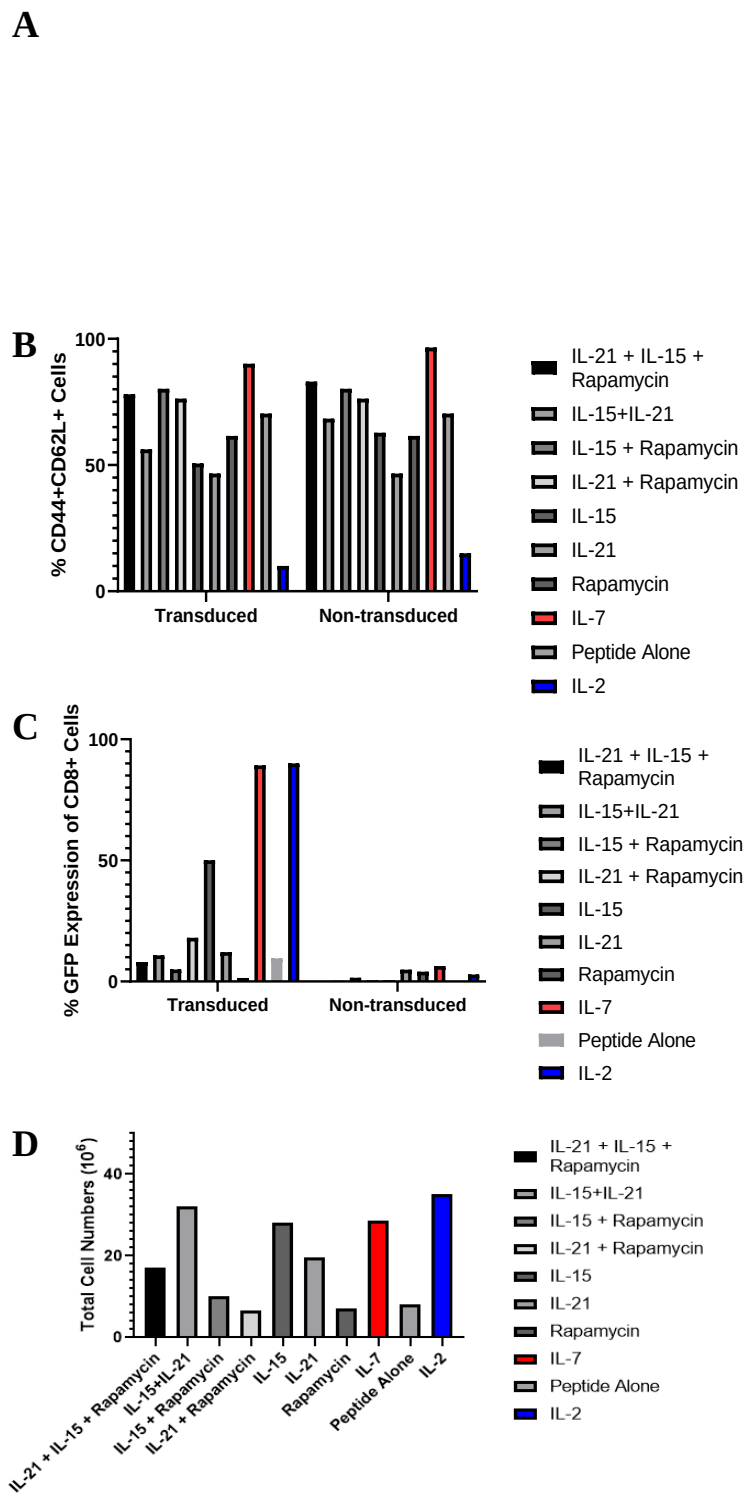

Figure 3.1: Modifications of T_{CM} protocol provides levels of memory population comparable to the standard protocol while heavily influencing transduction efficiency. P14 T cells are cultured from splenocytes with IL-7 and trace amounts of IL-2. **(A)** represents flow cytometry plots with the CD44, CD62L expression cross-gates and transduction efficiencies measured through direct staining (middle) and GFP expression (right). NT = non-transduced and TD = transduced. **(B)** All T cell cultures were stimulated using CD3/CD28 antibodies prior to transduction. Phenotyping takes place 7 days post transduction. Individual bars are representative of one replicate for the detailed culture conditions. **(C)** T cells were gated, 7 days post transduction, for effector and memory populations and transduction efficiency was measured via GFP expression for each T cell subset. **(D)** Cell cultures are initialized at 3 million cells per condition. On day 7, cell counts are completed and compared across multiple conditions. Measurement of culture conditions has been repeated in >10 instances.

3.1.3 Viability and cell yield are massively improved during T cell culture with IL-7

Two major aspects that lead to successful ACT lie within the number of T cells that are initially transfused into a patient and the health or viability of these transferred cells. To ensure that IL-7 cultured cells may potentially provide comparable downstream *in vivo* benefits to those seen in ACT with Wan lab protocol T cells, we must demonstrate healthy T cells in high numbers 7 days after spleen harvest. In Figure 3.1D, we demonstrate IL-7 culture yields significant cell growth numbers, comparable to effector cell culture, while maintaining the T_{cm} phenotype. Furthermore, we confirm that T cells remain healthy after 7 days of culture (Figure S3). The cell yield and viability compounded with T cell transduction and phenotype solidifies IL-7 as the cytokine supplement to be used for transduction. In consequence, all future work discussed in this dissertation utilizes IL-7 and trace amounts of IL-2, alongside CD3e and CD28e antibody stimulation as the culture condition of choice. Moving forward, the best measure for success in both phenotype and transduction efficiencies lies within a measure of receptor functionality. As such, we must then carry out functional assays that determine CAR presence alongside a proliferative capacity representing T_{cm} cells.

3.2 *In Vitro* Characterization of Dual-specific T cells

3.2.1 Creation of hBCMA expressing cell lines

First, to evaluate the therapeutic potential of Dual-specific T cells we gathered and created tumour cell lines that express one (gp33/hBCMA) or both target antigens. To confirm expression of hBCMA, flow cytometry analysis was performed through direct protein staining with a fluorescent antibody. After transduction, analysis indicates that B16F10 (Figure 3.2A) and B16gp33 (Figure 3.2B) cell lines express the hBCMA protein at significant levels.

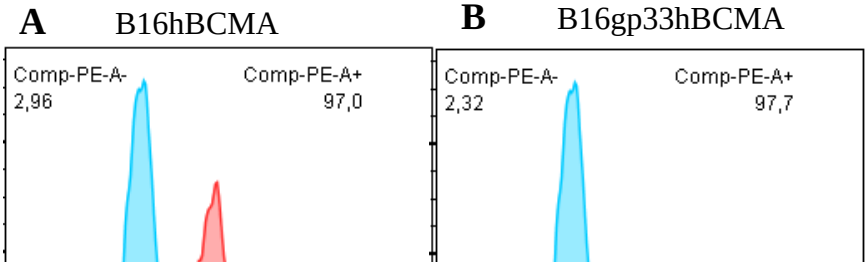

Figure 3.2: Lentiviral transduction of B16F10 and B16gp33 cell lines promotes high level of hBCMA expression. (A,B) hBCMA

3.2.2 Dual-specific T_{cm} cells expand and proliferate during co-culture with either TCR or CAR target expressing cells

Having established the ideal culture conditions for CAR expressing memory cells, we next sought to analyze the functionality of the T_{cm} cells. As mentioned previously, T_{cm} expand and proliferate[56] following stimulation through the cognate receptor thus, to demonstrate functionality of the transduced T_{cm} cells, we first use a proliferation assay. The proliferation assay will showcase division ability of the T cells – a direct measure of the proliferative capacity. To do so, on day 7 of T_{cm} culture, both TD and NT T cells are labelled with proliferation dye and co-cultured with target cells that contain target antigen (gp33 and/or hBCMA) for 3 days. Proliferation assay data is shown in Figure 3.3.

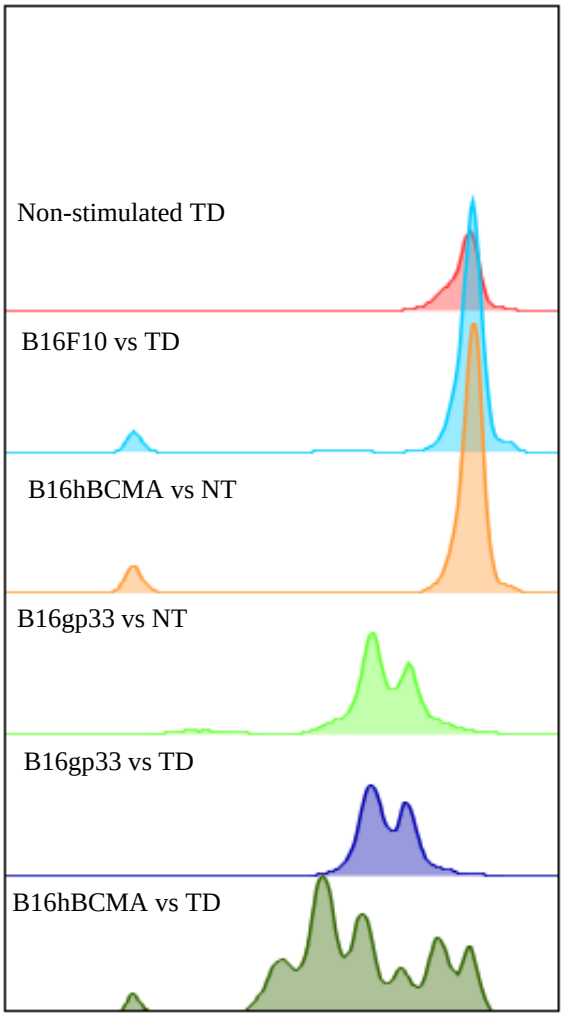

Figure 3.3: Against target antigen, TD and NT P14 T cells are capable of proliferating for multiple divisions *in vitro*. TD and NT P14 T cells are co-cultured with antigen expressing target cells after being stained with CTV proliferation dye to determine level of divisions/proliferation following stimulation. Each histogram is representative of a different set of conditions where-in the initial histogram (non-stimulated T cells) represent undivided cells. Peaks shifted and developed beyond this benchmark represent proliferation. Proliferation assays were completed successfully ~ 5 times in triplicate.

Here we observed the ability of transduced T cells to carry out target specific proliferation (Figure 3.3) *in vitro*. To represent the original cell generation, we have non-stimulated T cells in which we can reference as a point for cells that remain undivided, as seen with the red histogram. As expected, we observe that when culturing T cells against non-antigen target expressing targets, T cells remain undivided. Conversely, when cultured with a TCR or CAR target expressing cell line, T cells begin to divide for multiple divisions demonstrating T_{cm} function in TD and NT T cells. TD P14 T cells do not divide non-specifically in the presence of parental B16 cells, indicating target specific function with T cells harbouring a CAR. We can

replicate this data (Figure S4) with the same B16 cell lines or with plate bound hBCMA protein (Figure S4D), solidifying that these T cells function in a central memory manner.

3.2.3 Tumour target hBCMA expression and T cell hBCMA CAR expression is confirmed through an *in vitro* Killing Assay

To induce anti-tumour function, T cells must be able to elicit cytotoxic activity against target expressing cells to induce apoptosis. Following confirmation of T_{cm} function in a proliferation assay, we determine whether effector cells equipped with a CAR functions correctly in the presence of hBCMA expressing target cells. Killer function is measurable with a killing assay, as described in section 2.6.

In Figure 3.4, it is observed that both P14TD and NT effector T cells function as intended in that dose-dependant killing of antigen specific targets is seen in B16gp33, B16hBCMA and B16gp33hBCMA cell lines (Figure 3.4 A-D). Consequently, we have verified that transduction of the hBCMA CAR provides the T cell with the ability to kill at a similar or heightened capacity when compared to cytotoxicity elicited through the native TCR (Figure 3.4B). Evidence from this killing assay suggests that transduction of a secondary receptor improves killer function of T cells at lower cell numbers (Figure 3.4D) when targeting two antigens simultaneously. It may also be noted that memory cultured T cells, do not exhibit killing function against antigen target expressing cells within a 24-hour period.

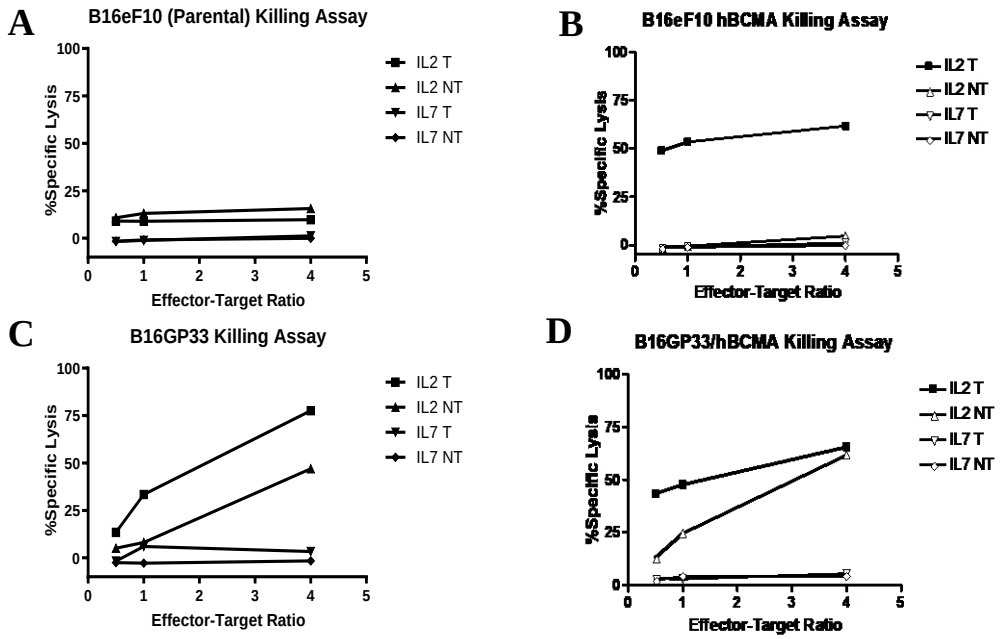

Figure 3.4: Effector cells exhibit killing capacity when equipped with the hBCMA CAR. The killing assay was completed over a 12-hour period in a 96 well plate and killing was measured via flow cytometry. (A) Against antigen negative target cells (B16eF10), minimal killing was seen across all T cell co-cultures. (B) Killing is illustrated in the IL-2 transduced cell lines, effector cells containing the hBCMA CAR, against B16h-BCMA. (C) Both transduced and non-transduced IL-2 cells exhibit killing through the native gp33 TCR against B16 expressing gp33. (D) Substantial killing in both transduced and non-transduced IL-2 cells can be seen against B16-gp33-hBCMA cells. Killing assay was completed once.

