COMBINING CAR-T CELLS AND ONCOLYTIC VIRUSES FOR CANCER IMMUNOTHERAPY

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By
HEATHER VANSEGGELEN, B.Sc.

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AUTHOR:	Heather VanSeggelen, B.Sc. (Carleton University)
SUPERVISOR:	Dr. Jonathan L. Bramson
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— ABSTRACT —

INTRODUCTION: Immunotherapy continues to garner strong support for use in the treatment of cancer. Adoptive transfer therapies offer a promising approach to combating metastatic disease. In addition, viruses can also be exploited to drive antitumor immunity and tumor destruction. While the use of chimeric antigen receptor (CAR)-engineered T cells has shown dramatic clinical benefit for use in blood-based cancers, solid tumors remain a significant hurdle.

METHODS: We have investigated the use of multi-faceted immunotherapies combining CAR-T cells with oncolytic virotherapy. We have also evaluated how these therapies interact with pre-conditioning lymphodepletion regimes.

RESULTS: In **chapter 3**, we investigated the differences between three similar chimeric receptors targeting NKG2DL. Upon adoptive transfer, we observed dramatic T cell-induced toxicity. In addition, there were stark differences in the severity of toxicity induced between different receptors or across different mouse strains, or if combined with pre-conditioning chemotherapy.

In **chapter 4**, we tested the ability of oncolytic vaccines to boost engineered T cells through their natural antigen receptor. While CAR-T cells could be boosted via oncolytic vaccines, prolonged T cell engraftment and successful oncolytic vaccine boost required pre-conditioning chemotherapy. Further analysis revealed a lack of antitumor function of the CAR-T cells *in vivo*.

In **chapter 5,** we evaluated loading CAR-T cells with oncolytic viruses (OVs). Loading of CAR-T cells with OV did not impair CAR expression or functionality of the T cells. In addition, CAR target recognition did not impact the ability of OV-loaded cells to deposit OVs onto tumor targets. CAR-T cells loaded with OV also displayed enhanced antitumor functions as compared to either treatment alone.

CONCLUSIONS: The research described in this thesis reveals important information into the interactions between CAR-T cells and OVs, and how pre-conditioning regimes may influence responses from either or both therapies. Overall, our research offers novel insight into future CAR-T cell therapeutic developments.

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While the saying is technically "it takes a village to raise a child," I truly believe successful completion of graduate studies takes at least that many people. Maybe more. I have been extraordinarily fortunate to be surrounded by such a great "village," without whom this process would have been considerably more difficult.

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— LIST OF ABBREVIATIONS AND SYMBOLS —

α	alpha
β	beta
Δ	delta
γ	gamma
ζ	zeta
μ	micro
Ab (s)	antibody (ies)
ALL	acute lymphoid leukemia
APC (s)	antigen-presenting cell (s)
CAR	chimeric antigen receptor
CD#	cluster of differentiation
cDNA	complementary DNA
CEA	carcinoembryonic antigen
CLL	chronic lymphoid leukemia
CSF	colony stimulating factor
CTL (s)	cytotoxic T lymphocyte (s)
CTLA-4	cytotoxic T lymphocyte antigen-4
CTX	cyclophosphamide
DAMP (s)	danger associated molecular pattern (s)
DC (s)	dendritic cell (s)
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
effLuc	enhanced firefly luciferase
FACS	fluorescence-activated cell sorting
FAP	fibroblast activation protein
FasL	Fas ligand
FBS	fetal bovine serum
FDA	Food and Drug Administration
Foxp3	forkhead box P3
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

G-CSF	granulocyte colony stimulating factor
GFP	green fluorescent protein
GM-CSF	granulocyte/monocyte-colony stimulating factor
HSCT	haematopoietic stem cell transplant
HSV	herpes simplex virus
iCAR	inhibitory chimeric antigen receptor
ICS	intracellular cytokine staining
IFN (s)	interferon (s)
IL	interleukin
LCMV	lymphocytic choriomeningitis virus
mAb (s)	monoclonal antibody (ies)
MAGE-A3	melanoma-associated antigen 3
MAGE-A12	melanoma-associated antigen 12
MART-1	melanoma antigen recognized by T cells 1
M-CSF	monocyte colony stimulating factor
MEM-F11	minimal essential medium F11
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MHC I	major histocompatibility complex I
MHC II	major histocompatibility complex II
MOI	multiplicity of infection
NDV	Newcastle disease virus
NK cell	natural killer cell
PAMPs	pathogen-associated molecular patterns
PBMC (s)	peripheral blood mononuclear cell (s)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-1	programmed death 1
PD-L1	programmed death ligand 1
PD-L2	programmed death ligand 2
PFU	plaque-forming unit
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute

scFv	single chain antibody fragment
SEM	standard error of the mean
TAA (s)	tumor-associated antigen (s)
TCR	T cell receptor
Th	T helper
TIL (s)	tumor-infiltrating lymphocyte (s)
TK	thymidine kinase
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TNF-related apoptosis-inducing ligand receptor
Treg (s)	regulatory T cell (s)
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VGF	vaccinia growth factor
VSV	vesicular stomatitis virus
VV	vaccinia virus
vvDD	double deleted vaccinia virus
WT	wild type

— DECLARATION OF ACADEMIC ACHIEVEMENT —

The work presented in this thesis is the result of my research efforts over the past 6 years as a graduate student at McMaster University. The experiments described here and the resulting data were designed and interpreted through a collaborative effort between my supervisor, Dr. Jonathan Bramson and myself. I was the primary researcher on all described experiments, with additional collaborations and aid provided as described below.

The studies presented in **chapter 3** were the result of collaborative efforts primarily involving Joanne Hammill and myself. We collaborated to plan and carry out all animal studies, T cell cultures, *in vitro* assays and flow cytometric analysis as described, and analyzed the resulting data. The cloning of CARs was performed as follows: the NKz-CAR was designed and constructed by Daniela Tantalo; Joanne Hammill designed and I constructed the NKz10-CAR; and Galina Denisova designed and constructed the NK28z-CAR. Daniela Tantalo provided additional technical assistance for *in vitro* cultures. Statistical analyses of serum cytokine data was performed by Dr. Anna Dvorkin-Gheva. Mouse pathology analysis was performed by Dr. Jacek Kwiecien, with immunohistochemistry staining of tissues performed in the Core Histology Facility at MIRC by Mary Jo Smith and Mary Bruni.

The research presented in **chapter 4** is the result of a collaboration primarily involving Joanne Hammill, Dr. Jennifer Bassett, and myself. I conducted the bulk of the animal studies, cell cultures, and *in vitro* assays, with

support from Joanne Hammill and Dr. Jennifer Bassett. Recombinant VSV vectors were generated and prepared by Natasha Kazhdan. Galina Denisova constructed the HER2-CAR. The VEGFR2-CAR was designed by Galina Denisova, and constructed by Dr. Dannie Bernard. Joanne Hammill generated the luciferase-expressing VEGFR2-CAR. Dr. Stephanie Swift provided additional technical support.

The data presented in **chapter 5** was collected through experiments primarily conducted by me. Daniela Tantalo and Aryay Afsahi provided me with engineered human T cell cultures. Dr. Kyle Stephenson and Carole Evelegh provided additional technical assistance.

— CHAPTER 1 —

Introduction

1.0 Cancer

1.1 The etiology of cancer

Despite significant advancements in long term survival due to improved detection and treatment options, cancer remains one of the leading causes of death worldwide[1]. In Canada alone, an estimated 21 patients were diagnosed each hour in 2013, illustrating the need for development of effective cancer therapies[2]. Successful treatment regimes will depend on an increased understanding of the tumor, from its origins to the mechanisms that allow for uncontrolled cell growth and resistance to current therapies. Cancer arises when normal cells develop abilities to proliferate uncontrollably, bypassing the regulatory mechanisms that control homeostatic growth. Many of these abilities develop from mutations, which can arise through a number of mechanisms. Genes contributing to cancer development can be inherited, as in the case of familial colon cancers and breast cancers[3,4]. Carcinogenesis can also result from accumulated damage caused by environmental exposure to carcinogens such as pollution or cigarette smoke, or by infections with viruses such as human papillomavirus or hepatitis virus[2,5]. The degree of genetic mutations required to make a cell cancerous varies greatly across types of cancer, with leukemias having relatively low mutation numbers while melanoma and lung cancers show high quantities of somatic mutations[6]. This is unsurprising, as cigarette smoke contains over 60 known carcinogens that contribute to the high concordance between smoke exposure and lung cancer

development[6,7]. Melanomas and other skin cancers are also highly associated with UV exposure, which induces multiple types of DNA lesions[8].

While the malignant cells themselves comprise the bulk of a tumor, there are a multitude of non-cancerous cells involved in the growth of a tumor. Tumors develop as a heterogeneous mix of fibroblasts, myeloid cells, and vascular components that provide necessary structure and blood flow to drive tumor growth[9]. In addition, the tumor cells themselves are frequently heterogeneous, accumulating individual mutations as they divide, resulting in a spectrum of mutations and cell populations across a single tumor. One of the hallmarks of more serious cancer diagnoses is the spread of cancer cells to distal sites in a process known as metastasis[10]. These secondary tumors are significantly harder to treat, as they tend to show even greater heterogeneitiy compared to their primary tumor[11]. This ultimately results in poor prognosis, with metastatic disease responsible for 90% of cancer deaths in patients with solid tumors[9,11].

1.2 Conventional cancer treatments

Current standard cancer treatments include surgery, chemotherapy, radiation, or some combination of these. Surgical removal of the primary tumor remains the most effective treatment for localized disease, particularly when combined with chemotherapy or radiation[12]. There are several different classes of chemotherapy drugs, differing in structure and origin. Most chemotherapies target cellular functions required for mitosis and thus will affect any rapidly

proliferating cell[13]. Radiotherapy also aims to kill the tumor by producing DNA damage, delivering controlled doses of ionizing radiation to the local tumor site[14]. These therapies can both be used to complement surgical resection, delivered either in the neo-adjuvant or adjuvant setting (pre- or post- surgery, respectively) to de-bulk the tumor or eliminate residual disease[14,15]. However, the non-specific nature of these therapies contribute to the significant side effects including nausea, vomiting, flu-like symptoms, and hair loss frequently observed during treatment[15,16]. Furthermore, tumor cells often evolve resistance to both chemotherapy and radiation, limiting the potential benefits of these therapies in treating advanced cancers[17,18]. With the ever-increasing rates of cancer diagnosis and death, it is evident that new therapeutic options are required.

2.0 The immune system and cancer

There is mounting evidence to suggest that both innate and adaptive immune responses play an important role in the success of chemo- and radiotherapy treatments[13]. A variety of chemotherapeutic agents have been shown to drive antitumor immune responses via enhancing antigen uptake, processing and presentation, activation of natural killer (NK) cells, as well as inhibiting regulatory cell populations[13,19]. Likewise, irradiation can increase immune cell homing to tumors and promote T cell proliferation and subsequent memory T cell development[13]. The concept of immune-driven tumor clearance has existed almost as long as conventional therapies, beginning with observations that the presence

of an infection could coincide with spontaneous tumor regression[20,21]. The first noted therapeutic investigation of this phenomenon was William Coley's use of "Coley's toxins" as a cancer treatment comprised of heat-killed streptococcus bacterium, which proved to be mildly effective in treating several cancer types[20]. With ever-increasing knowledge of the interplay between tumors and the immune system, the area of cancer immunotherapy has rapidly evolved into targeted therapies. In this section, the basic processes of immune activation will be discussed, with a particular focus on the generation of T cell responses.

2.1 Initiating an immune response

Human health and survival are persistently challenged by a wide variety of pathogens, cancers, or inappropriate immune responses to either self or innocuous molecules[22]. In order to combat these threats, the immune system has evolved a complex network of cells, receptors, and soluble mediators to protect against tissue damage and cell death. The multi-faceted components of the immune system act in concert to eliminate invading microbes or transformed cells, minimize bystander cell death and generate long-lived memory of the target.

The innate immune system serves as the first line of defense, functioning as both a barrier (via physical barriers like the skin or mucosa) and as a rapid cellular response capable of recognizing signs of danger[23]. Innate immune cells bear germline-encoded receptors capable of recognizing pathogens via pathogen-associated molecular patterns (PAMPs), or transformed or infected cells via

danger-associated molecular patterns (DAMPs)[23]. Recognition of these molecular structures elicits rapid responses from the innate immune system, resulting in both an acute immune response to eliminate the danger and activate the second line adaptive immune response[23].

2.2 NK cell immunity

NK cells are lymphocytes that operate under the umbrella of the innate immune system. NK cells are governed by a multitude of concomitant signals received through activating and inhibitory receptors[9,11,24]. The activation of NK cell-mediated cytolysis is determined by the balance of activating and inhibitory signals delivered to the NK cell[25,26]. Most NK cell activating receptors function in a complex, where the receptor that binds target ligands doesn't signal itself but relies on adaptor proteins to transduce activating signals[26]. Many of the NK cell receptors are also expressed on activated T cells, including NKG2D, DNAM-1, and CD94[24,25]. However, on T cells these receptors function predominantly as costimulatory or adhesion molecules instead of cytotoxicity-triggering receptors[24,25].

NKG2D functions as one of the primary activating receptors in NK cells, recognizing a variety of ligands whose expression is induced upon cellular stress[25,27,28]. NKG2D is capable of pairing with two different adaptor proteins: ITAM-containing DAP12, and DAP10[26,29]. In CD8+ T cells, NKG2D associates with DAP10 and functions as a costimulatory receptor[29]. In human NK cells,

association with DAP10 (DAP12 in murine NK cells) drives cytotoxic responses to target cells[29]. Numerous cell surface ligands can bind NKG2D[20,21,25,30]. In humans, known ligands include MICA, MICB, and the ULBP proteins 1-4, while in mice, ligands include RAE-1 family members, H60 and MULT1[20,24,25,31-33]. These ligands have been shown to be upregulated following cellular stresses induced by viral infections or transformation, explaining why NK cells play an important role in antiviral and antitumor immunity[22,27,32,33].

While NK cells rely on a careful balance between activating and inhibitory signals to determine the fate of the interacting cell, the inhibitory signals dominate[24]. These inhibitory receptors carry immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that function by recruiting SHP-1 and SHP-2 phosphatase enzymes to shut off signaling through the activating receptors[24,34]. Inhibitory NK receptors recognize MHC class I family members through a number of receptors recognizing specific allelic variants[34,35]. Recognition of MHC-I molecules on a target cell surface will disarm the NK cell despite the presence of activating ligands, confirming the strong inhibitory signal from this interaction[22,36]. The presence of MHC-I serves as a sensor for NK cell-mediated attack, as many cells attempting to evade T cell-mediated immunity (such as virally infected or tumor cells) will downregulate MHC-I antigen presentation[22,25]. This, in combination with activating ligand expression, renders these cells sensitive to NK-mediated cytotoxicity[25,37,38]. NK cell inhibitory receptors belong to several protein families, including the killer cell immunoglobulin-like receptors

(KIR) expressed in humans, the Ly49 receptors expressed in mice, and the shared CD94/NKG2 receptors[22,25]. Each of these families encode a number of different proteins that bind to both classical and non-classical MHC class I molecules[22,30].

When the proportion of signaling is greater through the activating receptors than the inhibitory receptors, the NK cell will rapidly mobilize its cytotoxic machinery. While NK cells and T cells recognize target cells via different methods, they share common killing mechanisms. NK cells primarily utilize perforin and granzymes to induce apoptosis of their target cells, and are also able to secrete IFNγ[22,25]. Interestingly, in addition to pre-packaged cytolytic granules containing perforin and granzymes, NK cells express "pre-formed" transcripts for IFNγ, so that cytokine synthesis can begin immediately upon NK cell activation[39-43]. The specific combination of activating receptors that are triggered on the NK cell will dictate the polarization of cytolytic granules, degranulation, and killing of the target cell[24,42,44,45].

2.3 Activating a T cell response

The adaptive immune response relies on the innate immune system for initial activation of cells that bear highly specific receptors that recognize antigens presented to them by the innate immune cells[22]. T cells are one of the critical players of the adaptive immune response, and express a wide array of highly individualized surface receptors known as T cell receptors (TCR). Each of these receptors results from a series of somatic rearrangements at the genomic level,

resulting in individual cells capable of recognizing virtually any pathogen[22]. During development T cells undergo careful selection processes to ensure cells that are highly reactive to self-antigens are eliminated, preventing unwanted autoimmunity[22]. Following this selection, naïve T cells migrate to secondary lymphoid organs to await activation.

