DEVELOPING AN ASSAY TO IDENTIFY TUMOUR SENSITIZING COMPOUNDS

DEVELOPING A HIGH THROUGHPUT ASSAY TO INVESTIGATE CHEMICAL AGENTS WHICH SENSITIZE TUMOUR CELLS TO KILLING BY CAR ENGINEERED T CELLS

By

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Abstract

Cancer immunotherapy is emerging as a powerful tool in the treatment of cancer. Multiple clinical trials have established that infusion of tumour-specific T cells can cause regression of advanced tumours and prevent tumour relapse. While tumour-specific T cells are typically rare, engineering methods have been developed to introduce tumour-specific receptors into T cells and engender peripheral blood T cells with the ability to kill tumour cells. These engineering successes notwithstanding, tumour cells demonstrate variable sensitivity to T cell attack. Therefore, to maximize the impact of the engineered T cells, it is necessary to develop therapeutic strategies that render tumour cells sensitive to immune attack. For my thesis research, I sought to develop a high throughput screening assay that would allow me to screen chemical libraries for agents that sensitize tumour cells to T cell attack. My ultimate goal is to define chemical agents that effectively sensitize tumour cells to T cell attack but display a better toxicity profile than existing chemotherapies. To this end, I developed a screen where resistant tumour cells were exposed to T cells engineered with chimeric antigen receptors and positive hits were defined as agents that could enhance killing of the tumour cells. My work explored both murine and human systems and I ultimately decided to use human cells for my screen. Multiple methods for measuring tumour cell killing were evaluated, many tumour lines were screened and I optimized the conditions for generating large numbers of engineered T cells for the screen. The net result of my thesis work is a miniaturized assay that is ready for high throughput screening.

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2-Me - Beta-mercaptoethanol
5-FU - 5-Flourouracil
aAPC – Artificial antigen presenting cell
ACT – Adoptive cell transfer
APC – Antigen presenting cell
BSA - Bovine Serum Albumin
CAR – Chimeric antigen receptor
cRPMI – complete RPMI
DARPin – Designed ankyrin repeat protein
DC – Dendritic cells
FBS - Fetal bovine serum
GM-CSF -
hchNKG2Dζ - Human chimeric NKG2D CAR
HSA – Human serum albumin
HER2 - Human epidermal growth factor receptor
IRES - Internal ribosomal entry site (IRES)
ITAM - Immunoreceptor tyrosine-based activation motif
L-glut – L-glutamine
LDH - Lactate dehydrogenase
mchNKG2Dζ - Murine chimeric NKG2D CAR
MFI - Mean fluorescence intensity
MOI - Multiplicity of infection
NCI – National Cancer Institute
NEAA – Non-essential Amino Acids
NGFR - Nerve growth factor receptor
NK – Natural killer cell
NKT – Natural killer T cell
PAP - Prostatic acid phosphatase
PBMCs - Peripheral Blood Mononuclear Cells
Pen/strep - Penicillin/Streptomycin
PFA – Paraformaldyhe
PD-L1 - Program death ligand -1
RFU – Relative fluorescent units
rhIL-2 - Recombinant human IL-2
RT – Room temperature
scFv - Single-chain variable fragment
SSA - Side scatter area
T cells – T lymphocytes
TCR - T cell receptor
TIL – Tumour infiltrating lymphocytes
TNFα - Tumour-necrosis factor alpha
TRAIL - Tumour necrosis factor-related apoptosis-inducing ligand

IFNγ - Interferon gamma
Z’ – Z prime

1.0 Introduction

1.1 Cancer

Cancer is a heterogeneous disease defined as abnormal cell proliferation within a particular area of the body. As the disease progresses, cancer cells can invade other tissues by traveling through the blood and lymphatic system, through a process known as metastasis. Within Canada, nearly 30% of all deaths are attributed to cancer, making it the leading cause of death (Canadian Cancer Society). With the addition of 200,000 new Canadians to be diagnosed with cancer this year, it is clear that there is a growing need for the development of new therapeutic strategies.

1.2 Biology of Cancer

The complexity of cancer starts with the notion that it is not just a single disease, but many diseases. A cancer can be categorized by where it originates in the body (skin, lung, breast etc.). Often, along with the general categorization of the originating organ, the cancer can be further subdivided into additional categories based on where in the organ it originated, whether it has infiltrated surrounding areas, and its expression of specific proteins. For example, breast cancer can arise in the milk ducts or lobules. Therefore, breast cancer can be further subdivided into ductal carcinomas (duct origin) or lobular carcinomas (lobe origin). Depending on how the disease progresses, further classification of the disease can be given, ex. if the cancer starts to spread, then a patient's cancer may be defined as infiltrating ductal carcinomas or infiltrating lobular carcinomas. Building on the complexity, depending on the expression of specific receptors, estrogen, progesterone or HER2 receptors, additional classification of breast cancer can be given[1].

Cancers arise when a normal cell acquires multiple mutations within its genetic material, allowing the cell a growth advantage, the ability to bypass inhibitory growth signals, and create a niche conducive for the cells' uncontrollable proliferation[2]. While genetic predisposition (inherited mutations) is a main cause for cancer development, there is a strong link between external factors and cancer development. These external factors include lifestyle choices such as tobacco usage and diet, as well as environmental factors such as chemical and radiation exposure and exposure to infectious organisms[3]. Taken together, over an individual's lifetime, all these different factors have a profound influence on tumour formation.

1.3 Standard Treatments for Cancer

The three standard treatments for cancer patients are surgery, chemotherapy and radiation. The use of these three therapies is determined by the type and stage of their cancer. Surgery was first employed in the early 1800's as an effective approach for removing a tumour, and in some cases achieving a cure. Innovations in surgical technique over the next century also provided oncologists with a better way to understand the development of cancer, as exemplified by the radical mastectomies of breast tissue in the late 1800's, where surgeons were better able to visualize the breast tumour and the locations it could spread. This newfound knowledge revealed that surgery required tailoring to the stage of cancer the patient had[4]. Unfortunately, certain tumours cannot be removed fully due to the potential of excessive damage to the body. In these cases, debulking the tumour (removing as much tumour mass as possible) can be accompanied by postoperative (adjuvant) radiation or chemotherapy. Radiation therapy was developed

in mid 1900's, and employed a direct beam of energy aimed at the tumour, sparing normal tissue[4]. Successful clinical application of radiation as a main treatment and as an adjuvant therapy after tumour resection, has been documented with many different types of localized cancers, particularly for breast cancer patients after receiving a mastectomy[5]. If radiation therapy is not possible, as in the case of disseminated disease, chemotherapy can be used as an alternative. Chemotherapy, a term devised by Paul Ehrlich in the early 1900s, was first used for the treatment of infectious disease. Conventional chemotherapeutics are chemical agents, which are non-specific, and target cells with high proliferation rates. The first case of chemotherapy use for the treatment of cancer was documented in World War II era. Mustard gas was originally used as a weapon during the war, but with the observation of depleted bone marrow of exposed soldiers, scientists tested the use of nitrogen mustard to treat patients with lymphomas. These studies with nitrogen mustard lead to the development of the current day chemotherapeutic, cyclophosphamide[6]. Since the early 1900's, many different types of chemotherapeutic agents have been developed and, depending on their mechanisms of action, they can be subdivided into different categories, these include DNA-damaging chemicals, antimetabolites, and mitotic spindle poisons[7]. Depending on the location, stage and the health of the patient, oncologists will prescribe a treatment plan tailored to the patients needs.

Although these strategies have been shown to be effective, patients with metastatic disease, that which has spread form the primary location around the body, do not fair well with these treatments, exemplifying a need for new robust treatments.

1.4 Origins of Cancer Immunotherapy

Observations pertaining to the role of the immune system in cancer detection and clearance have been made as far back as the late 1800's, with the work of Dr. William Coley. Coley performed intratumoural injections of inactive or live bacterial cultures, including *streptococcus pyogenes*, in an effort to augment a patient's own antitumour immunity. The premise behind this technique was to engage antibacterial phagocytes to also kill bystander tumour cells[8]. Although success was sporadic, there was documentation of significant tumour regression, however, due to the possibility of infection, safer treatments such as chemotherapy, radiation and surgery were preferred. Nevertheless, these initial observations were monumental in establishing the field of cancer immunotherapy.

The immune system can be divided into two active lines of defense. The first line of defense against the tumour is carried out by the innate immune system, which has a non-specific response. As time progresses, specialized cells from the innate immune system (antigen presenting cells (APCs)) will activate the adaptive immune system, using materials from the tumour. Ultimately generating adaptive immune cells, which will be able to seek out and destroy cells only expressing those tumour antigens[9]. The main goal of cancer immunotherapy is to generate an active immune attack against the tumour. While the immune system is complex, composed of many different cell types, each playing a role in host defense against the tumour, T cells, a member of the adaptive immune system, have been shown to play a prominent role in antitumour defense.

1.5 Antitumour Immunity: Role of T cells

T cells can be subdivided into two types of cells, $CD8^+$ T cells and $CD4^+$ T cells, based on the expression of surface proteins. $CD8^+$ T cells, otherwise known as killer T cells, play an essential role in directly recognizing and lysing tumour cells. $CD8^+$ T cells will recognize tumour specific antigens expressed on tumour cells presented on major histocompatibility complex (MHC) I molecules. Upon recognition, $CD8^+$ T cells have an arsenal of cytotoxic mechanisms to directly induce apoptosis in the tumour cells[9].

Upon target detection, $CD8^+$ T cells will release cytotoxic mediators such as perforin and granzymes; perforin creates holes in the target cell membrane, allowing the granzymes to enter into the target cells, where they will initiate caspase-dependant apoptosis[10]. Additionally, $CD8^+$ T cells can release cytotoxic cytokines such as IFNγ and TNFα. IFNγ, in part, functions by increasing the expression of MHC I and II molecules on the surface of target cells, which can increase target recognition, leading to enhanced target clearance[11]. TNFα operates though binding death receptors (TNF receptor 1 and 2) on the target cell, while also regulating the activities of other immune cells[12]. Additionally, T cells also utilize contact-dependent cytotoxicity by triggered cell death of a target cell by binding a death receptor on the target cell. The Fas receptor, found on target cells, can initiate caspase-dependant apoptosis when bound by Fas ligand on T cells[13]. The release of these potent apoptosis inducing mediators is tightly regulated to limit the potential of autoimmunity[10].

$CD4^+$ T cells play a large role in recruiting and orchestrating the activities of other immune cells, such $CD8^+$ T cells. $CD4^+$ T cells are defined by several different lineages; in particular T_H1 cells are required for the full activation and maturation of $CD8^+$ T cells.

$CD4^+$ T cells can also produce chemokines, which recruit innate immune cells such as NK cells and macrophages to the tumour bed for additional tumour eradication. Not all $CD4^+$ T cell subsets contribute to antitumour immunity, T_{regs} are $CD4^+$ derived T cells, and primarily function to suppress antitumour immunity[14] though the production of immunosuppressive cytokines.

In order to generate tumour specific T cells, dendritic cells (DCs), a type of antigen presenting cell and a member of the innate immune system, within the tumour bed sample antigens derived from dead tumour cells. These tumour antigens can be either mutated proteins within the tumour, or normal proteins that are overexpressed on tumour cells. Next, DC will travel through lymphatic vessels, and will enter secondary lymphoid organs, such as lymph nodes, to activate T cells through the presentation of tumour antigens. After expansion in the lymph nodes, these tumour specific T cells will migrate from the lymphoid organs to the tumour bed where they can specifically lyse tumour cells[15].

Ultimately, the development of an antitumour response is limited by immunological processes, known as central and peripheral tolerance, that prevent T cells from attacking healthy tissues. Central tolerance is a feature of the immune system to prevent the generation of T cells with the potential to generate autoimmune disorders against self-antigens[16]. As a result, during T cell development, T cells, which possess high affinity binding for self-antigens, are removed while selecting for T cells with low affinity for self-antigens. Once T cells exit the thymus, they are then subject to peripheral tolerance where DCs sample healthy tissues and render T cells that react to healthy tissue antigens

non-functional[9]. Thus, the generation of a natural T cell immune response against the tumour is limited, and therefore, different ways to enhance the efficacy of T cells, *ex vivo,* have been highly investigated.

1.6 Cancer Immunotherapy

A number of different strategies have been investigated which utilize T cells (either directly, or in directly) to target tumour cells. These include vaccines, immunomodulatory antibodies, and adoptive transfer of tumour specific T cells. These strategies have shown to be successful in both the experimental and the clinical setting.

Therapeutic vaccines, those that are administered after cancer has developed, have found modest success in the clinics. Provenge (sipuleucel-T) was approved by the FDA for the treatment of advanced prostate cancer. Provenge is a personalized vaccine where the patient's blood is used as a source for generating APCs, which are cultured with a fusion protein, prostatic acid phosphatase (PAP) and GM-CSF (granulocyte-macrophage colony stimulating factor). PAP is an antigen associated with prostate cancer cells, while GM-CSF stimulates the maturation of APC. Conceptually, these APC will take up the fusion protein and stimulate the generation of antitumour T cells once infused back into the patient. Clinically, treatment with Provenge did not show extensive tumour shrinkage, but, the vaccine improved patient survival by 4 months[17].

In addition to vaccines, the FDA has approved the use of Ipilimumab, a blocking antibody against CTLA4, for the treatment of metastatic melanoma (relapse or initial therapy). CTLA4 is a receptor that is unregulated on the surface of T cells following activation. Ligation of CTLA4 blocks T cell function, presumably as a mechanism to

prevent the development of autoimmunity that could result from an overly robust T cell response. The success of Ipilimumab relies on the patient already having generated tumour specific T cells, as Ipilimumab acts as a signal to reignite T cell antitumour function, after it has been previously suppressed[18]. It has been shown that melanoma patients have a high level of lymphocytes within tumours[19], increasing the likelihood of efficacy for this type of cancer. Ipilimumab was shown to increase the survival of responding patients, and these responses were still on going three years following treatment[18,20]. With the success of the immunemodulator Ipilimumab for melanoma treatment, other T cell immunomodulating antibodies have been investigated; these include both agonistic and blocking antibodies. PD-1 is a negative regulator on T cell membrane used to curb T cell function, while its binding partner, PD-L1, is found on tumour cells (melanoma, ovarian, and renal). Blocking antibodies against these two proteins are in clinical development, one clinical trial treating advanced melanoma (Nivolumab) has shown that responding patients had an average life extension of 16.8 months, with 43% of patients alive 2 years following treatment[21].

One of the major challenges in generating antitumour immunity is doing so in an immunosuppressed environment. Cancer patients receiving treatment, whether it is radiation or chemotherapy, are immune compromised (inability to generate immune response). Because these therapies target cells that divide rapidly, white blood cells from the bone marrow are compromised, and a patient's ability to generate antitumour T cells is hampered. To overcome the challenges of de novo tumour specific T cell generation,

strategies have been investigated which assess the transfer of tumour specific T cells into a patient; this is referred to as adoptive cell transfer (ACT).

ACT involves the isolation and transfer of autologous T cells into a patient to treat disease[22]. A number of different ACT strategies have been developed and have been evaluated in the clinics with promising results. One particular strategy involves the isolation of **tumour infiltrating lymphocytes (TIL)**. In patients with melanoma[23,24], it has been shown that populations of tumour specific lymphocytes can be isolated from a resected tumour. These tumour specific lymphocytes can be expanded *ex vivo* (growing up to 10^{11} TIL), prior to infusion back into the patient. Prior to infusion these patients receive either chemotherapy or radiation to deplete their white blood cells to create a niche for cell engraftment. TIL isolated and expanded in this way has been shown to have melanoma-specific activity, in a recent study of 93 patients with progressive disease, objective responses were observed in 72% of patients (with prior radiation treatment), and 19 of the 93 patients were tumour-free 3 years following treatment[25].

As stated previously, generating antitumour T cells requires overcoming immunological tolerance to self-antigens. T cell tolerance to self-antigens is a major hurdle in generating anti-tumour immunity, which limits the availability of T cells for ACT. To overcome this hurdle, T cells can be engineered to express recombinant tumour-specific receptors, which direct the specificity and function of that T cell against antigens on the tumour.

One approach is to engineer T cells with T cell receptors (TCR) that are known to interact with tumours. This approach first requires the identification of the genetic

sequence of the tumour-specific α and β T cell receptor chains. This can be done by isolating T cell clones from a patient, stimulating them with tumour antigen and isolating out the T cells that responded to stimulation. As mentioned previously, tolerance mechanisms limit the generation of high affinity T cells. Therefore, the αβTCR chains isolated by this method would have low affinity for the tumour target antigen. Researchers have shown that mutating these isolated αβTCR chains to enhance their affinity for the target is possible[22]. Clinically, αβTCR chains have been studied in melanoma patients, with αβTCR chains generated against the melanoma antigen MART-1. Two different clinical trials showed objective responses in 13% of 31 patients and 33% of 20 patients[26,27]. The disadvantage to using αβTCR chains is that as a mechanism to avoid immune detection, tumour cells will down regulate the expression of MHC I molecules on its surface. As a result, T cells genetically engineered with αβTCR chains will not be able to detect the tumour cells[22].

As a strategy to overcome the issue of TCR availability and MHC expression, T cells can engineered with chimeric antigen receptor (CARs), which are chimeric transmembrane proteins which contain an a*ntigen-binding domain* fused to *intracellular signaling domains*[28] (Figure 1*)*. The antigen-binding domain, which is often derived from antibodies, is able to recognize whole antigen on the tumour surface, and is therefore not affected by the expression of MHC molecules on the tumour, providing CARs an advantage over transgenic αβTCRs. The antigen-binding domain is then fused to a hinge region to provide flexibility and a transmembrane domain to enable signal transduction. On the intracellular side of the receptor, signaling components from T cell activating

receptors (ex. CD3ζ, CD28, CD137) are included to trigger T cell cytotoxic effector functions following CAR ligation[29]. The most successful application of CAR technology has been against hematological malignancies, specifically malignant B cells. Both normal and malignant B cells express the protein CD19, several clinical trials treating patients with CD19 specific CAR T cells have shown phenomenal results[30,31]. In one particular study by Grupp *et al*, two patients with late stage acute lymphoblastic leukemia (ALL) showed complete remission after treatment. Once infused into patients, CAR-T cells showed robust proliferation, 2 months after treatment malignant cells were no longer present, while evidence of persistent CAR T cells were observed. This study shows the possibility of achieving long-lasting responses using engineered T cells. As a side effect of targeting CD19, patients exhibit on-target/off-tumour affects, as normal B cells also express CD19, patients remain with no B cells after therapy, however, this side-effect of therapy is manageable[31].

Because CARs are not MHC restricted, and offer higher affinity target binding compared to αβTCR chains, we have chosen to study CAR engineered T cells.