3.2.3 *In Vitro*, Dual-specific T_{cm} cells can differentiate and carry out target specific killing

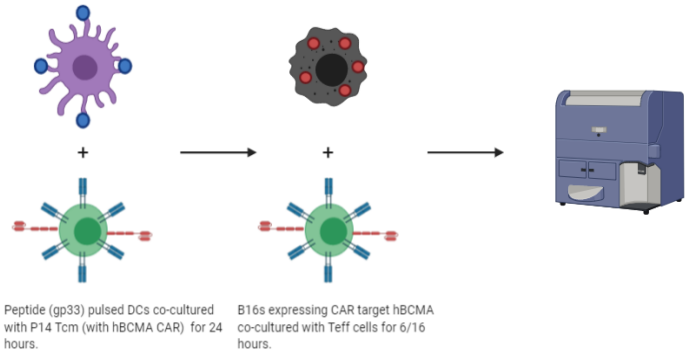

Figure 3.5: Visual representation of re-stimulation assay. Effectors cells are co-cultured with target cells for 24h.Then, target cells are stained with viability dye and cultured with effector cells where levels of death are analyzed through flow cytometry to determine T cell efficacy.

During the *in vivo* response, T_{cm} are made to boost, differentiate, and carry out cytotoxic activity following injection of OVV. Previous work has shown that T cells of a memory

phenotype expand and carry out anti-tumour activity during *in vivo* experimentation. This function is carried out solely by the native TCR following culture using the Wan lab memory protocol. Before moving to *in vivo* work, we wanted to mimic an *in vivo* scenario where IL-7 cultured T_{cm} are boosted and differentiated through the native TCR followed by execution of effector functions through both TCR and CAR. The re-stimulation assay was designed to mimic the *in vivo* conditions more closely wherein T_{cm} cells will be stimulated via exogenous gp33 through the TCR, differentiated to an effector state and then used to kill tumours via TCR and CAR (Figure 3.5). Simultaneously, we may also observe maintenance of CAR expression following the differentiation of the T cell.

As described in section 2.9, T_{cm} cells are generated with high expression of hBCMA CAR and co-cultured with peptide pulsed DCs for re-stimulation to promote differentiation and expansion. Figure 3.6A&B demonstrate the shift from a central memory phenotype to an effector phenotype after 24 hours of co-culture with gp33 pulsed DCs. Importantly, GFP expression remains high following differentiation, attesting to the persistence of CAR transduction.

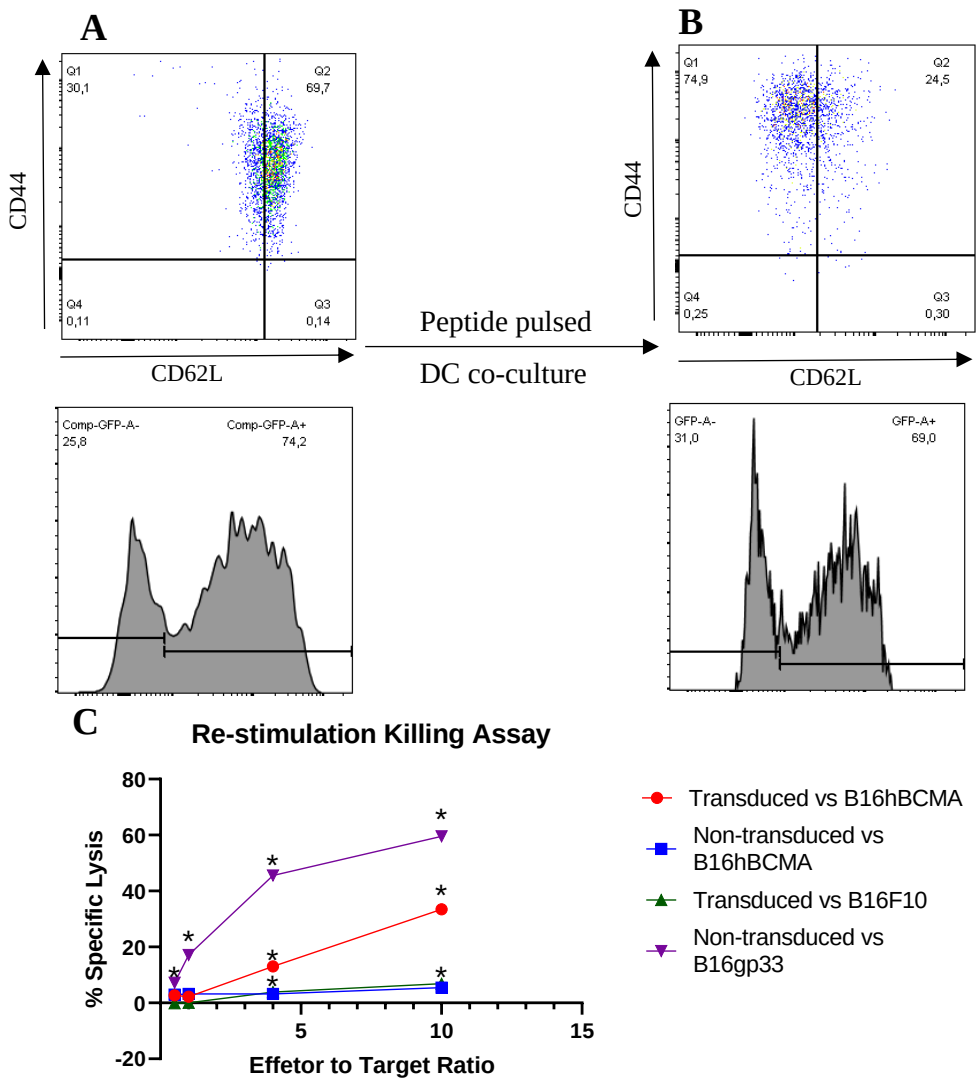

Figure 3.6: Re-stimulated TD T_{cm} demonstrate effective killing capacity against hBCMA or gp33 expressing target cells when compared to non-specific re-stimulated T cells. (A) Flow cytometry analysis of T cells prior to co-culture with DC2.4s pulsed with gp33 peptide. Each dot plot represents levels of CD44 and CD62L expression while each histogram is gated for transduction efficiency when compared to non-transduced cells. **(B)** Flow cytometry analysis is done following re-stimulation. **(C)** Killing assay carried out over a 6-hour period in a 96 well plate with killing analyses completed via flow cytometry. Results shown in duplicate with *P<0.05 by independent T-test. Re-stimulation was completed 3 times in triplicate.

Subsequent killing assays performed with the re-stimulated T cells reveals target specific killing through both TCR and CAR. TCR mediated cytotoxicity results in statistically significant differences at 0.5:1, 1:1, 4:1 and 10:1 effector:target ratios over the NT cells co-cultured with B16hBCMA and TD cells cultured with B16F10. CAR mediated killing results in statistically significant differences at 4:1 and 10:1 effector:target suggesting that over a 6 hour period, re-stimulated T cells are able to kill in response to cell lines that express gp33 or hBCMA *in vitro*.

3.3 *In Vivo* Analysis of Dual-specific T cells during ACT + OVV Therapy

3.3.1 Implanted B16gp33hBCMA tumours maintain high levels of hBCMA expression

To carry out therapy against dual-target expressing solid tumours, we must ensure that our model maintains expression of our newly introduced secondary antigen, hBCMA. hBCMA expression is not native to the murine system and the murine and human copies of the BCMA protein share approximately 62% homology in amino acid sequences[146]. The differences in proteins may result in the initiation of an endogenous anti-hBCMA immune response, with subsequent rejection of B16gp33hBCMA tumours or loss of hBCMA expression. To confirm hBCMA expression is maintained following implantation, we introduce B16gp33hBCMA into C57BL/6 mice. Once mice reach endpoint (Tumour volume >1000mm^3), we harvest the tumour and process it to measure hBCMA protein expression via flow cytometry. Flow cytometry analysis reveals that hBCMA expression in harvested tumours is similar to expression seen in *in vitro* cultured B16gp33hBCMA tumours (Figure 3.7). The comparison between the two, indicates that hBCMA expression remains at a high level following engraftment of tumours, thus making hBCMA an available target for treatment. It must be noted that typical study of the B16 tumour model requires an intradermal injection of tumours of 1x10^5 cells 7 days prior to therapeutic intervention. The introduction of the hBCMA antigen requires a modification to the protocol as it was found that B16gp33hBCMA tumours do not grow following injection of 1x10^5 cells. To compensate for this, experiments require an intradermal injection of 7.5x10^5 B16gp33hBCMA cells (Figure 3.11A).

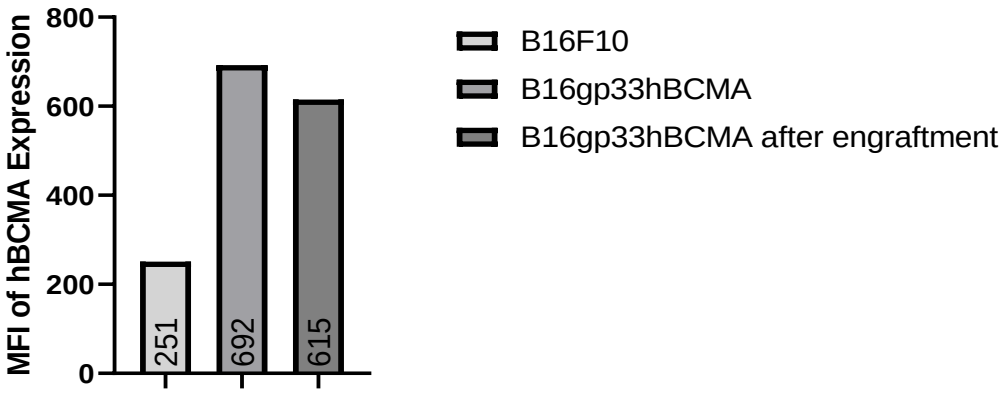

Figure 3.7: **B16gp33hBCMA tumours maintain hBCMA following engraftment.** $7.5x10^5$ B16gp33hBCMA tumours are implanted in C57BL/6 mice and tumours are extracted once tumours reach endpoint. Flow cytometry analysis is carried out with an anti-hBCMA antibody that measures for surface expression of hBCMA protein.

3.3.2 Preliminary analysis of Dual-specific T cells reveals high levels of functionality after boosting during ACT + OVV therapy

The next step in moving to an *in vivo* model of study was to determine whether the IL-7 cultured T_{cm} acts similarly to the T_{cm} cells utilized in typical Wan lab ACT protocol. The difference in culture protocols may modify the *in vivo* capacity of T_{cm} to proliferate and eliminate tumours following OVV stimulation. To investigate T cell functionality, a small scale experiment was initiated (n=3) comparing TD T cells and NT T cells in tumour free animals. Due to a mix-up during the injection process, the non-boosted control group contained one subject of study. To evaluate T cell function, T cell analysis was performed 5 days post OVV injection, and every subsequent 7 days, to evaluate presence of transferred cells and the magnitude of T cell response delineated by IFNγ expression. Each time point (Figure 3.8B, C) illustrates significant levels of transferred cell presence via the congenic marker Thy1.1 in addition to strong T cell functionality evidenced through significant IFNγ expression in TD and NT cells contrasted with the non-boosted control. When compared to Wan lab protocol T_{cm}

(Figure S1), levels of IFNγ closely mimic those observed in Figure 3.8C, alluding to potential success in curing tumour bearing mice.

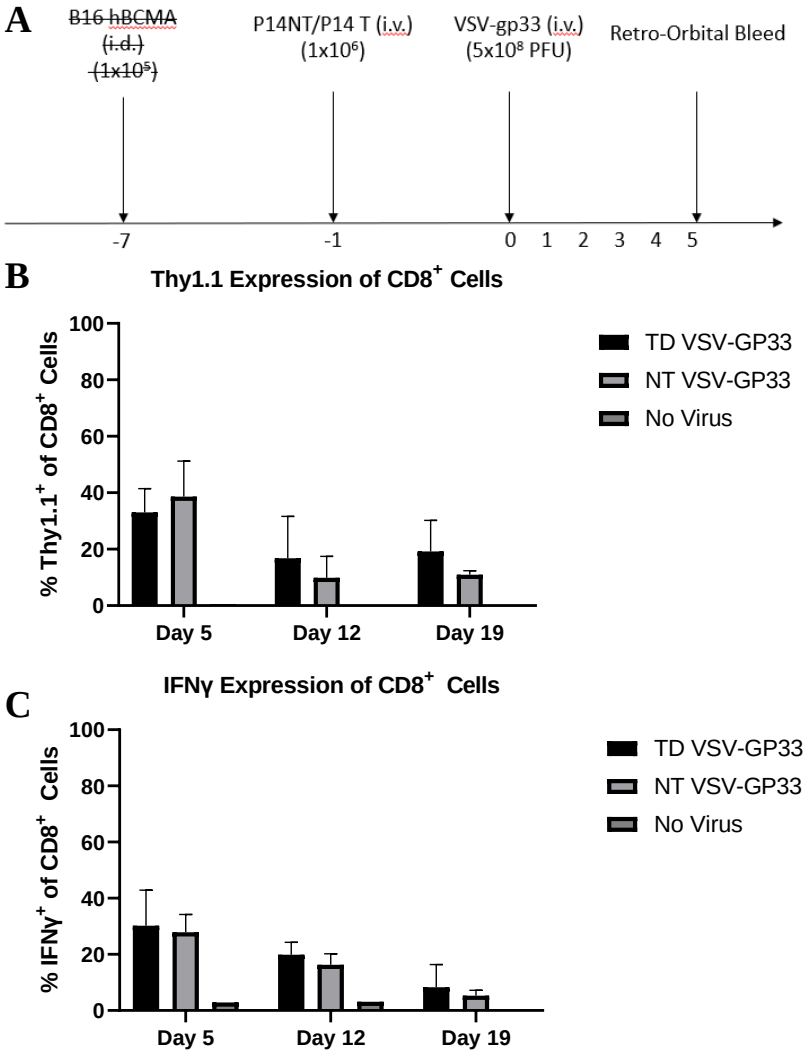

Figure 3.8: **IL-7 Cultured T_{cm} are boosted following ACT + OVV combination therapy. (A)** TD and NT P14 T cells are transferred I.V. followed by VSVgp33 boost 1 day later. Blood is collected on day 5 and every subsequent week following to measure for T cell function. n=3 for TD, NT groups, no virus n=1 **(B)** Surface staining of blood samples is completed to identify the Thy1.1 congenic marker to represent frequency of transferred cells. **(C)** IFNγ expression is observed following peripheral blood re-stimulation with gp33 peptide. Experiment was completed once.