Antigen presenting cells (APCs) play an important role in T cell-mediated immunity, and are required to present peptide antigens to naïve T cells in conjunction with major histocompatibility complex (MHC) molecules. Dendritic cells (DCs) function as the primary APC for the initiation of adaptive immunity[37,38]. Naïve T cells are activated upon recognition of a specific peptide-MHC complex presented by a DC. Signaling through the TCR is mediated via the associated CD3 hexameric protein complex, which consists of one of each of CD3δ and CD3γ proteins, and two copies of each CD3ε and CD3ζ proteins[22]. The CD3ε and CD3ζ protein contains sequence motifs referred to as immunoreceptor tyrosine-based activation motifs (ITAMs)[22]. Phosphorylation of these ITAMs initiates a complex signaling cascade involving a multitude of protein tyrosine kinases that culminates in calcium mobilization and the nuclear translocation of many transcription factors including NFAT (nuclear factor of activated T cells) and NFκB (nuclear factor κB) (**Fig. 1.1**)[39,41-43]. This ultimately results in actin reorganization and the transcription of genes required for T cell growth and differentiation[42,44]. APCs also dictate which T cells will become activated. CD4+ T cells will be activated upon recognition of peptide in complex with MHC class

II molecules (MHC-II), while CD8+ T cells recognize peptide complexed with MHC class I (MHC-I) molecules[38]. For naïve T cells, TCR triggering alone is insufficient to fully activate the cell. Indeed, T cells require two additional signals in order to become fully differentiated, activated effector cells; the absence of these signals can result in T cell unresponsiveness (anergy), deletion via apoptosis, or induction of a tolerogenic phenotype[46,47]. When a DC has recognized a PAMP or DAMP, the cell upregulates secondary costimulatory molecules such as CD80 and CD86[37]. These molecules provide a secondary signal to the antigen-specific T cell via binding to the CD28 receptor expressed on the T cell[42]. This signal is transduced via PI3K/AKT signaling and functions to inhibit the nuclear export of NFAT, promoting T cell survival (**Fig. 1.1**)[42]. In addition, CD28 ligation boosts multiple TCR-driven signaling pathways, such as the PLCγ pathway and enhancing calcium mobilization (**Fig. 1.1**)[48,49]. This secondary activation signal functions to induce T cell proliferation, mediated in part through IL-2 production and upregulation of the IL-2 receptor to allow for autocrine proliferation signaling[42]. There are a plethora of additional costimulatory molecules that become upregulated following TCR signaling, serving to potentiate the proliferative response as well as aid in development of T cell memory. The TNFR family, including 4-1BB and OX40, are well-characterized costimulatory receptors that play a role in T cell survival and cytokine production[50]. Stimulating an activated T cell through OX40 increases the level of cytokine production and prolongs expression of survival signals[51-53]. Similarly, engaging 4-1BB on

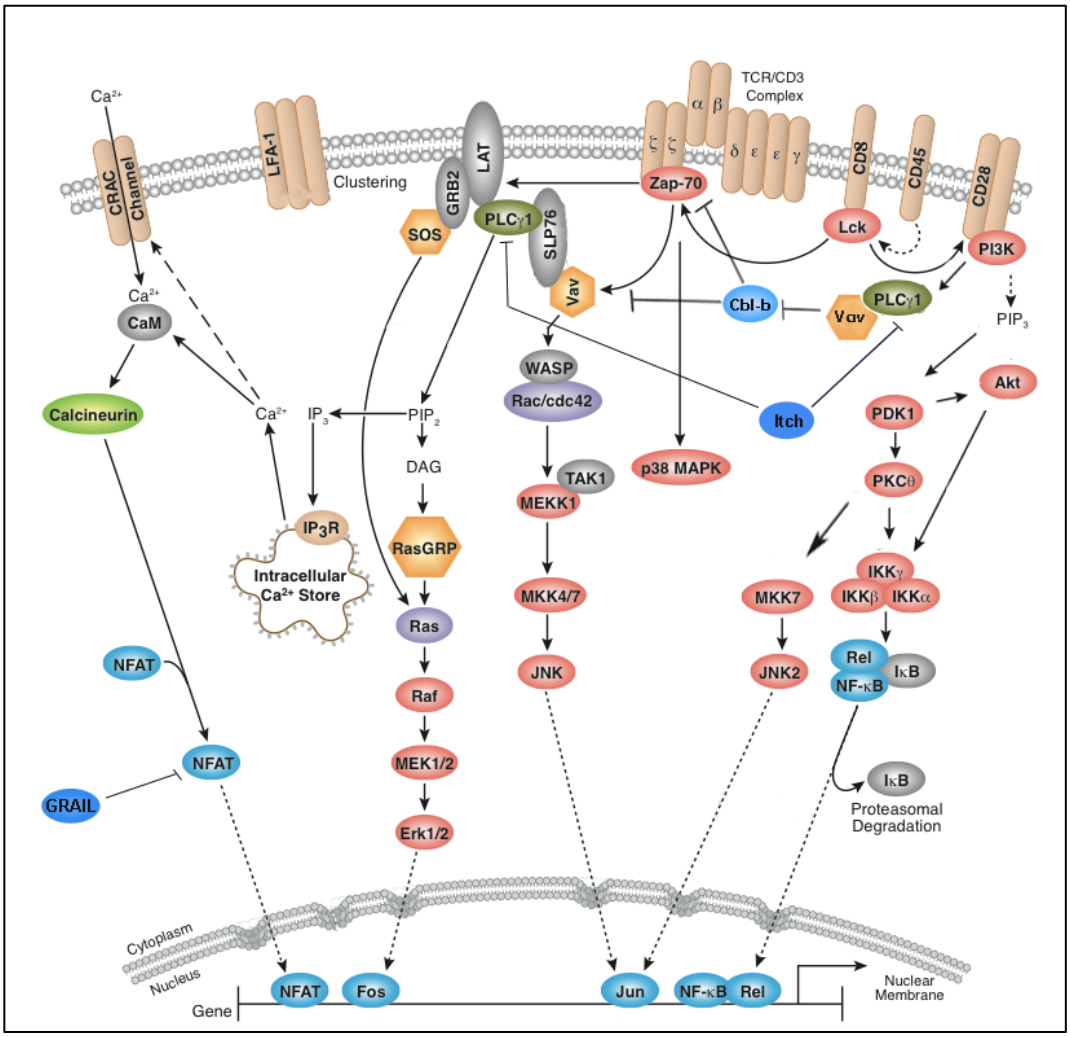


Figure 1.1: T cell signaling during activation. Recognition of peptide-MHC complexes by the TCR-CD3 complex, as well as costimulatory molecule binding induces multiple signaling cascades that results in nuclear translocation of NFAT and NFκB among others. (Figure adapted from Cell Signaling Technology[54])

activated T cells also promotes T cell survival and enhances cytokine production and effector function[50,55,56].

2.4 The 3rd activation signal and effector T cell responses

While the primary and costimulatory signaling receptors are capable of promoting T cell expansion and survival, a third signal is also required for differentiation and development of cellular effector functions. The third activation signal is derived from the cytokine environment present during T cell activation. Upon receipt of all three activation signals, naïve T cells become fully activated and can go on to proliferate and exert their effector functions.

CD8+ T cells can gain full effector functions following activation in the presence of IL-12, as well as type I interferons (IFNs)[57,58]. Activated CD8+ T cells are primarily responsible for killing target cells via contact-dependent cytotoxicity, leading to the common designation of "cytotoxic T lymphocytes" or CTL. Upon recognition of peptide-MHC class I complexes on the surface of the target cell, the CD8+ T cell polarizes numerous proteins and pre-formed lytic granules towards the site of interaction, referred to as the immunological synapse[59-61]. These granules contain membrane-permeabilizing perforin and apoptosis-inducing granzymes[60-62]. Perforin release into the immunological synapse results in the formation of pores within the target cell membrane, allowing for entry of granzymes that activate caspase-mediated apoptotic signaling cascades[63]. An alternate mechanism of contact-mediated cytolytic

activity occurs via FasL (on the CTL) binding the death receptor Fas on the target cell and resulting in apoptosis[62,64]. Similarly, binding of the death receptor TRAIL-R (on the target cell) by its ligand TRAIL (on the CTL) induces apoptosis of the target cell[65]. Cytotoxic T cells can also kill target cells indirectly via secretion of effector cytokines such as IFNγ and TNFα[66]. These inflammatory cytokines can modulate both immune and non-immune cells to result in target cell destruction. In particular, IFNγ signaling induces upregulation of MHC expression and increases antigen presentation, and as well as inhibits virus replication[66,67]. TNFα can mediate apoptosis via triggering of the death receptor TNFR[66,67]. Both IFNγ and TNFα are responsible for modulating responses from additional cells, such as activation of macrophages or NK cells, or differentiation of CD4+ T cell subsets[38,68].

CD4+ T cells have diverse roles in adaptive immune responses, as evidenced by the numerous CD4+ T cell subsets that exhibit distinct functions following activation. CD4+ T cell activation occurs via recognition of peptide presented on MHC II by APCs[38]. For CD4+ T cells, the third signal dictates the cell's differentiation pathway, which manifests through the cytokines the CD4+ T cell can produce upon subsequent ligation of its TCR. For example, Th1 CD4+ T cell differentiation occurs in response to IL-12, and results in a CTL-like effector phenotype with the production of IFNγ, TNFα, and IL-2 as discussed above[69]. The diverse cytokines produced by CD4+ T cell subsets are important for modulating the activities of other immune cells. Th1 responses are important for

protection against intracellular bacteria and viral infections via activation of macrophages, and enhancement of CD8+ T cell and NK cell cytolytic functions[69]. Th1 responses have also been implicated in autoimmune settings, including Crohn's disease[70], graft-versus-host disease[71], and rheumatoid arthritis[72]. Th2 differentiation is driven by the presence of IL-4, with effector Th2 cells characterized by production of IL-4, IL-5, and IL-13[69]. These cytokines play important roles in B cell isotype switching to IgE, as well as eosinophil recruitment and activation[69]. In this way, Th2 CD4+ T cells contribute to protection against extracellular threats such as helminth infections[3,4,73]. Inappropriate Th2 responses have been largely associated with allergic inflammation[74], as well as asthma[75]. Th17 CD4+ T cells (differentiated via TGFβ and IL-6 presence) are responsible for production of cytokines such as IL-17 A/F, IL-21 and IL-22, which go on to modulate the functions of endothelial cells, fibroblasts and macrophages amongst others[5,76-78]. Importantly, these pathways culminate in neutrophil recruitment and stimulate the production of additional granulocytes via G-CSF and GM-CSF[6,79]. These cells have been implicated in protecting against infections at mucosal surfaces, as well as perpetrating several autoimmune pathologies including rheumatoid arthritis, asthma, and inflammatory bowel disease[79,80]. In addition to these highly studied Th subsets, there are more recently identified subsets such as Th9 or Th22 cells that also play important roles in the adaptive immune response, with new subsets and their specific functions continuing to be investigated.

2.5 Regulatory T cells

While many subsets have immunostimulatory characteristics, CD4+ T cells can also function as suppressive cells in the form of regulatory T cells (Tregs). Tregs play an important role in homeostasis and protection against unwanted autoimmunity. The high level of expression of CTLA-4 on Tregs outcompetes CD28 on naïve T cells for binding with B7 molecules on the APC, resulting in the naïve T cell only receiving signal 1 in the absence of signal 2, leading to an anergic response[38,81]. Tregs suppress T cell and APC function through other mechanisms also, including production of immunosuppressive cytokines, direct cytolytic activity, disruption of metabolism, and suppression of antigen presentation functions[6,7,82,83]. In particular, the secretion of IL-10 and TGF-β function to inhibit effector T cell function, while Tregs can act as IL-2 "sinks," effectively starving effector T cells[8,82]. The importance of Tregs function in homeostasis is best illustrated in mouse models or humans lacking functional FoxP3 (the master transcription factor that defines the Treg program), which display severe systemic autoimmunity[9,83]. However, in a tumor environment, elevated Treg frequencies are associated with poor outcome in breast or ovarian cancer or melanoma, amongst others[10,84-86]. As such, the ability to limit Treg impact on cancer immunotherapy has become an important area of research[11,87,88].

2.6 T cells and cancer

There have been numerous studies in both pre-clinical models and clinical trials showing that T cells (both CD4+ and CD8+) are capable of mounting antitumor responses[38,89-92]. Antitumor T cell responses can be directly generated or augmented through vaccination strategies using viruses encoding tumor associated antigens (TAA)[46,47,93-95]. Alternatively, these responses can be indirectly generated via dendritic cell-based vaccination platforms[37,96,97]. This relies on provision of DCs carrying tumor antigens to prime existing tumor-reactive T cells[37,96,97]. Cancer vaccines have shown only modest clinical activity. Nevertheless, Dendreon's "Sipuleucel-T" vaccine recently became the first FDA-approved immunotherapeutic for prostate cancer[98]. This therapy involves infusing PBMCs (including APCs) that have been pulsed with a fusion protein that incorporates a prostate antigen fused to granulocyte-macrophage colony stimulating factor (GM-CSF) to activate the APCs, ultimately resulting in immune activation against the target antigen[98].

The cytolytic activity of CD8+ T cells in antitumor immunity has been found to operate via perforin/granzyme release as discussed previously, with additional work suggesting that the secretion of IFNγ can sensitize tumor cells to apoptosis induction via death receptor ligation[42,99-102]. The role of CD4+ T cells in antitumor responses is multi-faceted, as CD4+ T cells support CD8+ T cell activity, perform their own cytolytic functions, and secrete cytokines that can potentiate the antitumor response via recruitment and activation of additional

immune cells[48,49,103-107]. Tregs in the tumor microenvironment appear to counteract the anti-tumor effects of both CD8+ and CD4+ T cells. Thus, it is unsurprising that there has been significant research into enhancing T cell-mediated antitumor responses, as well as identifying mechanisms of tumor resistance to T cell function.

2.7 Cancer fights back: mechanisms of immunosuppression and evading the immune response

The immune system's ability to recognize and destroy tumor cells is commonly referred to as cancer immunosurveillance[108]. The immune system also plays an important role in driving the tumor's growth over time. The developing tumor undergoes a process termed "immunoediting," where tumor cells that can evade immune detection grow selectively whereas the cells that are susceptible to immune attack are deleted from the growing mass[57,58,108]. Immunoediting involves three separate phases: elimination, equilibrium, and escape. The elimination phase involves immune recognition followed by destruction of tumor cells[59-61,109]. During this phase, tumor variants that are capable of surviving the immunological onslaught will emerge. This represents the equilibrium phase, where the immune system is able to control the outgrowth of the tumor[60-62,110]. Over time, tumor cells escape elimination due to stochastic selective advantages that make them resistant to immune attack and the tumor forms. Immune escape can occur through a number of mechanisms, including antigen loss and down-regulation of MHC

molecules for antigen presentation which ultimately renders the tumor "invisible" to T cell-mediated clearance[63,110]. Tumor cells and infiltrating immune cells co-operate to produce a variety of factors capable of suppressing antitumor T cell responses[66,111]. Tregs and tumor cells both produce IL-10 and TGF-β, which potentiate anti-inflammatory Treg functions, inhibit pro-inflammatory APC functions, and prevent T cells from both proliferating and exerting cytolytic functions[66,67,112-114]. Tumors also often contain high levels of adenosine, which promotes tumor growth via angiogenesis stimulation, and also limits T cell function[66,67,115]. High local production of catabolic enzymes (ie. arginase, nitric oxide synthase, indoleamine 2,3-dioxygenease) depletes arginine and tryptophan, which in turn inhibits immune cell function partly by impairing protein production[38,112,116].

In addition to the multitude of secreted factors that function to suppress incoming immune attack, the cells within the tumor express immunosuppressive receptors and ligands. Due to repeated activation, T cells in the tumor express CTLA-4, which outcompetes CD28 for costimulatory ligand binding and instead transduces an inhibitory signal designed to turn off immune responses[38,112]. As discussed above, expression of CTLA-4 on Tregs can also inhibit effector T cell function[81]. Signaling through CTLA-4 can drive production of active indoleamine 2,3-dioxygenease within the tumor environment from APCs, potentiating the immunosuppressive functions of this receptor[69,112]. Activated T cells also upregulate the PD-1 receptor, which upon binding ligands PD-L1 and PD-L2

negatively impacts TCR signaling, effector responses and overall cell survival[69,117]. As PD-L1 can be found overexpressed on the surface of many tumor cells, this pathway represents an important mechanism of tumor-induced immunosuppression[112]. Immune activation within the tumor (specifically IFNγ production) exacerbates the local immunosuppressive environment, as IFNγ drives expression of PD-L1 on tumor cells[118]. These data indicate that even successfully mounting an immune attack against the tumor can negatively impact overall therapeutic success.

With our increased understanding of the local immunosuppressive environment of the tumor, therapeutic strategies have been developed to reverse this suppression. Several therapies including conventional chemo- and radiotherapies have proven successful at reducing the frequencies of Tregs, subsequently promoting antitumor immunity in both murine models and human patients[87,88,112,119]. More impressively, monoclonal antibodies (mAbs) have been developed to antagonize immunosuppressive receptors on T cells. Anti-CTLA-4 (Yervoy) has been approved for use in melanoma treatment, and is undergoing extensive testing for use in numerous other cancers[120-122]. Likewise, targeting the PD-1 signaling axis via blockade of PD-1 or its ligand PD-L1 has shown efficacy in treating non-small cell lung cancer, melanoma, and renal-cell cancer in early phase clinical trials[123-125]. In fact, the results with anti-PD1 in melanoma have been so impressive, the FDA has granted rapid approval to Merck's anti-PD1 "Keytruda" for patients with advanced melanoma. As these two inhibitory

pathways operate through different mechanisms, there has been increased investigation into the combination of both anti-CTLA-4 and anti-PD-1 blockade, with early clinical trials showing evidence of clinical activity in 65% of patients[126]. These studies indicate that immune checkpoint blockade represents an effective strategy for enhancing antitumor immunity that may function in concert with additional immunotherapies.

3.0 Adoptive transfer as a cancer therapy

Adoptive cell transfer (ACT) studies provide the best evidence that T cells can be used to fight cancer. This process involves the generation or isolation of tumor-reactive T cells, expansion of these cells to high numbers *in vitro*, followed by infusion into the tumor-bearing individual[127]. ACT overcomes the requirement for *in vivo* T cell response generation and expansion, allowing for transfer of as many as 10^{11} cells in a very short period of culture time[128]. Great strides have been made in utilizing T cells as a cancer immunotherapy, with over 33 clinical trials launched in the past year alone[129]. This section will discuss some of the different methods used for producing tumor-reactive T cells for ACT.

3.1 Donor lymphocyte infusions with stem cell transplant therapies

One of the earliest methods for utilizing T cells as an immunotherapy is in the setting of allogeneic haematopoietic stem cell transplants (HSCT) for treatment of leukemia[130]. In particular, it has been accepted that T cells within the

graft are capable of mounting a robust "graft-versus-leukemia" (GVL) effect[130]. However, the presence of T cells in the graft has also been identified as the source of graft-versus-host disease (GVHD)[131]. To reduce the risk of GVHD while maintaining the GVL effect, donor lymphocyte infusions were introduced[131]. This therapy involves a T cell-depleted bone marrow transplant, followed by the transfusion of donor-derived lymphocytes[132]. This process delays the introduction of donor lymphocytes until a time at which the initial graft tolerance has been established[131]. There is a fine balance however between GVHD and GVL, as the response to donor lymphocyte infusion is highly correlated with the presence of GVHD[133]. This is an inherent risk with cancer immunotherapy, as will be discussed in future sections.