1.7 Engineering T cell Specificity: Chimeric Antigen Receptors

In general, all CARs have been designed in an effort to recapitulate the T cell activating signals from a single receptor. In the simplest scenario, T cells require two signals to become activated, and fully functional. The first signal is provided by the CD3 complex that associates with the TCR. The CD3 complex is composed of 4 subunits and the CD3ζ subunit, in particular, is responsible for activating downstream kinases needed to trigger T cell activation[9]. T cells require a second signal, costimulation, provided by

receptors like CD28, CD134 and CD137. These secondary signals promote T cell growth, differentiation and survival[9]. If T cells only receive the CD3 signal, they become anergic with limited functionality[28]. Initially, first generation CARs contained only CD3ζ as the intracellular signaling domain. These first generation CARs had limited antitumour effect, presumably because these CARs were only delivering signal 1[29]. Second generation CARs combined various primary signals (ex. CD3ζ and FceRI) with various secondary signals (ex. CD28, CD134, CD137) [28]. These second generation CARs, with both signaling components have proved to be superior in terms of increased proliferation of T cells and up-regulation of anti-apoptotic proteins, resulting in enhanced survival[32]. The various CARs demonstrate similar and contrasting properties but the preferred configuration remains to be determined.

The antigen binding domain of CARs, much like the intracellular signaling components, can differ between receptors. Most commonly used is the single-chain variable fragment (scFv), which is derived from the antigen binding domain of immunoglobulins. Receptor binding domains can also be used to target CARs. We have been particularly interested in the application of the NKG2D receptor ligand-binding domain to target CARs against adenocarcinomas. NKG2D is expressed on NK cells, NKT cells and some $CD8^+$ T cells. While NKG2D can trigger cytotoxic responses from NK and NKT cells, it only provides a costimulatory signal to CD8+ T cells and cannot trigger their cytotoxic function. Several groups have published data regarding the potential of a human chimeric NKG2D CAR[33,34], which contained NKG2D as the extracellular binding domain and transmembrane domain, fused to intracellular CD3ζ. Researchers have shown

the functionality of these CAR T cells against MICA/B (NKG2D-ligands) positive tumour cells, engineered T cells retained endogenous T cell function, as CAR engagement resulted in release of the proinflammatory cytokines IFNγ and TNFα[33].

The Bramson lab has also investigated an alternate ligand binding domain known as a DARPin, which stands for designed ankyrin repeat protein. Ankyrin repeat proteins are a class of binding proteins which are highly abundant in the human genome and whose biological function is to facilitate protein-protein interactions. To create DARPin molecules, the genetic sequence of hundreds of ankyrin repeat proteins were aligned, allowing for the identification of conservative amino acids, which are essential for ankyrin protein structure. With the base conserved amino acid structure, point mutations could be made to introduce different amino acids in the variable amino acid positions, to create libraries of DARPins that can be screened against specific targets. Once specific binders are identified for a specific target, rational mutagenesis can be used to isolate DARPins with very high affinity for the target (in the nM range) [35].

For the research that will be discussed in my thesis, I have focused on two different CARs: 1) one that employs the NKG2D receptor and 2) one that employs a DARPin specific for HER2[36], a protein that is commonly overexpressed on adenocarcinomas[37]. Details of these CARs can be found in the Materials and Methods.

1.8 Genetic Engineering of Lymphocytes for CAR expression

A variety of methods have been used to engineer T cells to express CARs, including retrovirus vectors, lentivirus vectors, plasmid DNA and RNA. I will restrict this discussion to retrovirus and lentivirus methods since those are the techniques employed

for my thesis. Viral vectors have been preferentially used to generate clinical grade product because of their ability to integrate into the host genome and yield long-term expression of the CAR. Retroviral vectors are only able to transduce proliferating lymphocytes. Lentiviral vectors are more attractive compared to retroviral vectors, as they are able to transduce non-dividing cells as well[22,38]. The main safety concern with using viruses that integrate DNA into the host genome is the potential of transformation of patients' primary cells. This concern was exemplified by a study where retrovirus vectors were used to restore the IL-2 receptor in hematopoietic stem cells in X-linked SCID patients and 4 of the 9 patients developed T cell leukemia due to the transgene inserting near a proto-oncogene[39]. In the case of retrovirus-transduced T cells, patients treated have been shown to possess long lasting modified T cells with no observable mutagenesis decades after the procedure, indicating a lower risk of mutagenesis with transduction of mature T cells[40,41]. Data from clinical use of lentiviral vector transduction of mature T cells is not as extensive as retroviral vectors, however, one study examining the use of lentivirus to modify $CD4^+$ T cells extracted from HIV+ patients, showed that four years post adoptive transfer, no patients acquired leukemia or adverse heath effects associated with the treatment[42]. Also, others have shown that lentivirus integration is not random and they do not integrate near proto-oncogenes[42,43].

1.9 The Great Escape: Tumour Cell Resistance to Lysis by Immune Cells

As stated previously, T cells utilize a variety of effector functions to eliminate a target through the induction of apoptosis in the target cell[44]. Tumour cells have been shown to have defects in apoptotic pathways, presumably as a mechanism to support

survival and growth of the tumour. Thus, while genetic-engineering of T cells may overcome limitations in the availability of tumour-specific T cells, it cannot overcome the intrinsic resistance of tumour cells to T cell killing through modulation of apoptotic pathways.

Tumour cells have exploited mechanisms of resistance used by normal cells to prevent apoptosis by immune cells. For example, human T cells express intracellular serpin proteinase inhibitor 9 (PI-9) (inhibits granzyme B) within the cytoplasm to protect themselves from undergoing apoptosis induced by their own granzyme B. Studies of human T cell non-Hodgkin lymphomas found that 90% of the cases investigated expressed PI-9[45]. Additionally, PI-9 has also been shown to be highly expressed in human non small cell lung carcinoma cell lines[46]. Overexpression of PI-9 ultimately leads to tumour escape. In addition, the immune system utilizes a diverse array of death receptors, including Fas and TRAIL death receptors to kill target cells[44]. Engagement of TRAIL released by T cell with their cognate receptor on the target cell, results in an intracellular signaling cascade leading to caspase-8 activation and apoptosis induction in the target cell. Tumours can evade TRAIL-mediated killing by down regulating TRAIL receptors and/or an up regulation of decoy receptors (receptors which bind TRAIL, but lack intracellular signaling death domains) [47,48]. Tumour cells have also been reported to overexpress anti-apoptotic proteins such as cellular FLICE-inhibitory protein (cFLIP), which interferes with apoptosis induced by both perforin/granzyme B[49] and death receptors[50]. ***Therefore, to maximize the effect of the adoptive T cell therapy with CAR T cells, it will be necessary to maximize the sensitivity of tumours to T cell killing***.

1.10 Immunostimulatory Effects of Chemotherapy

Conventional chemotherapy drugs can sensitize tumours to killing by T cells and NK cells. This occurs by increasing expression of antigens[51], and by bypassing the mechanisms of tumour cell resistance (as described previously) [52,53]. Pretreatment of primary colon carcinoma cell lines with chemotherapy drugs enhanced the expression of Fas (a death receptor) on tumour cells, resulting in increased susceptibility to $CD8^+$ T cell attack[54]. Additionally studies showed that treatment of colon cancer cell lines with chemotherapy could enhance susceptibility to TRAIL-induced apoptosis and decrease the expression of cFLIP[55]. Thus, certain chemotherapeutic agents possess the ability to enhance antitumour immunity by eliminating immunosuppressive factors but most importantly, engaging the lytic capacity of $CD8^+$ T cells through sensitization of tumour cells.

1.11 Identifying Novel Compounds to Sensitize Tumour cells to T cell Lysis

The use of chemotherapy to sensitize tumour cells to lysis by T cells has been well established. However, these chemotherapeutic agents are very toxic and often lead to long-term complications[56]. The most common toxicities associated with chemotherapy are diarrhoea, mucositis, and haematological toxicity (neutropenia) [56,57], in addition to specific toxicities associated with individual chemotherapy agents[56,58]. To maximize the antitumour effect of T cell therapies, we believe that novel sensitizers are required with a better toxicity profile.

High throughput screening has become an important scientific tool for the discovery of new drugs and new applications for existing drugs. Large libraries containing hundreds

of chemicals can be screened for activity against a disease target in a short amount of time. The concept of screening chemical libraries for biological activity in miniaturized assays is not new; the first inclusion of "high throughput screening" in a pubmed article dates back to 1991. Since then, high throughput screening has become more widespread, with both academic and industry facilities housing capable handling systems. The process of high throughput screening starts with establishing an assay that is robust, can be miniaturized and is sensitive enough to detect changes in the target once exposed to screening agents. After screening chemical compound libraries, hits are identified. Hits are compounds that exhibited a response in the biological assay. Through additional assessment of drug properties (activity *in vivo* and *in vitro*), lead compounds from the hits are selected and further studied. The lead compounds are optimized for their potency, safety and physiochemical properties before they can be considered candidates for drug development and clinical trials[59]. Chemistry and biology converge at optimizing a lead compound for enhanced potency and selectivity for a target. Through the process of analyzing the structure-activity relationship (SAR) of the hit compound, researchers are able to understand how the activity of the compound is encoded within the chemical structure of the compound. SAR analysis is essential for developing the hit compound to increase its potency, increase its affinity for the desired target, and limiting its off target effects[60].

We propose to utilize high throughput screening to identify compounds less toxic than conventional chemotherapeutic agents, which are able to sensitize human tumour cells to lysis by human CAR-engineered T cells.

The work detailed here outlines the progress made in designing an assay to be used to analyze the sensitizing properties of bioactive compounds (molecules with activity against a biological target) against apoptosis resistant tumour cells, using CAR T cells.

2.0 Materials and Methods

2.1 Murine CAR T cells

2.1.1 Retroviruses

I constructed a retrovirus plasmid encoding the murine chimeric NKG2D CAR (mchNKG2Dζ) based on previously published work[61]. The mchNKG2Dζ consists of a fusion between NKG2D receptor and the intracellular portion of CD3ζ (**Figure 2A**). The cDNA encoding the complete mchNKG2Dζ was ordered from Genscript and provided in the pUC57 vector. The mchNKG2Dζ cDNA was removed from pUC57 and subcloned into the pRV2011 vector (gift from Dr. B Rabinovich, MD Anderson, Texas) using restrictions sites EcoRI and BglII. The final plasmid (pRV2011_chNKG2D_CD3ζ-sentman) contained the CAR, along with Thy1.1 separated by an internal ribosomal entry site (IRES), a sequence which allows the expression of two protein products from the same mRNA transcript[62].

Retroviruses were packaged by transient transfection on Plat-E cells[63] (grown in DMEM containing 10% fetal bovine serum (FBS), 50mg/mL normacin, 10mM HEPES and 2mM L-glutamine (L-glut)). Plat-E cells were plated at a density of $1.2x10^6$ cells in a T75 growth flask in 10mL of medium for 72 hours. The Plat-E cells were then transfected with 10 ug of pRV2011_chNKG2D_CD3ζ-sentman and 10 ug of pCL-Eco (plasmid expressing gag, pol and env from the ectotropic subgroup of murine leukemia virus) [64] using Lipofetamine 2000 (Life Technologies). All transfection reagents were diluted in 4.5mL Opti-MEM (Life Technologies). 24 hours after transfection, medium containing Lipofectamine 2000, was removed form Plat-E cells, followed by addition of 10mL fresh

media (detailed above). After 48 hours (post transfection), viral supernatant (10mL) was harvested and concentrated 100X using Amicon Ultra 100K Centrifugal filter (Millipore) for 18min at 3000rpm.

2.1.2 Engineering Murine Splenocytes to Express mchNKG2Dζ

Spleens were harvested from Balb/c mice and treated with 1X ACK (Ammonium-Chloride-Potassium, 8g/l NH_4Cl, 1g/l $KHCO_3$ and 3.7g/l EDTA $Na_2 2H_2O$) solution to remove red blood cells. The cells were plated at $3x10^6$ cells in a 24 well dish per well in 1mL of T cell medium (RPMI 1640 containing 10% fetal bovine serum (FBS), 2mM L-glut, 1X Non-essential amino acids (NEAA), 10 000 U/mL penicillin, 10ug/mL streptomycin (pen/strep), 55nM Beta-mercaptoethanol (2-Me), 1mM sodium pyruvate and 10mM HEPES). T cells were subsequently activated by the addition of recombinant human IL-2 (rhIL-2) at 400U/mL and hamster anti mouse CD3 (0.3 ug/mL) and incubated for 24 hours at 37ºC and 5% CO_2 prior to transduction.

To transduce splenocytes, 100uL of 100X concentrated retrovirus (described in Section 2.1.1) along with 2ug/mL Lipofectamine 2000 and 1.6ug/mL polybrene was added to each well. The splenocytes were centrifuged at 2000rpm at 32ºC for 1.5 hours. Cells were than incubated for 2-4 hours at 37ºC and 5% CO_2 prior to the addition of 500uL T cell medium supplemented with 400 U/mL rhIL-2 and incubated overnight at 37ºC and 5% CO_2. The transduction procedure was repeated 24 hours after the first transduction as described above.

T cells were maintained at 1-1.5x10^6 cells/mL for the next 6 days prior to analysis. Whenever fresh T cell medium was used to dilute cells, it was supplemented with 400U/mL rhIL-2.

2.2 Human CAR T cells

2.2.1 Generation of CAR constructs

A human chimeric NKG2D CAR (hchNKG2Dζ) [65], and a CAR that recognizes human HER-2 (hHER2-CAR) were created based on previous work[36,66]. Much like the mchNKG2Dζ, hchNKG2Dζ is also a fusion protein containing both the human NKG2D receptor and the cytoplasmic portion of human CD3ζ (Figure 2A-B), which contains immunoreceptor tyrosine-based activation motif (ITAM) responsible for T cell signaling. The hHER2-CAR antigen-binding domain is based on the G3 (H10-2-G3) DARPin published by Zahnd et al. The hHER-2 CAR is a chimera where the G3 DARPin was fused to a CAR scaffold composed of the human CD8a extracellular hinge, the transmembrane and intracellular domains of human CD28 and the intracellular domain of human CD3ζ (Figure 2C).

The cDNAs encoding each of these CARs were separately subcloned, using restrictions sites NheI and AscI, into the pCCL vector, a lentivirus genome vector contains both a 5' and a 3' LTR region (modified to render virus self-inactivating by removal of the enhancer region) to allow incorporation of flanked CAR into the target cell[38]. This vector expresses the CAR cDNA under the control of elongation factor 1-alpha promoter for ectopic expression. The vector also expresses a truncated version of

the nerve growth factor receptor (NGFR) protein under the control of CMV promoter to serve as a marker of transduction.

2.2.2 Lentivirus

To package lentivirus particles encoding CAR constructs, 293TM cells were transfected with 4 plasmids using 120ul of Lipofectamine 2000 reagent per 60ug of plasmid DNA (per one 15cm dish = $8x10^6$ cells) in 293TM transfection media (RPMI 1640 containing 10 000 U/mL penicillin, 10ug/mL streptomycin, 0.1mg/mL normacin, 2mM L-glut, 10mM HEPES). The following plasmids and amounts were used: 32ug of CAR plasmid (pCCL with CAR), 6.25ug of pRSV-Rev (contains Rev protein which regulates gene expression), 12.5ug pMDLg-pRRE (encodes gag and pol), and 9ug pMDG2 (encodes VSV-G (vesicular stomatitis virus) which is expressed on viral surface to enable viral entry) [38]. After 16 hours, the transfection media was removed and replaced with 293TM transfection media containing 1mM sodium butyrate. After 30 hours, the cell culture medium was harvested and stored at 4ºC overnight.

The culture medium, which contains the lentivirus, was spun at 3000rpm for 20min to pellet debris, followed by filtration through a 0.45micron filter. Viral supernatant was than centrifuged for 1 hour and 40min at 28 000rpm at 4ºC in an Optima L-90K Ultracentrifuge (Beckman Coulter) using a SW32 Ti rotor (Beckman Coulter). The medium was subsequently decanted and the pellet, which contains the lentivirus, was resuspended in cold PBS, and stored at -80ºC.

To determine the titer of lentivirus, $3x10^4$ 293TM were plated in a 24 well plate in 0.5mL of 293TM transfection media. After 3 hours, lentivirus was added to each well at

10^{-2}-10^{-6} serial dilutions in a volume of 0.5mL. After an additional 5 days, the 293TM cells were vigorously pipetted to detach cells and suspended in FACS buffer + 2.5mM EDTA. To determine the titer, NGFR expression (marker of transduction) was measured by staining the cells with anti-NGFR-PE (BD Pharmingen, Cat# 557196) for 30min at RT. Cells were fixed in 2% paraformaldehyde (PFA) for 15 mins at RT and the specimen was acquired on a BD LSR II Flow Cytometer followed by data analysis using FlowJo software. To calculate the titer, flow data was plotted based on counts vs. NGFR-PE, and gates were set based on a mock-transduced sample (no virus added). Titer was determined using the equation: Viral titer (Transducing Units/ml) = ((# of 293TM) * (% NGFR positive cells) * (Dilution Factor)) / 100.

2.2.3 Transducing Human T cells using CAR containing Lentivirus

To isolate healthy donor human peripheral blood mononuclear cells (PBMCs), blood samples were spun at 1500 rpm for 5 min to separate plasma. After removing the yellow upper layer, the remaining blood was distributed into Leucosep tubes (containing Ficoll). After the addition of an equal volume of PBS, Leucosep tubes were spun for 10 min at 2150 rpm at RT. The white interface was harvested carefully, followed by dilution using PBS (as much as possible). The tube was then centrifuged for 10 min at 1500 rpm at RT. The supernatant was removed, and cell pellet was resuspended in 20mL of PBS. Samples were then centrifuged for 5 min at 1500 rpm at RT. The pellet was resuspended in 12.5% human serum albumin (HSA) (Sigma, resuspended in RPMI 1640), followed by the addition of an equal volume of freezing media (12.5% HSA + 20% DMSO). Samples were distributed into chilled cryopreservation vials and stored in overnight freezing

container at -80ºC prior to long term storage in liquid nitrogen. To generate CAR T cells, PBMC from liquid nitrogen storage were thawed into T cell medium (same as murine T cell medium) with the addition of 200U/mL rhIL-2 (Peprotech) and 10ng/mL rhIL-7 (Peprotech). To activate T cells, anti-CD3/anti-CD28 coated beads (Dynabeads Human T-Activator, Gibco) were incubated with PBMCs at a 1:1 ratio. PBMCs were incubated in a U-bottom 96 well plate overnight at 37ºC and 5% CO_2 ($1x10^5$ PBMCs per well).