3.3.3 *In vivo* therapy mediated exclusively through the CAR against B16hBCMA tumours is unsuccessful in promoting therapeutic benefit

To create success of dual-specific therapy against solid tumours, *in vivo* anti-tumour function is mediated through both TCR and CAR against gp33 peptide and hBCMA protein respectively. Previously (Figure 1.3), it was shown that therapy carried out through the TCR alongside OVV boost results in tumour control followed by relapse. To define CAR functionality *in vivo* we look to recapitulate similar outcomes following treatment using TD P14 T cells B16hBCMA, a single antigen expressing tumour cell line. 7 days prior to OVV treatment, $7.5x10^5$ B16hBCMA cells were injected intradermally (n=5). ACT and OVV therapy follow on 8 and 9 days post tumour injection respectively (Figure 3.9A).

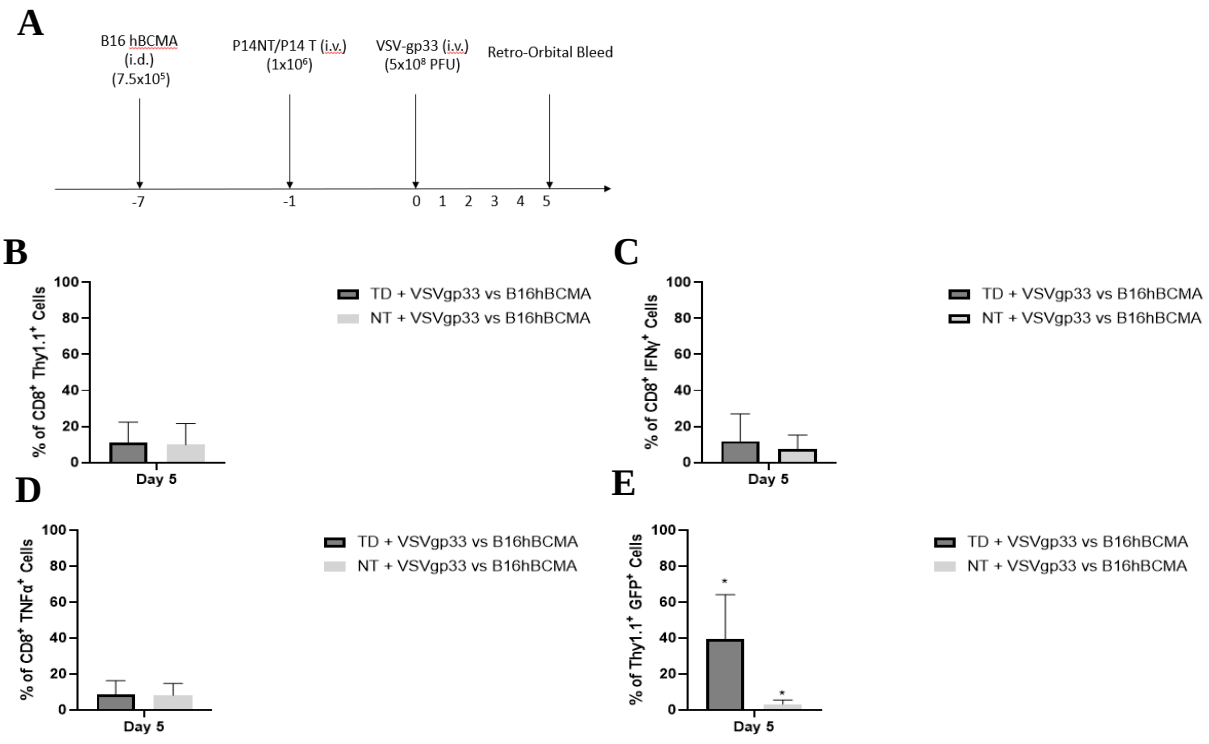

Figure 3.9. **T cell presence, T cell function and maintenance of CAR in transferred cells is observed 5 days following OVV therapy.** C57BL/6 mice were inoculated i.d. with B16hBCMA cells prior to adoptive transfer with dual-specific T cells and specific OVV treatment 24 hours later. Blood is collected on day 5 post injection of OVV and every subsequent 7 days following. T cell analysis reveals frequency of transferred cells **(B),** magnitude of total $CD8^+$ T cell response **(C, D)** and CAR expression (**E).** Statistical analysis is completed through independent T test vs NT + VSVgp33 control .*P<0.05. Experiment was completed once.

Two experimental groups are observed where one group receives TD T cells while the second receives NT T cells. T cell analysis (Figure 3.9B) reveals presence of T cells through Thy1.1 expression in both groups alongside moderate levels of T cell function captured by a moderate IFNγ and TNFa response (Figure 3.9C,D). Distinctively, the level of GFP expression, representative of CAR expression displays statistically significant differences, confirming maintenance of CAR expression following T cell transfer (Figure 3.89).

Despite evidence of T cell response displayed by ICS (Figure 3.9C,D) minimal tumour control is observed in mice receiving TD T cells (Figure 3.10A). Comparison between the group of interest and the control (Figure 3.10B,C) reveals absence of significant difference in survival between the two groups, indicative of poor therapeutic efficacy mediated by the CAR against B16hBCMA tumours. *In vivo* the CAR is incapable of mediating tumour control however, in combination with a secondary receptor (TCR), it may still provide additional therapeutic benefit, providing the basis to continue working with the dual-targeting T cells in an *in vivo* setting.

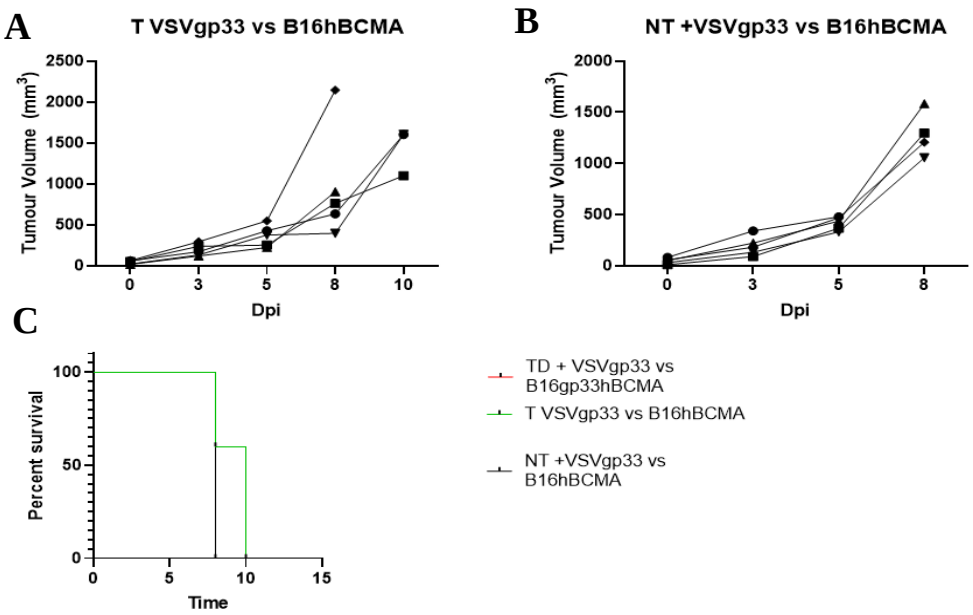

Figure 3.10: CAR mediated therapy is ineffective in initiating tumour control (A, B) Tumour volumes of mice measured, starting on day of OVV injection. A tumour volume of approximately 1000m^3 was used as an endpoint for the experiment. Each line represents one single mouse in the experiment group. **(C)** Survival curve of each experimental group. n=5 and data is analyzed using a log-rank (Mantel-cox) test *P<0.05.

3.3.4 Prior to palpable tumour growth, Dual-specific T cells can prevent or prolong tumour growth during combination therapy

Next, we sought to determine whether dual-specific T_{cm} in combination with OVVs would provide additional benefit over conventional ACT, OVV therapies in inducing complete and durable remission in a solid tumour model. 7 days prior to OVV treatment, B16gp33hBCMA cells were injected intradermally (I.D.). Subsequent ACT and OVV therapy follow, alongside blood sample analysis beginning on day 5 (Figure 3.11A). Prior to OVV injection, minimal tumour growth was observed, seen in Figure 3.11 B, C. The lack of tumour growth resulted in modifications of the experimental hypothesis from looking for therapeutic response and durable remission in tumour bearing mice to prevention of tumour growth in mice lacking palpable tumours. Minor tumour growth (<20mm^3) was visible in the group prior to receiving P14T,

VSVgp33 therapy and following treatment, remission is observed. From Figure 3.11 C, we show that NT P14 T cells alongside VSVgp33 are incapable of preventing tumour growth as all 5 mice reached endpoint (>1000mm^3). Simultaneously, the non-boosted T cells evoked a similar outcome, where all 5 mice reached endpoint (Figure 3.11D). Dual-specific T cells prevent tumour growth in 3/5 mice, displaying prolonged survival for more than 50 days with statistically significant differences when compared to the control(Figure 3.11E).

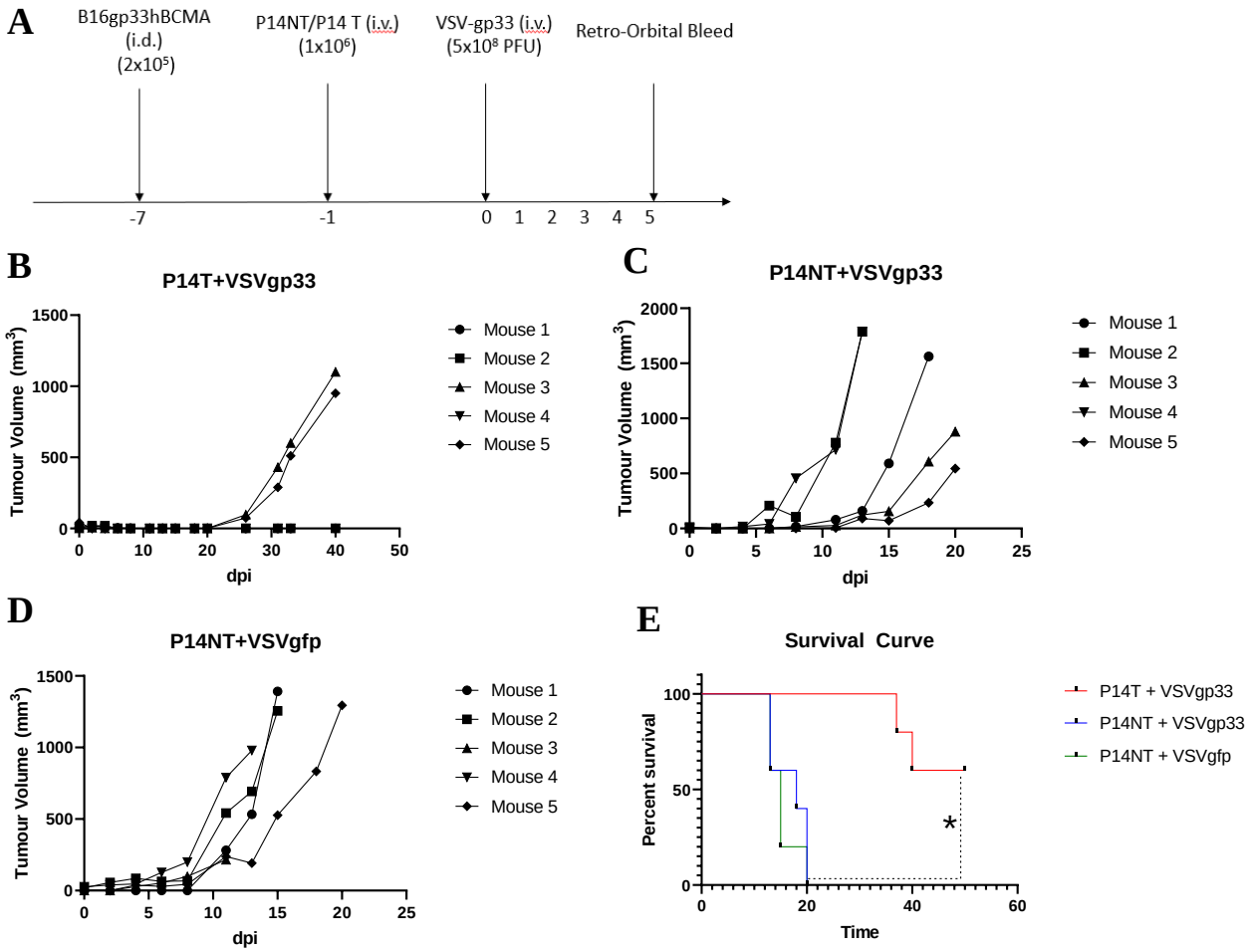

Figure 3.11: Combination of Dual-specific T_{cm} and VSVgp33 prevents tumour growth 7 days post tumour injection. (A) C57BL/6 mice were inoculated i.d. with B16gp33hBCMA cells prior to adoptive transfer with dual-specific T cells and specific OVV treatment 24 hours later. **(B, C, D)** Tumour volumes of mice measured, starting on day of OVV injection. A tumour volume of approximately 1000m^3 was used as an endpoint for the experiment. Each line represents one single mouse in the experiment group. **(E)** Survival curve of each experimental group. n=5 and data is analyzed using a log-rank (Mantel-cox) test *P<0.05. Experiment was completed once.