3.2 Tumor infiltrating lymphocyte (TIL) therapy

The use of TIL for adoptive immunotherapy has shown impressive results in the treatment of metastatic melanoma[89,134]. This therapy involves the isolation of lymphocytes from tumors (TILs), selection of tumor-reactive TILs, expansion *ex vivo* and adoptive transfer back into the patient[89]. Through the *ex vivo* expansion, large quantities of tumor-reactive cells can be generating from relatively small starting samples. Current "rapid expansion protocols" are capable of inducing an 1800-fold expansion of TIL (on average) from melanoma tissues in as little as 14 days[135]. In the clinic, TIL therapy combined with chemotherapy has shown objective response rates of 49-51%[128,136,137]. This response rate increases to

72% when combined with high dose irradiation prior to ACT[128,136,137]. TIL transfer therapies are typically combined with provision of supportive IL-2 infusions to promote T cell survival and engraftment. Toxicities following TIL therapy are more often a result of the IL-2 than the T cells themselves[89,137]. The combination of lymphodepletion, adoptive transfer, and exogenous IL-2 support has been shown to allow for significant proliferation and persistence of transferred TIL *in vivo*, showing a greater than 1000-fold expansion following ACT[128]. The inclusion of lymphodepleting regimes in TIL therapy has been shown to decrease Tregs, as well as liberate homeostatic cytokines such as IL-7 and IL-15, resulting in increased T cell persistence and overall efficacy[138-140]. In addition, total body irradiation functions to increase gut permeability and results in microbial translocation and collection in lymphoid tissues, where they serve to activate endogenous APCs and ultimately promotes antitumor immunity[141].

The primary pitfall of TIL therapy is that TIL must be culturable from a resected tumor[89]. This has proven to be a considerable challenge for non-melanoma cancers, which often have few TIL. Further, TIL cannot be cultured from every melanoma specimen[138,142]. Finally, even when TIL can be cultured, the resulting cells do not always show specific cytolytic activity against their originating tumor, rendering the cells unusable[138,142,143]. The tumor-reactivity of TIL represents an important hurdle, as one example of TIL expanded from GI cancer metastasis showed fewer than 3% of TIL were reactive to the tumor[143]. Work is currently underway to evaluate the use of TIL in other cancer types.

Those candidates include tumors whose prognosis positively correlates with T cell infiltrate, as well as those with potential immunogenic targets such as HPV-induced cancers[138]. Indeed, HPV-derived TIL therapy is currently being tested in Phase 1 clinical trials, with preliminary responses showing 2/9 treated patients achieving a complete, ongoing response[144]. Thus the use of TIL may be promising for at least a select subset of cancers from which these cells can be generated.

3.3 TCR-engineered T cells

With the difficulties faced expanding tumor-reactive cells from tumor tissues, alternate methods of antitumor T cell generation/isolation have been explored. Identification of specific T cell clones that recognize tumor antigens has led to the idea of engineering a tumor-specific TCR into peripheral T cells to confer tumor reactivity[145,146]. T cells can be readily transduced using retroviral or lentiviral vectors with up to 80-90% efficiency, providing the tools necessary to generate large numbers of tumor-reactive T cells[128]. In addition, the affinities of engineered TCRs can be enhanced to yield highly tumor-reactive T cells[128,147]. Tumor-specific TCR can also be engineered to enhance surface expression and prevent mis-pairing with the cell's native TCR chains. A recent study evaluating the use of a TCR recognizing cancer/testis antigen NY-ESO-1 observed objective responses in 4/6 or 5/11 patients with synovial cell carcinoma or melanoma respectively[148]. Several trials have shown success in treating melanoma patients with TCR-engineered T cells recognizing melanoma antigen MART-1, ranging

from 30-69% objective response rates[149,150]. As more tumor-reactive TCR are identified, this therapy may become more broadly applicable, but the reliance on HLA-matching currently prevents "off-the-shelf" T cell TCR engineering.

3.4 Chimeric antigen receptor (CAR)-engineered T cells

As mentioned previously, tumor cells often display diminished levels of MHC, which reduces their visibility to conventional TCRs. In the late 1980's, Eshhar *et al*. described the generation of receptors termed "T-bodies" that coupled antigen recognition via antibody fragments with either the CD3ζ chain or the γ-chain from the IgE receptor[151,152]. These receptors removed the conventional MHC restriction, allowing T cells to bind surface-expressed antigens and induce T cell activation via the intracellular signaling domain[153]. The first described CARs containing one signaling domain are referred to as first-generation CARs[154]. The use of CD3ζ as the dominant activating signal in CARs has been validated by studies showing that CARs containing this domain show superior antitumor responses and overall longer-lived activity as compared to CARs based on the Fcγ receptor[155]. First-generation CAR-T cells have proven effective at mediating tumor clearance in murine models of B cell lymphoma[156], colon cancer[157], HER2-positive cancers[158], as well as neurological tumors such as medulloblastoma[159] and glioblastoma[160] among others. However, further analysis revealed that these CARs were limited in their capacity to induce T cell proliferation, survival, and ultimate long-term antitumor immunity[155,161,162].

Indeed, human trials utilizing first-generation CARs targeting a variety of antigens have only produced modest results [163-165].

Next generation CARs included the addition of costimulatory signaling domains. Provision of costimulation via the CAR has been shown to significantly increase T cell survival, proliferation and cytokine production[165]. Utilizing the signaling domain of CD28 allows for T cell proliferation *in vivo*, increased production of IL-2 and IFNγ, and upregulation of antiapoptotic genes, promoting T cell survival[155,166-169]. The combination of CD28 and CD3ζ signaling shows superior antitumor efficacy when compared to CD3ζ alone in treating murine B cell lymphomas[167] and colon carcinoma[169,170], as well as showed enhanced expansion and persistence in human lymphoma patients[166]. The ideal choice of costimulatory domain for CARs remains to be established. One report suggests that inclusion of CD28 within the CAR may impair antitumor efficacy if the tumor has high levels of infiltrating Tregs[171]. Costimulatory domains from CD137 (4-1BB), OX40 and ICOS have also been evaluated[172-174]. While it has been reported that CD28 provided the greatest proliferative and cytokine responses [175], others have had great success with 4-1BB-containing signaling domains[172,176,177]. Clinically, significant complete responses have been observed using CARs recognizing CD19 containing either CD28 or 4-1BB costimulatory domains[178-182]. A study from the NCI observed 8 complete remissions and 4 partial responses out of 15 patients with advanced B cell cancers treated with a CD19-specific CD3ζ-CD28 CAR[182]. Other groups have reported similar successes with this

combination utilizing variations on T cell expansion methods, type of pre-conditioning, and cytokine support[183]. Treating B cell malignancies with CD19-specific CARs signaling via CD3ζ-4-1BB have also shown dramatic successes, with 4/5 patients (2 adult, 2 pediatric) showing complete responses and an additional partial response[179,180]. More recently, the use of this CD19-specific CAR has been reported to induce complete responses in 90% of patients (27/30) with relapsed or refractory ALL, with sustained responses occurring in 67% of patients[184].Currently ongoing trials with this CAR have reported preliminary results of 5/24 and 7/24 respective complete and partial responses in CLL patients also[185-188]. Overall, second-generation CAR-T cells are resulting in significant antitumor responses in hematological malignancies.

As the inclusion of costimulatory domains has proven to enhance CAR-T cell survival, persistence and function, several groups have investigated the addition of multiple costimulatory domains to the CAR. Thus, third-generation CARs bear the CD3ζ activating domain coupled to two costimulatory signaling molecules. Third-generation CARs that include CD28-OX40, or CD28-4-1BB as their costimulatory signals have been evaluated for their improvements over their second-generation CAR counterparts[189-192]. Combining CD3ζ, CD28 and OX40 signaling into the same receptor was able to enhance *in vitro* CAR-mediated clonal expansion of the engineered cells even in the absence of exogenous IL-2, promoted tumor-specific lysis, and was able to significantly delay tumor growth in murine models[189,192]. Similarly, the use of CD3ζ-CD28-4-1BB CARs have

shown enhanced *in vivo* persistence and antitumor functionality in murine xenograft models[190,191]. These CAR-T cells were also observed to undergo antigen-independent proliferation, which may pose a concern for off-tumor effects if the receptor is signaling in the absence of antigen recognition[191]. A CD20-specific CAR bearing CD3ζ, CD28 and 4-1BB signaling domains was capable of inducing two complete responses and one partial response in patients with mantle cell or follicular lymphoma[193]. Altogether, these studies illustrate the importance of including some form of costimulation when utilizing CARs to redirect T cell specificity, while the choice of which costimulatory molecule to include may still be unclear.

3.5 The risks of engineering T cell specificity

Engineered T cells, be it via TCR or CAR, have shown increasing evidence of clinical efficacy in a variety of disease sites. However, these studies have also delivered unexpected toxicities resulting from the use of high affinity, highly activating receptors. Toxicities can generally be one of two types: 1) on-target/off-tumor toxicity resulting from target antigen expression on normal tissues, or 2) off-target toxicity in tissues lacking the target antigen[90].

Murine models of TCR-engineering have highlighted the risk of mis-paring of the endogenous TCR chains with the engineered chains, resulting in off-target autoimmune lethality[90,194]. This has yet to be demonstrated in human trials, but does remain possible[90]. The trials targeting MART-1 using an engineered

TCR both observed on-target toxicity, including destruction of healthy melanocytes in the eyes, skin and ears[149,150]. Similar results were obtained following treatment of metastatic colorectal cancer patients with TCR-engineered T cells recognizing human carcinoembryonic antigen (CEA), with all patients showing strong decreases in serum CEA levels, while also experiencing severe transient colitis[195]. The severity of the colitis resulted in trial suspension although the colitis eventually resolved, presumably as a result of T cell quiescence over time[195].

The most severe toxicities observed to date were produced by high-affinity anti-MAGE-A3 TCR-transduced T cells, as lethal adverse events occurred in two independent trials [196,197]. In a trial where a high-affinity MAGE-A3-TCR was tested in melanoma, synovial cell sarcoma, or esophageal cancer patients, 5/9 patients did achieve tumor regression[196]. However, one patient developed Parkinson-like symptoms and two additional patients developed severe cerebral inflammation and subsequently died[196]. This toxicity was attributed to a previously unidentified cross-reactive protein MAGE-A12 found to be expressed in the brain[196]. Using a different, affinity-matured MAGE-A3-specific TCR in a separate trial aimed to treat melanoma and myeloma patients also resulted in lethal toxicity[197]. The first two patients treated with these T cells developed cardiogenic shock and died within the first few days of ACT[197]. This was determined to be caused by unidentified cross-reactivity between the MAGE-A3 TCR and the cardiac muscle-expressed protein, titin, which was attributed to the

enhanced affinity of the TCR[197,198]. Thus while TCR-engineered T cells have considerable potential for treating a number of different cancers, care must be taken when selecting antigen targets, particularly when artificially enhancing the TCR affinity.

Utilizing CARs to recognize tumor cells has also shown its shortcomings. Positive outcomes following treatment with CD19-reactive CARs are often associated with a complete loss of the B cell compartment, as that these CARs are not actually tumor specific and will eliminate all CD19+ cells[178,179,184,186,199]. This is remedied however by long-term immunoglobulin replacement therapy, as the persistence of CD19-CAR T cells also prevents the B cell compartment from reconstituting[90]. These studies also found significant, albeit transient, toxicities including high fevers and severe cytokine-release syndrome requiring interventions with cytokine blocking treatments[179,180,186,187]. These side effects were attributed to tumor lysis, and as such represent on-target CAR-T cell functionality resulting in toxic sequellae[179,182,200].

The use of third-generation CARs has also proven potentially concerning when utilizing a CAR recognizing HER2 combined with CD28, 4-1BB and CD3ζ signaling domains[201]. A patient with metastatic colon cancer was treated with a single infusion of 10^{10} HER2-CAR T cells, which induced respiratory distress within 15 minutes of infusion, to which the patient succumbed 5 days later[201]. This toxicity was attributed to both the high numbers of cells infused as well as the low level of endogenous expression of HER2 on the lung epithelium, resulting

in a dramatic cytokine response upon target recognition[201]. This study has since informed trial protocols whereby many trials now employ a dose-fractionation scheme where the total cell dose is broken into smaller increments in order to monitor for toxicity prior to infusion with the full cell dose. Similar to the TCR-engineered T cell reports, these studies indicate that careful monitoring and considerable attention must be dedicated to potential off-tumor, on-target toxicities that may arise from using such cytotoxic cell therapies.

4.0 Oncolytic Viruses

4.1 Viral Oncolysis

An oncolytic virus (OV), by definition, is one that is capable of replicating selectively within tumor cells, resulting in cell death[202]. The concept of using viruses as a cancer therapeutic has been studied for over a hundred years, based on early observations that leukemia patients who contracted influenza experienced short-lived cancer regressions[203]. Other reports observed regressions of leukemia, Hodgkin's disease and Burkitt's lymphoma following measles or varicella infections, furthering the idea that certain viruses are capable of attacking tumor cells while leaving most healthy cells untouched[203]. Early clinical trials during the period of 1949 to 1974 attempted to treat cancer with Hepatitis B, West Nile, or Mumps viruses; however, the outcomes on tumor growth were inconclusive and the virus infections resulted in undesirable pathologies[203]. Significant advancements in cell culture, such as the development of *in vitro* virus

propagation and genetic engineering techniques, have resulted in the generation of non-pathogenic viruses with enhanced tumor cell tropism[203]. Virus modifications have provided enhanced oncolytic capabilities in the form of either attenuated replication in normal cells, or increased replication in tumor cells[204]. Additionally, exogenous genetic sequences can be added to the viral genome to improve tumor tropism or manipulate the immune system's response to the virus[203,205].

Early studies of OV therapeutics used wild type, non-engineered viruses[206]. Some of these proved quite effective. Tumor regression following treatment with vaccinia virus displayed greater durability than those achieved by adenovirus or mumps virus treatments[206]. However, to drive more tumor-tropic viral replication, viral engineering became a prominent research focus[203]. In general, these modifications can be applied to two broad types of tumor selectivity: 1) enhancing the IFN sensitivity of the virus, or 2) attenuation of viral virulence genes[202]. Several viruses that do not cause human illness are used as oncolytic viruses due to their sensitivity to IFN, as healthy cells readily activate the antiviral IFN pathways in response to infection and mediate effective viral shut down[202,207,208]. The IFN response is frequently dysfunctional or absent in tumors, allowing viruses such as reovirus, Newcastle Disease virus (NDV), or vesicular stomatitis virus (VSV) to readily replicate in and lyse tumor cells, while healthy cells can control virus replication and spread[202,207,209,210]. Modification or removal of viral virulence genes has also been shown to enhance OV tumor tropism. Deletion of the thymidine kinase (TK) gene from herpes simplex virus

(HSV) attenuated virus replication in non-dividing cells, but did not impair replication in tumor cells[203,211]. Removal of the same TK gene has also proven effective in the case of poxviruses[212]. Deletions in viral components designed to block IFN signaling have also been found to confer IFN hypersensitivity and subsequently enhance viral oncolytic capacity[213].

4.2 Oncolytic viruses and immune stimulation

The antitumor properties of OVs are not solely attributed to their oncolytic capacity. The majority of OVs drive potent antitumor immunity, potentiating the overall tumor destruction[214]. There are a number of different mechanisms through which OVs stimulate both innate and adaptive immune responses. Firstly, viruses themselves are pathogens that bear PAMPs or danger signals to the innate immune system, which activates local innate immunity[215]. OV are also capable of influencing the tumor microenvironment, modulating the cytokine environment to promote a pro-inflammatory profile and promoting tumor infiltration by T cells and NK cells[215-219]. The act of oncolysis also results in the release of a plethora of tumor-associated antigens (TAA) into the tumor microenvironment[215]. Thus the immunogenic cell death induced by OVs provides both the stimulus and the target antigens needed to drive a robust adaptive immune response to the tumor antigens. The immune-mediated tumor destruction induced by OVs also supports the use of OVs to treat tumors that may be less susceptible to OV infection[215,216].

A detailed list of different OVs and their immunological mechanisms of action can be found in Melcher *et al*[220].

OVs can be further engineered to express immunostimulatory genes, such as T cell costimulatory molecules, chemokines for leukocyte recruitment, and cytokines aimed at activating a variety of immunological functions[221]. The inclusion of GM-CSF is one of the most common transgenes in oncolytic viral vectors[221]. GM-CSF is a haematopoietic growth factor that has been shown to increase antigen presentation, DC maturation and migration, and inflammation[222]. Encoding this gene within an OV results in localized cytokine production within the tumor and ultimately aims to enhance antitumor immunity[223]. The oncolytic JX-594, a TK-mutant vaccinia strain engineered to express GM-CSF, has been evaluated in clinical trials across a number of disease sites. In a Phase I trial of renal cell cancer, lung cancer and melanoma patients, antibody-mediated cell cytotoxicity correlated with overall survival, with the presence of T cell responses also detected via the presence of antibody class switching[224]. Treatment of patients with primary or metastatic liver cancer achieved partial responses or stable disease following intratumoral administration of JX-594, with responses also observed in non-injected tumors[225]. While a follow up Phase II study failed to show survival benefit of the virus when administered intratumorally for hepatocellular carcinoma treatment, JX-594 has proven safe for intravenous infusion and results in the destruction of the tumor vasculature, leading to tumor cell death[226-228]. Ongoing clinical studies aim to evaluate intravenous treatment of

ovarian carcinoma using JX-594, illustrating the broad potential of tumor types that can be targeted using OV[229]. The GM-CSF-engineered herpes virus talimogene laherparepvec (T-VEC) has shown significant clinical activity through Phase III clinical trials. This virus has deletions of the ICP34.5 and ICP47 genes, which are involved in virulence and immune detection, respectively[230]. Clinical studies in melanoma have compared T-VEC to GM-CSF alone. Phase II results and interim analysis from Phase III trials have revealed an objective response rate of 26% following T-VEC treatment, with 11% of patients experiencing complete regression[230-232]. In addition, regressions were observed in both injected and non-injected tumors, indicating this OV was capable of inducing systemic antitumor immunity[231]. Immunologically, tumor biopsies revealed the presence of tumor-reactive T cells as well as a reduction in immunosuppressive cell populations within the tumors of treated patients[233]. These data indicate that T-VEC is capable of inducing antitumor immune responses that may be contributing to the efficacy of this treatment. This OV is currently under application for approval as a therapeutic for melanoma, which would make it the first approved oncolytic immunotherapy to reach the public market.