After overnight incubation, 100uL of media was removed from each well, followed by addition of virus at the desired multiplicity of infection (MOI) determined for optimal transduction efficiency. T cells were transduced at either an MOI of 1 for hchNKG2Dζ T cells, MOI of 5 for NGFR T cells (contains no CAR construct) or MOI of 3 for hHER2-CAR T cells. After 24 hour incubation, T cells were fed with 100uL T cell medium supplemented with 200U/mL rhIL-2 and 10ng/mL rhIL-7. To maintain T cells, fresh media with cytokines were added three times a week and T cells were scaled up to a new vessel based on $1x10^5$ cells per cm^2 growth area. After 12 days in culture T cells were analyzed for function and CAR expression.

2.3 Phenotype and Functional Analysis of Human and Murine CAR T cells

2.3.1 Phenotype Analysis

Flow cytometry was used to visualize the expression of the CAR constructs on T cells. $1x10^6$ cells were resuspended in FACS buffer (PBS + 0.5% BSA (Bovine Serum Albumin)), and Fc receptors on T cells were blocked using Fc-Block (BD Pharmingen) for 15min at 4ºC.

To measure mchNKG2Dζ on mouse T cells, T cells were stained using the surface stain anti-CD4-PeCy7 (BD Pharmingen, Cat# 552775), anti-CD8-PerCP-Cy 5.5, (BD Pharmingen, Cat# 561109), anti-NKG2D-APC (eBioscience, Cat# 17-5882-82) and anti-Thy1.1- FITC (eBioscience, Cat# 11-0900-85). Cells were incubated with antibodies for 30min at 4ºC.

To measure hchNKG2Dζ on the human T cells, T cells were incubated with anti-NGFR-PE (BD Pharmingen, Cat# 557196) and anti-NKG2D-APC (BD Pharmingen, Cat# 562064) for 30min at RT. After staining with antibodies, hHER2-CAR T cells and hchNKG2Dζ were fixed using 2% PFA for 15min at RT. All T cell data was acquired on a BD LSR II Flow Cytometer and data analyzed using FlowJo software.

hHER2-CAR T cells were incubated with HER2-Fc protein (R&D Systems) for 45 min at RT. To detect HER2-Fc bound to hHER2-CAR, goat antihuman anti-IgG-PE (Jackson ImmunoResearch, Cat# 109-115-098) was incubated with hHER2-CAR T cells for 30min at RT. To detect NGFR protein (only on hHER2-CAR T cells), a separate sample was incubated with anti-NGFR-PE (BD Pharmingen, Cat# 557196) for 30min at RT.

2.3.2 *Intracellular Cytokine Assay*

To stimulate T cells, a U-bottom 96 well plate was coated with appropriate ligands for the specific CARs. 2000ng/mL murine Rae-1β-Fc (specific ligand for mchNKG2Dζ T cells) (R&D Systems) or 1000ng/mL HER2-Fc (specific ligand for hHER2-CAR T cells) (R&D Systems) was added to each well in 200uL PBS, the plate was stored at 4ºC overnight. The next day, each well was rinsed two times with cold PBS. $0.4x10^6$ CAR T

cells were added to each well, along with 0.2uL/ well GolgiPlug (BD bioscience). CAR T cells were incubated at 37ºC and 5% CO_2 for 4 hours. The plate containing CAR T cells was than spun down for 5min at 1500rpm, and fixed using 2% PFA, followed by storage in FACS Buffer + 2.5mM EDTA overnight at 4ºC.

Cells were stained for flow cytometry using FACS buffer and 1X BD Perm/Wash Buffer (BD Pharmingen, contains FBS and saponin) where indicated. CAR T cells from previous day were blocked for 15min at 4ºC using Fc-Block (BD Pharmingen).

To stain hHER2-CAR T cells the surface antibody cocktail included anti-CD4-PacificBlue (BD Pharmingen, Cat# 558116), anti-CD8-AF700 (eBioscience, Cat# 56-0086-82) and anti-NGFR-TexasRed (BD Horizon, Cat# 563452), T cells were incubated for 30min at 4ºC in FACS buffer. To stain mchNKG2Dζ T cells the surface antibody cocktail included anti-CD4-PerCP-Cy5.5 (BD Pharmingen, Cat# 550954), anti-CD8-AF700 (eBioscience, Cat# 56-0081-82) and anti-Thy1.1-FITC (eBioscience, Cat# 11-0900-85), T cells were incubated for 30min at 4ºC in FACS buffer. CAR T cells were permeabilized and fixed using 100uL of Cytofix/Cytoperm (BD bioscience) (per sample) for 20min at 4ºC. CAR T cells were washed with 1X BD Perm/Wash Buffer.

To stain internal cytokines, permeabilized hHER2-CAR T cells were stained using anti-IFNγ-APC (BD Pharmingen, Cat# 554702), and anti-TNFα-PECy7 (BD Pharmingen, Cat# 557647) for 30min at 4ºC in 1X BD Perm/Wash Buffer. To stain internal cytokines of mchNKG2Dζ T cells, anti-IFNγ-APC (eBioscience, Cat# 624076) and anti-TNFα-PECy7 (eBioscience, Cat# 557644), and cells were incubated for 30min at

4ºC in 1X BD Perm/Wash Buffer. Data was acquired on a BD LSR II Flow Cytometer and data analyzed using FlowJo software.

2.4 Tumour cells

2.4.1 *Murine Tumour Cell Lines*

Four murine tumour cell lines were screened in the development of a high throughput screening protocol. 4T1.2 a mammary gland carcinoma, CT26, a colon carcinoma, D2F2 and D2F2/E2 (D2F2 cells engineered to express human HER2), both mammary gland carcinomas, were used for experiments. 4T1.2 cells were grown in RPMI 1640 (cRPMI) supplemented with 10% FBS, 10 000 U/mL penicillin, 10ug/mL streptomycin, 2mM L-glut, 10mM HEPES, and 55nM 2-Me, CT26 were grown in alphaMEM supplemented with 10% FBS, 2mM L-glut, 10 000 U/mL penicillin, 10ug/mL streptomycin, 0.1mg/mL normacin, D2F2 and D2F2/E2 cell lines were grown in hi glucose DMEM supplemented with 5% FBS, 5% cosmic calf serum, 2mM L-glut, 1X NEAA, 10 000 U/mL penicillin, 10ug/mL streptomycin, 55nM 2-Me, 1mM sodium pyruvate. After 2 passages, D2F2/E2 media was supplemented with 800 ug/mL Geneticin (G418).

2.4.2 Human Tumour Cell Lines

Four lung tumour lines (NCI-H322M, A549, EKVX, and NCI-H460) and five breast tumour lines (MCF-7, Hs578-T, MDA-MB-231, T47D, and BT-549) were used for these studies. All human tumour cells were grown in RPMI 1640 supplemented with 10% FBS, 10 000 U/mL penicillin, 10ug/mL streptomycin, and 2mM L-glut.

2.4.3 CAR Target Expression on Murine Tumour Cell Lines

To determine the expression of murine NKG2D ligands on 4T1.2, CT26, and D2F2/E2 cell lines, cells were stained and analyzed by flow cytometry. The cells were incubated with 1X citric saline buffer (135mM potassium chloride and 15mM sodium citrate) until detached (5-20min depending on cell line). Flasks were tapped vigorously to help detach cells. Cells were suspended in FACS buffer + 2.5mM EDTA at a concentration of $0.5x10^6$ cells/mL. Fc receptors on tumour cells were blocked with a treatment of Fc-Block diluted in FACS buffer + 2.5mM EDTA for 15min at 4ºC. Samples were subsequently stained with 0.5ug murine NKG2D-Fc (binds all NKG2D ligands) (R&D Systems) in FACS buffer + 2.5mM EDTA for 30min at 4ºC. Afterwards, goat antihuman anti-IgG-PE (Jackson ImmunoResearch, Cat# 109-115-098) was used to detect NKG2D-Fc protein in FACS buffer + 2.5mM EDTA for 30min at 4ºC. Negative control samples only stained with anti-IgG-PE were used to determine anti-IgG-PE non-specific binding. Data was acquired on a BD LSR II Flow Cytometer and data analyzed using FlowJo software.

2.4.4 Target Expression on Human Tumour Cell Lines

To determine the expression of CAR target proteins (either NKG2D ligands or HER-2) on tumour cell targets, flow cytometry was used. Cells were detached from plates as described previously for murine tumour cells. Cells were resuspended in FACS buffer + 2.5mM EDTA at a concentration of $0.5x10^6$ cells/mL. Fc receptors on cells were blocked with a treatment of 10% goat serum (Gibco) diluted in FACS buffer + 2.5mM EDTA for 30min at 4ºC. To measure human NKG2D ligands, cells were stained with

0.5ug human NKG2D-Fc (R&D Systems) in FACS buffer + 2.5mM EDTA for 30min at 4ºC. To measure human HER-2 expression, cells were stained with the trastuzumab monoclonal antibody (1/1000 dilution) in FACS buffer + 2.5mM EDTA for 30 min at 4ºC. Goat anti-human IgG-PE was then added to detect either NKG2D-Fc or trastuzumab, it was diluted in FACS buffer + 2.5mM EDTA, and cells were incubated for 30min at 4ºC. Negative control samples only stained with anti-IgG-PE were used to determine anti-IgG-PE non-specific binding. Data was acquired on a BD LSR II Flow Cytometer and data analyzed using FlowJo software.

2.5 Expansion of Cryopreserved CAR T cells

2.5.1 Freezing Human CAR-T cells for Storage in Liquid Nitrogen

Previously transduced hchNKG2Dζ T cells were frozen in FBS with 10% DMSO at a concentration of $8x10^6$ cells/mL in 1ml of freezing media. hchNKG2Dζ T cells were pelleted (1500rpm for 5min), resuspended in FBS, with DMSO added dropwise. Previously transduced hHER2-CAR T cells were frozen in 12.5% HSA with 10% DMSO at $4x10^6$ cells/ml in 1 mL of freezing media. hHER2-CAR T cells were pelleted (1500rpm for 5min), and resuspended in 12.5% HSA. An equal volume of 12.5% HSA+20% DMSO was then added to the suspension of hHER2-CAR T cells. The T cells underwent slow freezing at 1ºC per minute until -80ºC (~24 hours) using isopropanol before transfer to liquid nitrogen for permanent storage.

2.5.2 Expansion of Frozen Human CAR-T cells with Artificial Antigen Presenting Cells

24 hours prior to T cell co-culture, K562 cells or artificial antigen presenting cells (aAPC) K64-41BBL[67], were irradiated with 10 000 rads of gamma irradiation

(Gammacell 1000) in serum free media (RPMI 1640 with 10 000 U/mL penicillin, 10ug/mL streptomycin) at a concentration of $1x10^6$ cells/mL. After irradiation, cells were incubated overnight at 37ºC and 5% CO_2. On the day of the co-culture, cryopreserved CAR T cells were collected from liquid nitrogen storage and thawed in 37ºC water bath followed by a 3 hour incubation (to allow cells to adjust to culture before expansion) in 10mL T cell medium (10ng/mL IL-7 and 200U/mL IL-2) at 37ºC and 5% CO_2. T cell medium contained either 10% FBS or 5% human AB serum, depending upon the experiment. The irradiated K64-41BBL cells were loaded with either i) 0.5ug/mL anti-CD3 antibody (OKT3, eBioscience) and 0.5ug/mL anti-CD28 antibody (Clone 9.3, eBioscience) or ii) 500ng of HER2-Fc (per $1x10^6$ aAPC), for 10min at RT, after which, cells were not washed.

Thawed CAR T cells and antibody-loaded K64-41BBL (or K562) were co-cultured at a ratio of 2:1 ($3x10^6$ T cells and $1.5x10^6$ stimulatory cells) in a final volume of 4.5mls, in a single well of a 6 well plate. Two days after the start of the co-culture, fresh cytokines were added to the media (10ng/mL rhIL-7 and 200U/mL rhIL-2). One day later, the cells were collected and transferred to a T25 flask at a concentration of $0.5x10^6$ cells/mL in fresh T cell medium (10ng/mL rhIL-7 and 200U/mL rhIL-2). Every 2 days, the concentration of CAR T cells was adjusted to $0.5x10^6$ cells/mL by counting the cells and adding fresh T cell medium as needed. CAR T cells were kept in culture for 8 days post co-culture.

2.6 Killing Assays

2.6.1 *AlamarBlue*

AlamarBlue (Life Technologies) is a nontoxic, weakly fluorescent reagent used to assess the health of a target cell, in this case tumour cells. To establish the linear relationship between cell density and fluorescent signal, tumour cells were plated at different densities from $1.5x10^3$-$2x10^6$ tumour cells per well in a flat bottom 96 well plates in triplicate. 30 hours after plating, tumour cells were washed three times with 200uL of warm PBS. 100uL of 10% AlamarBlue reagent (diluted in T cell medium) was added to each well and incubated at 37ºC and 5% CO_2 for 3 hours. Fluorescence was read using the Tecan Safire fluorescent reader. Measurement parameters include $\lambda_{excitation}$ of 530 and $\lambda_{emission}$ of 595. It was determined that for all cell lines, both human and murine, the fluorescent signal was linear up to a density of 12 500 tumour cells per well. Therefore, to ensure that we were measuring a linear loss of cells in our assays, we conducted all of our T cell – tumour cell cultures with a density of 12 500 tumour cells/well.

To assess the ability of mchNKG2Dζ T cells to kill targets, tumour cells were seeded into 96 well plates at a density of 12 500 cells/well. After 24 hours, media was removed from each well, and mchNKG2Dζ T cells were added at a variety of T cell:tumour cell ratios (0.25:1 – 16:1). mchNKG2Dζ T cells were co-cultured with tumour cells for 6 hours. After, tumour cells were washed three times with 200uL of warm PBS. 100uL of 10% AlamarBlue reagent (diluted in T cell medium) was added to each well and incubated at 37ºC and 5% CO_2 for 3 hours. Fluorescence was read as described above.

2.6.2 *Calcein AM*

Calcein acetoxymethyl ester (Calcein AM) (Life Technologies) is a cell-permeant dye used to assess the lysis potential of CAR T cells. 50ug of Calcien AM was resuspended at a concentration of 1mM in DMSO. Tumour cells were resuspended in cRPMI at a concentration of $1.5x10^6$ cells/mL and Calcein AM was added to the cell suspension at a final concentration of 10uM. Cells were labeled for 30min at 37ºC and 5% CO_2. Cells were shaken every 5min, to prevent settling. After incubation, tumour cells were spun down (1500rpm for 5min) and washed with 10mL of cRPMI twice. Tumour cells were then plated in a U-bottom 96 well plate in 100uL. After 24 hours, mchNKG2Dζ T cells were added at a variety of T cell:tumour cell ratios (0.25:1 – 16:1) in 100uL. An aliquot of 100ul of 2% Triton X-100 was added to the positive control wells to determine the maximum release of Calcein AM. The mchNKG2Dζ T cells were co-cultured with tumour cells for 6 hours. After 6 hours, the plate was spun at 1500rpm for 5min, 100uL from each well was carefully drawn off and transferred to a new flat bottom 96 well plate. Fluorescence of the supernatant was read using the Tecan Safire fluorescent reader. Measurement parameters include $\lambda_{excitation}$ of 485 and $_{\lambda\ emission}$ of 530.

2.6.3 *CytoTox96*

The CytoTox96 assay (Promega) is a non-radioactive cytotoxicity assay, which provides a quantitative measure of cell lysis based on the release of lactate dehydrogenase (LDH), a cytosolic enzyme. Tumour cells were plated in a V-bottom plate in triplicate (100ul/well; 1.25 x 10^5 cells/ml). 24hours later, mchNKG2Dζ T cells were added to each well at a variety of T cell:target ratios (0.25:1 – 16:1) in 100uL. The mchNKG2Dζ T cells

alone were also assessed for their contribution to LDH release. In addition, 20uL of lysis solution (containing 9% (v/v) Triton X-100; provided with the kit) was added to maximum release wells. After 6 hours, the plates were spun at 250g for 4mins, and 50uL of supernatant was transferred to another 96 well flat bottom plate. 50uL of Substrate Mix (undefined and provided by the manufacturer) was added to 50uL of supernatant, and incubated for 30min at RT, protected from light. Finally, a 50uL aliquot of "stop solution" (1M acetic acid; provided with the kit) was added to each well, and absorbance was read at 490nM using the Tecan Safire.

2.7 Optimizing High Throughput Screening Components

2.7.1 Determining Tumour Cell Density

AlamarBlue was chosen to assess the killing ability of CAR T cells during screening. Using Beckman/Coulter high throughput screening machinery, the density of tumour cells plated per well for AlamarBlue had to be determined to ensure the signal was not saturated, and a loss in fluorescence of a sample would be proportional to a lose in tumour cells from the plate. 4T1.2 or A549 cells were plated in black polystyrene 96 well plate (Costar cat#3916) at densities ranging from $1.5x10^3$-$2x10^6$ cells per well using the Biomek FX (Beckman/Coulter). After 30 hours, plates were washed three times with 200uL warm PBS on the BioRAPTR (Beckman/Coulter), using the ELx405 plate washer (BioTek Instruments), followed by addition of 10% AlamarBlue reagent. 3hours after, fluorescence was detected using EnVision plate reader (Perkin Elmer) at $\lambda_{excitation}$ of 535nm and $\lambda_{emission}$ of 600nm.

2.7.2 Determining High and Low Control Compounds for Murine Screen

For high throughput screening, both a high and a low signal control compounds were required. DMSO was chosen for the high control (high fluorescence, maximal tumour cell viability). To determine the low control, 4T1.2 cells were plated in black polystyrene 96 well plate (Costar cat#3916) at a density of 12 500 cells per well in 100uL cRPMI (experiment not completed in high throughput). After 6 hours, gemcitabine (Sigma, G6423, resuspended in DMSO at 1mM) was added to a final concentration of 50uM, 5uM or 0.5uM to each well (in 100uL cRPMI). Cells were incubated with gemcitabine for 18 hours at 37ºC and 5% CO_2. Afterwards, media was removed and mchNKG2Dζ T cells were added to each well at T cell: tumour cell ratios of 0.1:1 to 2:1 in 100uL T cell medium. Wells not receiving T cells (gemcitabine treated 4T1.2 cells only) received 100uL of T cell medium. 6 hours after incubation at 37ºC and 5% CO_2, Plates were washed, 10% AlamarBlue was added and incubated overnight (~18 hours).