T cell analysis reveals an absence of adoptively transferred cells in both groups receiving NT T cells (Figure 3.12A). In addition to absence of Thy1.1 expression, we also observe an absence of IFNγ and TNFa expression. In contrast, we observe a presence of TD T cells within the peripheral blood, alongside a significant IFNγ and TNFa response (Figure 3.12A, B, C). CAR expression is maintained alongside persistence of transferred T cells however, expression does begin to drop after Day 5 (Figure 3.12D). It may also be noted persistence of TD T cells is weak, as presence of Thy1.1 drops drastically after day 5. These results suggest that the presence of dual-specific T cells may strengthen the resistance to developing palpable B16gp33hBCMA tumours despite not having a side-by-side comparison to single target therapy.

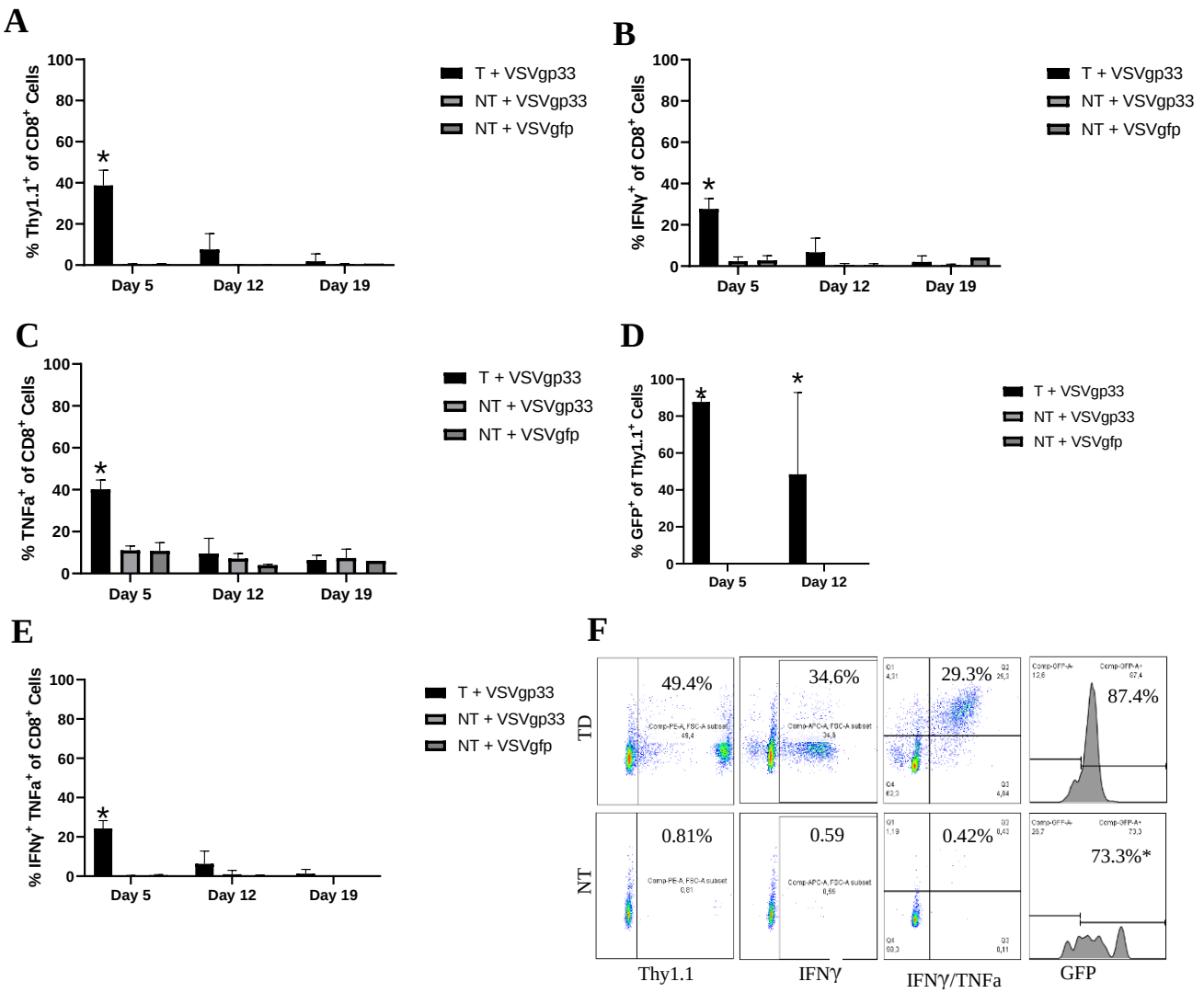

Figure 3.12: Expansion of and successful transfer of T cells is observed in TD T cells alone. Blood is collected on day 5 post injection of OVV and every subsequent 7 days following. T cell analysis reveals frequency of transferred cells **(B),** magnitude of total $CD8^+$ T cell response **(B, C, E)** and CAR expression (**D). (F)** Representation of flow cytometry analysis on Thy1.1 expression, IFNγ and TNFa expression and GFP (CAR) expression on blood samples in mice that received either TD or NT T cells. Statistical analysis is completed through independent T test vs NT + VSVgfp control.*GFP levels for NT cells is misrepresented due to low cell quantities seen of $thy1.1^+$ cells (less than 10). Realistically, GFP expression remains close to zero.

3.3.5 Comparison of TD and NT T cells against dual-antigen expressing target cells reveals no therapeutic benefit in dual-specific T cells

Further study on dual-specific T cells requires an *in vivo* model in which we can measure therapeutic efficacy of treatment after palpable tumour is formed. Following the procedure carried out in previous *in vivo* experiments we carry out blood sample analysis beginning on day 5 (Figure 3.13A). Every 2 days, beginning on day 0, tumour measurements are completed and used to calculate tumour volume, plotted in a line graph (Figure 3.13 B,C,D,E). Observations of tumour volumes reveals initial tumour remission of mice receiving TD or NT ACT followed immediately by relapse to which 4/5 mice reach endpoint (Figure 3.13B,C). Mice receiving non-boosting VSV (vsvgfp) demonstrate limited tumour control followed by rapid tumour growth leading to an early endpoint, earlier than 10 days post OVV injection (Figure 3.13D). This experiment also includes a group observing the effects that a non-specific CAR harbours on ACT. Interestingly, despite containing similar tools to the NT T cells, the her2 transduced T cells performed to a lesser extent, demonstrating rapid tumour growth where 4/5 mice reached endpoint at time point like mice without OVV boost (Figure 3.13E). Survival curves indicate statistically significant difference between the group receiving TD or NT T cells compared against the group absent of OVV boost however, no statistically significant difference is present between TD and NT groups, suggesting that dual-targeting T cells do not provide additional therapeutic benefit over their single target counterpart (Figure 3.13F).

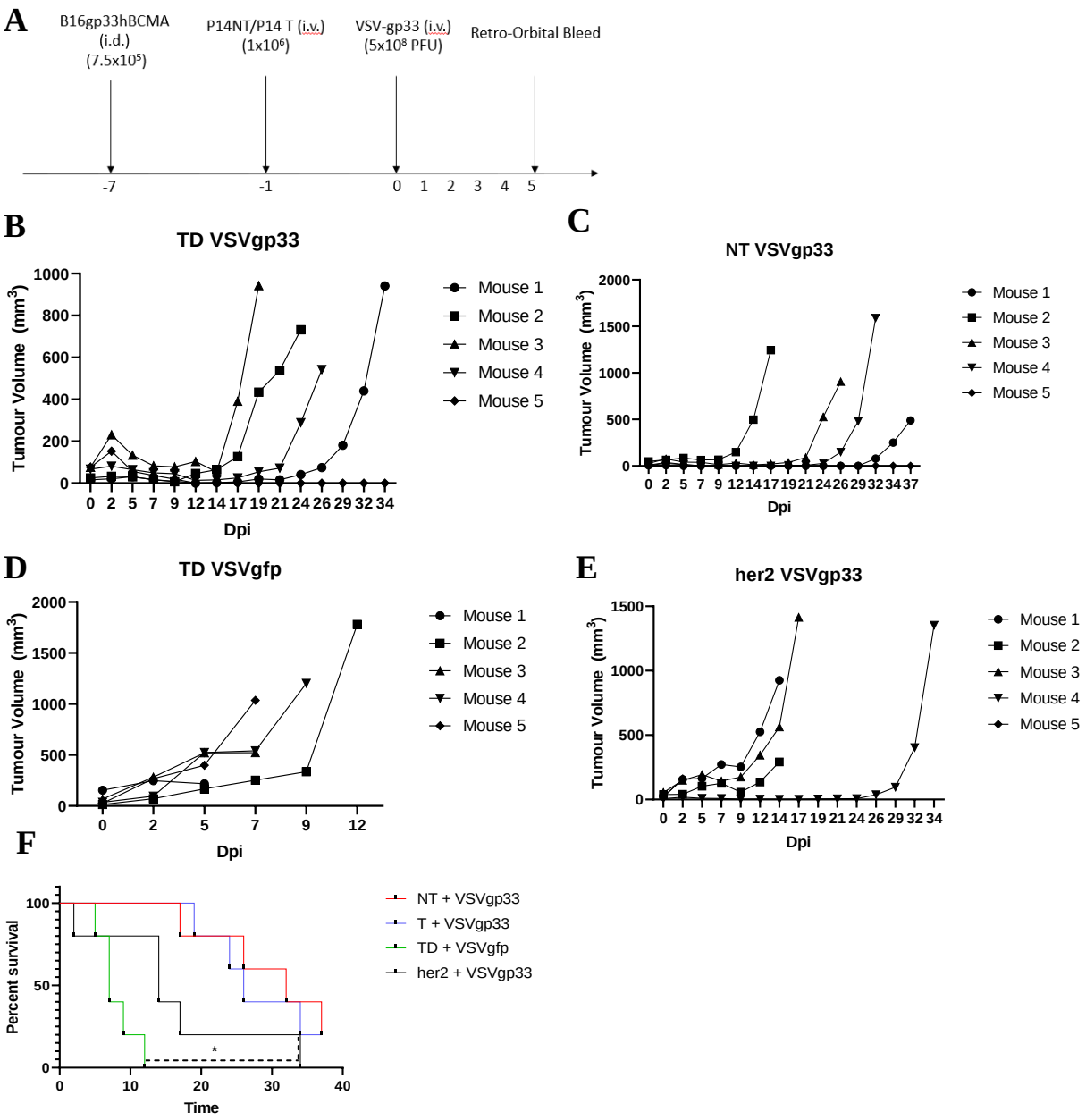

Figure 3.13: Combination of NT or TD T_{cm} and VSVgp33 initiates tumour remission followed by relapse. (A) C57BL/6 mice were inoculated i.d. with B16gp33hBCMA cells prior to adoptive transfer with dual-specific T cells and specific OVV treatment 24 hours later. **(B, C, D,E)** Tumour volumes of mice measured, starting on day of OVV injection. A tumour volume of approximately 1000m^3 was used as an endpoint for the experiment. Each line represents one single mouse in the experiment group. **(F)** Survival curve of each experimental group. n=5 and data is analyzed using a log-rank (Mantel-cox) test. *P<0.05. Experiment was completed once.

T cell analysis performed from these mice reveal observable presence of transferred cells in the periphery 5 days following OVV boost in NT and TD groups (Figure 3.14A). Average presence of TD T cells is notably lower than that of NT T cells indicating lesser levels of T cells in the periphery following transfer, despite nearly identical cell viability prior to transfer suggesting that transfer of IL-7 T_{cm} cells remains inconsistent. Subsequent T cell analysis on days 12 and 19 uncover poor T cell persistence after day 5 of analysis. Cytokine analysis reveals high levels of IFNγ expression in groups receiving OVV boost – resulting in statistically significant differences when compared to IFNγ levels in non-boosted ACT (Figure 3.14B). Similarly, TNFa expression is increased follow OVV boost – observable 5 dpi (Figure 3.14C). Consequentially, as T cell persistence is poor, levels of cytokine expression is poor post 5 day analysis (Figure 3.14 B,C). CAR expression is still present in mice receiving transduced T cells (Figure 3.14D), albeit at a lower expression compared to GFP expression levels of T cells prior to injection.

These findings suggest that therapy with dual-specific T cells does not provide additional therapeutic benefit over the NT T cells counterpart. A possible cause for lack of efficacy may lie within additional ANR followed by hBCMA CAR treatment. To test this, we extract relapsed tumours and perform flow cytometry on processed tumours to measure levels of hBCMA following *in vivo* treatment. Flow cytometry analysis demonstrates that treatment of B16gp33hBCMA tumours leads to downregulation of hBCMA surface expression (Figure 3.14E). Interestingly, hBCMA expression is dampened whether T cell treatment contains the hBCMA CAR (TD) or is lacking the hBCMA CAR (NT) and thus, it is not certain that downregulation of hBCMA expression is a result of T cell treatment.