4.3 Oncolytic viruses as a vaccination platform

The inclusion of immunomodulatory molecules such as cytokines or costimulatory molecules within an OV provides a non-specific immune stimulation geared at activating pre-existing tumor-reactive cells, or driving novel

responses to tumor antigens exposed following OV lysis. However, the immune response produced by oncolysis is typically of low magnitude[234]. To augment the adaptive immune response generated by OV therapy, OVs have been investigated as potential vaccine vectors (termed *oncolytic vaccines*)[234]. This process results in upregulation and release of specific tumor antigens within the pro-inflammatory environment induced by oncolysis, promoting antigen uptake and presentation and ultimately culminating in the generation of T cell responses to the tumor[234]. Encoding tumor-associated antigens (TAAs) within OVs has proven effective at promoting CD8+ T cell priming to tumor antigens, leading to enhanced antitumor immunity[235,236]. Additionally, oncolytic vaccines have shown considerable success when used to boost existing immune responses[237,238]. The oncolytic vaccines generate a robust anti-TAA response, particularly in hosts that have been primed with a heterologous vaccination[221,235]. The use of OVs as part of the vaccination platform also results in tumor de-bulking through oncolysis, providing the adaptive T cell response with a reduced tumor mass to eliminate[221]. Indeed, antitumor T cell responses play a pivotal role in the successful elimination of tumors when using TAA-encoded OVs in pre-clinical models[239]. Overall, oncolytic vaccines capture both robust anti-tumor activity via oncolysis and a powerful antigen-specific immune response via genetic immunization, yielding a potent anti-cancer agent.

5.0 Models used in this thesis

The research described in this thesis has focused on the development of T cell-based adoptive transfer therapies for cancer therapy. We have engineered T cells to express CARs that direct T cell reactivity to surface-expressed tumor antigens. In addition, we have evaluated the impact of combining these CAR-T cells with OVs to enhance antitumor activity. This section describes the different CARs and OVs that have been utilized in my research.

5.1 CARs and their targets

During my PhD research, I investigated a number of different CARs. A brief description of the CARs and their antigen targets is provided here. Detailed schematics illustrating the individual components of each CAR, as well as methods for their construction can be found in **Chapter 2**.

5.1.1 HER2-CAR

The bulk of the research described in Chapters 4 and 5 employed a CAR targeting the human HER2 protein, which is overexpressed on ~30% of breast and ovarian cancers and, to a lesser extent, non-small cell lung cancer, bladder, colorectal, and gastric cancers[240-243]. The HER2-CAR was first described in the early 1990's following the cloning of the HER2-specific scFv from the FRP5 monoclonal antibody[244-246]. For use in human T cells, we utilized a HER2-CAR provided by Dr. Philip Darcy (Peter MacCallum Cancer Centre, Melbourne,

Australia)[247]. For use in murine T cells, we replaced the human CD28 and CD3ζ sequences in the CAR provided by Dr. Darcy with the corresponding sequences from the mouse proteins. The HER2-CAR has previously been reported to induce CD4+ and CD8+ T cell activation and cytolytic activity upon ligation by tumor targets resulting in complete regression of certain murine tumors[170,247-249].

5.1.2 VEGFR2-CAR

Some of the work in Chapter 4 employed a CAR recognizing the vascular endothelial cell growth factor receptor 2 (VEGFR2). This receptor plays an important role in vascular growth and angiogenesis upon binding its ligand, VEGF[250]. Overexpression of VEGFR2 has been described on the tumor vasculature and some tumor cells[251,252], making it an interesting therapeutic target[253-255]. The anti-VEGFR2 scFv was cloned from the mouse DC101 hybridoma[256]. We obtained the scFv sequence from Dr. Chinnasamy, synthesized the corresponding cDNA and replaced the HER-2 scFv sequences in HER2-CAR with the DC101 scFv sequences to produce the VEGFR2-CAR. In previous reports, treatment of a variety of syngeneic mouse tumors with murine T cells engineered to express this VEGFR2-CAR produced significant inhibition of tumor growth, resulting in prolonged survival[256].

5.1.3 NKz-CAR

The work described in Chapter 3 focuses on comparisons of CARs based on the concept of using the NKG2D receptor as a ligand-binding domain. The NKz-CAR comprises the full-length NKG2D receptor fused to the CD3ζ signaling domain[257,258]. This CAR induces pro-inflammatory cytokine production and target cell death upon ligand binding, ultimately inhibiting tumor growth in murine models[257-260]. In addition, surface expression of NKz on murine T cells is dependent on the endogenous expression of adaptor protein DAP10[257].

5.1.4 NKz10-CAR

As the NKz-CAR relies on endogenous expression of DAP10 for surface expression, we evaluated the use of a retroviral vector expressing both the NKz-CAR and DAP10 (referred to as the NKz10-CAR). The DAP10 molecule functions as the signaling portion of the NKG2D receptor[261], and thus overexpression of this protein was hypothesized to increase expression and activity of the NKz-CAR. The use of the NKz10-CAR has been described in human NK cells, where the chimeric receptor was capable of inducing significant upregulation of NKG2D on the NK cell surface, and enhanced the cytolytic activity of the cells in response to tumor cell recognition[262]. Human NK cells engineered with the NKz10-CAR were capable of suppressing the growth of tumors in a xenograft model of osteosarcoma, while control NK cells could not, indicating this receptor provides antitumor function[262].

5.1.5 NK28z-CAR

The NKz-CAR is effectively a first generation CAR, although it is hypothesized that ligation of the CAR will provide costimulatory signals through the NKG2D-mediated association with DAP10. To determine whether a conventional second-generation CAR might have better T cell activation properties than NKz, we constructed the NK28z-CAR where the extracellular domain from NKG2D was used in place of the scFv used in the above-described HER2- and VEGFR2-CARs. Previous studies evaluating a similar NKG2D-CD28-CD3ζ-CAR construct in Ewing's sarcoma[263], and a related NKG2D-4-1BB-CD3ζ-CAR construct in ovarian cancer[264] described efficient target cell killing by both CD4+ and CD8+ T cells engineered with the CARs. These CAR constructs have not been evaluated *in vivo*.

5.2 Oncolytic viruses

5.2.1 Vesicular Stomatitis Virus

VSV is a single-stranded, negative-sense RNA virus belonging to the *Rhabdoviridae* family[265,266]. The virus predominantly affects rodents, cattle, swine, and other hoofed animals, while human infection is generally limited to those in frequent contact with these animals[266]. Description of VSV infections in farm animals date back to the early 1800's, with low rates of human infection and subsequent antibody seropositivity outside of endemic areas of the Southwestern USA and Central America[265]. In 2000, VSV was observed to preferentially infect

tumor cells due to intrinsic defects in IFN-signalling in tumor cells[207]. The M protein of VSV is involved in shutting down the IFN response through blockade of IFNβ mRNA nuclear export[204]. It was discovered that deletion of the methionine 51 in the VSV M protein further enhanced the sensitivity of the virus to IFN, rendering it highly attenuated in any cell type capable of producing and responding to IFN[204]. By preventing this shut down of IFN production, the ΔM51 mutant is substantially attenuated in healthy cells with intact IFN responses[204]. The anti-tumor activity of VSVΔM51 variant was the same as wild type, but the VSVΔM51 variant had a much improved toxicity profile[204]. Studies from our group have shown that VSVΔM51 viruses function as effective oncolytic viruses as well as vaccine vectors[235,237,267]. These data combined with the lack of pre-existing immunity to the virus in the general population makes VSV a strong candidate for immunotherapy development.

5.2.2 Vaccinia virus

Vaccinia virus (VV) has been long understood to drive potent immune responses, having been widely used in vaccination strategies leading to the eradication of smallpox[268]. VV is a large, enveloped, double-stranded DNA virus that belongs to the *Poxviridae* family[269]. The large size of the VV genome has facilitated the engineering of a variety of different vaccine vectors carrying antigens, cytokines, as well as immunostimulatory molecules[270]. Clinical investigations of VV for cancer therapy date back to the 1970's, however the

mechanism of action of regressions was unclear and suspected to involve activation of immune cytolytic activity[271,272]. The inherent lytic nature of VV provides the basis for development of this virus as an oncolytic[212]. While VV has been shown to also naturally target tumor cells, genetic modifications have proven to increase the tumor tropism and safety of oncolytic VV[212,270]. In particular, the deletion of the thymidine kinase (TK) gene encoded within the virus forces the virus to rely on cellular TK for replication[212]. This gene is transiently expressed in normal cells during growth, however is constitutively expressed at high levels in cancer cells[212]. The additional deletion of VV's vaccinia growth factor (VGF) results in even greater tumor cell specificity and reduces pathogenicity in healthy tissues[212,270]. This "double-deleted" vaccinia (vvDD) has shown significant oncolytic effects in murine tumor models, and is currently undergoing clinical evaluation[212,270].

6.0 Scope of described research

The overall objective of my PhD thesis research was to develop adoptive transfer therapeutics for cancer utilizing CAR-T cells, as well as investigate the potential for use in combination with oncolytic virotherapy. The research objectives set forth during my studies were based on previous findings from the Bramson laboratory reporting on adaptive immune responses and cancer immunotherapy.

6.1 Previous work by the Bramson lab relevant to thesis work

Initial studies by our group observed that CD4+ and CD8+ T cells could mediate prophylactic tumor protection in the setting of recombinant adenovirus immunization[273-275]. However, vaccination alone was incapable of producing marked regression of existing tumors despite a strong immune response (approx. 5% of circulating CD8+ T cells were specific for tumor antigen)[93,94]. The tumors retained antigen expression even in the face of the vaccine-induced immune response, demonstrating that antigen loss was not responsible for the lack of therapeutic effect of the vaccine. Tumor-specific T cells within the tumor were defective in their ability to produce cytokines and degranulate, providing some explanation for the lack of therapeutic effect[94]. Further investigations revealed that the tumor itself rapidly adapted to the immune attack following vaccination by upregulating numerous immunosuppressive pathways to counteract the T cell response[118]. Ultimately, we determined that the slow expansion of the immune response following vaccination strategies does not permit a sufficiently robust T cell response to control the tumor before the adaptation event resulted in suppressed local immunity. By increasing the availability of tumor-reactive T cells prior to vaccination via adoptive transfer methods, we determined that the immunosuppression could be overcome and lead to tumor regression[118]. This work demonstrates the importance of driving a sufficiently robust and rapid T cell attack against the tumor in order to achieve therapeutic success. Since there is conceivably no upper limit to the number of T cells that can be infused at a single

time, the focus of the lab shifted to ACT in an effort to deliver a sufficiently large bolus of T cells to overcome the adaptive response of the tumor.

6.2 Research Objectives

The research conducted during my graduate studies has been framed around three research objectives described in Chapters 3-5 of this thesis as described below:

Objective 1: Investigate the utility of NKG2D-based CARs for therapeutic treatment of breast cancer, and the differences between receptors containing different signaling elements. Results pertaining to these studies are presented in **Chapter 3**.

Objective 2: Examine the combination of CAR-T cells and oncolytic virus boosting through use of dual-specific T cells. Results pertaining to these studies are presented in **Chapter 4**.

Objective 3: Evaluate the ability of CAR-T cells to function as oncolytic virus carriers. Results pertaining to these studies are presented in **Chapter 5**.

— CHAPTER 2 —

Materials and Methods

Mice

Six- to eight-week-old female BALB/c, C57BL/6 or albino C57BL/6 (C57BL/6N-Tyr$^{c\text{-}Brd}$/BrdCrCrl) mice were purchased from Charles River Breeding Laboratories. Dr. Pamela Ohashi kindly provided spleens from P14 transgenic mice. SMARTA transgenic mice were bred in the Central Animal Facility at McMaster University. All animal studies have received approval by the McMaster University Animal Research Ethics Board.

Cell lines

The murine breast tumor cells D2F2 and D2F2/E2 (provided by Dr. Wei-Zen Wei, Barbara Ann Karmanos Cancer Institute, Detroit, MI, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 5% cosmic calf serum, 1mM sodium pyruvate, 2mM L-glutamine, 0.1mM non-essential amino acids, 10mM HEPES, 55nM β-mercaptoethanol, 100 U/ml penicillin and 100μg/ml streptomycin. To ensure stable expression of human HER2 was maintained, D2F2/E2 cells were further supplemented with 800μg/ml G418 (Sigma). The murine breast tumor line 4T1.2 and the colon carcinoma line MC38 engineered to express human HER2 (MC38-HER2) were maintained in RPMI 1640 medium containing 10% FBS, 2mM L-glutamine, 10mM HEPES, 100 U/ml penicillin and 100μg/ml streptomycin. The murine melanoma line B16F10 was cultured in MEM-F11 media containing 10% FBS, 2mM L-glutamine, 1x vitamin solution, 1mM sodium pyruvate, 0.1mM non-

essential amino acids, 55nM β-mercaptoethanol, 100 U/ml penicillin and 100μg/ml streptomycin.

The human lung tumor cells A549 and the human breast tumor cells T47D were maintained in RPMI 1640 medium containing 10% FBS, 2mM L-glutamine, 10mM HEPES, 100 U/ml penicillin and 100μg/ml streptomycin. Vero cells were cultured in α-modified Eagle's medium with 10% FBS, 2mM L-glutamine, 100 U/ml penicillin and 100μg/ml streptomycin. CV-1 cells were cultured in DMEM with 10% FBS, 2mM L-glutamine, 100 U/ml penicillin and 100μg/ml streptomycin. For vaccinia titrations, CV-1 medium used 2% FBS instead. All cells were cultured at 37°C with 5% CO2.

Generation of CAR retroviral vectors

A human anti-HER2 CAR was kindly provided by Dr. Philip K. Darcy (University of Melbourne, Victoria, Australia) [170]. An in-house variant was generated for use in murine T cells, in which the human sequences from the HER2-CAR were replaced by equivalent murine sequences. This HER2-CAR was comprised of a single chain antibody fragment (scFv) specific for human HER2[244], a short marker epitope of *c-myc*, the hinge region from murine CD8, the transmembrane and cytoplasmic portions of murine CD28, and finally the cytoplasmic portion of murine CD3ζ (**Fig. 2.1a**). These components were cloned into the MSCV-based retroviral vector pRV2011-oFL, kindly provided by Dr. Brian Rabinovich (MD Anderson Cancer Centre, Houston, Texas)[276].

The anti-VEGFR2-CAR construct was constructed from the scFv derived from the DC101 monoclonal antibody specific for mouse VEGFR2 described previously[256]. The scFv genetic sequence was kindly provided by Dr. Steven Rosenberg (NCI, Bethesda MD), which was used to synthesize the VEGFR2 scFv cDNA (GenScript, NJ, USA)[256]. The scFv was cloned onto the hinge and signaling domains from the above described HER2-CAR.

The NKz-CAR construct was generated according to [257], where the cytoplasmic region of murine CD3ζ was fused to full-length murine NKG2D via DNA synthesis (GenScript, Piscataway, NJ, USA). The shuttle plasmid pDONR222 oTK-P2A-oFL-T2A-eGFP (kindly provided by Dr. Brian Rabinovich, MD Anderson Cancer Centre) was modified by deleting oTK-P2A and replacing eGFP with NKz, resulting in pDONR222 oFL-T2A-NKz (oFL = firefly luciferase [276],T2A = *Thosea asigna* virus self-cleaving 2A peptide [277]). The oFL-T2A-NKz expression cassette was transferred into the retroviral vector pRV100G (also provided by Dr. Rabinovich) by LR recombination (Gateway LR Clonase II Enzyme Mix, Life Technologies). Production of the NKz10-CAR construct was similar; pDONR222 oTK-P2A-oFL-T2A-eGFP was modified by replacing oTK with full-length murine DAP10 and eGFP with the synthesized NKz sequence, resulting in pDONR222 DAP10-P2A-oFL-T2A-NKz (P2A = porcine teschovirus-1 self-cleaving 2A peptide [277]). LR recombination transferred DAP10-P2A-oFL-T2A-NKz into pRV100G. The NK28z-CAR was constructed by modifying an existing second generation CAR vector; HER2scFv28z. In

short, an scFv specific for HER2, a marker epitope from c-myc, the membrane proximal hinge region of murine CD8, the transmembrane and cytoplasmic regions of murine CD28, and the cytoplasmic region of murine CD3ζ were fused together and cloned into the retroviral vector pRV2011 oFL [276](also provided by Dr. Rabinovich) in place of firefly luciferase, leaving the IRES and Thy1.1 sequences intact. The extracellular portion of NKG2D was amplified out of the NKz-10-CAR plasmid using the primers NKDG2DF: 5'-GTTCAAGGAGACATTTCAGCCTGTG-3' and NKG2DR: 5'-ACAGCTCTCTTCATACAAATATAGGTATTC-3' and inserted into the HER2scFv28z vector in place of the scFv and c-myc to generate pRV2011-NKG2D-CD8-CD28-CD3ζ-IRES-Thy1.1 (**Fig. 2.1a**).

All CARs were constructed in the pRV2011-oFl vector, which also contained the congenic marker Thy1.1, which was used to identify transduced cells by flow cytometry, as well as identify adoptively transferred cells *in vivo* (**Fig. 2.1b**). CAR-constructs used for *in vivo* imaging studies were inserted into the pRV2011-oFL vector in place of Thy1.1, leaving the enhanced firefly luciferase (effLuc) sequence intact (**Fig. 2.1c**). Each individual component was separated by an IRES sequence. One exception was the NKz10-CAR, which was cloned into the pRV100g vector (also provided by Dr. Brian Rabinovich) in order to facilitate inclusion of the NKz CAR, DAP10, and effLuc sequences, separated by P2A and T2A sequences respectively (**Fig. 2.1a**).