2.7.3 Murine screen: Z prime and Pilot Screen

A schematic of our high throughput screening strategy is depicted in Figure 3. Before undertaking high throughput screening, a Z prime experiment was conducted to evaluate the suitability of our assay for high throughput screening. To complete the Z prime, 12 500 4T1.2 cells were plated in 100uL cRPMI in each well of the black polystyrene 96 well plate (Costar cat#3916) using the Biomek FX (Beckman/Coulter). 6 hours after, half the plate(columns 1-6) received 1uL of DMSO, while columns 7-12 received a final concentration of 10uM gemcitabine (1uL of 1mM at a final volume of 100uL) using the Biomek FX (Beckman/Coulter). Cells were incubated with DMSO or

gemcitabine for 18 hours at 37ºC and 5% CO_2. Afterwards, media was removed and mchNKG2Dζ T cells were added to each well at an T cell to target ratio of 1:1 in 100uL T cell medium using the BioRAPTR (Beckman/Coulter) and incubated for 6 hours at 37ºC and 5% CO_2. Plates were washed, 10% AlamarBlue was added and incubated overnight (~18 hours). Plates read as previously described in *2.7.1 Determining Tumour Cell Density*.

After completing the Z prime, a pilot screen was conducted screening 320 compounds (Canadian compound collection plate #91). To complete this screen, 12 500 4T1.2 cells were plated in 100uL cRPMI in each well of the black polystyrene 96 well plate (Costar cat#3916) using the Biomek FX (Beckman/Coulter). 4T1.2 cells were incubated for 6 hours at 37ºC and 5% CO_2, to allow tumour cell attachment. Test compounds (from Canadian Compound Collection) were added to each well in 1uL (final concentration of 10uM). Columns 1 and 12 of the 96 well plate did not receive test compound, but received high control (DMSO) or low control (10uM gemcitabine) as described previously. Each test compound (10uM in DMSO) was assessed in duplicate. Cells were incubated with test compound, DMSO or gemcitabine for 18 hours at 37ºC and 5% CO_2. Afterwards, media containing compounds was removed and either 100uL of T cell medium (to determine test compound toxicity) or mchNKG2Dζ T cells (at T cell: tumour cell ratio of 1:1 in 100uL T cell medium) were added to each well using the BioRAPTR (Beckman/Coulter) and incubated for 6 hours at 37ºC and 5% CO_2. Plates were washed, 10% AlamarBlue was added and incubated overnight (~18 hours). Plates read as previously described in *2.7.1 Determining Tumour Cell Density*.

3.0 Results

3.1 Developing a High Throughput Screening Assay for Murine CAR-T cells

3.1.1 Generation of Functional Murine CAR-T cells

To generate murine CAR T cells, murine splenocytes were transduced using a retrovirus. The retrovirus vector we employed encodes both the CAR cDNA and a cDNA for a reporter protein, Thy1.1, which was used to determine transduction efficiency. We cannot directly measure the expression of the mchNKG2Dζ CAR by flow cytometry since our antibody cannot distinguish between the NKG2D CAR protein and endogenous NKG2D protein on the T cell surface. However, we can measure mchNKG2Dζ CAR expression indirectly as elevated expression of NKG2D on the cell surface, which only occurs in mchNKG2Dζ CAR engineered T cells. Figure 4 depicts the expression of the mchNKG2Dζ CAR on $CD8^+$ T cells. Based on the Thy1.1 expression, approximately 50% of T cells transduced with mchNKG2Dζ expressed the CAR and Thy1.1 (Figure 4, left panel). Figure 4, right panel shows NKG2D expression on activated, but non-transduced T cells.

While the phenotypic data supported the likelihood that the CAR was expressed on the cells, we needed to confirm that the CAR was functional. We next evaluated whether the mchNKG2Dζ T cells could become activated (assessed based on the production of interferon gamma (IFNγ) and tumour-necrosis factor alpha (TNFα) [10]), following exposure to the CAR ligand. For this assay, we used the protein Rae1β, a cognate ligand for the murine NKG2D[68] receptor. MchNKG2Dζ T cells were incubated for 4 hours with Rae1β protein, and cytokine production was measured using intracellular cytokine assays

and analyzed by flow cytometry (Figure 5). In addition to Rae1β, mchNKG2Dζ T cells were also incubated with the protein HER-2, which does not bind the CAR, as a negative control. MchNKG2Dζ $CD8^+$ T cells responded to Rae1β (Figure 5, right panel) based on the production of both TNFα and IFNγ (20% of cells produced both cytokines), but did not respond to HER-2, as there were no cytokines produced above background levels (Figure 5, middle panel). These results indicate that mchNKG2Dζ-engineered T cells are functional and respond to NKG2D ligands.

3.1.2 Killing Assay: Measuring the Killing Capacity of Murine CAR-T cells

While cytokine production is a convenient way to measure T cell functionality, we would like to use tumour cell killing as the output for our high throughput screen, since this is an important property of antitumour T cells. We compared three different tumour viability detection assays, Cytotox96, AlamarBlue, and Calcein AM, to determine which would be most suitable for our high throughput screen. Cytotox96 and Calcein AM were chosen based on published reports indicating they are suitable replacements for ^{51}chromium release assay, the most widely used cytotoxicity assay to assess T cell killing[69,70]. AlamarBlue was chosen, as there were several published reports of its use for high throughput screening[71,72]. These three assays were compared in a head to head experiment using mchNKG2Dζ T cells and D2F2/E2 murine mammary tumour cell line as a proof-of-principle, and to determine which assay could translate best to high throughput screening. All three assays confirmed that mchNKG2Dζ T cells were cytotoxic against D2F2/E2, however too different degrees (Figure 6). The Cytotox96 assay, which measures release of the cytoplasmic protein LDH, showed the highest

measurement of cytotoxicity, with >100% cytotoxicity at T cell:tumour cell ratio from 1:1 to 8:1. Despite the apparent high sensitivity of this assay, there were numerous negative attributes: 1) the Cytotox96 assay had a low signal to noise ratio, 2) it required the most experimental steps, 3) it indicated cytotoxicity of >100%, which is mathematically impossible unless the assay was measuring LDH from both tumour cells and T cells, and 4) it was the most costly. Calcein AM, which measures the release of a previously internalized fluorescent dye, yielded the lowest signal of the three assays and displayed poor sensitivity. AlamarBlue showed an intermediate level of T cell cytotoxicity, with maximal cytotoxicity (~97%) occurring at 2:1, and not exceeding the mathematical limit of 100% cytotoxicity at any T cell:tumour cell ratio. Based on these results, AlamarBlue was chosen for the screen, because of the large differential between high and low fluorescence values, tight replicates and experimentally, most amenable to high throughput.

3.1.3 Identifying a Murine Target Cell Line for the High Throughput Screen

The goal of our screen is to identify chemical compounds that will render tumour cells sensitive to T cell killing. Therefore, we decided to identify a tumour line that was resistant to T cell killing as the basis for our screen. As a first step, we confirmed the expression of NKG2D ligands on the tumour cell lines available in our lab (Figure 7A).

To measure sensitivity to cytolysis by mchNKG2Dζ T cells, the three tumour lines were co-cultured with the CAR T cells and tumour cell viability was assessed using the AlamarBlue assay. All three cell lines exhibited differential sensitivity to killing by mchNKG2Dζ T cells (Figure 7B). 4T1.2 cells were most resistant with 90% tumour

viability at the highest T ccell:tumour cell ratio, while D2F2/E2 cells were most sensitive to killing. We thought that the differential susceptibilities to mchNKG2Dζ T cell killing might be due to a differential expression of NKG2D ligands on each cell line. Indeed, the 4T1.2 expressed the lowest amount of NKG2D ligands (MFI = 2,438) followed by CT-26 (MFI = 6,747) and D2F2/E2 (MFI = 10,358) demonstrating that killing correlated with the level of NKG2D ligand expression (Figure 7A).

3.1.4 Preparing for High Throughput Screening using Murine Cells

To prepare for high throughput screening, both high control (high fluorescence) and low control (low fluorescence) reagents needed to be determined. The purpose of having both of these controls is to determine the sensitivity of the assay, allowing us the ability to determine if a test compound is a hit, based on reference to the high and low control fluorescent values. The high control selected was DMSO, a standard reagent that would have no sensitization properties, and thus would result in a high fluorescent value, indicating maximal 4T1.2 viability. The low control would result in a low fluorescent value, indicating tumour cell death. For the low control, a compound, which has low toxicity alone, but was able to sensitize 4T1.2 cells to killing by CAR T cells was required. 4T1.2 cells were exposed to gemcitabine (a known chemotherapeutic drug) alone at different concentrations (1mM to 500nM) for 18 hours, prior to viability detection using AlamarBlue. From these dosages, three concentrations (50uM, 5uM and 0.5uM) with low toxicity in 4T1.2 cells were selected for further characterization.

To determine if gemcitabine at 50uM, 5uM and 0.5uM was able to sensitize 4T1.2 cells to killing by mchNKG2Dζ T cells, 4T1.2 cells were exposed to gemcitabine for 18

hours. Afterwards, drug treated 4T1.2 cells were co-cultured with mchNKG2Dζ T cells for 6 hours. AlamarBlue was subsequently added to the wells, and cell viability was assessed. It was determined that all three doses of gemcitabine were effective at sensitizing 4T1.2 cells to killing by mchNKG2Dζ-T cells (Figure 8). At a ratio of 1:1 (mchNKG2Dζ T cell: 4T1.2 cell), non-treated 4T1.2 cells were resistant to death by mchNKG2Dζ T cells (90% viable 4T1.2 cells after 6 hours co-culture), however, if 4T1.2 cells were exposed to 50uM or 5uM of gemcitabine (~80% 4T1.2 cell viability) for 18 hours prior to co-culture with mchNKG2Dζ T cells, 10% of 4T1.2 were viable after co-culture at a 1:1 T cell to target ratio (Figure 8). For high throughput screening, it was determined that 10uM gemcitabine (to be consistent with the test compound concentrations) would be used as the high control, and a T cell to target ratio of 1:1 would be used.

To utilize the AlamarBlue assay for high throughput screening, the quantity of 4T1.2 cells to be plated per well was determined to avoid over saturation of the fluorometer used for the high throughput screening facility. It was determined that 12 500 4T1.2 cells per well yielded a strong fluorescent signal without saturating the fluorometer.

3.1.5 Z prime and Pilot High Throughput Screen Using Murine Cells

To ensure that the killing assay, using 4T1.2 cells was amenable for high throughput screening, a Z prime (Z') (a statistical measure to assess the quality of the AlamarBlue assay with 4T1.2 for high throughput screening) was calculated. For the Z' experiment, 4T1.2 cells were plated in 96 well plates, 6 hours afterwards, 4T1.2 cells were exposed to either DMSO (high control) or 10uM gemcitabine (low control) for 18 hours, afterwards,

cells were incubated with AlamarBlue reagent. The purpose of this experiment is to ensure there is sufficient difference between the high and low control fluorescent values (taking into consideration standard deviation). Using our murine assay set up, it was confirmed that our assay was excellent for high throughput screening, based on a Z' of 0.71 (Figure 9). We conducted a pilot screen of 320 compounds (Canadian compound collection plate #91), we found four molecules that seemed to sensitize 4T1.2 cells (Figure 10A-B). These compounds had an average fluorescent reading that was lower than gemcitabine (indicating better sensitizers). The molecules identified in our screen are small molecules which inhibit cellular proteins, such as #134 a Casein Kinase 1δ inhibitor (IC 261, Sigma #I 0658), #142 an inhibitor of IκBα (Bay 11-7082, Sigma #B5556) and #223 a natural product which has been shown to have anti proliferative and pro-apoptotic effects on cancer cell line (Sanguinarine chloride hydrate, Sigma #S5890). While the hits were of interest, validation experiments failed to confirm that these agents could sensitize targets to T cell killing.

To this point, the tumour cells were incubated overnight in AlamarBlue to measure viability. This incubation period was chosen for convenience. However, given the rapid proliferation rates of murine tumour cells, we became concerned that cells would grow during the 18 hour period and this growth could obscure our analysis. Therefore, we compared different incubation times with AlamarBlue to determine the minimal time required to achieve robust and sensitive signal. We chose short periods (1 hour, 2 hours, 3 hours and 3.5 hours) and found that 3 hours of incubation with AlamarBlue was sufficient to obtain a strong signal with a high signal:noise. We subsequently re-evaluated

the toxicity of gemcitabine on 4T1.2 cells. Strikingly, while 5uM gemcitabine demonstrated no toxicity following overnight exposure to AlamarBlue, we observed that this dose of gemcitabine killed 40% of the 4T1.2 cells when the incubation with AlamarBlue was reduced to 3 hours, confirming our concern that growth of the 4T1.2 during the overnight period was obscuring our data (Figure 11). Using this revised protocol with a 3 hour AlamarBlue incubation period, we confirmed that 5uM gemcitabine + CAR-T cells resulted in more tumour cell death than either agent alone, however, this effect was much less pronounced than we had previously observed with the overnight AlamarBlue incubation.

3.2 High Throughput Screening using Human Cells

My work to this point focused on murine cells. However, over the course of multiple discussions, I decided to adapt my screening assay to human cells as the output of the screen will ultimately be used in human. My work in the murine system had confirmed the principles of the high throughput screen and provided a basis for the development of a high throughput screen for chemical agents that sensitize human tumour cells to killing by CAR-T cells. The next section of my thesis will focus on the development of a high throughput screening assay with human cells.

3.2.1 Generation of Functional Human CAR-T cells

Human T cells were engineered with the lentivirus vectors that express both the CAR and NGFR. At an MOI of 1, we could achieve >90% transduction of human T cells with the hchNKG2Dζ CAR vector based on NGFR expression (Figure 12, left panel). Similar to the murine T cells, we could not distinguish the NKG2D CAR from the

endogenous NKG2D receptor. However, we did note an elevation in the surface expression of NKG2D on the cells engineered with the hchNKG2Dζ CAR vector compared to T cells engineered with a control vector that expresses only NGFR (Figure 12, right panel), which indirectly confirms surface expression of the hchNKG2Dζ CAR.

Since the murine data suggested that killing of tumour targets may be related to the level of target expression, we decided to perform our screen with CAR T cells specific for two different targets. In this way, we should avoid hits that reflect agents that stimulate upregulation of just NKG2D ligands. We chose HER-2 as the second target as this protein is a well-defined tumour target. To optimally transduce T cells with hHER2-CAR lentivirus it was determined an MOI of 3 was required. Flow cytometry staining for hHER2-CAR and NGFR expression simultaneously was not possible because both antibodies were conjugated to the PE fluorophore. However, surface expression of the hHER2-CAR is easily resolved on human T cells when compared to control T cells, so coincident staining for both proteins is not needed. Flow cytometry analysis of the hHER2-CAR and NGFR separately resulted in similar positivity, 67.2% and 65% positive cells for hHER2-CAR and NGFR, respectively (Figure 13A-B).

To confirm the functionality of the CAR T cells, we stimulated the hHER2-CAR T cells with either recombinant HER-2 (hHER2-CAR T cell specific ligand) or Rae-1β (non specific ligand). As expected, the hHER-2-CAR T cells responded to HER-2 but not Rae-1β (Figure 14). When exposed to HER-2, 28.3% of hHER2-CAR T cells produced both TNFα and IFNγ, with a substantial population producing only TNFα (23.7% of cells)

(Figure 14, right panel). hHER2-CAR T cells did not produce cytokine above background to non-specific ligand Rae1β-Fc (Figure 14, left and middle panel)**.**

3.2.2 Human Tumour cells

The Bramson lab is particularly interested in tumours of the lung and the breast. Therefore, to identify target cells for our high throughput screen, we investigated lung tumour and breast tumour cell lines in the National Cancer Institute (NCI)-60 human tumour cell line panel, which was designed for cancer drug discovery, and contains a diverse array of tumour types, including colon, lung, ovarian, breast, renal, melanoma, to name a few[73]. We defined the following criteria for the ideal target line:

- Express CAR T cell target ligands (HER-2 and NKG2D ligands),
- Easy to grow to ensure enough cells for each screen,
- Exhibit resistance to killing by CAR T cells.

Table 1 summarizes the expression of the ligands on the tumour cell lines, indicating that all cell lines tested expressed both ligands.

Cell lines were initially tested for sensitivity to killing by hchNKG2Dζ T cells using the AlamarBlue assay. Based on their sensitivity to hchNKG2Dζ T cells, two cells lines were selected, A549 (human non small cell lung carcinoma) and MDA-MB-231 (breast carcinoma) because they were the least sensitive to killing, Table 1. Unlike the murine tumour lines, the level of NKG2D ligand expression on the tumour cell did not seem to influence killing as the A549 has a higher expression of NKG2D ligands compared to MCF-7, NCI-H460, and MDA-MB-231, however exhibited less sensitivity to

hchNKG2Dζ T cells. These two cell lines, in addition to the most sensitive cell line, T47D, were then tested to examine sensitivity to killing by hHER2-CAR T cells, to ensure that the cell lines selected were universally resistant to both types of human CAR T cells. A549 exhibited similar resistance to killing by hHER2-CAR T cells. While MDA-MB-231, exhibited an intermediate level of resistance, between A549 and the sensitive cell line T47D. In terms of HER-2 expression, sensitivity to killing by hHER2-CAR T cells was correlated with ligand expression; T47D expressed the most HER2, while A549 expressed the lowest levels.

Thus, A549 appears to be generally resistant to killing by CAR T cells. Therefore, we have chosen to employ A549s for our primary screen. To avoid hits that are specific to a given CAR-ligand pairing, we will screen first with hHER2-CAR-T cells and then confirm all hits using hchNKG2Dζ T cells. Given the relative resistance of the MDA-MB-231 cells, we will use that line to corroborate hits generated with the A549 screen.

3.2.3 Developing a Method for Human CAR-T cell Expansion

Since it will take several weeks to screen the library of compounds, we will need a consistent source of CAR T cells for the screen. Unfortunately, we are working with primary T cells, which complicate matters because each day in culture can change the cells' attributes. Therefore, we would prefer to generate one batch of T cells that would be used for the entirety of the screening period to ensure consistency. The cells would need to be cryopreserved to ensure that they were always cultured for the same period. Therefore, we needed to optimize the protocol for cryopreservation and thawing.

Initially, we cryopreserved the CAR T cells in a standard freezing medium, FBS with 10% DMSO. This method yielded cells with approximately 50% viability following thaw. To increase the CAR T cells viability; we employed an alternate freezing medium that has been used to cryopreserve peripheral blood mononuclear cells with great success (RPMI 1640 containing 12.5% human serum albumin (HSA) and 10% DMSO). Using this alternate cryopreservation medium, the viability of the CAR T cells was increased to 80-90% viability following thaw. Therefore, this freezing medium was selected for further experiments.

CAR T cells proliferate following ligation of the CAR. Therefore, to increase the availability of our CAR T cells for the screen, we opted to trigger proliferation following thaw to expand the available number of T cells for the screen. Artificial antigen presenting cells (aAPC) have been constructed for such a purpose. To create an aAPC cell line, K562 (a cell line generated from an erythromyeloid cell line) were engineered to express the high affinity Fc receptor (CD64) that can be used to bind antibodies and Fc-fusion proteins to the cell surface[74,75]. The cells have also been engineered to express 4-1BBL which provide a costimulatory signal that enhances T cell proliferation[74,75], these aAPC cells are called K64-41BBL. The aAPC naturally express NKG2D ligands and the Fc receptor allows for binding of the HER2-Fc fusion, so these cells can be used to stimulate both NKG2D-CAR T cells and hHER2-CAR T cells.