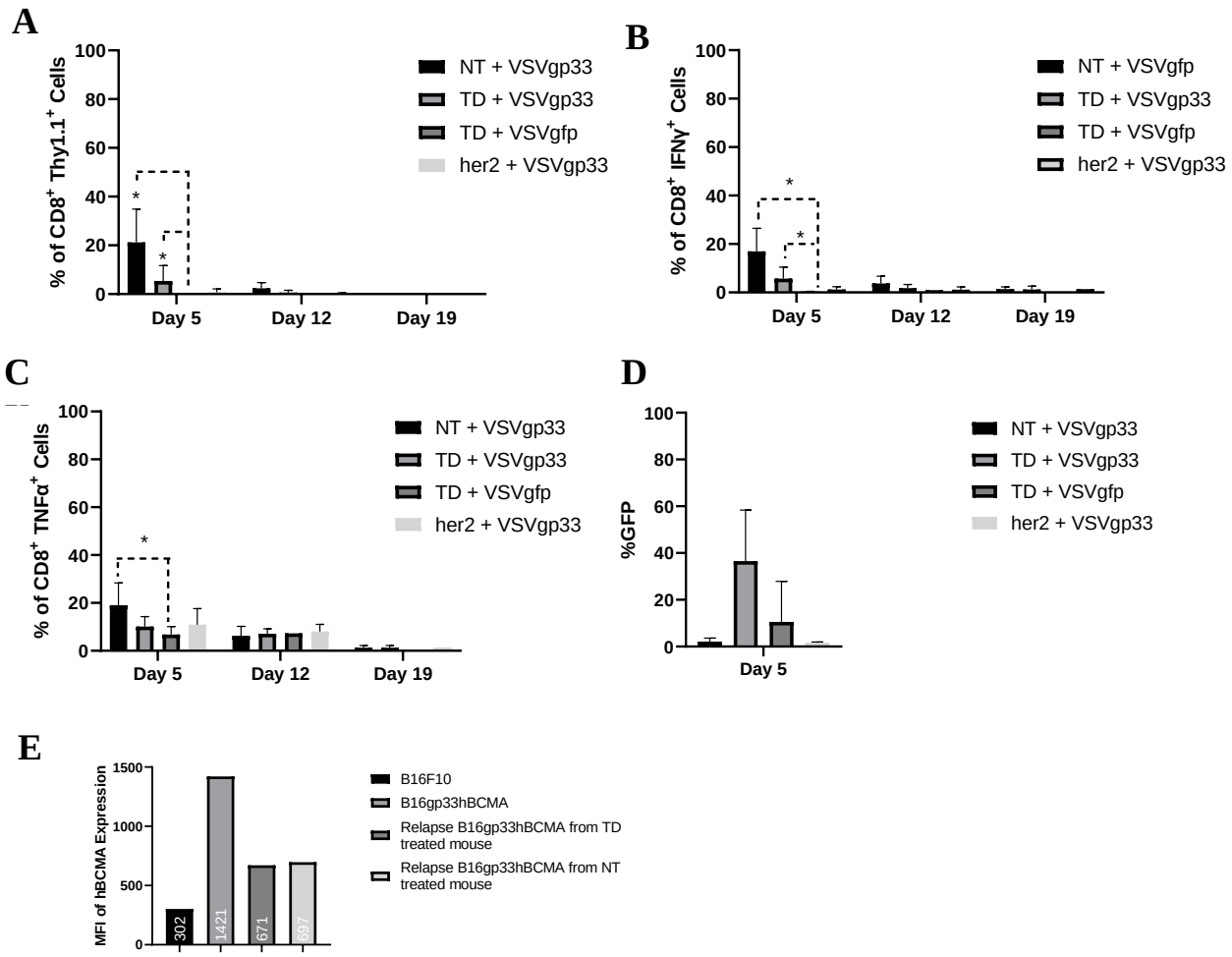

Figure 3.14: Expansion and successful transfer of T cells is observed in both TD and NT T cells. Blood is collected on day 5 post injection of OVV and every subsequent 7 days following. T cell analysis reveals frequency of transferred cells **(B),** magnitude of total $CD8^+$ T cell response **(B, C)** and CAR expression (**D). (E)** Flow cytometry analysis of hBCMA expression following extraction of relapsed B16gp33hBCMA tumours. Statistical analysis is completed through independent T test vs T + VSVgfp control .*P<0.05.

Chapter 4

4. Discussion

The use of ACT in cancer therapy has repeatedly shown success in multiple malignancies at both a preclinical and clinical level[70–72]. Despite these successes, the effect seen from ACT is heavily dampened when applied to the therapy of solid tumours. In both hematological and solid malignancies, multiple issues can arise – attributed to poor T cell persistence, insufficient T cell quantity, insufficient T cell quality, toxicity, tumour mutagenesis and tumour heterogeneity[58,59,98]. To overcome T cell originating issues, it was determined that early progenitor T cells or T cells of a memory-like phenotype, provides improved cell persistence and cell numbers[52,53]. To produce T_{cm}, our lab has previously described a protocol (IL-15, IL-21, rapamycin) where we can generate a large population of viable memory T cells. Alongside T_{cm} therapy, we combine OVV therapy to produce synergy in the form of T cell boosting such that T cells expand and differentiate following exposure to target antigen. This combination has proven effective in multiple tumour models (Figure 1.2). Despite the success, we have seen the propensity of this therapy to give rise to tumour relapse, in the form of ANR (Figure 1.3), thus creating a new problem in which the currently described therapy is rendered ineffective. This effect is observable not only in Wan lab ACT, OVV protocol but in many other forms of immunotherapy seen in clinical settings where the therapy in question targets one single antigen[102,103]. For the purpose of combatting ANR, this project focuses on tumour heterogeneity and strategies to overcome this concern.

To combat tumour heterogeneity, several strategies are in development that allow for targeting of multiple antigens. One such therapy, dual-specific T cells, harness the power of ACT and CAR therapy by combining them into one entity to produce T cells capable of recognizing

two distinct tumour antigens. While the concept of dual-specific CAR T cells has been described previously in literature, producing dual-specific T_{cm} cells and boosting with an OVV is a concept that requires further study[123–125]. In this context, we provide the ACT, OVV protocol another avenue of attack to in principle, prevent ANR. The CAR provides additional benefit such as circumventing MHC downregulation and potential immunosuppression, further enhancing the ability of the T cell in ACT. Most importantly, we reduce the likelihood of development of antigen negative variants as the probability of tumour cells not expressing 1 of the 2 target antigens is low, creating a strategy to combat tumour heterogeneity.

The first deliverable for this project was developing a culture protocol for the generation of T_{cm} T cells capable of carrying a secondary CAR receptor. For study, a model commonly used in the Wan lab utilizes the P14 transgenic mouse line that harbours T cells that have been enhanced to primarily express a TCR targeting the LCMV gp33 epitope. The gp33 epitope has been genetically engineered into the B16F10 melanoma model where this epitope acts as a neoepitope for therapeutic studies. Our lab has also generated many variations of OVVs expressing the gp33 epitope for *in vivo* boosting. In this study, VSVgp33 is the OVV of choice as it provides potent tumour killing and immune stimulation[126]. Above all else, we have provided pre-clinical evidence that the combination between P14 T cells and VSVgp33 elicits strong *in vivo* therapeutic response when targeting B16gp33, indicated in Figure 1.2. The secondary receptor to be transduced on P14 T cells, is the CAR targeting hBCMA. The hBCMA CAR was graciously provided to us from Rebecca Burchett of the Bramson lab (Figure 2.1). The CAR was previously characterized which consists of the hBCMA ScFv domain linked to the CD3 intracellular domain and a CD28 co-stimulatory domain to induce activation of T cells. Thus, dual-specific T cells targeting gp33 and hBCMA were designed.

Through previous work from a former Master's student, the difficulty to transduce IL-15, IL-21, rapamycin cultured T cells was described (Figure S8). Despite the ability for this protocol to induce a memory phenotype, further study revealed a poor readiness for transduction (Figure 3.1C). Therefore, the first undertaking for this project was to optimize our culture conditions for maintaining a memory culture population while increasing the transduction efficiency. It was hypothesized that one or multiple of IL-15, IL-21, rapamycin and peptide stimulation was responsible in inhibiting retroviral transduction. Indeed, it was found that culturing with IL-7 alongside a small dose of IL-2, produced a desirable outcome in terms of transduction, phenotype, yield, and viability.

Observations made from culture condition modifications, reveal several details regarding the effects of different modifications seen in culture protocol. First, we note that peptide stimulation of harvested splenocytes, reduces transduction efficiency (Figure S2). It was evident that peptide stimulation, that typically induces high levels of memory phenotype, leads to reductions of GFP expression in all culture conditions, including IL-2 cultured T cells (Figure S8). Reasoning as to why low transduction efficiency is a by-product of peptide stimulation is unknown as the comparisons between peptide stimulation and CD3, CD28 stimulation is uncommon. Speculation on this issue points to the direct stimulation potency of the CD3e and CD28e antibodies[127]. A possibility may arise from the activation strength of the CD3e and CD28e antibodies where rapid proliferation, that may be caused by strong activation, is a typical requirement for transduction of cells[128]. In this scenario, the combination of memory culture cytokines and the potentially weaker epitope stimulation was sufficient to impair T cell transduction.

Further investigation into cytokine selection reveals several differences dependent on the cytokine cocktail. In summary, inclusion of rapamycin heavily improves proportion of memory cells as well as cell health, while reducing cell numbers (Figure 3.1 B,D Figure S3). The inclusion of IL-15 or IL-21 in the absence of rapamycin induces poor cell health however, cell yield and transduction efficiency are marginally improved. Interestingly, IL-7 culture proved to deliver the superior quality and quantity of T cells as yield and transduction efficiency is comparable to that of IL-2 cultures while T_{cm} proportions and cell health are comparable to that of Wan lab protocol.

Literature review of the culture components reveals extremely important details pertaining to retroviral transduction. Rapamycin is a mTor inhibitor where mTor is a protein kinase involved in pushing forward the cell cycle. Inhibition of mTor, limits the cell cycle and divisions thereby reducing cell transduction due to the nature of the retrovirus requiring high levels of proliferation for successful replication[129]. It has also been shown that mTor inhibition can lead to improved viral clearance[129]. With exception of the IL-15 & IL-21 cytokine cocktail, all conditions involving IL-21 saw low cell yield, attributed to the nature of the IL-21 cytokine. In culture, IL-21 has shown to modulate T cell differentiation such that T cells remain in a minimally differentiated phenotype by shifting the T cells into an immunometabolism state designed to promote survival[130,131]. In contrast, the addition of IL-15 improves cell yield and GFP expression however, these cells were more prone to exhibiting poor survival. As with the nature of IL-2, IL-15 acts to promote proliferation in T cells, albeit at a lower intensity[132]. In particular, the major difference between IL-15 and IL-2 lies within the ability for IL-15 to reduce T cell differentiation to allow for minimally differentiated T cells. Thus, a combination of IL-21 and IL-15 pulls benefits from both cytokines – creating T cells that have higher rates of growth

and transduction while reducing differentiation. Interestingly, the addition of IL-21 to IL-15 culture did not improve T cell viability substantially thus making IL-15 + IL-21 culture a less likely candidate for dual-specific T cells.

IL-7 as a culture component draws comparison to the use of IL-15 in that IL-7 pushes proliferation of a less differentiated phenotype of T cells[133]. In the field, it has been demonstrated multiple times that IL-7 as a cytokine is a regulator of survival and homeostasis of CD8 T cells, thus limiting differentiation of T cells during use in *ex vivo* T cell culture[133–135]. IL-7 cultures completed during transduction demonstrate what is stated in literature (Figure 3.1B), thus confirming that IL-7 drives naïve T cell proliferation while reducing differentiation. In addition, trace amounts of IL-2 (0.001 ng/mL, 1000x diluted) are added as a supplement to ensure T cell health and yield. Most importantly, there is no major distinction between TD and NT cultures indicating that retroviral transduction does not modify T cell quality in any manner.

To confirm T_{cm} functionality of the dual-specific T cell, we conduct proliferation assays, a standard in determining the expansion ability of T_{cm} cells. Figure 3.3 demonstrates proliferative ability, measured as divisions, of T cells following co-culture with various targets. Here we confirm target specific receptor function in the TD T_{cm} cells. Importantly, TD T cells do not appear to undergo ligand-independent divisions indicating minimal presence of tonic signalling, a common concern found with CAR bearing T cells[136]. Properties of tonic signalling include exponential expansion, constitutive cytokine release and continuous differentiation in absence of target ligand[136]. Affirmation that CAR expressing T_{cm} cells are absent of exponential expansion suggests that tonic signalling is absent.

Furthermore, we look to ascertain that after transduction TCR function is preserved. It has been shown that dual-expressing TCR/CAR T cells may experience antagonistic therapeutic

effects[137]. Current understanding of the effects of CAR transduction on TCR bearing T cells and primary receptor activation on dual-specific T cells is not well defined. For example, activation of one receptor may promote premature exhaustion of T cells, lowering therapeutic efficacy *in vivo*[137]. Therefore, we must remain vigilant in documenting differences viewed within TD T cells versus their NT counterparts. Comparing NT and TD cells in the presence of B16gp33 cell lines, we confirm identical proliferative function thus corroborating that TCR function remains untouched after transduction. Despite evidence demonstrating that downstream events such as proliferation and cytotoxic activity can differ based on the mode of stimulation[138], our dual-specific T cells observe no significant reductions in function. Still, the TCR or CAR may prove to be differentially successful in one aspect of function (e.g. killing) *in vivo*, potentially hindering the downstream ability for the secondary receptor to remove antigen negative tumour variants. *In vivo* study may look to observe the differences in both a proliferative capacity and killing capacity when comparing function through the CAR versus the TCR as well as whether boosting through one receptor leads to premature exhaustion in dual-specific T cells.

In the context of cancer therapy, the main function of the T cell is not to proliferate but rather kill the targets. Use of the flow-based killing assay confirms that when effector T cells are fitted with a CAR and co-cultured with target expressing tumour cells, we observe killing in a target specific manner (Figure 3.4). Interestingly, in target cells expressing two target antigens, major differences are observed at lower effector:target ratios with decreasing divergence as co-culture ratios increase (Figure 3.4D). Again, we provide further evidence that equipping a T cell with a secondary receptor does not inhibit function of the primary receptor and we also improve effector cell function when T cells express both receptors against the B16gp33hBCMA tumours. Differences in cell death indicate increased capacity for killing in T cells equipped with the CAR

during co-cultures with lower concentrations of effectors cells. As numbers of effector cells increase, differences in death become negligible indicating saturation of the killing capacity in T cells. This points to a receptor redundancy at increased numbers of effectors due to inaccessibility to target cells and overcrowding of effector cells. Despite the potential redundancy, *in vitro* killing assays do not closely mimic *in vivo* settings and as such, the results observed here may be taken with a grain of salt. Most importantly, this assay is carried out with effector cells where typical *in vivo* experimentation will be completed using T_{cm} cells.