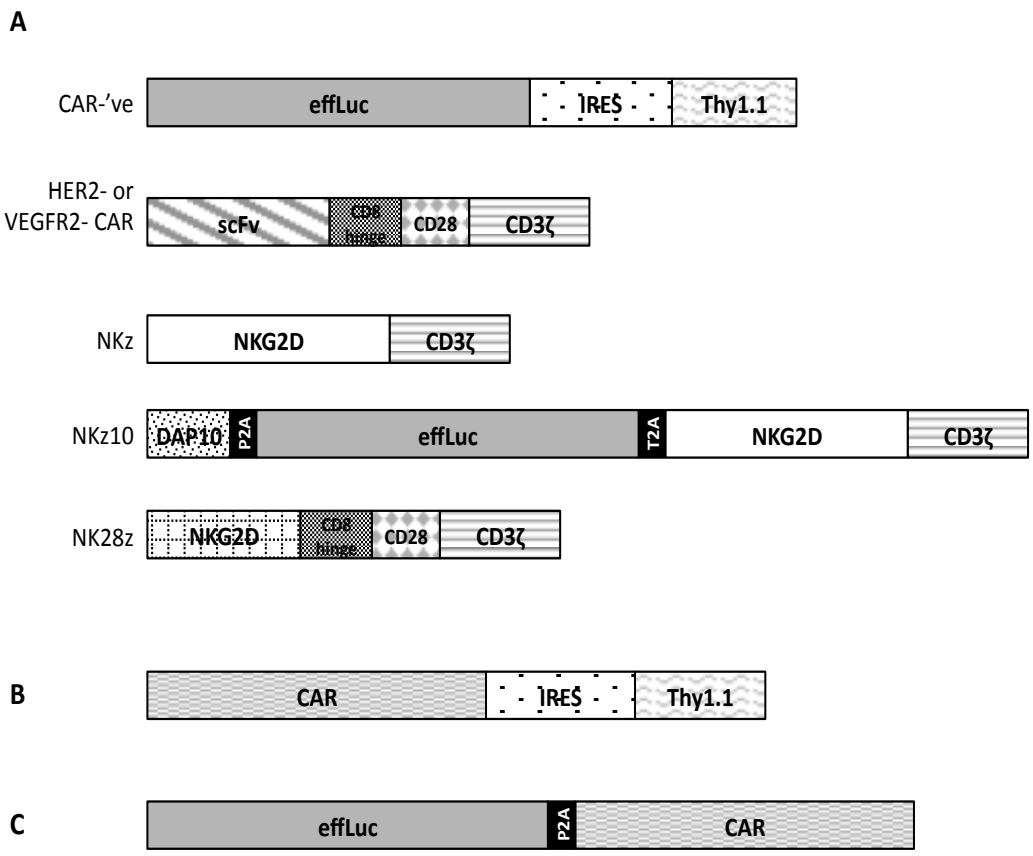


Figure 2.1: Schematics of the CAR constructs utilized throughout this thesis. (a) Individual CAR constructs. (b) CAR-Thy1.1 vector used for transduction efficiency evaluation, and identification of adoptively transferred cells. (c) CAR-effLuc vector used for *in vivo* imaging studies.

Murine ecotropic retroviruses were packaged by transfecting Platinum-E (PLAT-E) cells with retrovirus vectors and the helper plasmid pCL-Eco using Lipofectamine 2000 (Life Technologies) as described previously[276,278]. Retrovirus supernatants were harvested at 48 hours post-transfection, concentrated 10x using Amicon Ultra 100K Centrifugal filters (EMD Millipore), and used immediately to transduce murine T cells. Negative control murine T cells (CAR-'ve) were prepared using the same methods, using viruses were prepared from transfections with the pRV2011 oFL plasmid, which lacks the CAR cDNA.

Generation of human lentiviral vectors

For human T cell engineering, the human HER2-CAR (described above) was cloned into the lentiviral vector pCCL-ΔNGFR vector kindly provided by Dr. Megan Levings (University of British Columbia, Canada)[279]. Lentiviruses were produced by four-plasmid transfection of HEK 293T cells as described previously [280,281]. Virus titers were determined using serial dilutions on 293T cells and evaluated by flow cytometric staining for NGFR. Negative control human T cells (CAR-'ve) were prepared using the same methods, with viruses were prepared from transfections with the pCCL-ΔNGFR plasmid, which lacks the CAR cDNA.

Murine T cell transduction

Splenocytes from BALB/c or C57BL/6 mice were isolated and cultured in RPMI 1640 supplemented with 10% FBS, 10mM HEPES, 2mM L-glutamine,

0.1mM non-essential amino acids, 0.1mg/ml normocin (Invivogen), 1mM sodium pyruvate, and 55nM β-mercaptoethanol. Splenocytes were activated with 0.1μg/ml each hamster anti-mouse CD3 (clone 2C11; BD Biosciences) and hamster anti-mouse CD28 (clone 37.51; BD Biosciences), and cultured in the presence of 60 IU rhIL-2 (Peprotech). After 24 hours, T cells were transduced via spinfection, whereby $3x10^6$ cells were incubated with 100μl of concentrated retroviral supernatant in the presence of 1.6μg/ml Polybrene (Sigma) and 2μg/ml Lipofectamine 2000 (Life Technologies). Cultures were centrifuged at 2000rpm at 32°C for 90 minutes. Cells were incubated at 37°C for 2-4 hours and fed with fresh medium and IL-2. After 48 hours, T cells were scaled up into larger flasks containing fresh medium and 60U/ml IL-2. Four days after activation, T cells were stained for CAR expression and used for both *in vitro* and *in vivo* experiments.

For dual-specific T cell experiments, the above protocol was followed, with the exception of T cell activation. P14 splenocytes were activated by addition of 1μg/ml GP33-43 peptide, while SMARTA splenocytes were activated with 10μg/ml GP61-80 peptide instead of the anti-CD3/CD28 antibodies.

Human T cell transduction

Human peripheral blood cells (PBMCs) were isolated from healthy donors. T cells were activated using anti-CD3/CD28 Dynabeads (Gibco) in the T cell media described above, in the presence of 100IU/ml IL-2 and 10ng/ml IL-7

(Peprotech). Twenty-four hours after activation, T cells were transduced with an MOI of 1 of the indicated lentiviruses. T cells were expanded in T cell medium with 100IU/ml IL-2 and 10ng/ml IL-7, feeding with fresh medium and cytokines every 2-3 days, and loaded with OVs 14 days after activation.

Tumor challenge, pre-conditioning, ACT and* in vivo *monitoring

Murine T cells engineered with the indicated RVs were harvested 4 days after activation, and prepared for intravenous (i.v.) injection of 10^7 viable cells in 200μl of sterile PBS.

For VEGFR2-CAR experiments, C57BL/6 mice were challenged intradermally with 10^5 B16F10 cells in 30μl sterile PBS as described previously[94]. For HER2-CAR-T cell experiments, C57BL/6 mice were challenged intradermally with $5x10^5$ MC38-HER2 cells in 30μl PBS. BALB/c mice were challenged either subcutaneously in the flank or orthotopically in the 4th right mammary fat pad with $3x10^5$ 4T1.2 cells in 50μl PBS. Adoptive transfer occurred between 5-14 days post-inoculation depending on the tumor model used.

In experiments using pre-conditioning cyclophosphamide (CTX) treatment, CTX (Sigma) was reconstituted in sterile PBS and administered intraperitoneally (i.p.) at 150mg/kg 24 hours prior to ACT. Experiments that used pre-conditioning sublethal irradiation exposed animals to 550cGy administered via ^{137}Cs γ-irradiation source (Gamma Cell 40; Nordion, Kanata, ON, Canada).

For dual-specific T cell boosting experiments, animals received two doses

of $2x10^9$ PFU VSVΔM51-GP33/61 i.v. with the first at 6 hours post ACT, with the second dose 48 hours post ACT.

For toxicity experiments, animals were monitored at least twice daily, evaluated on physical appearance, behavior, and reaction to stimulus. Mice showing signs of toxicity were supported with hydrogel and food on the cage floor. Temperatures were assessed by rectal probe at the indicated time points.

Bioluminescent imaging

Mice were anaesthetized using Isoflurane (Pharmaceutical Partners of Canada Inc., Richmond Hill, ON) and received 150mg/kg D-luciferin (Perkin Elmer, Woodbridge, ON) i.p. Animals were monitored for 14 minutes, after which images were taken using the "open filter" setting for 30s using the IVIS 200 Spectrum Imager (Perkin Elmer). Total flux signal was quantified with LivingImage 3.2 software (Perkin Elmer).

Intracellular cytokine staining (ICS)

Both murine and human CAR-T cells were stimulated using plate-bound recombinant human HER2-Fc chimera (1000ng/ml in PBS; R&D Systems), recombinant murine Rae1β-Fc chimera (4000ng/ml in PBS; R&D Systems), recombinant murine VEGFR2-Fc (1000ng/ml in PBS; R&D Systems), or vehicle control (as indicated) for 4 hours at 37°C in the presence GolgiPlug™ protein transport inhibitor (BD Pharmingen). Following stimulation, cells were

resuspended in 5% FBS (in PBS) and stored at 4°C overnight.

Flow cytometry antibodies and analysis

For murine T cell work, we used the following BD Bioscience antibodies: mouse Fc-Block™, anti-CD4 (clone RM4-5), anti-CD8a (clone 53-6.7), anti-IFNγ (clone XMG1.2), and anti-TNFα (clone MP6-XT22). The following anti-mouse antibodies were purchased from eBiosciences (San Diego, CA): anti-CD8a (clone 53-6.7) and anti-NKG2D (clone CX5). Recombinant murine NKG2D-Fc (R&D Systems) was used to stain for NKG2D ligands, and detected with goat-anti-human IgG (Jackson ImmunoResearch). Viability staining was performed using the Molecular Probes LIVE/DEAD® Fixable Near-IR dead cell stain kit (Life Technologies). For human T cell staining, we used the following BD Bioscience antibodies: anti-CD4 (clone RPA-T4), anti-NGFR (clone C40-1457), anti-IFNγ (clone B27), and anti-TNFα (clone MAb11). The anti-CD8a clone OKT8 from eBiosciences was also used. Recombinant human HER2-Fc (R&D Systems) was used to stain for HER2-CAR expression on both murine and human T cells, and detected with goat-anti-human IgG (Jackson ImmunoResearch). Intracellular cytokine stains were performed using the cytofix/cytoperm reagent and associated protocol (BD Biosciences). Data were acquired on a FACSCanto or LSRII (BD Biosciences) and analyzed using FlowJo software (TreeStar).

Multiplex cytokine analysis

We quantified 32 murine chemokines and cytokines using the Mouse Discovery Assay® (Eve Technologies Corp, Calgary, AB, Canada). Serum samples were derived from terminal retro-orbital blood samples processed as per Eve Technologies Corp. recommendations. The multiplex assay was performed by Eve Technologies using the Bio-Plex 200™ system and the Milliplex® Mouse Cytokine/Chemokine Magnetic Bead Panel Kit according to their protocol. The 32-plex panel included: Eotaxin, G-CSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNFα, and VEGF. The assay sensitivities of these markers range from 0.3-33.3pg/ml. Individual analyte values can be found in the Milliplex protocol. Heat maps were created using the HeatMapImage (version 6) module available on Gene Pattern (http://genepattern.broadinstitute.org/gp/ pages/index.jsf). Luminex data were preprocessed using the "affy" package in R, with RMA background adjustment and quantile normalization procedures [282]. Resulting cytokine expression values were transformed to the $\log_2$ scale. Linear models were fit for each cytokine using the 'limma' package in R to test for differential expression for pre-specified contrasts [283]. P-values for each contrast were obtained for each cytokine and adjusted for multiple comparisons using the Benjamini-Hochberg procedure[284]. After pre-processing, we confirmed that samples were separated into homogeneous groups matching experimental groups, and performed Principal

Component Analysis (princomp function from 'stats' package, R) with all 32 cytokines.

Immunohistochemistry

Tissues were prepared for veterinary necropsy via whole body formalin perfusion as described previously [285]. After fixation in 10% neutral buffered Formalin, tissues were paraffin embedded, sectioned and stained using hematoxylin and eosin at the Core Histology Facility, McMaster Immunology Research Centre (MIRC).

RNA extraction from murine tissues and quantitative real-time PCR

Lungs were perfused with PBS, excised and snap frozen in liquid nitrogen prior to storage at -80°C. Tumors were excised and snap frozen by the same method. Tumors and lungs were homogenized in Trizol (Life Technologies) using a Polytron PT1200C (Kinematica) and total RNA was extracted according to the manufacturer's protocol. Reverse transcription was carried out using Superscript III First-Strand (Life Technologies) according to the manufacturer's specifications. Quantitative PCR was performed using Perfecta SYBR Green SuperMix, ROX (Quanta Biosciences), with samples run on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Data for target genes were analyzed via the delta/delta CT method, with GAPDH serving as an endogenous control.

qRT-PCR primer sequences

Gene	Primer Sequences
IFNγ	Fwd: CTTGAAAGACAATCAGGCCATC
	Rev: CAGCAGCGACTCCTTTTCC
TNFα	Fwd: AAATAGCTCCCAGAAAAGCAAG
	Rev: CTGCCACAAGCAGGAATGAG
TCRα	Fwd: TCACCAAAGCAGACAAGAGAAC
	Rev: GCAGGAAGGGAATGGAAAC
effLuc	Fwd: CCACCCTGTTCAGCTTTTTC
	Rev: AGAATGGCGCTTGTGGTCTC
GAPDH	Fwd: AGGAGCGAGACCCCACTAAC
	Rev: GGTTCACACCCATCACAAAC

Oncolytic Viruses and T cell loading

We utilized the interferon-sensitive ΔM51 mutant of VSV expressing GFP (VSVΔM51-GFP) kindly provided by Dr. Brian Lichty (McMaster University, Hamilton, Ontario, Canada)[286]. The VGF-, TK- double deleted vaccinia virus (vvDD) expressing GFP was provided by Dr. Andrea McCart (University of Toronto, Ontario, Canada)[270] and Dr. John Bell (University of Ottawa, Ontario, Canada). Murine and human CAR-T cells were loaded with OV following the same protocol. CAR-T cells were incubated with a multiplicity of infection (MOI) of either 0 (mock) or 3 of the indicated virus for 3 hours at 37°C. Cells were

washed 4 times in PBS at 4°C, resuspended in T cell growth medium and used in the described experiments.

Virus titrations

All samples taken for virus titrations were frozen at -80°C prior to titration, and thawed only to titrate virus. To determine vaccinia titrations, confluent CV-1 cells were infected and visualized using crystal violet as described previously [287].

Virus titers for VSV were determined utilizing confluent Vero cells in 60mm dishes. Serial virus dilutions were prepared and added to Vero cells in a 100µl volume for 45 minutes. After allowing for adsorption, 3ml of prepared agarose overlay was added (1:1 of 1% agarose, 2x F11 medium with 20% FBS). Plaques were counted at 24 and 48 hours later.

In vitro cytotoxicity assay

Murine D2F2, D2F2/E2, or 4T1.2 cells, or human A579 or T47D tumor cells were used for *in vitro* cytotoxicity assays. These assays were performed by co-culturing varying ratios of transduced T cells with $1.25x10^4$ target cells per well in triplicate in 96-well flat-bottom plates in a 200µl volume. After 6 hours, plates were washed three times with PBS, and 100µl of 10% alamarBlue® (Life Technologies) in T cell media was added. For assays evaluating combined killing between OV and CAR-T cells, plates were incubated for 24 hours, after which T cells were washed off, and 10% alamarBlue® was added. Three hours later,

fluorescence was measured with excitation at 530nm and emission at 590nm using a Safire plate reader (Tecan). Tumor cell viability was calculated as the loss of fluorescence in experimental wells compared to untreated target cells.

Statistical analysis

Student's *t* tests were used to compare data between two groups. One and two-way ANOVA were used for analysis of more than two groups, with Bonferroni post-hoc tests used to evaluate significance between groups and appropriate controls. Results were prepared using GraphPad Prism 5. Significant differences between means was defined as: * $p<0.05$, ** $p<0.01$, *** $p<0.001$; n.s= not significant.

— CHAPTER 3 —

Systemic toxicities associated with CAR-T cells that target NKG2D ligands

Introduction

Treating patients with T cells that are engineered to express tumor-specific receptors has proven to be a clinically efficacious form of immunotherapy. In particular, the use of chimeric antigen receptors (CARs) to direct T cells to attack tumors has shown significant promise in clinical trials[129,179-181,183,200]. The goal of these receptors is to target surface-expressed antigens that are either restricted to or overexpressed on tumor cells, eliminating the conventional T cell receptor requirement for antigen presentation on MHC molecules. One method of generating CARs fuses native proteins, which naturally ligate proteins on the surface of tumor cells, with the intracellular signaling domains required to induce T cell activation. Ligands for the natural killer group 2 member D (NKG2D) receptor are numerous and have been shown to be frequently upregulated on many cancer types[33,260,263,288,289]. Additionally, NKG2D ligand (NKG2DL) expression can be induced or upregulated on tumor cells through the use of already approved drugs such as spironolactone, allowing for further target enhancement[290].

Using a CAR comprised of NKG2D fused to the CD3ζ TCR signaling domain enables T cells to recognize any of the several natural NKG2DL, and exert their cytolytic functions[257,258,289,291]. While NKG2D is an activating receptor on natural killer (NK) cells, it functions primarily as a costimulatory receptor on activated CD8+ T cells[31,292-294]. In both murine and human T cells, or human NK cells, signaling through the NKG2D receptor is mediated through an adaptor

protein, DAP10[31]. This adaptor protein activates the PI3-K and Grb-2 pathways, much like the T cell costimulatory molecule CD28[293,295]. Research has revealed that the inclusion of costimulatory domains in CARs enhances T cell efficacy and persistence post-adoptive transfer[166,167,171,Song:2011cv 173,191,193]. In that regard, fusion of full-length NKG2D with CD3ζ may provide costimulatory signals via the NKG2D portion of the receptor, in addition to the activation signal delivered through CD3ζ. In this manuscript, we investigated 2 distinct CARs based on the NKG2D receptor: 1) a fusion of full-length NKG2D with CD3ζ (NKz) and 2) a fusion of the NKG2D ligand binding domain to signaling domains from a conventional second-generation CAR composed of CD28 fused to CD3ζ (NK28z). Since surface expression of full-length NKG2D is dependent upon the DAP10 molecule[257,261], we also investigated whether co-expression of DAP10 along with the NKz fusion protein (NKz10) could further augment CAR activity in terms of cytokine secretion and cytolytic capacity; all of which play an important role in antitumor responses.