To expand CAR T cells in culture, stimulatory signals must be given to them, these signals are provided to CAR T cells by stimulatory agents which are loaded onto aAPC and subsequently presented to T cells (Figure 15). The cell line K562 express a high level

of NKG2D ligands, therefore, for our hchNKG2Dζ T cell culture, K562 do not need to be loaded with stimulating agents, as these CAR T cells will receive stimulation through the CAR by interacting with the NKG2D ligands on the aAPC. CAR positivity of K562 expanded hchNKG2Dζ T cells remained similar to primary culture (>90% CAR positive cells, data not shown) and only yielded a modest expansion of 2-3 fold (data not shown).

We had the option of stimulating the hHER2-CAR-T cells with either **anti-CD3/anti-CD28** or **specific ligands against the CAR** as stimulatory agents**.** Loading aAPC with a non-specific stimulating agent such as anti-CD3 and anti-CD28 would expand out all T cells in the culture, including T cells that were not transduced with the lentivirus. The other option, loading aAPC with specific ligands, will results in expansion of only CAR positive T cells. To determine which stimulatory agent would produce the most suitable CAR T cells for screening, we used hHER2-CAR T cells and tested the two different stimulating conditions, anti-CD3/anti-CD28 loaded K64-41BBL or recombinant HER2-Fc loaded K64-41BBL. The starting culture of hHER2-CAR T cells before expansion had contained ~50% transduced hHER2-CAR T cells (Figure 16A). After co-culture with anti-CD3/anti-CD28, the culture contained 41% transduced hHER2-CAR T cells (Figure 16B). The CAR-T cell culture which received HER2-Fc loaded K64-41BBL had an enriched population, 81% hHER2-CAR positive T cells (Figure 16C). In terms of the quantity of cells, both cultures had similar cell numbers. Taken together, HER2-Fc loaded K64-41BBL aAPCs would be used for hHER2-CAR T cell expansion to ensure that the CAR T cell culture used for screening contains the highest proportion of CAR positive T cells.

Our initial studies employed CAR T cells, which were grown in medium containing FBS, however, published reports on T cell expansion on aAPC detail the use of human AB serum in culture medium[74,75]. A head to head comparison evaluating hHER2-CAR T cells growth in media containing FBS or Human AB serum[75] was completed. hHER2-CAR T cells grown in Human AB serum had a 10 fold increase in cell number over 8 days in culture, while FBS grown hHER2-CAR T cells had a 6.3 fold increase in cell number over 8 days. The hHER2-CAR exhibited slightly enhanced expression on T cell surface when grown in FBS (~77% hHER2-CAR+ cells) compared to human AB serum (~73% hHER2-CAR+ cells), data not shown**.** To test the functionality of both hHER2-CAR T cell cultures, they were stimulated with HER2-Fc for cytokine expression analysis, and co-cultured with A549 cells to examine killing capacity. 28% of hHER2-CAR T cells grown in human AB serum produced both IFN-γ and TNF-α, while only 21% of FBS grown hHER2-CAR T cells produced both cytokines (Figure 17)**.** Human AB serum grown hHER2-CAR T cells also exhibited better killing ability against A549 compared with FBS grown hHER2-CAR T cells (Figure 18). In terms of T cell viability, by trypan blue stain (DNA dye, stains dying cells blue), human AB serum grown T cells were consistently more viable compared to FBS grown T cells (data not shown). Future T cell cultures will be grown using human AB serum.

3.3 Preparing for High Throughput Screening

3.3.1. Determining Minimal FBS Required for Tumour Cell Growth

Fetal bovine serum is added to cell growth media as a growth supplement due to the richness of growth factors contained within the serum. FBS also contains a high

concentration of serum albumin, a protein which normally functions as a carrier protein by binding to a wide variety of molecules[76]. Because of albumins high affinity for molecule binding, using FBS in medium for high throughput screening may affect the availability of the chemical compounds in our screen by binding them and making them unavailable to the target cells. To mitigate this affect, we determined the lowest percentage of FBS we could use in our assay, which would have limited affect of A549 viability, and ultimately hHER2-CAR T cell killing. A549 cells were grown in 10%, 5%, 2% or 1% FBS containing cRPMI for 24 hours. This period of time is equivalent to the time cells would be allowed to attach to 96 well plate, and exposed to compound during our high throughput screening assay. hHER2-CAR T cells were added in T cell medium containing 10% serum concentration.

In terms of the fluorescence readings, A549 cells alone, grown in 10%, 5% or 2% FBS exhibited similar readings, 25 900, 24 7000, and 25 2000, respectively. However, A549 cells grown in 1% FBS containing cRPMI had lower fluorescence readings (19 000) compared to the other three cultures, indicated lower metabolic activity. Comparing the sensitivity to killing by hHER2-CAR T cells, A549 cells grown in 10%, 5% or 2% FBS exhibited similar sensitivity to killing (Figure 19). However 1% FBS grown A549 exhibited increased sensitivity to hHER2-CAR T cells (Figure 19). Based on this data, for screening A549 cells will be cultured in medium containing 2% FBS, in an effort to mitigate potentially confounding effects of the serum albumin without compromising the integrity of our assay.

3.3.2 Establishing Tumour Cell density for High Throughput Screening

It was established that 12 500 A549 cells had to be plated for the AlamarBlue assay to ensure that loss in fluorescence would mean a proportional loss in tumour cell numbers. The same experiment was completed using the instrumentation and fluorescent reader used for the high throughput screening. After plating a variety of A549 cell densities, it was established that 8 000 and 16 000 cells per well gave fluorescent readings within the linear range (RFU vs. cell density), before the signal reached saturation. 16 000 cells per well had a larger signal to noise ratio (~15.4) compared to 8 000 cells per well (~11.1), however, the RFU were much closer to the saturation point.

To determine whether 8 000 or 16 000 cells per well would produce more reproducible results; we used both of these cell densities in killing assay with hchNKG2Dζ T cells. A549 were co-cultured with different quantities of hchNKG2Dζ T cells, T cell to target ratios ranging from 0.12:1 – 8:1 in five replicates. Results showed that replicate wells with 8 000 A549 cells had variable RFU readings (certain replicates exhibited 3 fold differences), while readings from replicate wells containing 16 000 A549 cells per well were more consistent. To ensure the assay produces the most reliable results, 16 000 A549 cells per well will be plated for screening.

4.0 Discussion

The focus of this work was to develop the components for a high throughput screen to identify compounds which sensitize tumour cells to killing by CAR engineered T cells. The first phase of this research involved murine cells and later switched to human cells, as we believed that the outcomes of a screen with human cells would be more meaningful.

The CAR technology is well established in the Bramson lab and preparing the T cells required for the screen was quite straightforward. Since we chose to use tumour cell killing as the readout for the screen, we carefully considered a variety of assays for measuring tumour cell viability in a high throughput setting. We compared three different methods for measuring T cell lysis/killing of tumour cells: Cytotox96, Calcein AM and AlamarBlue. The Cytotox96 assay measures the release of the cytosolic protein LDH as a consequence of cell lysis. While the assay appeared to be sensitive, it also revealed some peculiarities. Notably, the assay indicated greater than 100% cytotoxicity of tumour cells, which is not mathematically possible unless there was a source of LDH other than the tumours cells. Since LDH is present in both the tumour cells and the CAR T cells, the assay cannot discriminate between LDH released from the tumour cells or the CAR T cells within the experimental wells. While the assay does take into consideration the level of LDH released by CAR T cells themselves in non-experimental wells, these controls do not compensate for the larger LDH release from CAR T cells which may occur if the tumour cells in co-culture are inducing apoptosis in the CAR T cells. It has been established in the literature that as a means to ensure survival, tumour cells establish a counter attack against the immune system. One particular mechanism used by tumour

cells is the expression of Fas ligand, which when interacting with the Fas death receptor on the target cell (T cells in this case), can induce apoptosis[77]. It is possible that the D2F2/E2 tumour cells in this assay may have established a counter attack against the CAR T cells, resulting in a release of LDH by the CAR T cells in experimental wells. This release was much larger than the maximum release by tumour cells alone, resulting in the greater than 100% cytotoxicity.

Similar to Cytotox96, Calcein AM assay also measures tumour cell lysis based on release of an internal dye. However, unlike the Cytotox 96 assay that measures release of LDH, the Calcein AM assay relies upon labeling target cells with a fluorescent dye (Calcein AM). In this way, the only source of Calcein AM is the target cell, so the assay directly measures target cell lysis. Despite this attractive feature, we observed that the Calcein AM assay was quite insensitive. One of the major problems with the Calcein-AM assay was the spontaneous release of the dye by the tumour cells. Furthermore, the spontaneous release displayed day-to-day variability; anywhere between 15-35% spontaneous release was observed using the same cell line on different days. This high spontaneous release of the Calein AM resulted in a low signal:noise which likely explains the low sensitivity. As a result of these undesirable properties, we chose not to pursue this method for our high throughput screen.

Unlike the previous two assays which measure tumour cell lysis, AlamarBlue measures the metabolic activity of tumour cells. Since the CAR T cells are non-adherent, we developed an assay method where the CAR T cells were washed off the wells following the co-culture period and the AlamarBlue was used as a measure of the viable

cells remaining on the dish. The AlamarBlue method demonstrated very good signal:noise and killing did not exceed the theoretical maximum of 100%. We also found that the assay displayed low intra-experimental variability. While this assay does not measure lysis, per se, we believe it is a valid method for high throughput screening since the viability of the tumour cells following exposure to the CAR T cells is ultimately the most important parameter.

Initially, we cultured the tumour cells in the presence of AlamarBlue overnight following removal of the T cells. This time frame was chosen for convenience. Under this condition, we found that pre-exposure to apparently non-toxic doses of gemcitabine could greatly sensitize the murine 4T1.2 cell line to killing by mchNKG2Dζ T cells. We adapted the assay to the high throughput screening equipment and found an excellent Z'. We subsequently conducted a pilot screen and identified two compounds that appeared to sensitize the cells. Validation experiments failed to corroborate the screen. In parallel, we evaluated the optimal time frame for incubating the tumour cells with AlamarBlue and discovered that 3 hours was sufficient to achieve a strong, linear signal. Surprisingly, when we employed a shorter incubation time with the AlamarBlue, we discovered that the doses of gemcitabine that we had been using were actually toxic to the 4T1.2 cells and that there was little evidence of sensitization to CAR T cell killing. While we do not know the exact reason for this discrepancy, it is likely due to cellular growth in the overnight period as murine tumour cells divide very quickly. Regardless of the exact reason for the discrepancy, we decided to incubate the target for 3 hours in the presence of AlamarBlue for all future experiments to avoid further artifacts.

Following the studies to optimize the high throughput killing assay, we decide to adapt the murine system to human cells. We also decided to use two different CARs in our screen to avoid chemical agents that may influence killing in CAR-specific ways (ex. chemical agents that up regulate NKG2D ligands may be of value to hchNKG2Dζ T cells, but would not be of general value for CAR T cell therapies). To develop a screen with human cells, we required an appropriate human tumour cell that was resistant to CAR T cell killing. After screening nine human tumour cell lines, we determined that the non-small cell lung carcinoma cell line A549 was the most resistant cell line to killing by both hchNKG2Dζ T cells and hHER2-CAR T cells despite the expression of both HER2 and NKG2D ligands. Interestingly, A549 cells have a higher expression of NKG2D ligands compared to MCF-7, NCI-H460, and MDA-MB-231, but still remained more resistant to killing by hchNKG2Dζ T cells, this indicates that A549 cells must exhibit defects in apoptosis pathways, and its resistance to killing is beyond CAR T cell detection. Indeed, others have shown that A549 are resistant to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), an apoptosis inducing protein released by T cells[78], Ikediobi *et al* had completed sequence analysis of 24 known genes associated with cancer in the NCI 60 panel of tumour cells. They showed that A549 cells had mutation in cyclin-dependent kinase inhibitor 2A, Serine/threonine kinase 11 and KRAS[79]. The oncogene KRAS plays a role in the inability of certain therapies to induce apoptosis in KRAS-mutant non-small cell lung carcinoma cells[80]. KRAS may just be a single example of cancerous related mutation resulting in an acquired resistance to apoptosis induction.

Successful execution of the screen with human T cells requires a consistent batch of CAR T cells. Since screening the chemical libraries (Prestwick chemical library, BIOMOL natural products library, Sigma's LOPAC, and MicroSource spectrum collection) will require millions of CAR T cells, we decided to produce the T cells in a 2-step process. A single batch CAR-engineered T cells was produced and cryopreserved. The cryopreserved cells would then be used as a source of cells for each screen to ensure consistency between screens conducted on different days. This strategy requires optimization of the freezing process and the thawing process. FBS is commonly employed for cell cryopreservation, due to the relatively low cost. However, I determined that cells frozen in human serum albumin displayed better recovery following thaw than those preserved in FBS consistent with the observations made in a large scale study in 2012 across 31 labs, guided by the Cancer Immunotherapy Immunoguiding program. The study compared the use of serum and serum-free cryopreservation methods of human PBMC[81]. It has been suggested that the reason for albumins optimal cryopreservation ability lies within its ability to reduce cell swelling during cell storage. Albumin increases oncotic pressure (a pressure exerted by proteins) of the solution, leading to cell dehydration[82].

We examined the different strategies for expanding the CAR T cells using hHER2-CAR T cells. We compared two strategies: in one strategy the K64-41BBL were loaded with anti-CD3 and anti-CD28 to trigger proliferation of all T cells in the culture (both CAR-positive and CAR-negative), in the other strategy we loaded the K64-41BBL cells with HER-2-Fc to trigger proliferation of only hHER2-CAR-enginered T cells. While both strategies successfully promoted expansion of the T cell cultures, we chose to

use the specific activation method where the aAPCs were loaded with HER2-Fc for future work as this method resulted in an enrichment of the CAR-expressing T cells. A T cell culture with a low percentage of CAR T cells will inevitably perform poorer in a killing assay against a tumour cell line, presenting a misleading state of tumour resistance against the T cells. Choosing an enriched T cell culture for CAR engineered T cells will ensure that any lack of killing we observe is because of the tumour, not just because the T cells we are using do not express the CAR, and are therefore unable to lyse tumour cells.

We also evaluated the impact of serum on the CAR T cells - aAPC co-culture. We compared the commonly used FBS to Human AB serum[83]. hHER2-CAR T cells grown in Human AB serum exhibited a larger expansion in culture, produced more cytokines when exposed to antigens, enhanced cytotoxic activity against A549 cells, and had better viability throughout the 8 days in culture, compared to FBS grown hHER2-CAR T cells. A study by Dias *et al* examined the cytokine profile of human eosinophils when exposed to LPS when cultured in either human serum (autologous) or FBS. They noted that eosinophils grown in FBS had decreased cytokine production relative to eosinophils grown in human serum[84] similar to our observations with T cells. It is possible that human white blood cells respond better to human derived growth factors as compared to bovine derivatives, and thus produce more functional white blood cells.

5.0 Conclusion

With the variable success of cancer immunotherapies in the clinics, there is a need for the development of therapeutics to increase the effectiveness of the immune system attack on the tumour. Administration of a drug, that overcomes tumour apoptosis resistance mechanisms, prior to administration of an immunotherapy, would increase the efficacy of these treatments allowing for more successful outcomes.

The work completed here has led to the establishment of an assay to be used to screen test compounds for activity as tumour sensitizers. Starting with murine derived components, we established the assay protocol we would use to screen compounds.

While we had successfully started screening using murine cells, finding compounds with sensitizing activity against human tumour cells is more clinically relevant. The main challenge to using human derived components for screening was the generation of CAR engineered T cells that were phenotypically and functionally similar with every batch created. Here, we have developed a protocol encompassing, the generation of CAR T cells, the freezing and storage of CAR T cells and expansion on aAPC. This method allows us to prepare a single batch of CAR T cells, and use this batch to screen all 3900 test compounds. We have shown that with each expansion of a single aliquot of CAR T cells from the same initial batch, CAR T cell lysis of A549 cells is comparable, exemplifying the reproducibility of protocol.

Our future work will encompass examining the translation of human derived components into our high throughput screening assay by completing a Z' analysis. Based on our current work, we suspect to attain a high Z' value. We will than screen all

compounds from 4 compound libraries, the Prestwick chemical library, Sigma LOPAC, the spectrum collection (Microsource) and Screen-Well natural products library (Enzo Life Science).