The final method in which T cell function is analyzed is conducted with an assay designed to mimic the boosting T cell effect more closely in an *in vitro* setting. The assay that is referred to as the re-stimulation killing assay, pushes T_{cm} differentiation and proliferation utilizing peptide pulsed DCs. Subsequently, stimulated T cells are analyzed and used in a cytotoxicity assay as outlined in Figure 3.4. Initially, we look to determine whether CAR expression is maintained following re-stimulation. Typically, long-term persistence of T cells induces TCR downregulation however, long-term CAR downregulation has not been widely studied[139]. Decrease in CAR function and persistence following the boost would negatively impact future *in vivo* work as CAR mediated killing is required to prevent ANR. Within the re-stimulation assay, CAR functionality and presence remains intact at a significant level after 24 hours of *in vitro* re-stimulation signifying CAR persistence needed to prevent ANR (Figure 3.6B). Interestingly, levels of death are enhanced drastically via TCR related cytotoxicity (Figure 3.6C). This would indicate that re-stimulated T cells exhibit lesser killing capacity through the CAR when compared to the TCR despite maintenance of CAR expression. First these results suggest that once again, CAR transduction does not reduce TCR functionality in a killing capacity. Secondly, these results may imply that CAR function decreases following T cell

phenotype shift from T_{cm} to T_{eff}. Furthermore, we demonstrate that an increase in killing assay co-culture time, from 6 hours to 16 hours massively improves killing of CAR target cells during co-culture TD T cells (Figure S5). This serves as an indication for long term dual-specific T cell functionality provided that T cell persistence remains and that transferred cells maintain expression of the CAR during that period.

Following *in vitro* characterization of CAR transduced P14 cells, we sought to determine *in vivo* functionality of these cells. Therapeutic success is measured by the ability to reduce tumour load as well as the distinct cytokine profile of transferred cells. In particular, the ability for T cells to perform *in vivo* can be evaluated through expression of cytokines such as IFNγ and TNFa. Traditional ACT, OVV therapy observes tumour remission following 5 days of treatment (Figure 1.2) alongside measurable peak IFNγ expression of ~25% on day 5 of treatment (Figure S1). Taken together, these results serve as a benchmark of comparison for the newly generated T cell protocol to predict the *in vivo* success of treatment. A first attempt in treating C57BL/6 wild type mice bearing B16hBCMA cells reveals observable presence of transferred cells evidenced by Thy1.1 presence in the blood (Figure 3.8B) alongside levels of IFNγ (~25%) comparable to that of traditional ACT, OVV therapy (Figure 3.8C), a sign that the IL-7 cultured dual-specific T cells would be able to replicate the strong therapeutic outcomes seen in T_{cm} cell therapies.

The execution of this experiment revealed differential growth kinetics of the B16hBCMA tumour cell line in parallel to the B16gp33 cell line, which was used as a basis for injection. Typical injection in the B16gp33 model produces palpable tumours of around $50mm^3$ 7 days post injection (dpi). The b16hBCMA tumour model failed to produce palpable tumours 7 dpi (seen as day 0) thus preventing the surveillance of a true tumour regression model in the context of the CAR. Eventual tumour growth is observed (Figure S6), wherein mice reached endpoint rapidly

due to tumour volume. It is hypothesized that lack of tumour growth is a result of the introduction of a novel human antigen, preventing tumours from successfully grafting in a short period of time. hBCMA as a target for CAR ACT has shown varying levels of success in clinical trials[140,141], thus making it a promising target of study for mouse models. Despite this, the potential for a strong endogenous response is likely as human and murine BCMA proteins share 62% homology[142]. In this context, the use of a human antigen is not a direct representation of a xenograft however, the discrepancies seen within the two proteins may produce an initial endogenous T cell response preventing engraftment of hBCMA tumours like the rejection seen in a xenograft model[143]. Similar observations have been seen within the Wan lab in the B16 tumour model expressing human prostate-specific membrane antigen (hPSMA) where tumour engraftment requires increased cell numbers ($>1x10^5$) or CD8 depletion. To compensate for lack of growth, increasing the concentration of injected tumour cells and expanding the period of tumour growth, results in sufficient growth of tumours for experimentation.

Additionally, in the same experiment, it was discovered that the cells involved within this experiment tested positive for mycoplasma shortly after completion. Although the exact implications of mycoplasma contamination on experiments are unknown, it has been documented that mycoplasma reduces cell viability, thus reducing the engraftment potential of the implanted cell lines[144]. Furthermore, as with any exogenous factor and infection, introduction of mycoplasma into a healthy mouse will induce inflammation, again reducing the ability of the B16hBCMA to engraft into the site of injection due to excess immune recruitment to the site of injection[145]. Because of mycoplasma contamination, experimental cell lines were remade for future experimentation both *in vitro* and *in vivo*.

To further examine whether hBCMA expression results in an inability for tumours to successfully engraft, we carried out a secondary experiment where we observe whether hBCMA expression is maintained following tumour growth and no therapeutic intervention. Figure 3.7 reveals that increasing the dosage of tumour cells for experimental purposes does not hinder the expression of the hBCMA protein in B16gp33hBCMA tumours. This allows for further *in vivo* study on the hBCMA CAR to be completed however, the inability to grow tumours at the standard dose ($1x10^5$) is something to still consider further down the line.

To ensure CAR function *in vivo*, we carried out an experiment pitting the TD T cells against B16hBCMA tumours (single target therapy). Single target therapy mediated through the TCR (against B16gp33) has shown previous success in mediating tumour control. Single target therapy against hBCMA does not recapture the same effect (Figure 3.10) despite promising levels of T cell function (Figure 3.9). It is difficult to pinpoint the reasoning behind the failure to enact any level of tumour control through the CAR as literature has shown success of CAR therapy in multiple instances. We have also shown CAR functionality *in vitro*, eliminating the possibility that the CAR is non-functional. In this manner, the culprit for lack of therapeutic efficacy remains speculative due to lack evidence. For one, effective treatment of solid tumours using a CAR remains a challenge and requires additional modification to enhance therapy to improve the potential of treating a solid tumour[146,147]. Furthermore, in literature TCR therapy is typically not directly compared to CAR therapy in the treatment of solid tumours, however TCR therapy may show greater promise in the treatment of solid malignancies[148]. The results in Figure 3.10 and Figure 1.3 support this hypothesis in that ACT therapy mediated through the CAR may be weaker than ACT therapy mediated through the TCR. Another possibility lies within the lack of stimulation for an endogenous response against target antigen. A previous study by Dr. Scott

Walsh[87] reveals that the endogenous response initiated or boosted following the administration of OVV therapy is essential in preventing tumour relapse. This indicates that an endogenous immune response against target protein/peptide is generated following priming of T cells through the tumour. Generally, the primed endogenous response is unable to act on its own and would require an additional push to act such as therapeutic intervention or boosting. In our therapy, we administer VSV-gp33 as a boosting vector for our adoptively transferred T cells while the tumour being treated contains the hBCMA protein as a target. In this case, if the tumour does indeed prime an endogenous response, our subsequent OVV therapy would be unable to rescue the primed hBCMA T cells. If, for example, we utilize an OVV genetically engineered to express the hBCMA protein, we may then rescue the endogenous hBCMA response producing stronger anti-tumour function. Weakened anti-tumour efficacy of CARs against solid tumours in combination with a poor endogenous response may then result in the major difference seen with the CAR versus the TCR mediated therapies. Alone, the CAR may prove to be unsuccessful in this tumour model, but it remains possible that combining the CAR and TCR may result in improved therapeutic efficacy against B16gp33hBCMA tumours as both receptors may produce synergistic effects.

Successful T cell boosting following transfer of IL-7 cultured dual-specific T cells provides the basis to commence an experiment that determines whether dual-specific T cells induce durable therapeutic efficacy following initial tumour remission. This experiment was completed prior to the realization that injection $7.5x10^5$ tumour cells are required to see palpable tumour growth. Unfortunately, cell numbers were only doubled ($2x10^5$) to compensate for poor growth resulting in minor growth of tumours, suggesting that doubling of tumour cell numbers for a 7 day period is not sufficient to produce palpable tumours. As stated previously, the lack of

tumour growth resulted in modifications of the experimental hypothesis from looking for therapeutic response and durable remission in tumour bearing mice to prevention of tumour growth in mice lacking palpable tumours. Results presented in Figure 3.11 suggest that dual-specific T cells significantly increase the probability that growth of a tumour is subdued (60% survival vs 0%). Interestingly, GFP expression (Figure 3.12D) drops drastically indicating loss of CAR expression. This loss of CAR expression may predict the inability of the dual-specific T cells to protect against ANR, in the scenario that the gp33 target is lost however, a model where ANR is induced in monotherapies against B16gp33hBCMA needs to be established before we can determine whether ANR is preventable.

Additionally, the presence of NT T cells is undetectable in all mice receiving NT therapy suggesting that NT T cells did not enter the system following injection. To distinguish the beneficial effects of the dual-specific therapy against dual-antigen expressing tumour cell lines, we must provide evidence that monotherapy can produce initial remission followed by relapse. Thus, the experimental data provided from this experiment requires set controls, such as the NT T cells, to affirm the improved therapeutic effect. The explanation as to why NT T cells did not show presence after adoptive transfer remains unknown as prior to experimentation, NT T cells were confirmed healthy and of a T_{cm} phenotype through flow cytometry (Figure S8). We speculate that an unobservable factor reduced T cell viability prior to or during transfer to mice, thus preventing T cells from persisting after injection. From this, we draw that therapy using IL-7 ACT is inconsistent following T cell transfer. This result is not uncommon when observing T_{cm} ACT (unpublished data) and thus, consistency of results arises as another potential issue for experimentation. Despite the lack of NT T cells in this experiment, dual-specific T cells in ACT,

OVV therapy show efficacy in preventing growth of B16gp33hBCMA tumours and maintaining the outcome long-term.

Followed by success in preventing growth of tumours, we must now look to determine whether dual-targeting therapies induce complete regression illustrating the benefit of a secondary receptor. Unfortunately, we observed that dual-specific T cells were unable to prevent relapse in B16gp33hBCMA bearing mice (Figure 3.13). As such, we then sought to discover the cause of relapse in the case of dual-specific T_{cm} therapy. Successful therapy relies on maintenance of hBCMA expression in tumours after initial remission, persistence of T cells, maintenance of CAR expression and cytotoxic T cell function against relapsed tumours.

To carry out function through the CAR, hBCMA expression in tumours must remain following relapse. Loss of target protein, results in loss of CAR function thus rendering the secondary receptor ineffective. Tumour analysis reveals downregulation of hBCMA protein following tumour relapse – potentially explaining the failure of the dual-specific T cells (Figure 3.14E). Loss of target protein/antigen is not uncommon in both solid and liquid cancers as evidenced through work carried out in the Wan lab (Figure 1.3) as well as data produced in the field[149,150]. We observe that mice receiving the NT T cell therapy demonstrates tumour relapse following initial remission and interestingly, tumour processing and analysis reveals loss of hBCMA expression, like that of the mice receiving TD T cell therapy (Figure 3.14E). Loss of hBCMA expression may then be a result of a tumour primed endogenous immune response. As discussed, the implantation of hBCMA bearing tumours may prime an immune response to hBCMA, that is not capable of enacting complete remission on its own. In this instance, by administering therapy that directly targets the tumour (through the TCR and OVV) we enable tumour debulking, release of tumour antigens and reduction of immunosuppression. The

combination of these three effects may then potentially rescue the endogenous response against hBCMA while simultaneous therapeutic intervention is acting against gp33. The combination of these two factors may result in loss of both gp33 and hBCMA (Figure 3.14E) in which tumour relapse occurs and tumour control is lost. The fact that hBCMA is downregulated in both TD and NT treated mice is strong evidence that the endogenous response is responsible for loss of hBCMA expression rather than the CAR. It remains possible the CAR is capable of some therapeutic efficacy (evidenced by *in vitro* data); however, evidence points towards the CAR providing little therapeutic effect on its own (Figure 3.10), or in dual-targeting therapies.

Besides poor hBCMA expression of tumours following relapse, we observe poor persistence of T cells following therapy. In a clinical setting, a main source of relapse arises through poor T cell persistence allowing for tumours to escape therapeutic intervention[149,151]. Antigen positive relapse, where target antigen expression is maintained, is hallmark of poor T cell persistence as they fail to completely clear all antigen bearing malignant cells before exiting the system. Our therapy experiences both downregulation of hBCMA expression while T cell analysis (Figure 3.13A) also reveals minimal T cell presence on days 12 and 19 thus raising the additional concern of poor T cell persistence. As the TD treated mice experience relapse while tumour expression of hBCMA remains (albeit at a lower level), poor T cell persistence remains a plausible suspect for the failure of the dual-specific T cell therapy against B16gp33hBCMA. To rule out persistence as possible causes for inability to treat dual-target bearing tumours we must resolve the issue of poor T cell persistence past 12 dpi.

Whether the augmentation of ACT therapy through the addition of the CAR is beneficial for treating solid tumours remains a mystery, *in vitro* experimentation alongside preliminary *in vivo* data illustrating T cell function demonstrates that this combination maintains its potential. *In*

vivo results obtained when studying whether ANR is preventable has shown that tumours will relapse in an antigen negative manner following the administration of dual-targeting therapy, potentially due to multiple different factors. Factors include early loss of target antigens, poor T cell persistence and inconsistencies regarding T cell function and presence. As such, to accept that dual-specific ACT is unsuccessful, it is imperative that experiments are repeated to replicate the failure in preventing ANR since reproducibility of this failure remains to be seen (Figure S13). Additional study must also be performed to rule out lack of T cell function through the CAR and to also determine a method to further enhance dual-targeting therapies to overcome issues of persistence while also improving consistency of ACT administration. Potential enhancements include the addition of small molecule therapies such as HDAC inhibitors (MS-275) where members in the Wan lab have shown that MS-275 improves therapeutic outcomes in treatment of solid tumours[100] or through administration of a secondary dose of ACT at later time point to increase levels of transferred cells prior to relapse (Figure S13A). If the issue of persistence is resolved and other unanswered questions are cleared up, the studies completed in this dissertation, coupled with the groundwork laid by prior Wan lab students, may catapult the use of dual-targeting ACT therapy to the forefront of treatments aimed at eliminating solid malignancies.