Our results revealed that the functionality of the CARs was strain-dependent in murine T cells. Further, T cells expressing NKG2D-based CARs displayed *in vivo* toxicity, which was exacerbated when T cell infusion was combined with chemotherapeutic lymphodepletion. The NKz-CAR-T cells displayed the lowest toxicity *in vivo*, which suggests that this configuration may be amenable to clinical evaluation. These results revealed that NKG2D-based CAR-T cells can be highly toxic when delivered systemically and indicate that further research is

required to better understand how to deploy these CAR-T cells safely in the clinic.

Results

Inclusion of DAP10 in the retrovirus significantly enhances surface expression of NKz

To develop T cells recognizing NKG2D-ligands, we engineered murine T cells with one of three different NKG2D-based CAR retrovirus (RV) constructs: full length NKG2D fused to cytoplasmic CD3ζ (NKz), the same NKz-CAR with the addition of adaptor protein DAP10 to the RV construct (NKz10), or a conventional second-generation CAR that fuses the extracellular domain of NKG2D to a CD8-hinge region, CD28 transmembrane and cytoplasmic domains, and the cytoplasmic domain of CD3ζ (NK28z; **Chapter 2, Fig 2.1**). We utilized NKG2D cell surface staining as an indicator of NKG2D-CAR expression, as CAR-'ve T cells show very low levels of endogenous NKG2D expression (**Fig. 3.1a-b**). Engineering T cells with any of the three NKG2D-CAR RVs resulted in nearly 100% of both CD8+ and CD4+ T cells staining positive for NKG2D within three days of transduction for all three constructs in both strains tested, although expression on the BALB/c T cells was generally higher than the C57BL/6 T cells (**Fig. 3.1a-b, Fig. 3.2**). On a per-cell basis, the NKz10-CAR-T cells showed over 7- to 10-fold higher expression of NKG2D compared to the NKz construct,

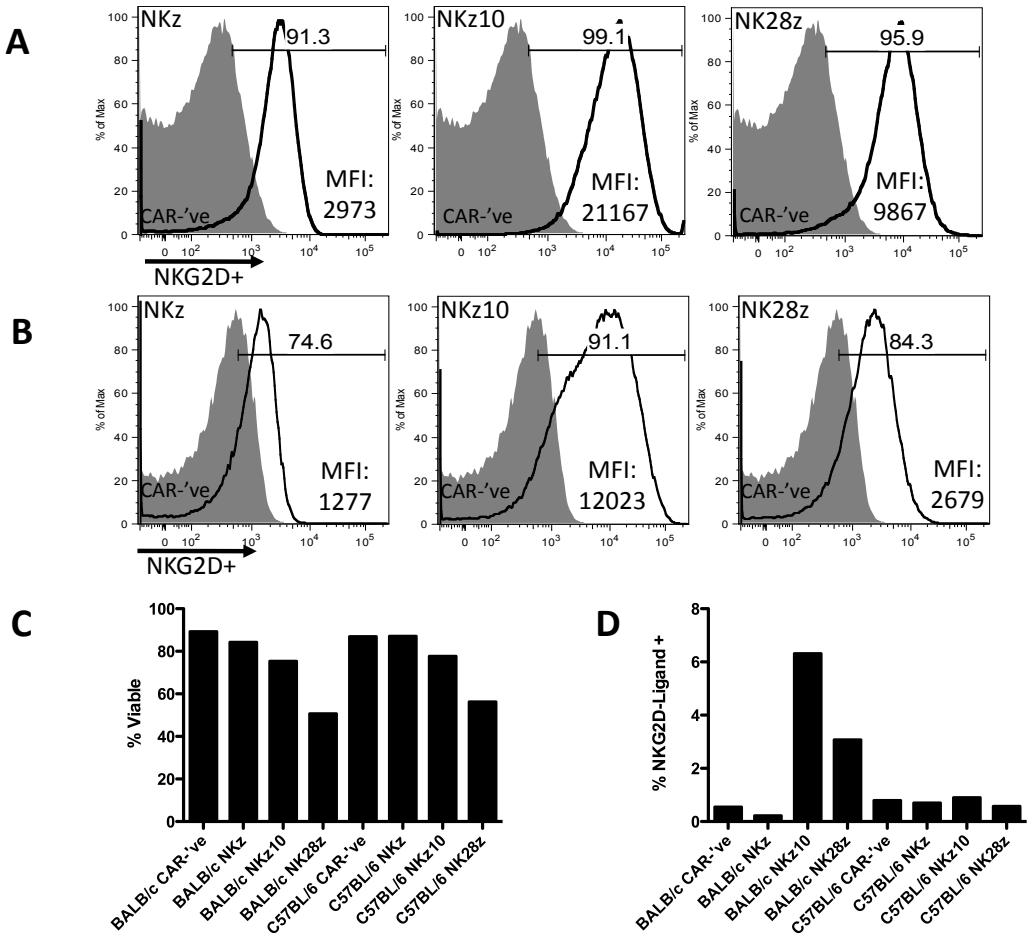


Figure 3.1: *In vitro* phenotypic profiles of NKG2D-ligand-specific chimeric antigen receptor (CAR)-engineered T cells. NKG2D expression on (a) BALB/c or (b) C57BL/6 CD8+ T cells was evaluated 3 days after transduction with the indicated CAR-containing retroviruses. Surface expression was determined using a fluorescence-minus one control of the anti-NKG2D – APC antibody, and compared to basal expression on control CAR-'ve T cells (shaded peaks). Mean fluorescence intensity and percentage of NKG2D+ CD8+ cells are shown. Data is representative of at least three independent experiments. (c) NK28z-CAR T cells show reduced viability compared to NKz or NKz10 CAR-T cells from both BALB/c and C57BL/6 mice as indicated by Live/Dead staining. (d) NKG2D-CAR cells differ in NKG2D-ligand expression, as indicated by staining using an NKG2D-IgG-Fc chimeric protein to detect ligand expression.

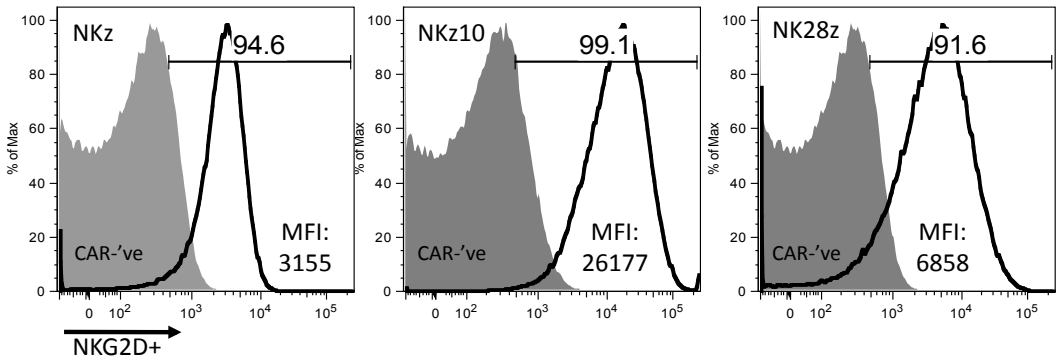


Figure 3.2: NKG2D-CARs are well expressed on murine CD4+ T cells. Expression of NKG2D on CD4+ T cells was evaluated 3 days post retroviral transduction using the indicated NKG2D-CAR retroviruses, with surface expression detected using an anti-NKG2D-APC antibody. Shaded peaks represent CAR-'ve T cell staining. Mean fluorescence intensity and percentage of NKG2D+ CD4+ cells are shown. Data is representative of at least three independent experiments.

indicating that the endogenous availability of DAP10 can limit surface expression of the NKz-CAR (**Fig. 3.1a-b**). The NK28z CAR showed an intermediate level of NKG2D surface expression, with 2-fold lower expression compared to NKz10 in BALB/c T cells (**Fig. 3.1a**) and 5-fold lower expression in C57BL/6 T cells (**Fig. 3.1b**).

NKG2D-CARs show strain-specific differences

We evaluated changes in CAR surface expression, T cell viability, and NKG2DL expression on the NKG2D-CAR-T cells between the three NKG2D-CAR constructs, as well as between two mouse strains. Interestingly, both BALB/c and C57BL/6 T cells showed the same changes in cell viability across NKG2D-CAR constructs; NKz-engineered T cells showed no reduction in viability compared to CAR -'ve T cells, NKz10-CAR-T cells showed a slight reduction in cellular viability, and NK28z-CAR-T cells had a considerably decreased viability (**Fig. 3.1c**). This viability pattern was consistent over the course of the culture period for all NKG2D-CAR T cells (*data not shown*). We observed considerable differences in the levels of NKG2DL on the CAR-engineered T cells between BALB/c and C57BL/6-derived cells (**Fig. 3.1d**). NKG2D-CAR-engineered C57BL/6 T cells showed comparably low levels of NKG2DL expression across all CAR groups (**Fig. 3.1d**). Conversely, CAR-T cells derived from BALB/c splenocytes showed varied expression of NKG2DLs; CAR –'ve and NKz cultures showed low levels of expression, whereas NKz10

and NK28z cultures showed 6% and 3% NKG2DL expression respectively. All cultures, including CAR-'ve controls, displayed a steady decline in the frequency of NKG2DL-positive T cells over time (*data not shown*). While we initially theorized that NKG2D-CAR-T cells may be selectively depleting NKG2DL-positive T cells over time in culture, CAR -'ve cultures, devoid of NKG2D-CARs, also demonstrated the same decrease. As such, the progressive reduction of NKG2DL positivity observed in our T cell cultures is unrelated to the presence of an NKG2D-CAR.

Despite having a significant increase in per-cell NKG2D expression, the NKz10-CAR did not demonstrate enhanced *in vitro* functionality in BALB/c T cells. In BALB/c T cells, all three NKG2D-CARs were similarly capable of producing the activation cytokines IFNγ and TNFα upon stimulation with recombinant Rae-1β, a well-defined NKG2D ligand (**Fig. 3.3a**). In addition, all three NKG2D-CARs were able to induce robust killing of murine breast tumor cells *in vitro*, with all BALB/c-derived NKG2D-CAR-T cells able to kill ~50% of tumor targets after 6 hours of co-incubation at only a 0.5:1 T cell to tumor cell ratio (**Fig. 3.3b**). Virtually all of the tumor targets were killed after co-culture of BALB/c NKG2D-CAR-T cells and tumor cells at a 2:1 ratio, illustrating the strong cytotoxic potential of these NKG2D-CAR-T cells (**Fig. 3.3b**).

In contrast, NKG2D-CAR-engineered C57BL/6 T cells showed considerably lower capacity to produce cytokines upon CAR-stimulation compared to BALB/c-derived CAR-T cells (**Fig. 3.3c**). Interestingly, while

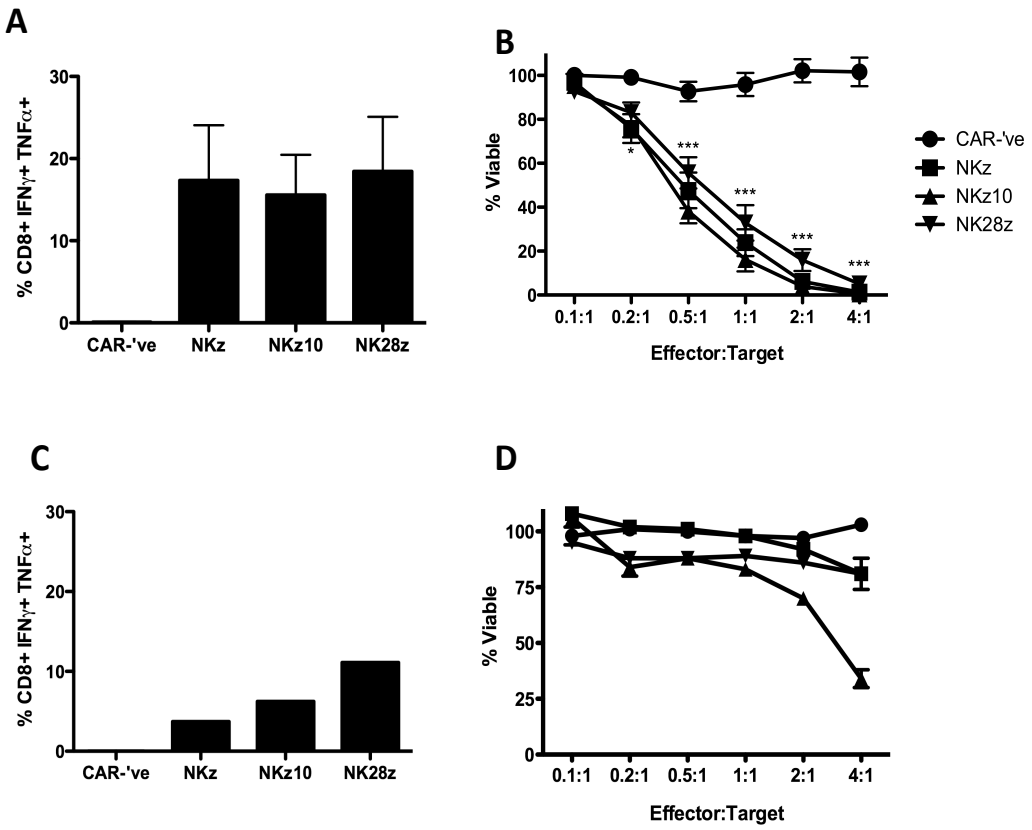


Figure 3.3: NKG2D-CAR-T cells *in vitro* functional profiles. (a-b) BALB/c or (c-d) C57BL/6 T cells were engineered with the indicated NK-CARs. (a) BALB/c T cells show equivalent production of IFNγ and TNFα in response to stimulation with plate-bound recombinant Rae1β-Fc fusion protein, while (c) C57BL/6 T cells show reduced cytokine production. Data is expressed as mean frequency ± SEM normalized to background levels from three independent experiments. (b) NKG2D-CAR BALB/c T cells show comparable capacity to kill 4T1.2 tumor cells *in vitro* using a 6-hour AlamarBlue assay at the indicated effector: target ratios. (d) C57BL/6 NKG2D-CAR- T cells show diminished *in vitro* killing. Mean frequency of viable tumor cells ± SEM from 3-4 independent experiments of triplicate wells is presented. *P < 0.05, ** P<0.01, ***P <0.001 as calculated against CAR-'ve.

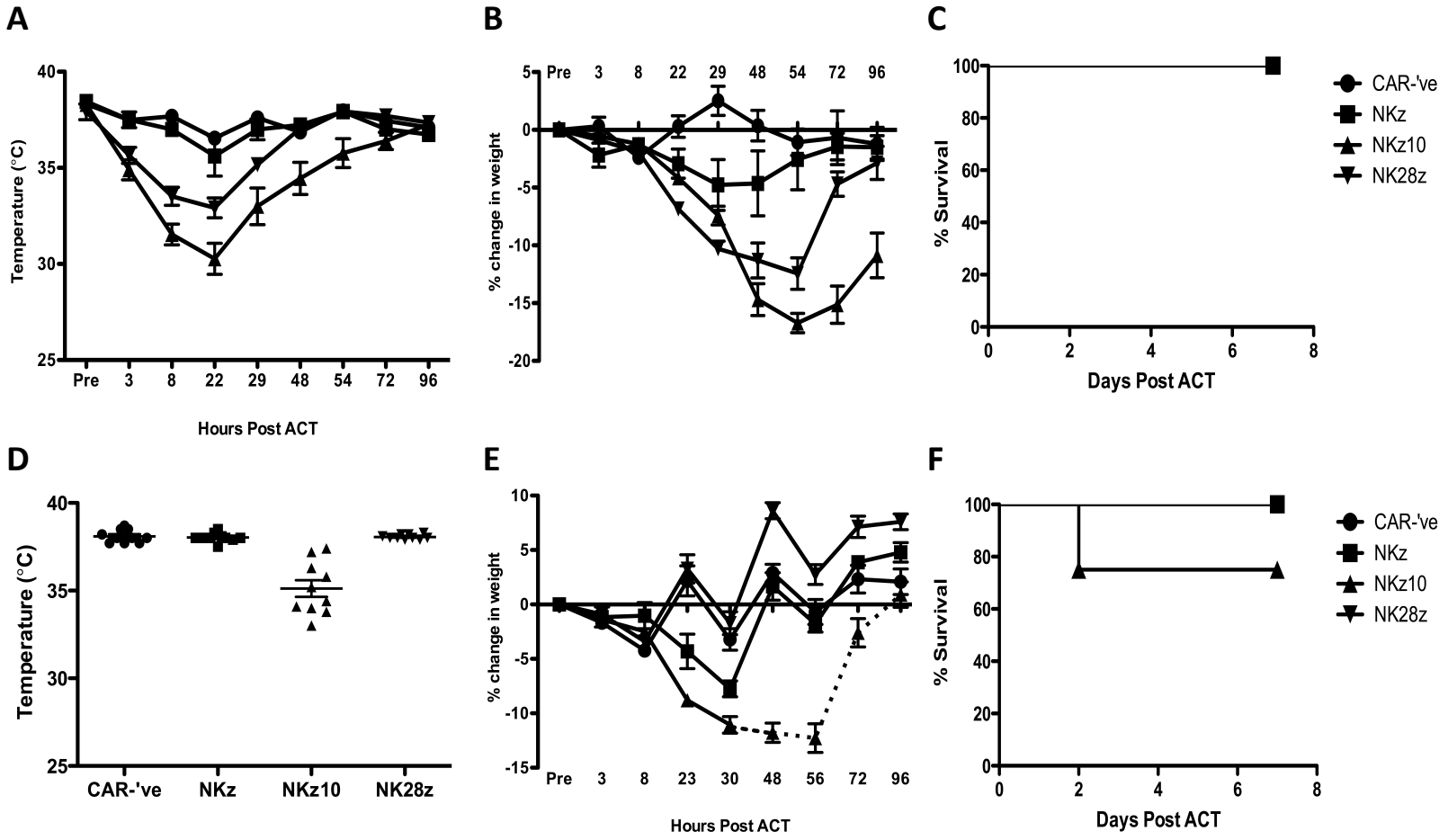
NKz10-CAR-T cells had the highest level of CAR expression in C57BL/6-derived cells, the NK28z-CAR-T cells showed the greatest cytokine production (**Fig. 3.3c**). This cytokine production did not translate to killing potential, as the C57BL/6 NK28z-CAR-T cells displayed weak cytotoxicity *in vitro* (**Fig. 3.3d**). Similarly, the C57BL/6-derived NKz-CAR T cells also exhibited weak cytotoxicity (**Fig. 3.3d**). While C57BL/6 NKz10-CAR-T cells were capable of killing tumor targets at higher E:T ratios, their activity was considerably diminished when compared to their BALB/c counterparts (**Fig. 3.3b,d**). Taken together, our data reveal striking strain-dependent differences in the functionality of the various NKG2D-based CARs, which were independent of observed strain-specific differences in NKG2D-CAR expression.