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7.0 Figures

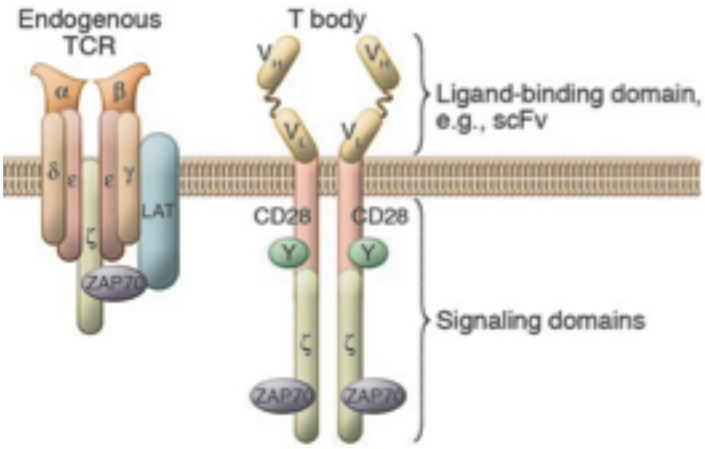


Figure 1: Comparison of the Structure of a Chimeric Antigen Receptor (CAR) and the T cell Receptor (TCR). CARs contain a ligand(antigen) binding domain, fused to intracellular signaling components, which mimic those found in the TCR complex. **Adapted from: June, C. Adoptive T cell therapy for cancer in the clinic. *Journal of Clinical Investigation*.2007; 117(6).**

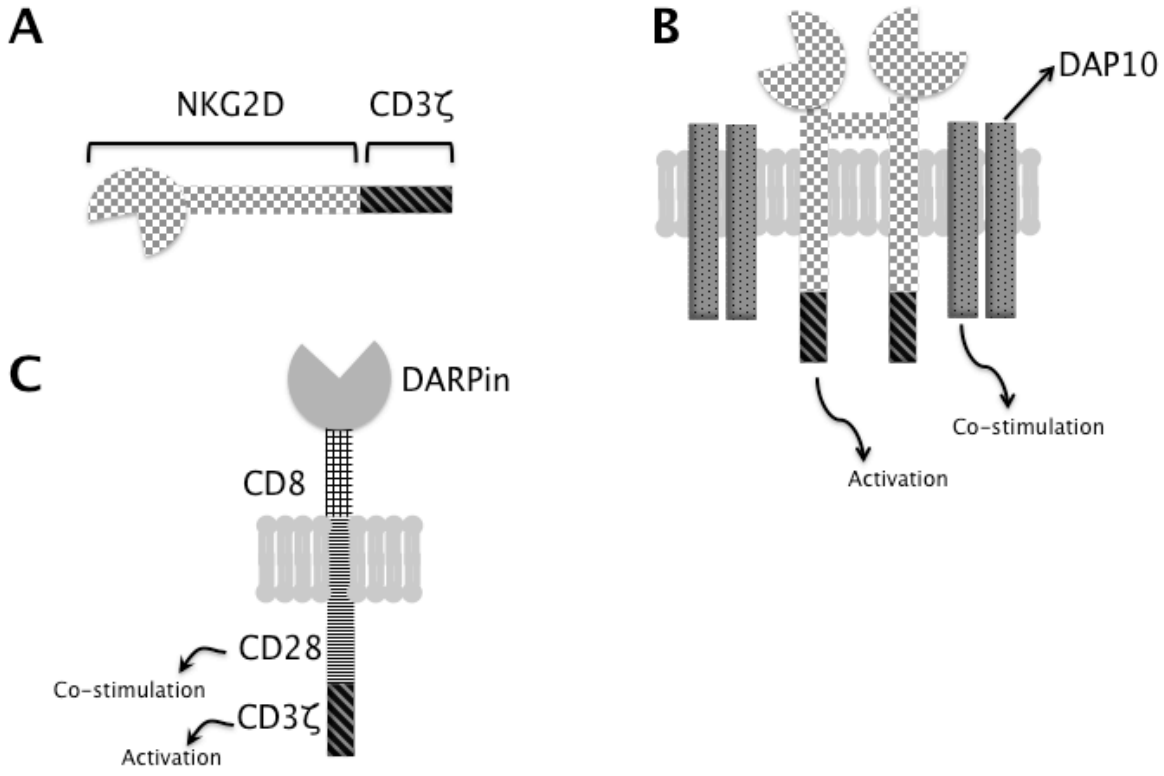


Figure 2: Schematic of all CAR constructs. A) Schematic of the constructs for both the murine and human NKG2D CAR. The fused CD3ζ protein in the CAR is only the intracellular portion of CD3ζ. **B)** Schematic of CAR configuration at cell membrane and signaling components of the murine and human NKG2D CAR. The signals provided to the T cell upon ligation of the chNKG2Dζ CAR are provided as follows, chNKG2Dζ CAR provides activation signals while endogenous DAP10 (necessary to stabilize NKG2D at cell surface) provides co-stimulation. **C)** Schematic of the hHER2-CAR. The DARPin protein is responsible for antigen binding, and is connected to the CD8 protein hinge (allows for CAR flexibility). CD28 and CD3ζ are intracellular and provide stimulatory signals.

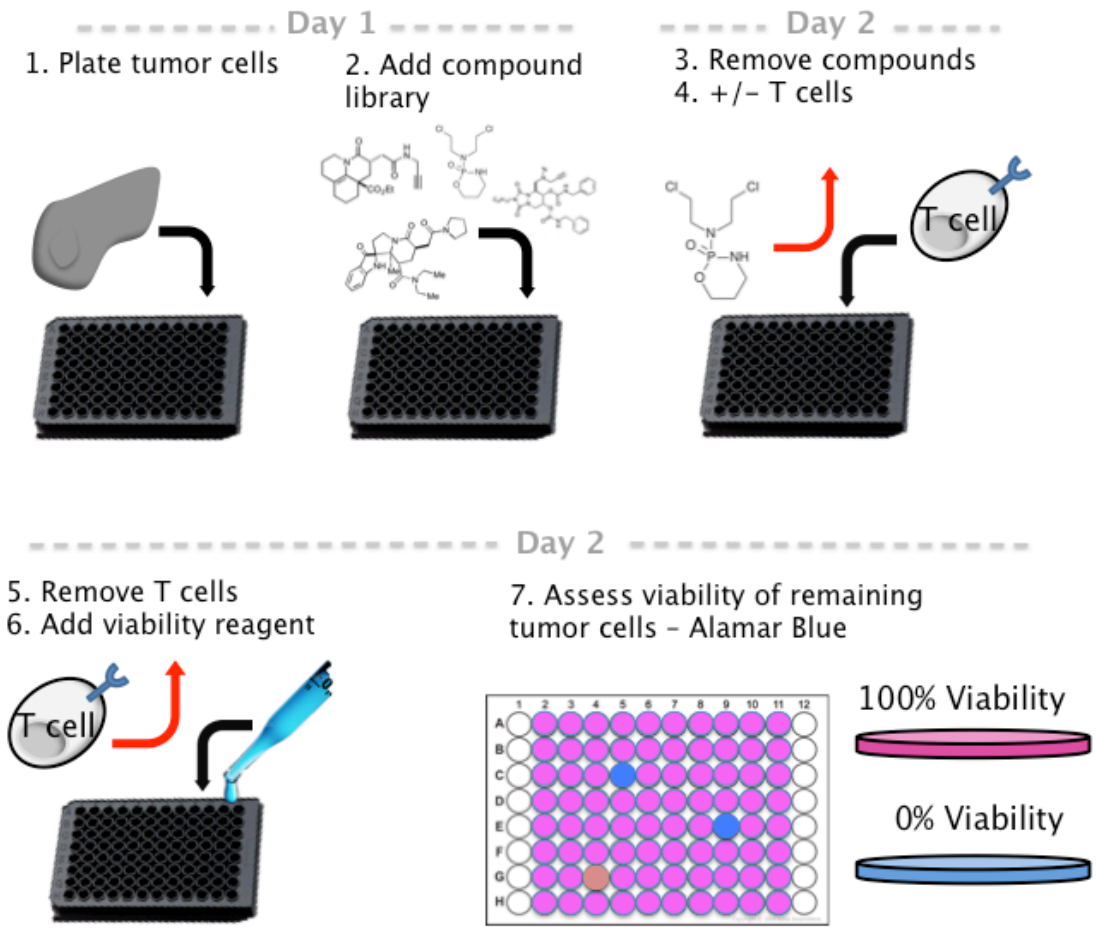


Figure 3: Overview of High throughput screening assay. 1) Tumour cells are plated in black flat bottom 96 well plates at a pre-determined density. 2) 6 hours later a single 10uM compound is added to each well. 3) After 18 hours of exposure to compounds, compounds are removed. 4) CAR T cells or media (to determine compound toxicity) are added to each well. 5) After 6 hours of co-culture, T cells are removed from the plate by 3 washes with PBS. 6) 10% AlamarBlue viability reagent is added to each well to determine the amount of viable tumour cells remaining. 7) After 3 hour incubation with AlamarBlue, fluorescence is read from the plates and data is analyzed.

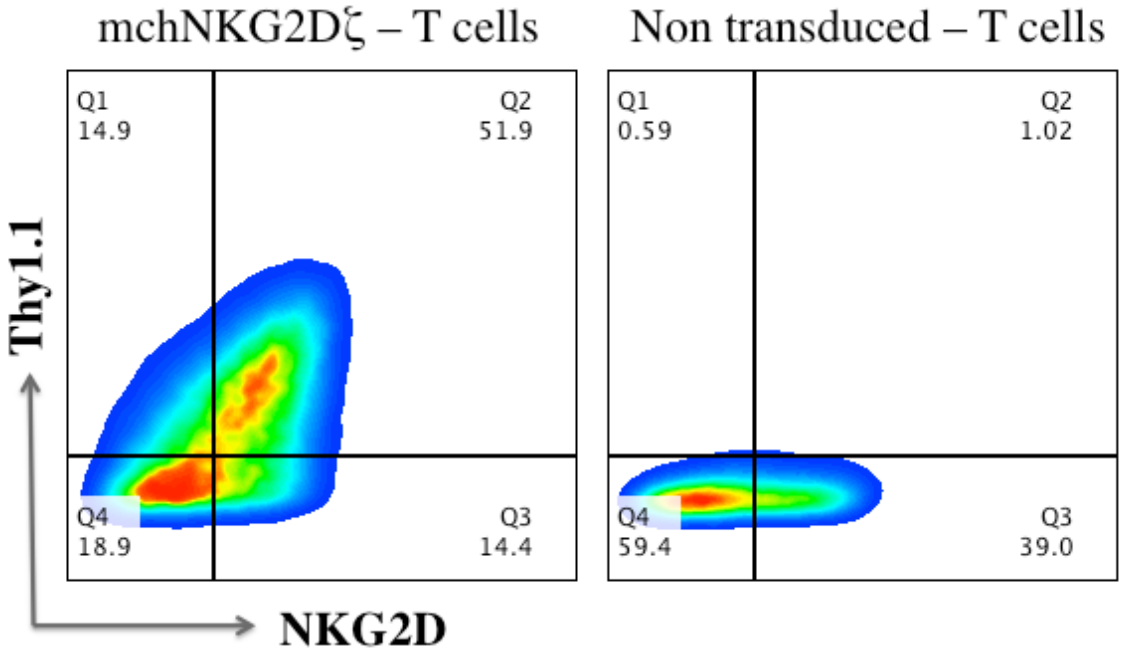


Figure 4: Phenotype stain of mchNKG2Dζ CD8$^+$ T cells and non transduced CD8$^+$ T cells. Using flow cytometry, we have shown that mchNKG2Dζ CAR is expressed on the cell surface, and had a transduction efficiency of 66.8%, with a total of 66.3% of T cells expressing NKG2D (both endogenous and CAR). Non transduced T cells contain a population (only 40%) which express endogenous NKG2D. Gating was used to select on CD8$^+$ CD4 $^-$ T cells.

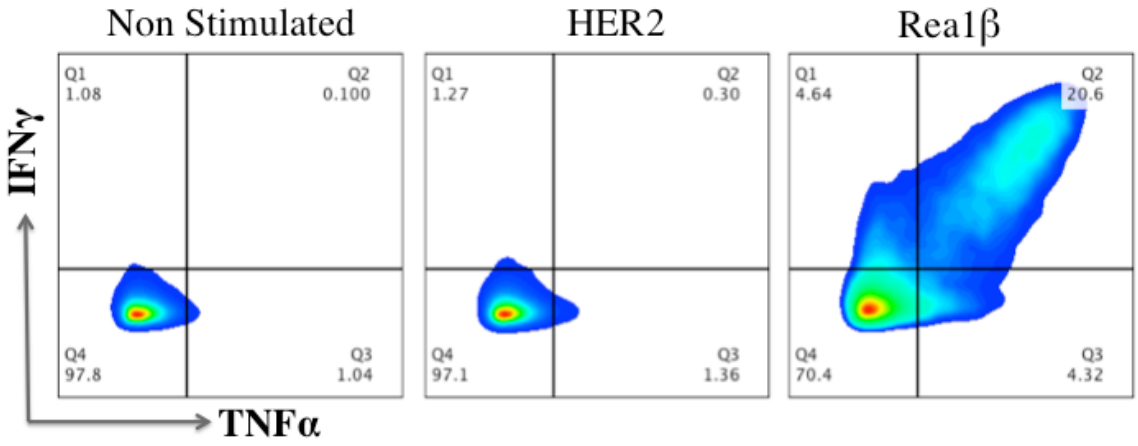


Figure 5: Functional stimulation of mchNKG2Dζ CD8⁺ T cells with plate bound ligand. mchNKG2Dζ T cells were stimulated with Rae1β (specific ligand) and HER-2 (negative control), or not stimulated at all for 4 hours and cytokine production was examined by flow cytometry. mchNKG2DζT cells were able to produce both TNFα and IFNγ in response to Rae1β but not HER2. Gating was used to select only $CD8^+$ $CD4^-$ T cells.

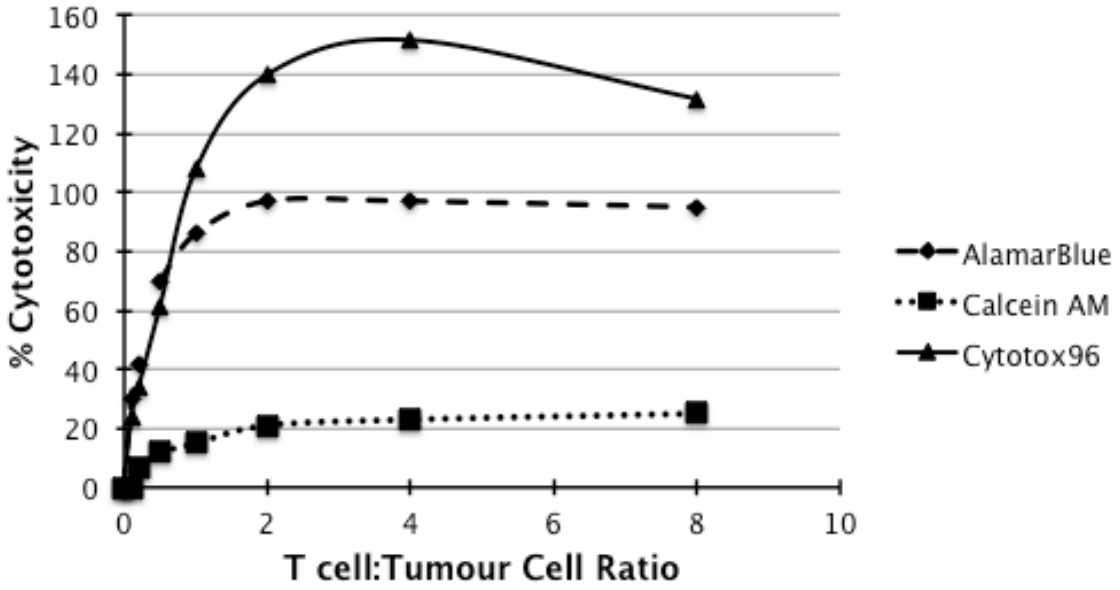
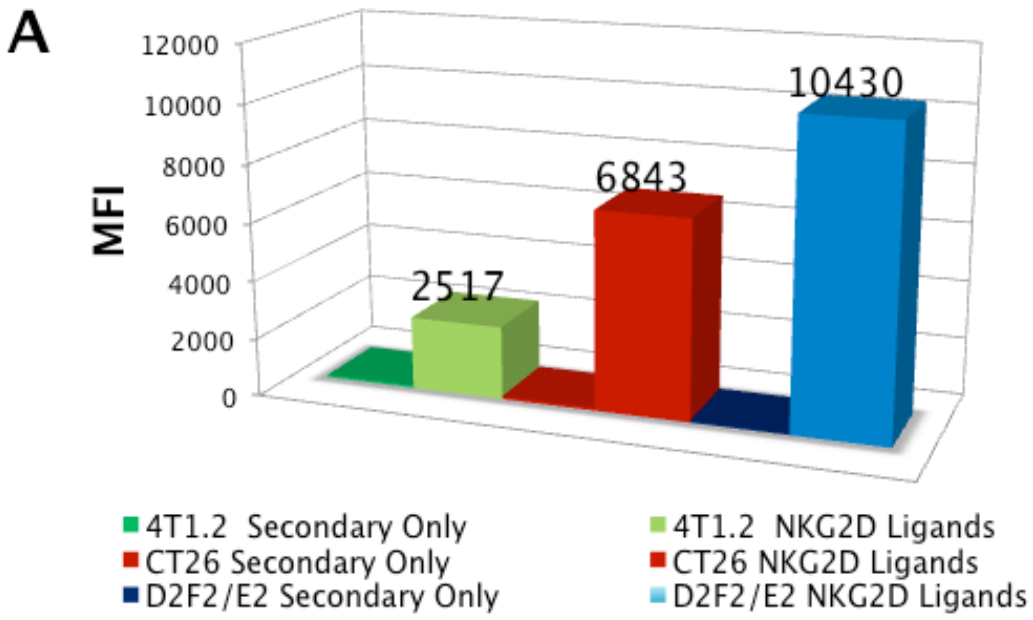


Figure 6: Comparison of three different assays to detect tumour cell death induced by CAR T cells. AlamarBlue, Calcein AM and Cytotox96 were used to detect the killing capacity of mchNKG2Dζ T cells against murine breast tumour cell line D2F2/E2 cells line. Each killing assay measures T cell killing differently, using % cytotoxicity (y-axis) vs. T cell:tumour cell ratios (x-axis) analysis, we show that each killing assay gave different readings on tumour cell death.


A
12000
10000
8000
6000
4000
2000
0
MFI
2517
6843
10430
4T1.2 Secondary Only
CT26 Secondary Only
D2F2/E2 Secondary Only
4T1.2 NKG2D Ligands
CT26 NKG2D Ligands
D2F2/E2 NKG2D Ligands

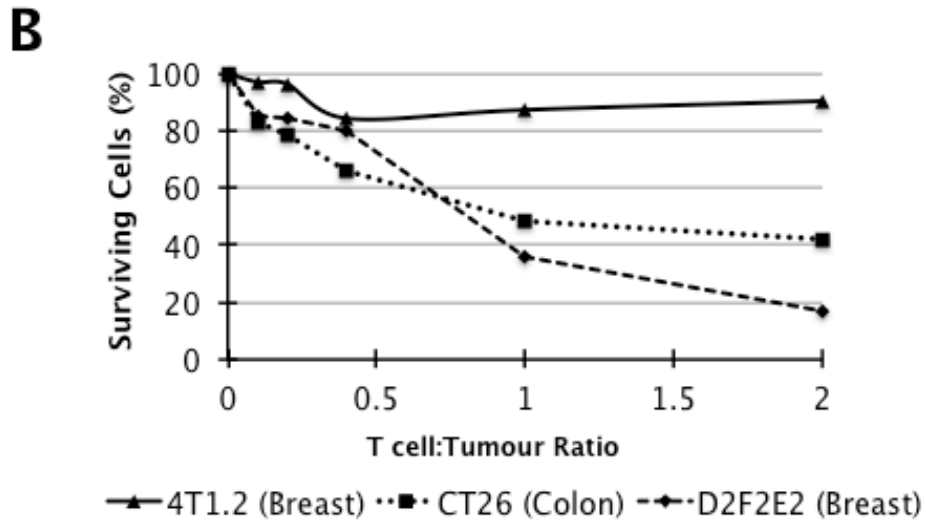

B
Surviving Cells (%)
100
80
60
40
20
0
0
0.5
1
1.5
2
T cell:Tumour Ratio
4T1.2 (Breast)
CT26 (Colon)
D2F2E2 (Breast)

Figure 7: Tumour cells exhibit differential sensitivity to mchNKG2Dζ T cell attack. A) Three tumour cell lines were tested for their sensitivity to mchNKG2Dζ T cell attack. D2F2/E2 are most sensitive, 4T1.2 are most resistant and CT26 lie in the middle. **B)** Tumour cell lines were stained (flow cytometry) for NKG2D ligands and it was shown that D2F2/E2 express the most, while 4T1.2 express the least (based on MFI).