5. Supplementary Figures

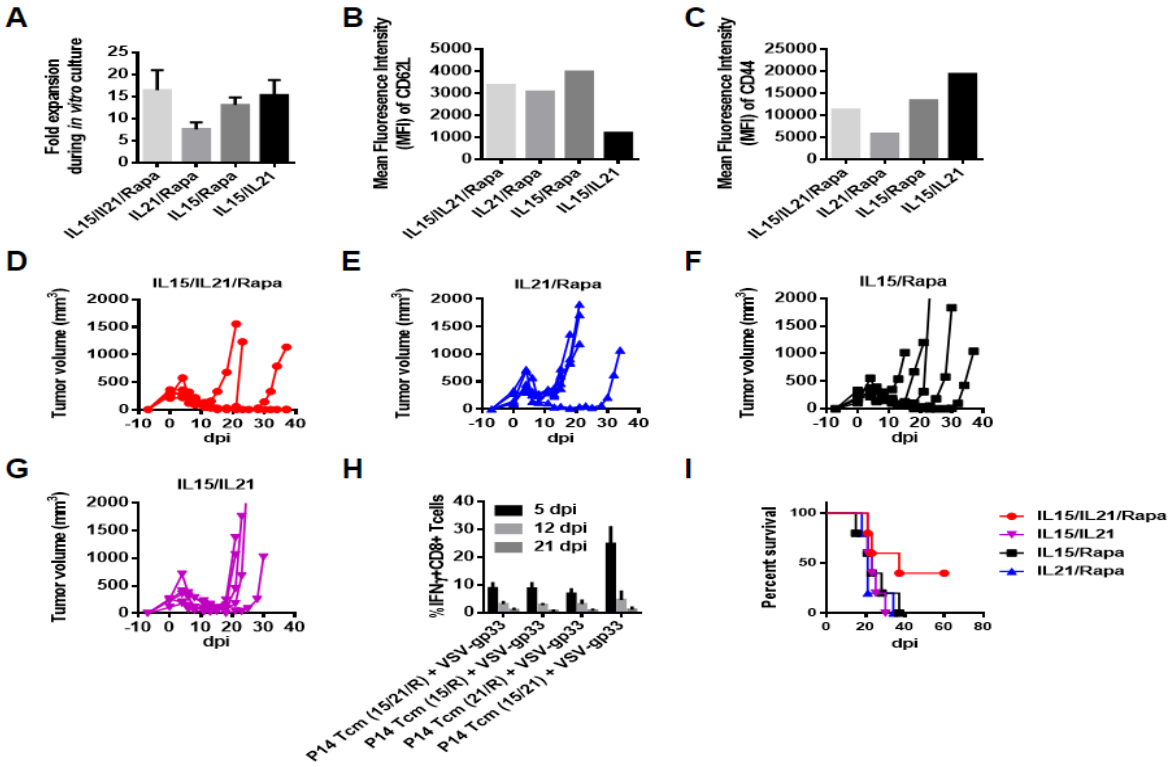

***Figure S1*: *In vivo* experimentation indicates IL-15, IL-21 and rapamycin as the optimal culture conditions in creation of T_{cm} cells for ACT + OVV combination therapy.** P14 T cells are cultured *ex vivo* with varying cell culture conditions and then tested *in vivo* with co-infusion of VSV-gp33 against the B16gp33 tumour cell line. **A** – Fold expansion of T cell cultures for detection of *ex vivo* cell growth. **B,C** – MFI of CD62L expression and CD44 expression where dual expression is representative of T_{cm} cells. **D-G** – *In vivo* tumour measurements observing tumour growth kinetics following ACT infusion. All therapies show initial remission followed by relapse in all culture conditions with exception to the Wan culture conditions. n=5. **H –** IFNγ expression on day 5,12,21 dpi. **I –** Survival curve of each experimental group. Best survival conditions are seen in the IL-15, Il-21, rapamycin group. All data from this experiment is generated by Dr. Scott Walsh and Lan Chen in the Wan Lab

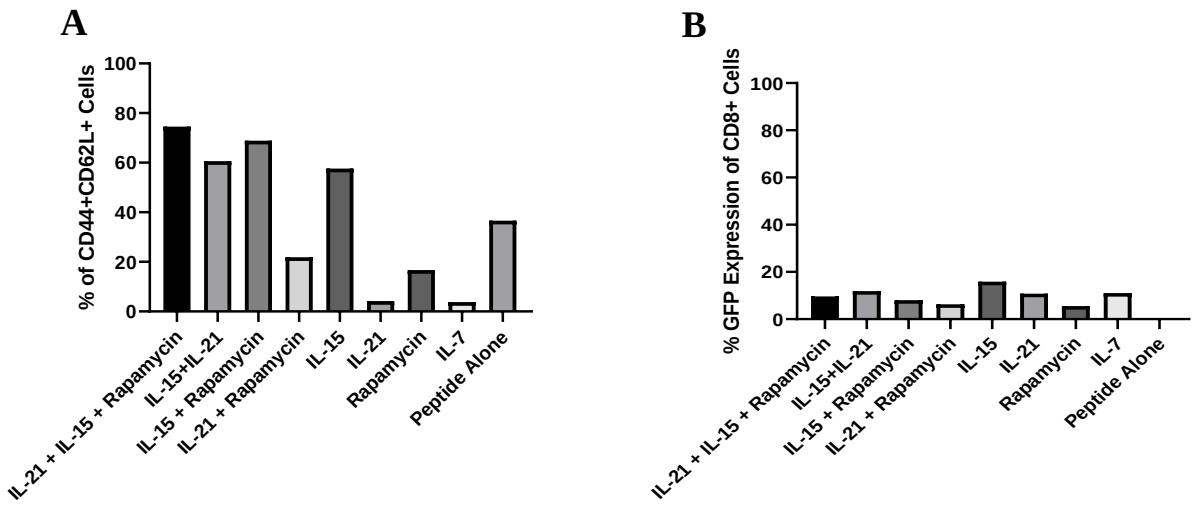

Figure S2: **Peptide stimulation during splenocyte culture improves memory cell proportions while simultaneously decreasing transduction efficiency. (A)** Across multiple conditions, generation of T_{cm} cells is successful while using peptide stimulation. **(B)** Peptide stimulation leads to massive decreases in GFP expression measured 6 days after initial splenocyte culture.

Condition x	% Memory	% Transduction	Other observations
IL-15+IL-21 + Rapamycin	>80%	<10%	Low cell number
IL-15	>50%	>10%	Poor cell survivability
IL-21 + IL-15	>50%	~30%	
IL-15 + Rapamycin	>80%	<10%	
Il-21 + Rapamycin	>40%	<10%	Poor survivability
IL-21	<50%	>20%	Poor survivability, low cell number
Rapamycin Alone	>60%	<2%	Low cell number
IL-2	<10%	>60%	
No cytokine	N/A	N/A	Cells don't survive

Figure S3: **Summary of general ability to induce T_{cm} and transduction in multiple culture conditions with CD3e and CD28e antibody stimulations.**

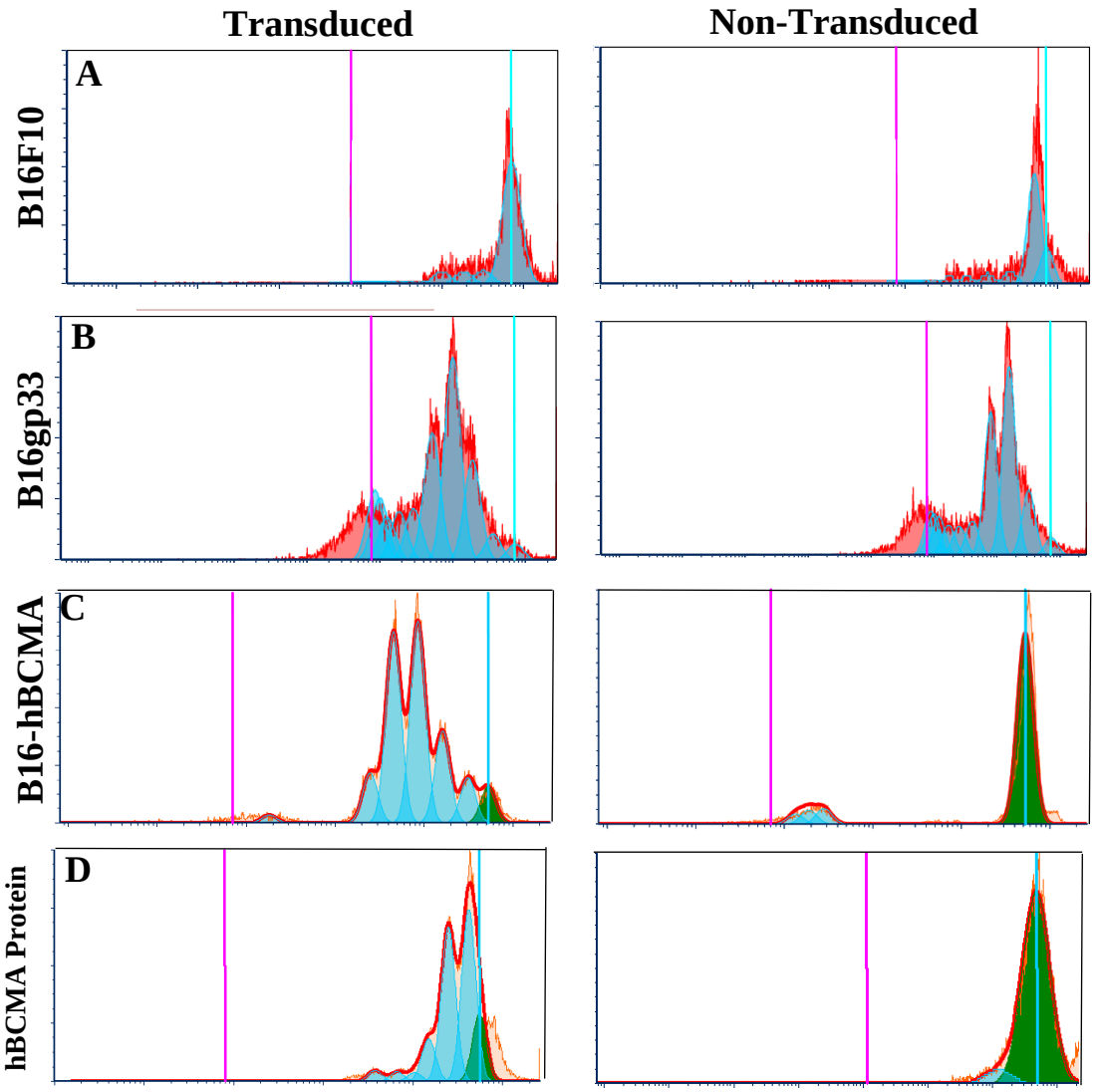

Figure S4: **A replicate of the proliferation assay, analyzed through a secondary software.** In this experiment, we see T and NT T cells co-cultured with B16F10 **(A)**, B16gp33 **(B)**, B16hBCMA **(C),** and hBCMA recombinant protein **(D)**. The initial cyan line represents stained, unstimulated V450 expression while the magenta line shows where background fluorescence lies. Peaks in between represent divisions seen in T cells. Co-cultures are analyzed after 3 days.

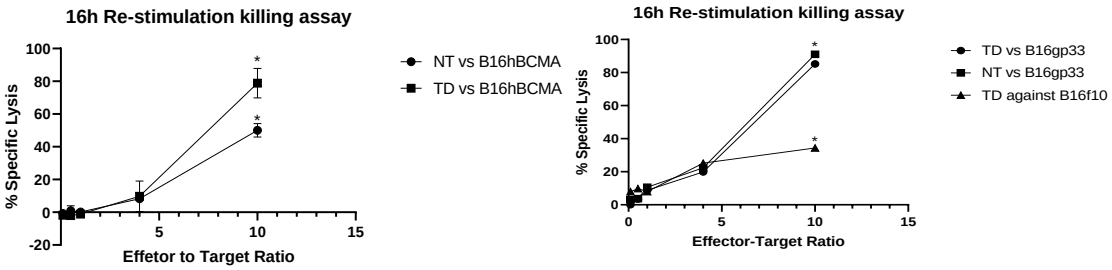

Figure S5: **16h re-stimulation killing assay revels higher levels of T cell killing with higher levels of non-specific target cell death.** An assay similar to the one completed in *Figure 3.5* is carried out for a longer period of time (16h). Herein, we observe higher non-specific levels of cell death, in both NT cells co-cultured with B16hBCMA and TD cells co-cultured with B16f10 parental cells. Differences in killing, remain statistically significant but to eliminate background death, co-culture time was shortened.

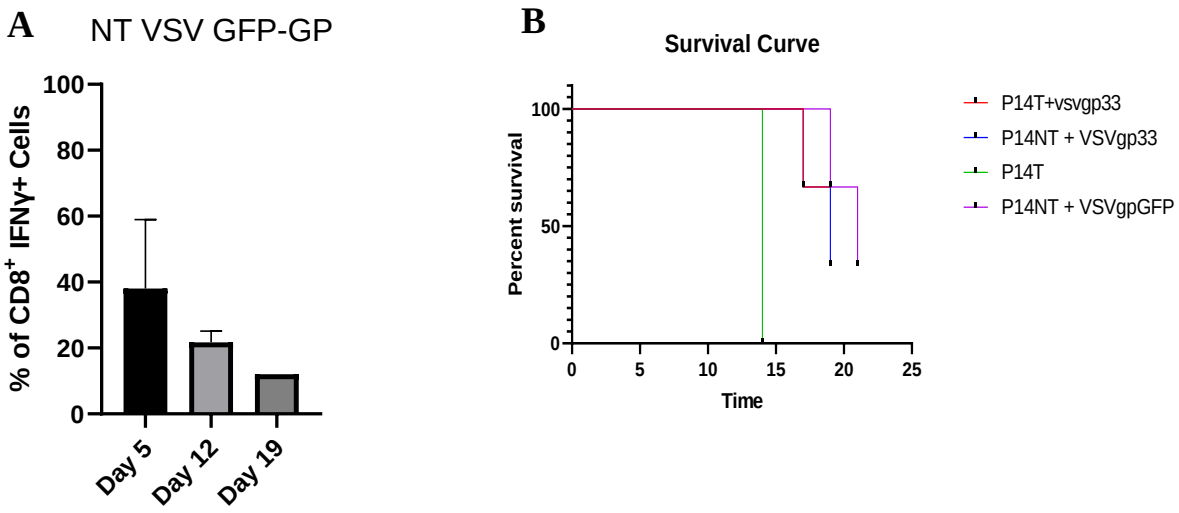

Figure S6: **VSVgpGFP boosting of T_{cm} *in vivo* leads to robust IFNγ expression following ex-vivo peptide stimulation.** During this *in vivo* experiment, a mix-up occurred where treatment with VSVgfp was substituted for treatment with VSVgpGFP, where VSVgpGFP contains the full length glycoprotein that harbours the gp33 epitope. In theory, this virus will act similar to the VSVgp33 virus in that it will produce a boosting effect. **(A)** Indeed, VSVgpGFP produces a boosting effect for the T cells as their presence is observed within the blood with immensely high levels of IFNγ expression on days 5, 12 & 19. **(B)** From the same experiment, survival curves were produced to illustrate ensuing growth of B16hBCMA tumours despite the lack of palpable tumour at the beginning of the experiment.