NKG2D-CAR-T cells can induce significant, acute toxicity upon adoptive transfer

We next investigated the functionality of NKG2D-CAR-T cells *in vivo.* For these experiments, we employed the 4T1.2 breast tumor model in BALB/c mice. Mice bearing established tumors were treated with cyclophosphamide (CTX), followed by infusion of NKG2D-CAR T cells. Strikingly, we observed dramatic clinical symptoms indicative of toxicity within just a few hours of adoptive transfer. To understand whether this toxicity was related to an over-exuberant antitumor immune response resulting from the NKG2D-CAR-T cell infusion, we adoptively transferred NKG2D-CAR-T cells into naïve, tumor-free animals and

monitored the mice closely for toxicities. Tumor-free mice still exhibited significant clinical symptoms, including hunched body posture, ruffled fur, and a lack of grooming, indicating that the NKG2D-CAR-T cells were producing off-tumor toxicities *in vivo*.

To better understand these off-tumor toxicities, BALB/c mice were infused with 10^7 NKz, NKz10, or NK28z CAR-T cells and toxicity was evaluated via changes in core body temperature, body weight, and overall survival (**Fig. 3.4a-c**). Despite similar *in vitro* functionality, the BALB/c-derived NKG2D-CAR-T cells displayed a distinct hierarchy of disease severity between the different CAR-T cells *in vivo*. The NKz10-CAR-T cells elicited the most significant toxicity, with core body temperatures dropping as low as 30°C within 24 hours of adoptive transfer (**Fig. 3.4a**). Additionally, these mice lost up to 17% of their body weight in less than 3 days post-adoptive cell transfer (ACT) (**Fig. 3.4b**). The NKz-CAR treated animals conversely showed no significant temperature drops, and only slight weight loss over the course of one week post-ACT (**Fig. 3.4a, b**). The NK28z-CAR-T cells induced an intermediate level of toxicity with respect to both temperature changes and weight loss (**Fig. 3.4a, b**). These toxicities were accompanied by significant clinical symptoms such as ruffled fur, hunched posture, labored breathing, and decreased activity that corresponded to the severity of temperature drops and weight loss (**Table 3.1**). Interestingly, these data parallel our observations of the differences in per-cell NKG2D-CAR expression *in vitro* (**Fig. 3.1a**), with the greatest expression and most severe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Pre 3 8 22 29 48 54 72 96
C
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Days Post ACT
CAR-'ve
NKz
NKz10
NK28z
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Temperature (°C)
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0 2 4 6 8
Days Post ACT
CAR-'ve
NKz
NKz10
NK28z

Figure 3.4: NKG2D-CAR-T cells induce differing levels of toxicity in mice. (a-c) Naïve BALB/c mice were treated with 10^7 NK-CAR-T cells intravenously as indicated (N=6). (a) Temperatures, (b) weight loss, and (c) survival were monitored over the course of 7 days post adoptive cell transfer (ACT). (d-f) Naïve C57BL/6 mice were treated as in (a) (n=5-10). (d) Temperatures are shown at the peak drop of 8 hours post ACT. (e) Weight loss and (f) survival were monitored over 7 days post ACT. Temperature and weight loss data are presented as mean ± SEM. Dotted lines indicate data from surviving animals.

Table 3.1: Mouse clinical observations following ACT into naïve BALB/c mice

	CAR-'ve	NKz	NKz10	NK28z
Ruffled	-	++	+++	++
Hunched	-	+	+++	+
Ocular Discharge	-	++	+++	+
Lack of grooming	-	+	+++	++
Labored breathing	-	++	++	++
Decreased activity	-	+	++	++

toxicities observed with NKz10-CAR-T cells. Despite these significant, acute toxicities, all mice survived the course of treatment, and recovered within 7 days of ACT (**Fig. 3.4c**).

We evaluated whether similar toxicities were observed in C57BL/6 mice. Interestingly, only the NKz10-CAR T cells displayed any detectable evidence of toxic effects (**Fig. 3.4d-f**). The temperature changes and weight loss in these animals were considerably more modest compared to those observed in BALB/c mice. The C57BL/6 mice did not display any overt clinical symptoms (**Fig 3.4d,e**, *data not shown*). The core body temperature changes observed in NKz10-CAR-T cell treated mice were variable, with some mice showing only mild temperature depression and others dropping to 33°C within 8 hours of ACT (**Fig. 3.4d**). Weight loss by NKz10-CAR-T cell treated mice was consistent; over the course of 3 days post-ACT C57BL/6 mice lost up to 12% of their body mass (**Fig. 3.4e**). Despite the appearance of reduced toxicity in C57BL/6 mice, ACT of NKz10-CAR-T cells was lethal in 25% of the treated animals within 48 hours of treatment (**Fig. 3.4f**). The NKz and NK28z CAR-T cells showed no significant toxicity or lethality in C57BL/6 mice. These data reveal a notable difference in severity of NKG2D-CAR off-tumor toxicity between the two strains of mice tested, with BALB/c mice exhibiting greater toxicity than their C57BL/6 counterparts.

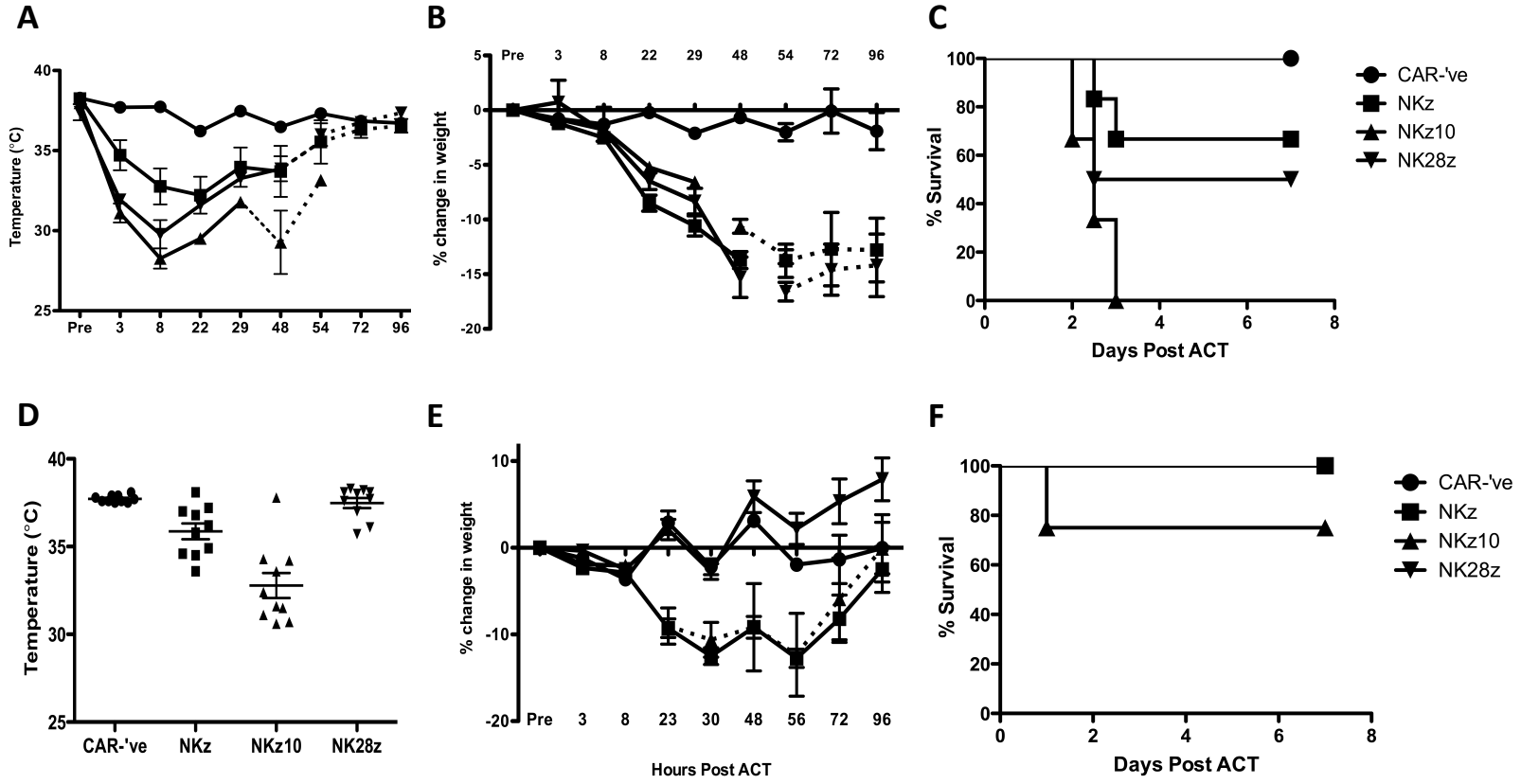
Pre-conditioning cyclophosphamide exacerbates NKG2D-CAR toxicity

In both pre-clinical models and clinical trials of CAR-T cells,

lymphodepletion regimes have proven to enhance the engraftment and persistence of CAR-T cells upon adoptive transfer[296-298]. In particular, pre-treatment with cyclophosphamide (CTX) has been found to be particularly effective. Since chemotherapeutic agents cause DNA damage and cell stress which can upregulate NKG2DL expression, we tested whether pre-treatment with CTX would influence toxicity following adoptive transfer of NKG2D-CAR-T cells. Strikingly, CTX pre-treatment significantly exacerbated the toxicity induced by all three NKG2D-CARs (**Fig. 3.5**).

In BALB/c mice, the NKz-CAR-T cells, which showed minimal toxicity in naïve mice, became very toxic, inducing core body temperature drops and weight loss comparable to those induced by the NKz10-CAR-T cells (**Fig. 3.5a, b**). Further, infusion of NKz-CAR-T cells in CTX pre-treated BALB/c mice resulted in 33% mortality (**Fig. 3.5c**). Toxicities produced by NKz10-CAR-T cells were also dramatically exacerbated, with temperatures dropping below 29°C within as little as 8 hours post ACT (**Fig. 3.5a**). Clinical symptoms were likewise exacerbated; mice demonstrated a complete lack of grooming and the appearance of ocular discharge. Most alarmingly, all mice treated with CTX and NKz10-CAR-T cells succumbed to the toxicities within 72 hours of ACT (**Fig. 3.5c**). As observed in naïve animals, NK28z-CAR-T cells showed an intermediate level of toxicity that was similarly worsened by CTX; 50% of animals in this treatment group succumbed to T-cell mediated toxicity (**Fig. 3.5c**).

Pre-conditioning chemotherapy also enhanced the toxicity of NKG2D-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Days Post ACT
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Figure 3.5: NKG2D-CAR-T cell toxicity is exacerbated by pre-conditioning with chemotherapy. Mice were treated with 150mg/kg cyclophosphamide (CTX) intraperitoneally 24 hours prior to adoptive transfer. (a-c) Balb/c mice were treated with 10^7 NK-CAR-T cells intravenously as indicated (n=6). (a) Temperatures, (b) weight loss, and (c) survival were monitored over the course of 7 days post adoptive cell transfer (ACT). (d-f) Naïve C57BL/6 mice were treated as in (a) (n=5-10). (d) Temperatures are shown at the peak drop of 8 hours post ACT. (e) Weight loss and (f) survival were monitored over 7 days post ACT. Temperature and weight loss data are presented as mean ± SEM. Dotted lines indicate data from surviving animals.

CAR-T cells in C57BL/6 mice (**Fig. 3.5d-f**). Mice treated with NKz-CAR-T cells showed significant weight loss of over 12% of pre-treatment weight (**Fig. 3.5e**), with average core body temperatures dropping below 35°C within 8 hours of T cell infusion (**Fig. 3.5d**). In the case of NKz10-CAR-T cells, 25% of the treated animals succumbed to their toxicities in less than 24 hours following ACT (**Fig. 3.5f**). Surviving animals varied in their weight loss, with some losing more than 18% of their body weight before recovering (**Fig. 3.5e**). Core body temperatures were also variable, with some of the survivors exhibiting little change, while others dropped below 31°C within 8 hours of ACT (**Fig. 3.5d**). While C57BL/6 mice failed to show any of the physical symptoms of toxicity observed in BALB/c mice (hunched posture, labored breathing, lack of grooming, etc.), chemotherapeutic pre-treatment prior to ACT of NKz10-CAR-T cells resulted in an observable increase in docility (noted upon handling the animals) in the C57BL/6 mice (*data not shown*). The NK28z-CAR-T cells showed no signs of toxicity, even when combined with CTX in C57BL/6 mice. These data reveal that the toxicities are dependent on both the strain and the CAR structure.

Adoptive transfer of NKG2D-CAR-T cells results in an acute cytokine storm in vivo

We next sought to investigate possible causes of this *in vivo* toxicity. Using a 32-plex cytokine array, we examined serum cytokine levels in BALB/c mice at 8 hours post ACT, both with and without pre-conditioning cyclophosphamide.

Strikingly, the majority of the cytokines evaluated were upregulated in the serum of mice receiving any of the NKG2D-CAR-T cells (**Fig. 3.6a**). Mice treated with NKz10-CAR-T cells showed the most dramatic upregulation, with all but four analytes increased compared to CAR -'ve controls (**Fig. 3.6a, Tables 3.2, 3.3**). These mice showed serum concentrations of over 4ng/ml of IFNγ, amongst others, indicating severe immune responses were occurring in these animals (**Fig. 3.6a, Tables 3.2, 3.3**).

Our previous observations indicated that NKz and NKz10 represented the lowest and highest observed toxicity and CAR-expression, respectively, with NK28z exhibiting an intermediate outcome. This pattern was reinforced through Principle Component Analysis of serum cytokine and chemokine levels. While each NKG2D-CAR-T cell treatment clustered tightly, regardless of CTX pre-treatment, each NKG2D-CAR-T cell cluster was separate from the others, with NK28z falling between NKz and NKz10 (**Fig. 3.6b**).

CTX pre-treatment enhanced the serum concentration of many cytokines/chemokines (**Tables 3.2, 3.3**). The observed serum concentration increases following CTX pre-treatment were NKG2D-CAR-T cell specific, as we did not observe any measureable changes between CAR-'ve ± CTX or PBS ± CTX control groups (**Fig. 3.6a, b**). The most dramatic effects of CTX were observed in mice treated with NKz-CAR-T cells and NK28z-CAR-T cells, where the concentrations of many cytokines were more than doubled by CTX pre-treatment (**Table 3.2, 3.3**). Overall, our data is indicative of a severe cytokine

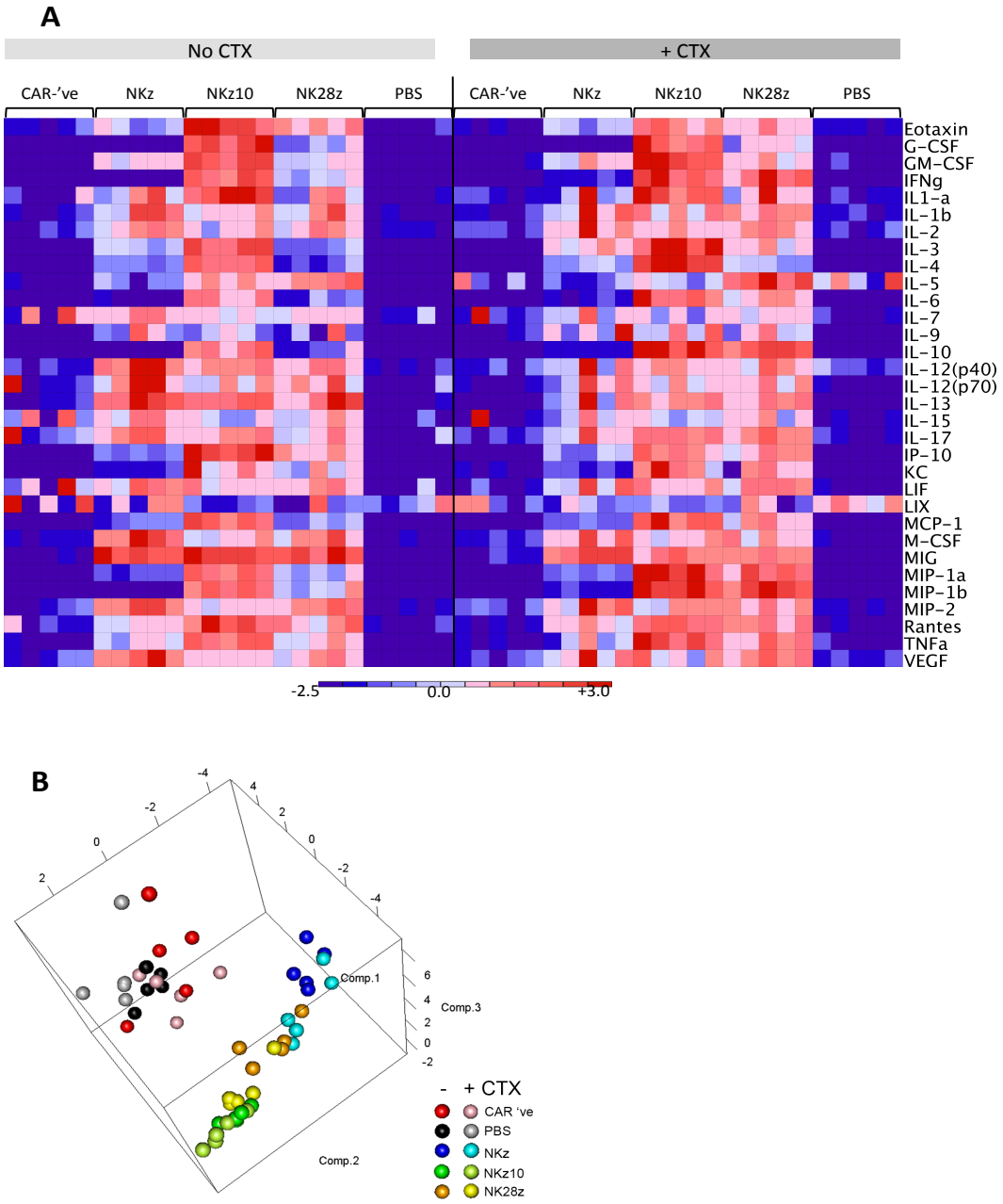

Figure 3.6: NKz10 CAR-T cells induce severe cytokine storm in BALB/c mice. Mice with or without pre-conditioning CTX were treated with 10^7 engineered T cells as indicated. Serum was collected at 8 hours post ACT and sent for Luminex analysis. (a) Heat map displaying the relative changes in serum cytokine concentrations from mice treated with CAR-'ve, NKz, NKz10 or NK28z CAR-T cells, or PBS control. Data was normalized by row (b) Principle component analysis of serum cytokine concentrations indicating clustering of treatment groups.