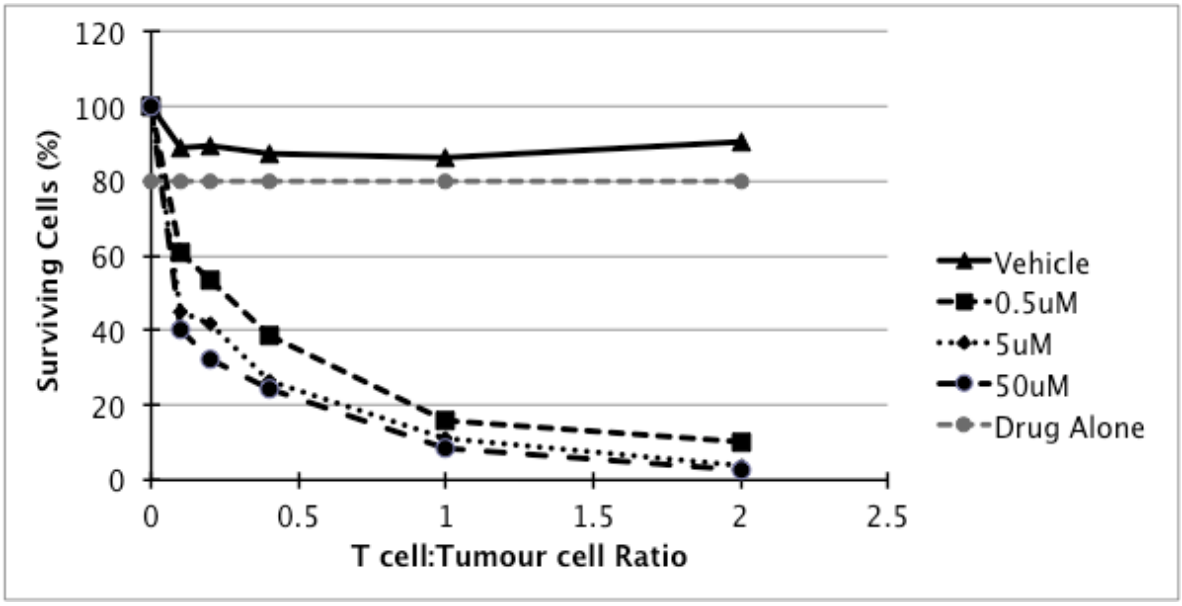


Figure 8: Sensitization of 4T1.2 tumour cells using Gemcitabine. 4T1.2 tumour cells were exposed to 0.5uM, 5uM or 50uM gemcitabine for 18 hours prior to exposure to mchNKG2Dζ T cells for 6 hours (gemcitabine removed prior to co-culture). Prior exposure to gemcitabine with low toxicity (50uM kills ~20% 4T1.2) renders 4T1.2 tumour cells more susceptible to killing by mchNKG2Dζ T cells.

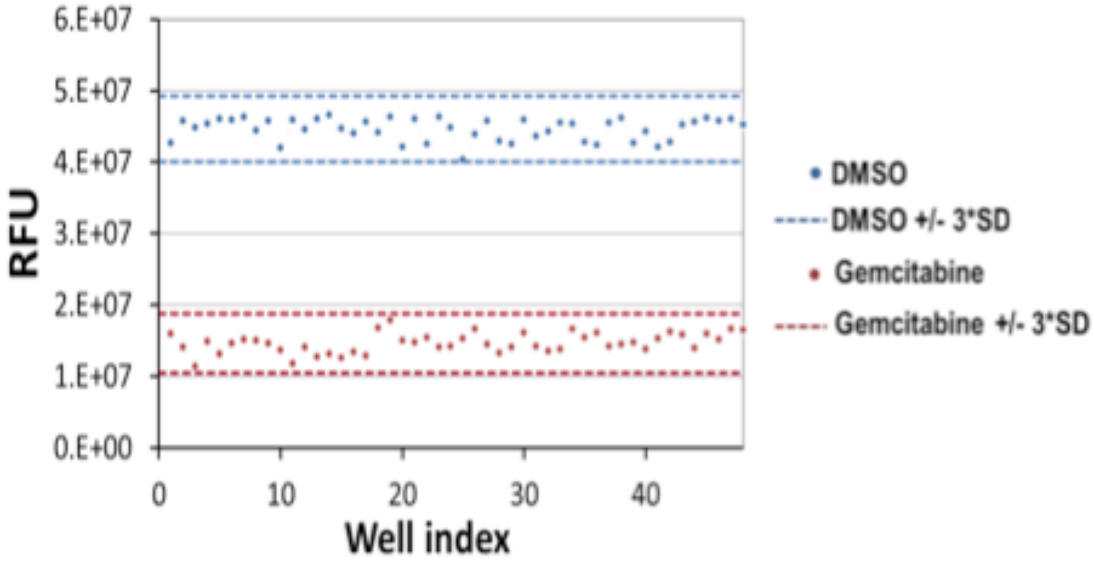


Figure 9: HTS results from initial Z' experiment. In our initial experiment to determine if our AlamarBlue assay is suitable for a high-throughput screen, we generated a Z' of 0.71 which is excellent. The figure depicts a high signal (DMSO) and a los signal (Gemcitabine) and 3 units of standard deviation. Figure and data generated by Cecilia Murphy.

A

	With T-cells			Without T-cells		
IDs	Ave. RFU of DMSO (high control)	Ave. RFU of gemcitabine (low control)	Avg. RFU with library cmpd	Ave. RFU of DMSO (high control)	Ave. RFU of gemcitabine (low control)	Avg. RFU with library cmpd
134	44,697,410	14,673,316	3,686,971	45,858,759	30,292,648	46,323,954
142	44,697,410	14,673,316	3,686,971	45,858,759	30,292,648	1,770,123
215	44,697,410	14,673,316	3,921,590	45,858,759	30,292,648	46,293,861
223	44,697,410	14,673,316	3,921,590	45,858,759	30,292,648	1,735,726

B

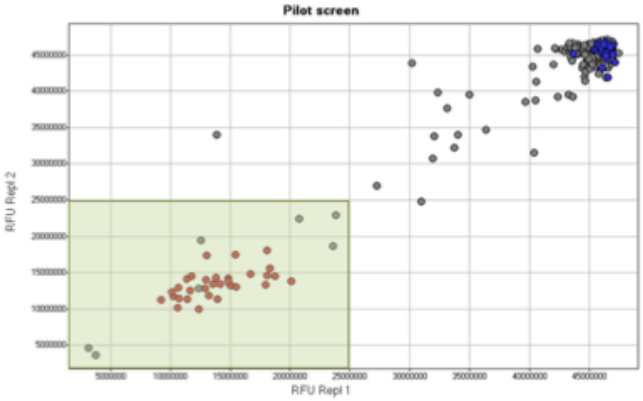


Figure 10: First HTS using 300 compounds and mchNKG2Dζ T cell produced four potential hits. A) Data profiling the response from the 4 hits from the pilot HTS. Each compound was tested in the absence of T cells, and with T cells. This way those compounds which are just toxic can be sorted out from those which sensitize. **B)** Red dots are the low signal control gemcitabine; blue dots are the high signal control (DMSO) while the grey dots are test compounds. The four hits produced better sensitization in comparison to gemcitabine (red dots). These hits were tested in a non-high-throughput killing assay but did not produce the same effect. Figures and data generated by Cecilia Murphy.

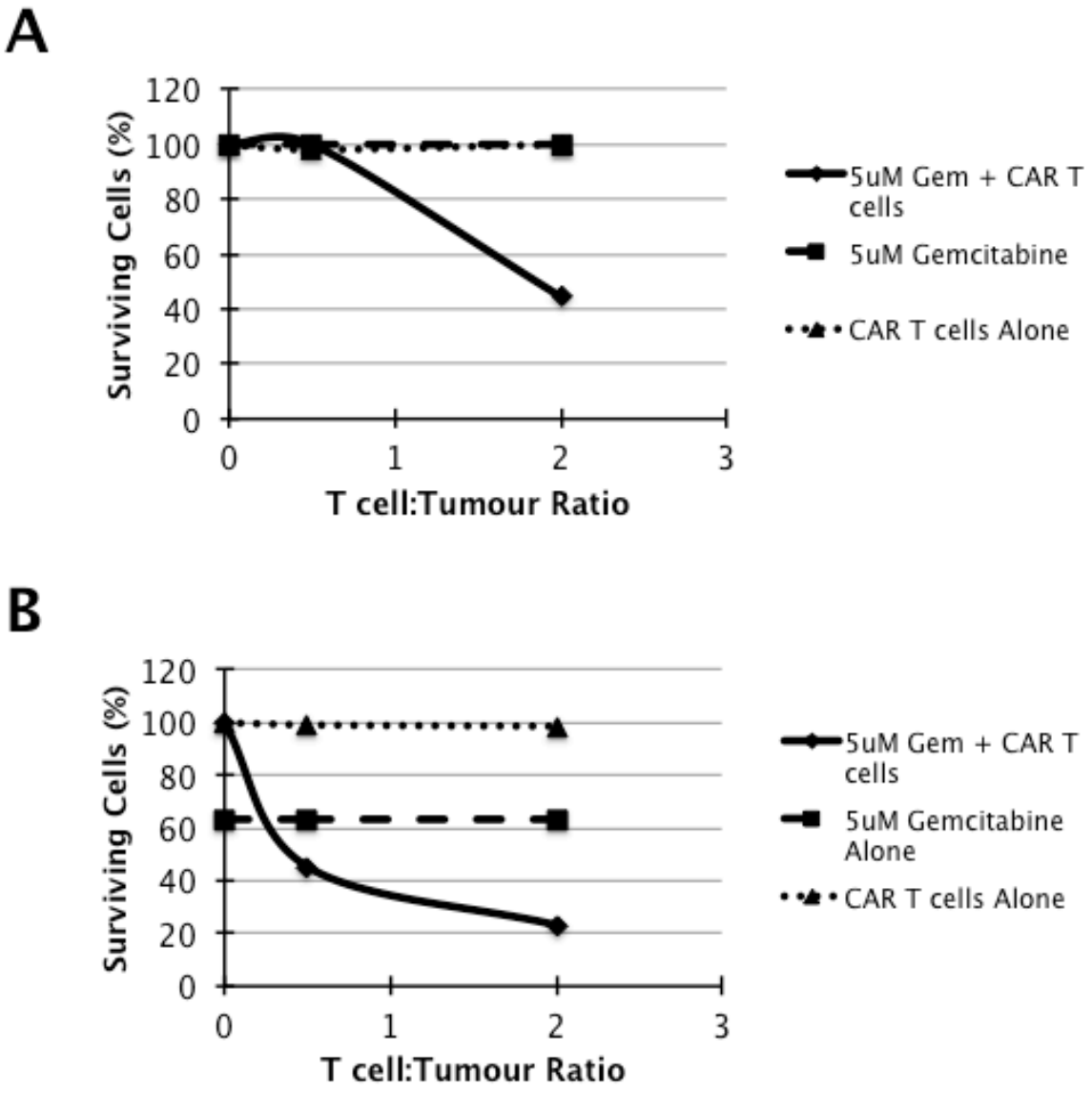


Figure 11: Sensitization Assays with 5uM Gemcitabine: Varying the AlamarBlue Incubation Time. Changing the AlamarBlue incubation time from overnight to 3 hours yielded significantly different results, in terms of % viability of tumour cells. **A)** Pretreatment of 4T1.2 cells with 5uM gemcitabine was determined to be non-toxic using an overnight AlamarBlue assay. **B)** However, the 3 hour AlamarBlue assay showed that 5uM gemcitabine was more toxic to 4T1.2 cells than originally thought. Because of overnight growth of tumour cells, the toxicity of gemcitabine was masked. For future high throughput screening, our assay will use a 3 hour AlamarBlue incubation.

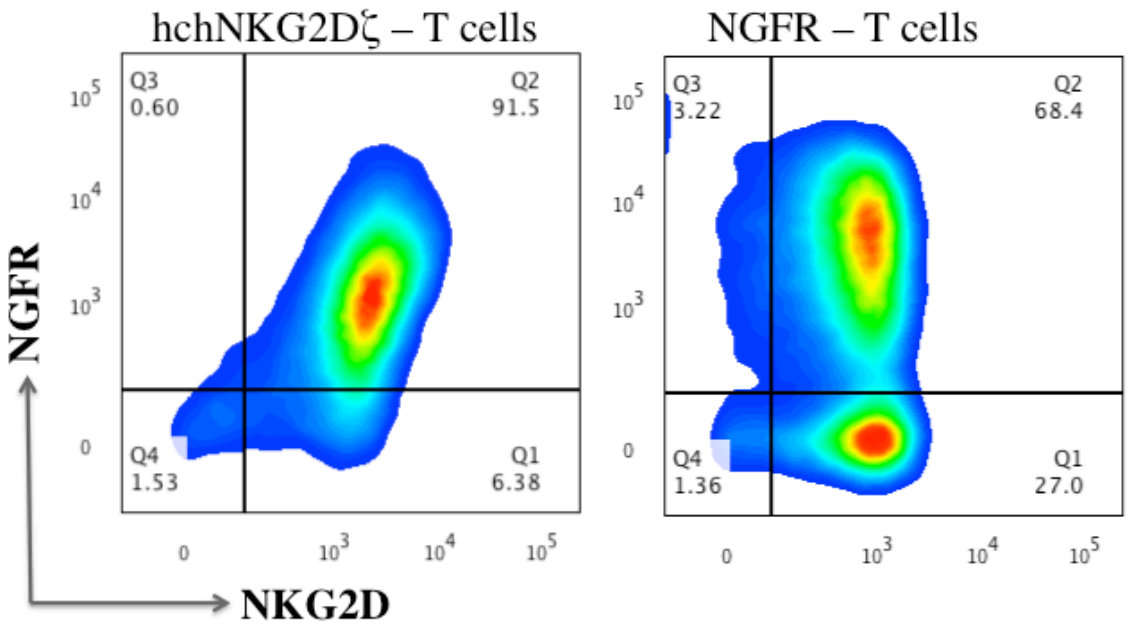


Figure 12: Phenotype stain of hchNKG2Dζ T cells and NGFR T cells. Using flow cytometry, we have shown that hchNKG2Dζ CAR is expressed on the cell surface, and had a transduction efficiency of 92% (left panel). NGFR transduced T cells exhibit 71% transduction efficiency (right panel). Based on the MFI of NKG2D, T cells transduced with hchNKG2Dζ have a higher MFI compared to NGFR transduction, indicating hchNKG2Dζ is expressed on the cell surface.

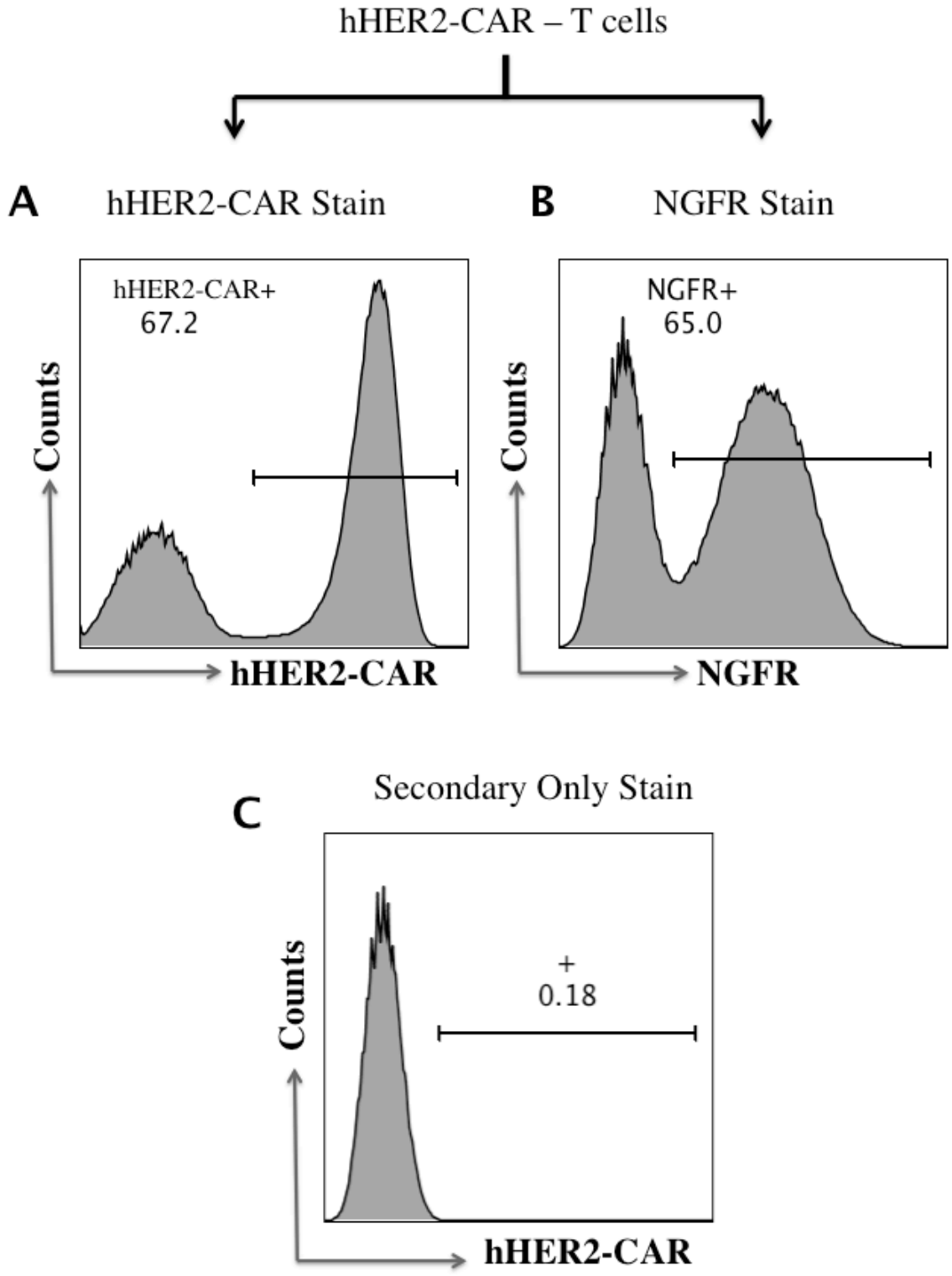

hHER2-CAR – T cells
A
hHER2-CAR Stain
hHER2-CAR+
67.2
Counts
hHER2-CAR
B
NGFR Stain
NGFR+
65.0
Counts
NGFR
C
Secondary Only Stain
+
0.18
Counts
hHER2-CAR

Figure 13: Phenotype stain of hHER2-CAR T cells. Using flow cytometry, we have shown that T cell transduced with hHER2-CAR exhibit a **A)** 67.2% population of T cells expressing the CAR, and **B)** 65% of T cells expressing NGFR. **C)** A two stage staining process is required to detect the hHER2-CAR. Recombinant HER2-Fc protein was used to detect the hHER2-CAR, and a generic antibody (IgG-PE), which binds the Fc portion of HER2-Fc, was used to detect the CAR. To ensure IgG-PE is not binding background proteins, a secondary only control was completed, and did not show background staining.