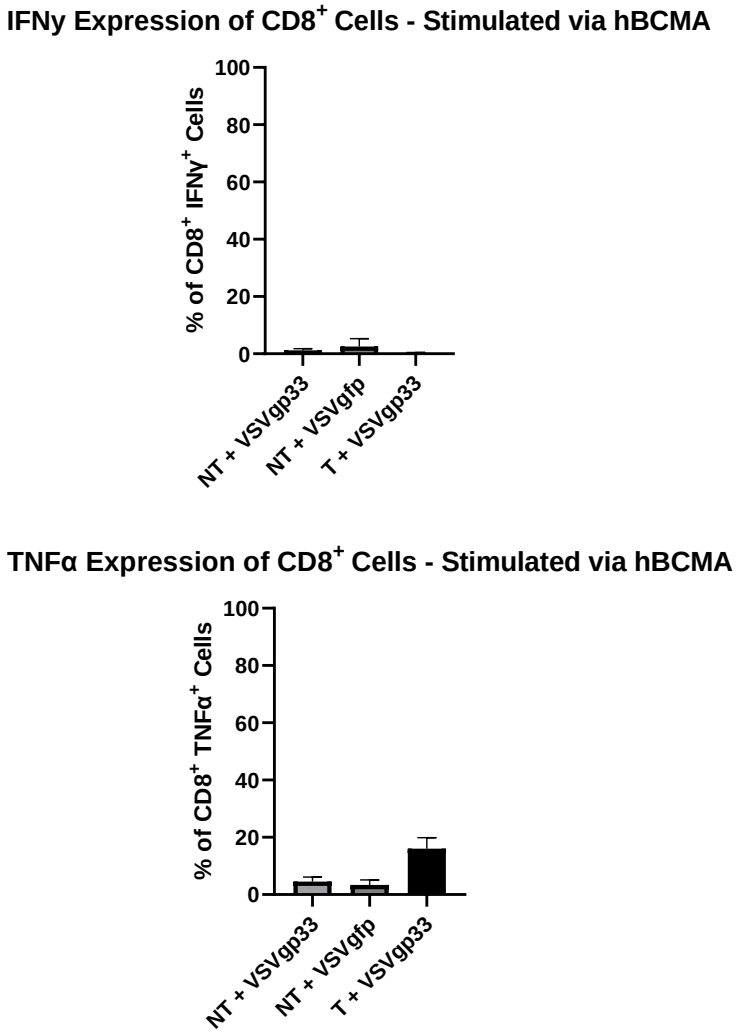

Figure S7: **Re-stimulation through CAR during ICS does not revel T cell functionality through the CAR.** To complete ICS, T cells need to be re-stimulated *ex vivo* to measure cytokine production. To confirm CAR function, hBCMA protein is provided to stimulate T cells through the CAR. Unfortunately, this method has not been perfected as we are currently looking for a method to better stimulate T cells through a CAR. In this instance, we used B16hBCMA as stimulation however, T cells were unable to produce IFNγ or TNFa despite evidence that the CAR is present through analysis of GFP expression.

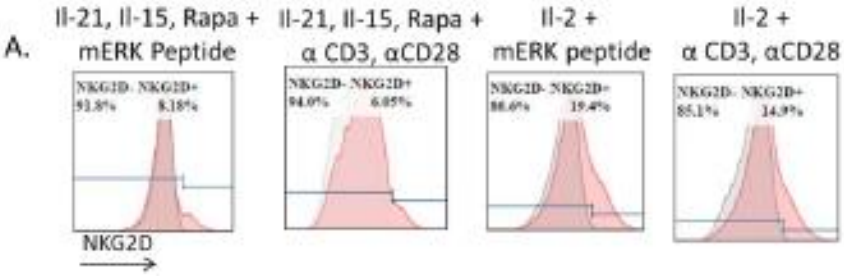

Figure S8: Initial work illustrating the difficulty in transducing memory culture T cells, done by Donald Bastin, a former Master's student in the Wan lab.

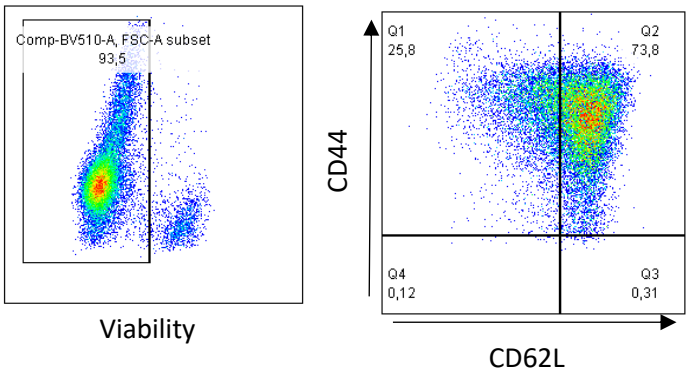

Figure S8: NT T cell viability and proportion of T_{cm} cells prior to injection.

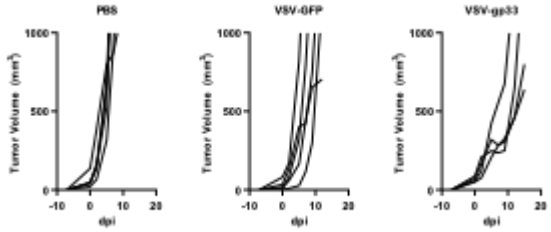

Figure S9: *In vivo* experimental data observing the effects of VSV-gp33 alone on tumour progression. Tumour growth is delayed slightly followed by immediate outgrowth where mice rapidly reach endpoint.

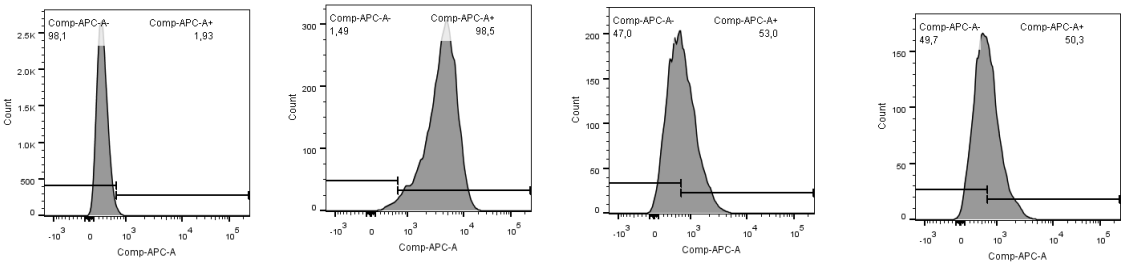

Figure S10: % of hBCMA expression in varying cell lines. From left to right: B16F10, B16gp33hBCMA, relapsed B16gp33hBCMA from TD treated animals, relapsed B16gp33hBCMA from NT treated animals. % hBCMA expression is drastically lowered following extraction of tumours from relapsed tumour bearing mice when compared to non-injected B16gp33hBCMA.

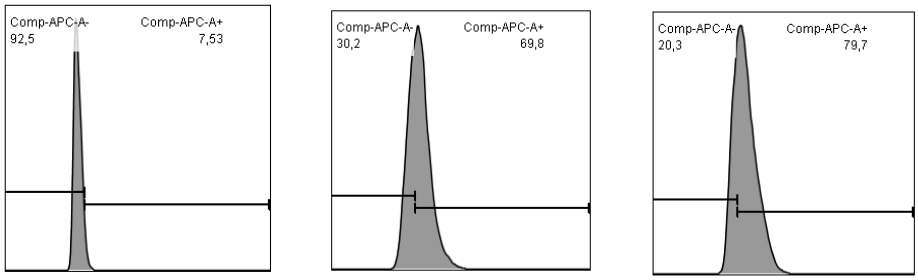

Figure S11: % of hBCMA expression in varying cell lines. From left to right: B16F10, B16gp33hBCMA from *in vivo* extraction, B16gp33hBCMA. % hBCMA expression is maintained followed implantation.

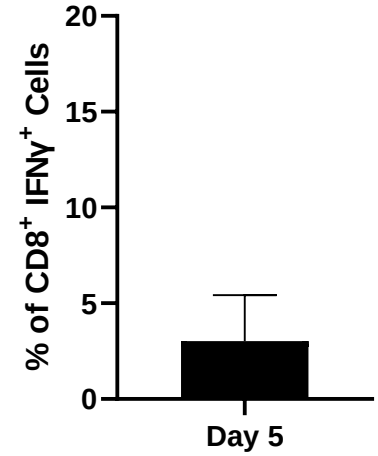

Figure S12: Endogenous response of $CD8^+$ T cells in the *in vivo* experiment seen in Figure 3.14. Endogenous response of T cells is low but not zero. Nonetheless, this information provides evidence that some level of rescue for a tumour-primed response (against gp33) exists to some capacity, potentially paving the way to explain why hBCMA is downregulated.

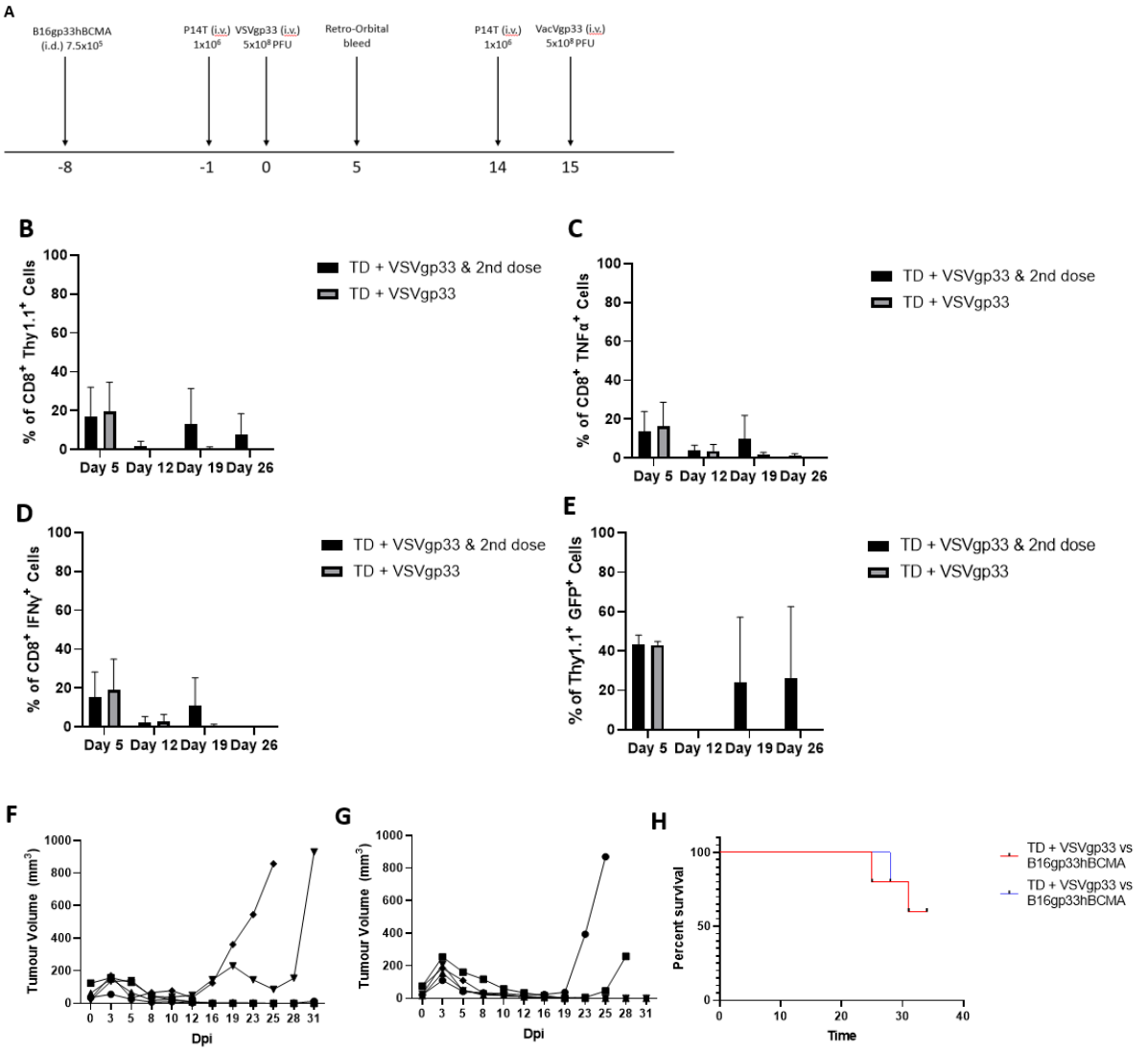

Figure S13: In an attempt to enhance persistence and longevity of therapy, we administered a second dose of ACT, OVV therapy to mice to determine if the secondary dose would prevent ANR. The primary dose of therapy remains the same as the other doses however, in the second dose of OVV treatment, we use a new OVV (Vaccinia virus – VacV) to avoid the natural immune response against our OVV in order to boost the T cells. In this experiment, we compared 2 groups, one group that received one dose of therapy where the second group received two doses of therapy. (B, C, D, E) We observe that both primary and secondary doses of T cells are successful and that T cells are functional based on ICS carried out on extracted T cells alongside persistence of the CAR on said transferred T cells. Importantly, we identify that T cell response is improved following administration of the second dose. Observation of tumour size reveals no significant differences in survival between the two groups. Interestingly, the group that received only one dose (as well as the group that received both doses) appears to show sustained remission without relapse in 3/5 mice after 1 dose of therapy, contrary to what was observed before (1/5 mice cured with 1 dose). Unfortunately, due to constrictions and time this experiment did not have any NT groups for comparison and so it is difficult to tell whether this therapeutic outcome was a sole result of the TCR therapy. Although no conclusion may be drawn from this, it does imply that repeat experiments comparing NT vs TD cells in therapy need to be conducted to confirm our initial experiment where we conclude that dual-specific ACT + OVV therapy is incapable of preventing ANR. Experiment was completed once.

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