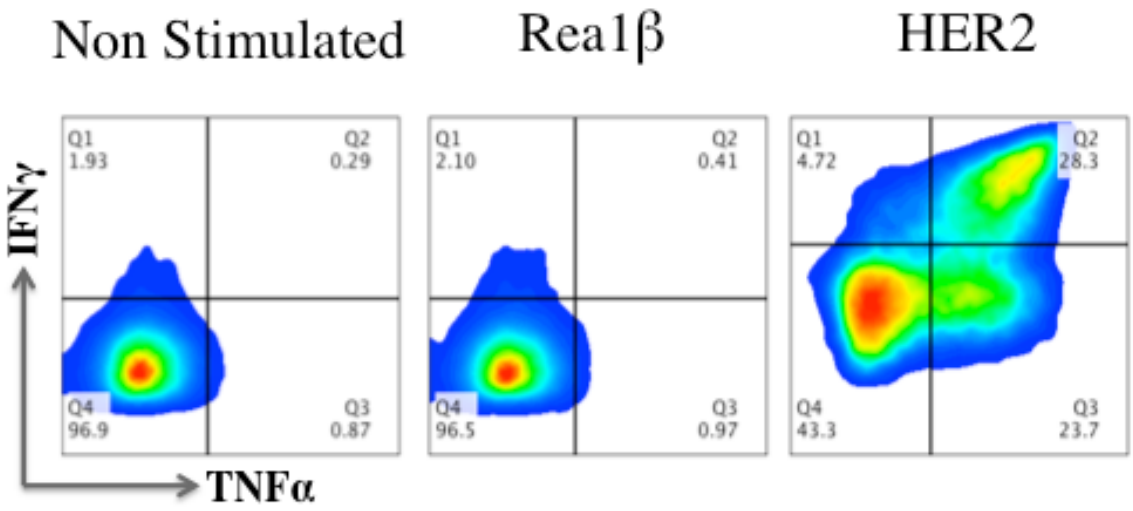


Figure 14: Functional stimulation of hHER2-CAR T cells with plate bound ligand. hHER2-CAR T cells were stimulated with Rae1β (specific ligand) and HER-2 (negative control), or not stimulated at all for 4 hours and cytokine production was examined by flow cytometry. hHER2-CAR T cells were able to produce TNFα and IFNγ in response to HER-2, but not Rae1β.

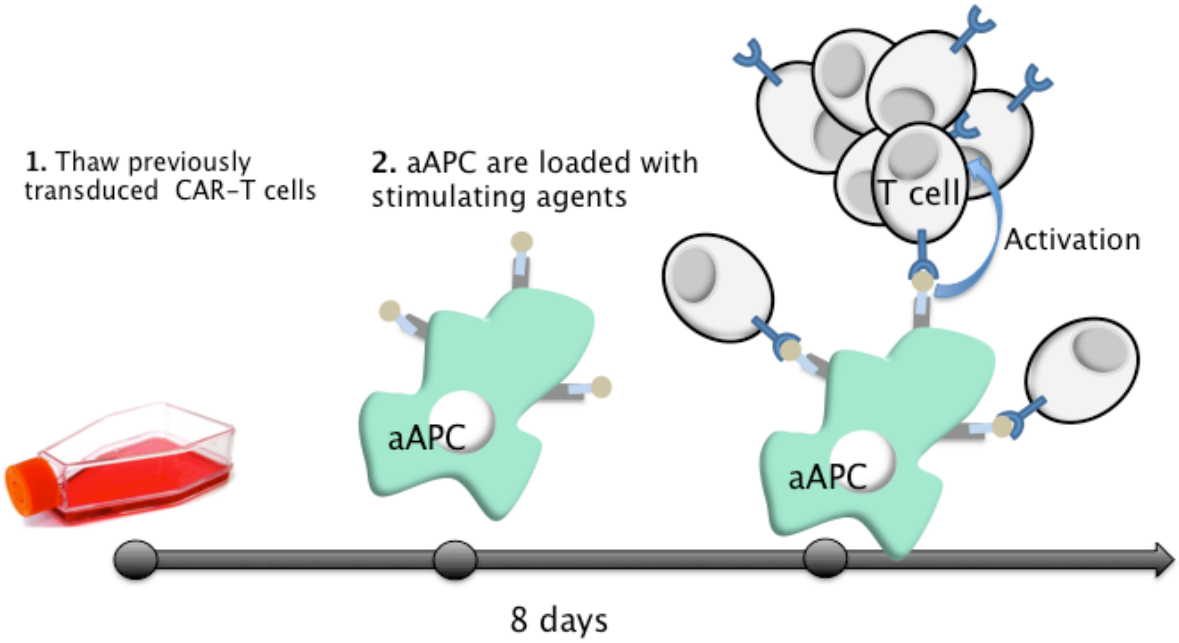


Figure 15: Expansion protocol for CAR T cell expansion on aAPC. Previously transduced CAR T cells are thawed and co-cultured at a ratio of 2:1 (T cells:aAPC) with aAPC. aAPC were loaded with stimulatory agents, either anti-CD3/anti-CD28 or CAR specific ligands, prior to co-culture. After 8 days in culture, expanded CAR T cells were used for experiments.

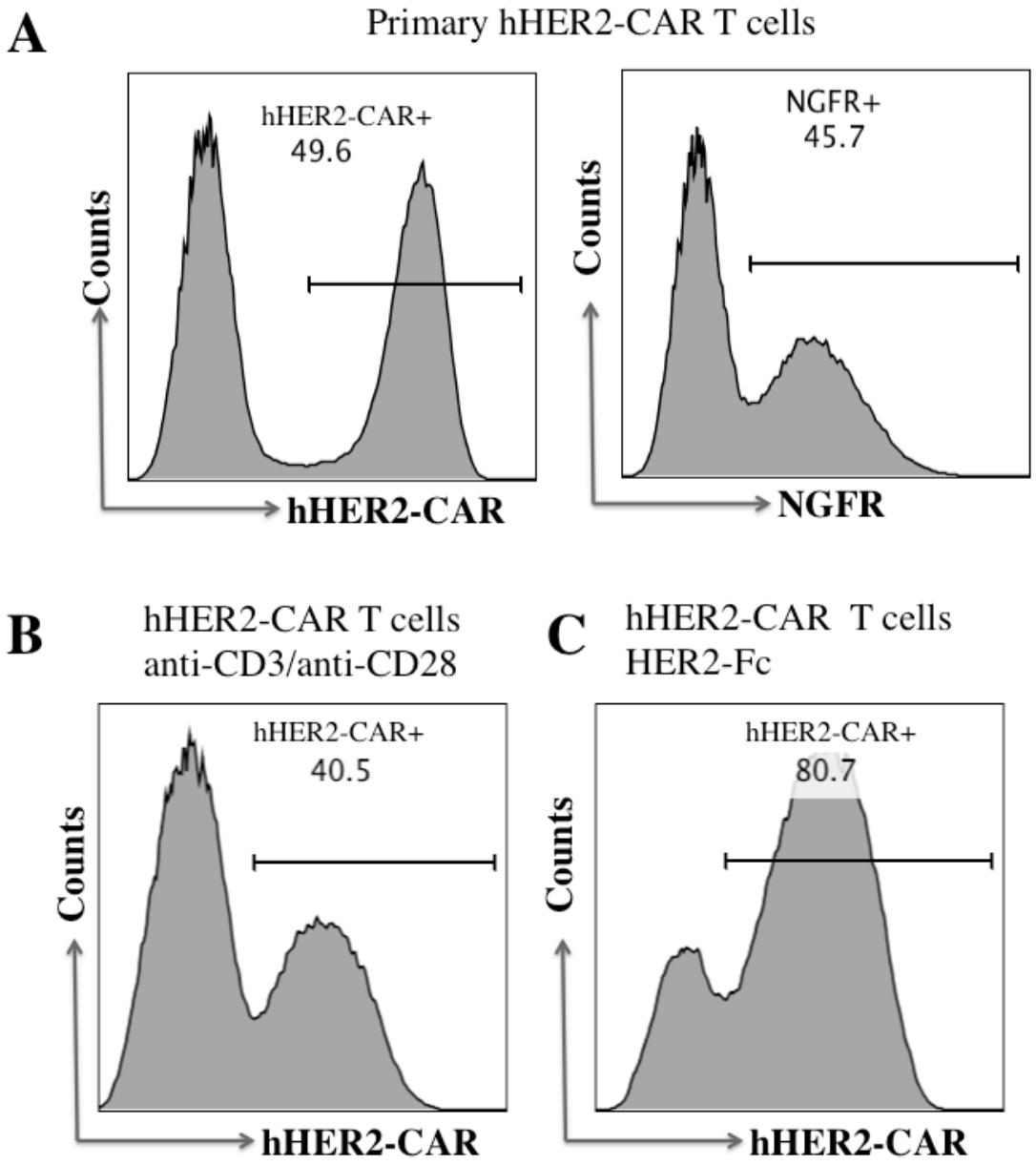


Figure 16: Phenotype analysis hHER2-CAR T cells expanded on aAPC. Using Flow cytometry, we examined the expression level of the hHER2-CAR. **A)** Prior to co-culture with aAPC, hHER2-CAR T cells were ~50% positive for the CAR (hHER2-CAR+). **B)** hHER2-CAR T cells expanded on aAPC loaded with anti-CD3/anti-CD28, resulted in 40% T cells expressing the CAR. **C)** hHER2-CAR T cells expanded on aAPC loaded with HER2-Fc, resulted in ~81% T cells expressing the CAR.

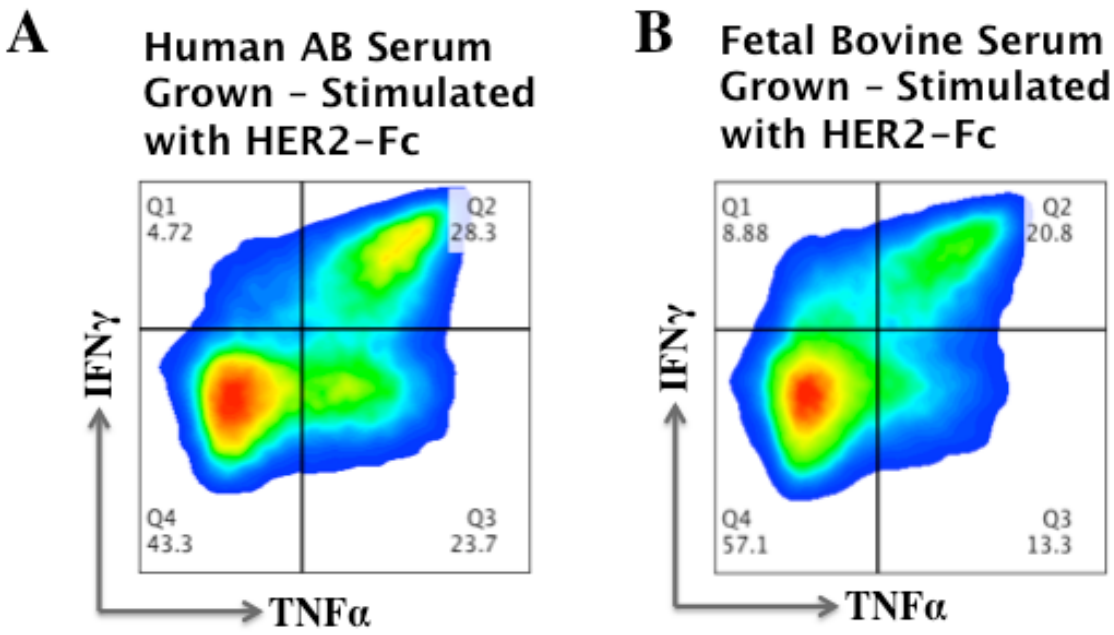


Figure 17: Functional stimulation of hHER2-CAR T cells grown in either FBS or human AB serum with plate bound ligand. hHER2-CAR T cells were expanded on aAPC (loaded with HER2-Fc) and grown for 8 days in medium containing either **A)** human AB serum or **B)** FBS. After 8 days, hHER2-CAR T cells were stimulated with HER2-Fc for 4hours. hHER2-CAR T cells grown in human AB serum produced more TNFα and IFNγ compared to FBS grown hHER2-CAR T cells.

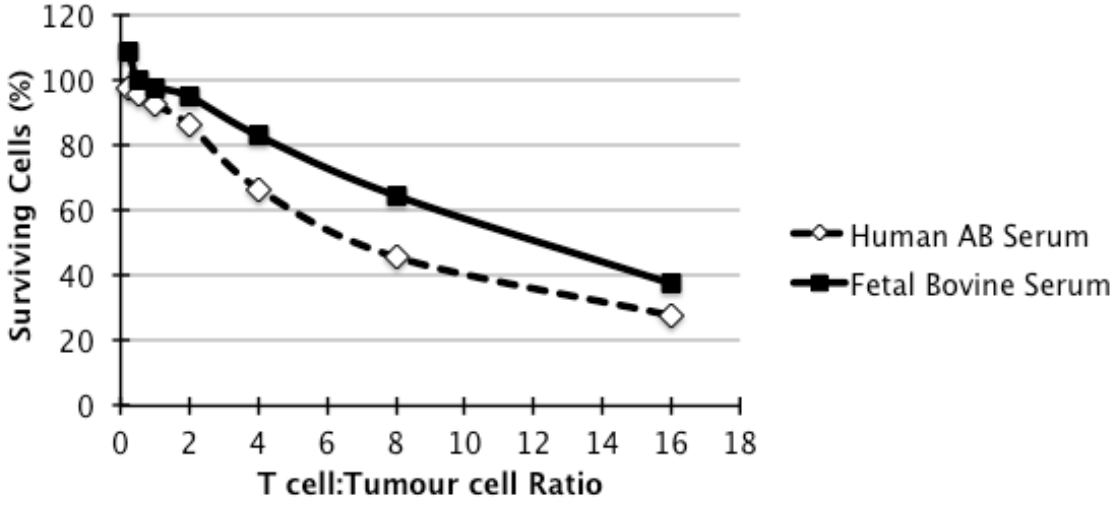


Figure 18: Analysis of differential media composition on killing ability of hHER2-CAR T cells. To test the killing capacity of both T cell cultures, A549 tumour cells were co-cultured with T cells for 6 hours, prior to analysis using AlamarBlue. hHER2-CAR T cells grown in human AB serum exhibited better killing of A549 tumour cells compared to FBS grown hHER2-CAR T cells.

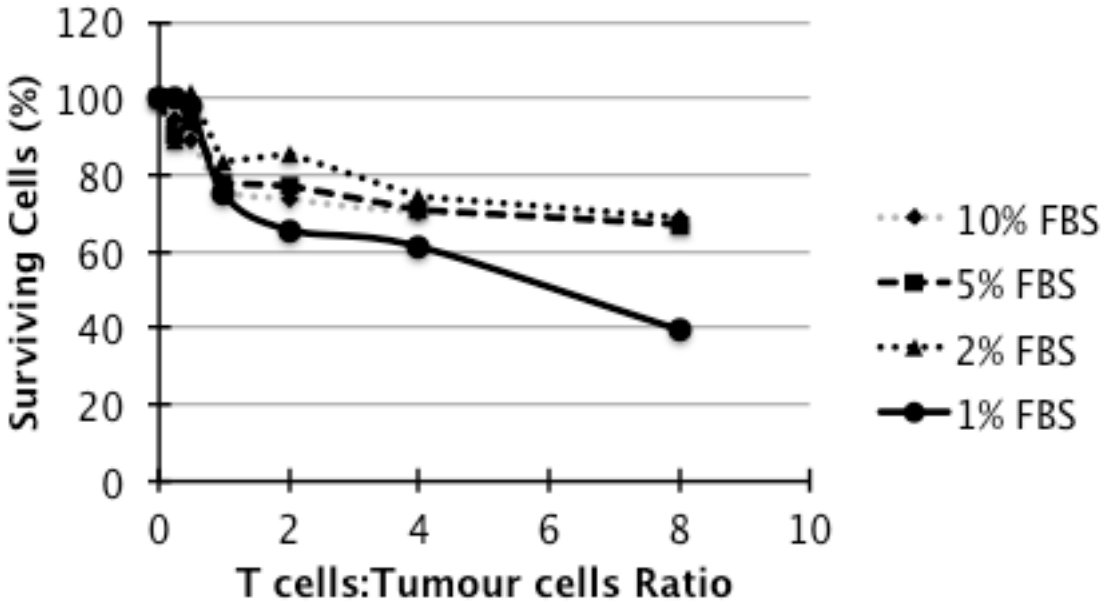


Figure 19: Differential killing of A549 tumour cells grown in variable serum levels. To determine the minimal amount of FBS which can be used in our high throughput screening assay, A549 tumour cells were grown in medium contain either 10%, 5%, 2% or 1% FBS, followed by co-culture with hHER2-CAR T cells for 6 hours. A549 viability was analysed using AlamarBlue. A549 cells exhibited similar killing by hHER2-CAR T cells up until 2% FBS.

8.0 Tables

Table 1: Individual tumour cell line MFI values for NKG2D Ligand and HER-2 expression and sensitivity to hchNKG2Dζ and HER2-CAR T cells.

	NKG2D Ligands	HER2	Sensitivity to Killing – hchNKG2Dζ T cells	Sensitivity to Killing – hHER2-CAR T cells
A549	5251.9	1953.5	+	+
MCF-7	26.2	57.3	+++	N/A
MDA-MB-231	2243	2306	++	+++
NCI-H460	847	1168	+++	N/A
T47D	13510	8258	+++++	+++++
BT-549	217	333.4	N/A	N/A
EKVX	5267	3238	N/A	N/A
MDA -MB-455	2211	16278.1	N/A	N/A
D2F2/E2 (Control for Flow Cytometry)	147	23695	N/A	N/A
K562 (Control For Flow Cytometry)	6393	48	N/A	N/A
Hs578-T	N/A	N/A	+++++	N/A

Where noted N/A cell lines were not tested based on insufficient cell numbers at time of experiment. Cell lines were not further examined based on the findings of A549 resistance.