Table 3.2: Serum cytokine concentrations 8h post ACT. Luminex analysis was performed on BALB/c serum samples collected 8 hours post ACT. Concentrations are presented as mean ± SEM of N=5 run in duplicate. Fold change was calculated relative to PBS-treated controls.

	No CTX							
	CAR-'ve		NKz		NKz10		NK28z	
CYTOKINE	[pg/ml]	Fold Change	[pg/ml]	Fold Change	[pg/ml]	Fold Change	[pg/ml]	Fold Change
Eotaxin	434.19 ± 28.17	1.38	757.31 ± 54.79	2.40	2365.86 ± 137.71	7.49	1405.26 ± 91.03	4.45
G-CSF	182.81 ± 32.73	2.01	463.54 ± 18.18	5.10	18355.48 ± 1493.15	201.93	2800.72 ± 464.96	30.81
GM-CSF	3.16 ± 3.16	3.16	75.93 ± 5.76	75.93	196.41 ± 10.12	196.41	67.20 ± 5.93	67.20
IFNγ	0.06 ± 0.06	0.06	97.51 ± 8.49	97.51	4040.56 ± 236.12	4040.56	1285.92 ± 146.14	1285.92
IL-1α	44.63 ± 14.78	2.24	132.58 ± 21.16	6.66	237.15 ± 15.23	11.92	105.25 ± 17.17	5.29
IL-1β	5.46 ± 2.14	1.56	60.66 ± 10.23	17.37	41.16 ± 4.52	11.78	48.42 ± 8.14	13.86
IL-2	10.41 ± 2.11	1.50	51.13 ± 5.16	7.36	37.43 ± 4.10	5.39	44.40 ± 5.50	6.40
IL-3	6.93 ± 2.80	2.06	286.96 ± 16.38	85.48	1021.21 ± 56.54	304.20	205.14 ± 20.17	61.11
IL-4	1.76 ± 0.80	2.76	21.26 ± 0.49	33.32	123.03 ± 4.72	192.84	16.55 ± 2.19	25.94
IL-5	9.44 ± 1.34	1.61	41.04 ± 5.30	6.97	104.61 ± 7.17	17.78	144.79 ± 12.54	24.61
IL-6	5.24 ± 1.37	4.20	22.65 ± 1.50	18.15	514.19 ± 48.20	412.01	79.64 ± 8.62	63.81
IL-7	22.44 ± 7.49	4.33	28.03 ± 3.13	5.40	18.10 ± 1.16	3.49	19.47 ± 1.44	3.76
IL-9	0.16 ± 0.13	0.63	22.20 ± 9.70	86.71	22.41 ± 10.51	87.55	18.32 ± 8.75	71.58
IL-10	16.69 ± 7.06	2.82	123.38 ± 5.90	20.86	2583.26 ± 220.78	436.80	588.91 ± 141.58	99.58
IL-12 (p40)	17.76 ± 2.29	1.33	93.08 ± 9.34	6.98	66.70 ± 4.23	5.01	63.34 ± 5.62	4.75
IL-12 (p70)	60.46 ± 30.07	5.54	147.74 ± 24.55	13.53	76.20 ± 9.52	6.98	108.17 ± 18.12	9.90
IL-13	54.23 ± 9.02	1.36	404.71 ± 26.21	10.14	382.07 ± 13.20	9.57	361.50 ± 39.98	9.06
IL-15	196.75 ± 60.61	10.94	220.40 ± 34.94	12.26	128.80 ±19.05	7.16	158.34 ± 23.14	8.81
IL-17	3.50 ± 0.99	2.98	5.37 ± 0.46	4.58	4.73 ± 0.42	4.04	4.05 ± 0.38	3.46
IP-10	119.58 ± 12.82	2.82	1191.45 ± 48.79	28.08	8763.98 ± 522.19	206.55	3422.32 ± 240.86	80.66
KC	30.48 ± 4.78	1.16	113.71 ± 7.29	4.34	1162.94 ± 315.50	44.42	422.39 ± 78.25	16.13
LIF	5.47 ± 1.72	4.51	7.29 ± 0.81	6.02	6.00 ± 0.50	4.96	7.00 ± 0.65	5.78
LIX	1755.92 ± 505.48	1.91	1066.31 ± 235.15	1.16	505.08 ± 45.30	0.55	805.97 ± 305.34	0.88
MCP-1	23.86 ± 4.47	1.18	290.99 ±20.67	14.39	2006.50 ± 134.65	99.26	385.33 ± 29.06	19.06
M-CSF	7.12 ± 1.65	1.47	70.55 ± 7.90	14.59	38.71 ± 3.88	8.00	50.89 ± 7.28	10.52
MIG	184.72 ± 35.25	8.72	3299.98 ± 145.92	155.74	3323.12 ± 93.95	156.83	3247.80 ± 154.06	153.28
MIP-1α	29.18 ± 6.10	1.39	121.60 ± 5.33	5.81	796.61 ± 45.80	38.08	267.13 ± 22.02	12.77
MIP-1β	19.04 ± 5.58	2.68	183.45 ± 3.88	25.79	4183.29 ± 296.51	588.04	1627.44 ± 202.10	228.77
MIP-2	33.22 ± 9.97	1.73	231.21 ± 19.43	12.07	133.15 ± 10.34	6.95	191.16 ±16.12	9.98
RANTES	29.73 ± 9.11	2.57	81.47 ± 4.42	7.04	161.05 ± 13.13	13.92	108.18 ± 10.28	9.35
TNFα	3.01 ± 1.15	6.80	24.07 ± 2.75	54.34	56.42 ± 2.81	127.35	29.22 ± 3.32	65.96
VEGF	0.55 ± 0.10	2.14	2.61 ± 0.27	10.10	1.62 ± 0.05	6.29	1.85 ± 0.19	7.16

Table 3.3: Serum cytokine concentrations 8h post ACT into CTX-pretreated mice. Luminex analysis was performed on BALB/c serum samples collected 8 hours post ACT. Concentrations are presented as mean ± SEM of N=5 run in duplicate. Fold change was calculated relative to PBS-treated controls.

	+ CTX							
	CAR-'ve		NKz		NKz10		NK28z	
CYTOKINE	[pg/ml]	Fold Change	[pg/ml]	Fold Change	[pg/ml]	Fold Change	[pg/ml]	Fold Change
Eotaxin	344.78 ± 26.65	0.93	742.30 ± 34.12	2.01	1523.53 ± 107.80	4.12	1154.83 ± 73.99	3.12
G-CSF	164.07 ± 16.00	1.77	523.85 ± 25.55	5.64	15625.04 ± 1681.74	168.21	4578.15 ± 548.72	49.29
GM-CSF	3.70 ± 2.49	0.64	81.86 ± 9.07	14.08	214.62 ± 11.23	36.91	96.58 ± 7.83	16.61
IFNγ	1.08 ± 0.72	2.88	361.31 ± 26.98	960.92	4802.61 ± 341.31	12772.90	4037.89 ± 429.08	10739.07
IL-1α	33.61 ± 5.37	1.01	109.46 ± 28.35	3.29	219.73 ± 20.74	6.60	151.47 ± 26.76	4.55
IL-1β	8.37 ± 1.32	1.13	56.53 ± 11.99	7.67	47.69 ± 5.41	6.47	51.48 ± 4.26	6.98
IL-2	13.07 ± 2.05	1.22	56.77 ± 11.26	5.30	43.91 ±2.69	4.10	54.67 ± 4.40	5.10
IL-3	4.11 ± 0.52	1.25	429.66 ± 34.59	130.87	1165.46 ± 82.10	355.00	418.17 ± 36.20	127.38
IL-4	0.62 ± 0.06	1.76	27.75 ± 1.71	78.40	183.40 ± 6.32	518.09	27.60 ± 1.77	77.95
IL-5	53.65 ± 18.93	0.51	37.67 ± 4.59	0.36	69.52 ± 6.37	0.67	211.56 ± 16.72	2.03
IL-6	1.93 ± 0.29	1.39	44.35 ± 5.76	31.93	751.15 ± 97.43	540.79	331.95 ± 34.36	238.98
IL-7	18.25 ± 8.77	5.36	21.21 ± 4.74	6.23	15.19 ± 2.25	4.46	19.93 ± 1.61	5.85
IL-9	2.23 ± 1.37	9.96	40.99 ± 14.18	183.00	30.58 ± 10.72	136.53	27.17 ± 10.15	121.31
IL-10	8.89 ± 1.48	1.91	266.35 ± 16.15	57.19	4185.06 ± 266.87	898.66	3189.75 ± 253.54	684.94
IL-12 (p40)	17.42 ± 2.88	0.80	63.02 ± 14.01	2.88	53.65 ± 3.50	2.45	64.58 ± 6.10	2.95
IL-12 (p70)	10.18 ± 1.84	1.47	101.99 ± 23.42	14.77	77.87 ± 8.95	11.28	99.18 ± 8.83	14.37
IL-13	26.91 ± 3.37	1.26	262.93 ± 58.43	12.27	290.18 ± 25.29	13.55	297.78 ± 24.10	13.90
IL-15	140.78 ± 87.42	8.03	156.52 ± 35.16	8.93	128.46 ± 18.07	7.33	160.92 ± 21.04	9.18
IL-17	1.35 ± 0.15	1.41	4.48 ± 0.66	4.68	5.66 ± 0.31	5.91	5.15 ± 0.41	5.38
IP-10	126.21 ± 8.31	2.73	1532.65 ± 127.84	33.15	4907.29 ± 397.68	106.13	3871.74 ± 406.00	83.73
KC	61.05 ± 9.63	2.57	132.36 ± 13.63	5.57	1180.65 ± 247.97	49.69	802.26 ± 162.40	33.77
LIF	1.25 ± 0.31	2.21	7.33 ± 1.17	12.98	6.87 ± 0.51	12.15	7.99 ± 0.61	14.13
LIX	944.88 ± 204.04	0.63	703.14 ± 100.35	0.47	623.66 ± 39.59	0.41	811.58 ± 175.70	0.54
MCP-1	36.27 ± 5.29	1.99	278.37 ± 23.55	15.24	2191.20 ± 246.48	120.00	1076.61 ± 122.42	58.96
M-CSF	10.82 ± 1.45	3.54	55.61 ± 7.37	18.21	37.11 ± 3.37	12.15	45.02 ± 3.79	14.74
MIG	374.52 ± 67.19	19.51	3019.26 ± 165.12	157.32	2416.97 ± 100.74	125.94	2416.42 ± 73.57	125.91
MIP-1α	38.13 ± 3.50	1.80	164.71 ± 4.50	7.76	1182.53 ± 86.41	55.73	802.65 ± 77.03	37.83
MIP-1β	32.78 ± 4.33	6.97	480.86 ± 24.25	102.22	6349.63 ± 281.83	1349.84	5302.19 ± 427.62	1127.17
MIP-2	46.54 ± 8.76	2.05	205.27 ± 33.37	9.03	147.62 ± 15.25	6.49	166.75 ± 9.29	7.33
RANTES	12.28 ± 3.10	3.04	83.80 ± 5.10	20.76	145.06 ± 3.58	35.94	118.60 ± 6.39	29.38
TNFα	3.17 ± 1.32	1.08	19.63 ± 3.64	6.70	67.25 ± 5.03	22.94	43.45 ± 4.06	14.82
VEGF	0.66 ± 0.09	1.14	2.22 ±0.37	3.82	1.79 ± 0.22	3.09	2.30 ± 0.18	3.96

storm induced by ACT of NKG2D-CAR-T cells that is exacerbated by pre-conditioning with CTX.

NKz10 CAR-T cells induce severe lung immunopathology

Given that the most substantial toxicities were observed in BALB/c mice treated with CTX and NKz10-CAR-T cells, we sought to further investigate the pathology elicited by this treatment. BALB/c mice were treated with CTX followed by adoptive transfer of 10^7 CAR-‘ve or NKz10-CAR-T cells and subjected to a comprehensive necropsy performed by a veterinary pathologist in a blinded fashion. NKz10-CAR-T cell-treated animals exhibited severe necrotizing pneumonitis, which was deemed to have been fatal in these animals (**Fig. 3.7**). No tissue pathology was observed in CAR-‘ve control animals. The lungs of NKz10-CAR-treated mice displayed severe perivascular edema, diffuse thickening of the alveolar septae, as well as heavy infiltration by neutrophils and mononuclear cells (**Fig. 3.7**). Additionally, these mice exhibited lymphocytolysis in the spleen, which indicated severe stress due to the inflammation in the lungs (*data not shown*). Taken together, our data suggests that NKG2D-CAR toxicity is driven by severe lung immunopathology.

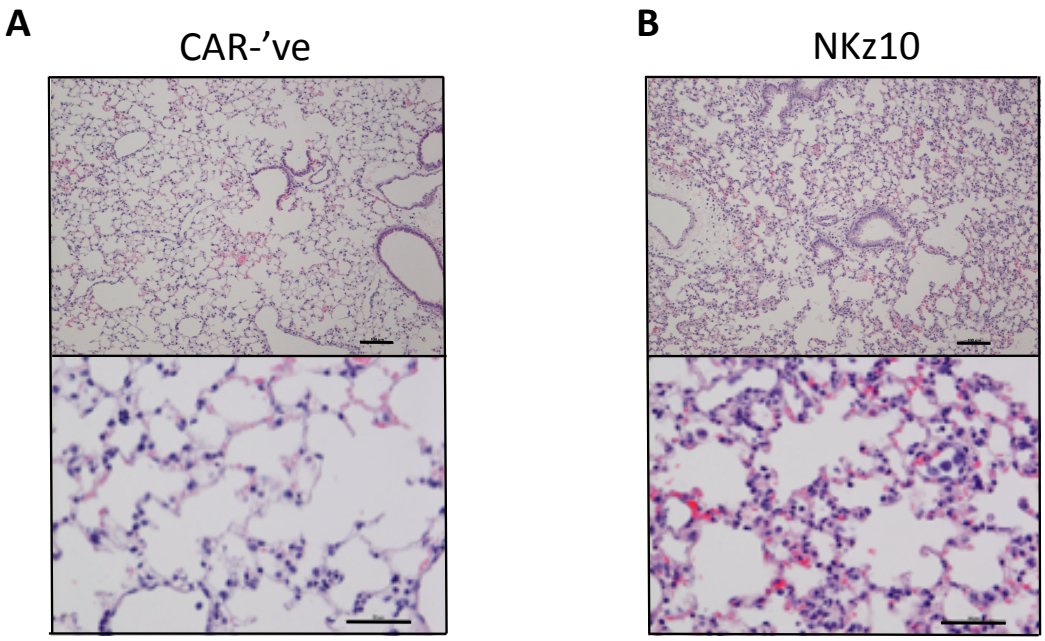

Figure 3.7: NKz10 CAR-T cells induce lethal inflammation in the lungs of BALB/c mice. Mice were treated with 150mg/kg cyclophosphamide, followed by intravenous injection of 10^7 (a) CAR-'ve or (b) NKz10 CAR-T cells. Animals were sacrificed for complete veterinary necropsy 24 hours later. H&E stained cross-sections of lung tissues from representative mice are shown. Top panel scale bars 100µm, bottom panel scale bars 50µm.

Discussion

On-target, off-tumor toxicity remains a primary concern for all immunotherapy approaches, especially given that the vast majority of tumor targets are not tumor-restricted in their expression [299]. As such, CARs require considerable pre-clinical testing to detect any detrimental side effects prior to their use in patients. Here, we examined the pre-clinical *in vitro* and *in vivo* functionality of three CARs based on the NKG2D receptor. Unexpectedly, we observed evidence of severe off-tumor toxicity upon testing these NKG2D-CARs *in vivo* in two different mouse strains. This previously unreported finding provides insight into considerations that must be taken into account prior to the clinical application of NKG2D-CARs.

When we adoptively transferred the three types of NKG2D-CAR-T cells into naïve BALB/c and C57BL/6 mice, we observed overt signs of toxicity, the severity of which was greatest when utilizing the NKz10-CAR (**Fig. 3.4**). Adoptive transfer of NKz10-CAR-T cells into BALB/c mice induced significant core body temperature decreases, weight loss, and dramatic physical symptoms. While the overt physical symptoms of toxicity, such as a lack of grooming, were absent in C57BL/6 mice, NKz10-CAR-T cells remained toxic, as NKz10-CAR-T cell treatment resulted in core body temperature decreases, weight loss, and even mortality. The cause of these observed toxicities is likely two-fold. First, expression of NKG2DLs is far from tumor-specific, and expression of these NKG2D-CAR targets on healthy, non-malignant cells offers opportunity for the

off-tumor, on-target activation of NKG2D-CAR-T cells under the right conditions. Secondly, we hypothesize that differences between the nature of the endogenous NKG2D receptor and NKG2D-CARs lead to off-tumor activation of NKG2D-CARs, but not NKG2D, upon binding to non-tumor NKG2DLs.

The range of homeostatic NKG2DL expression at both the transcriptional and protein levels is currently unclear in both mice and humans. Multiple reports have established NKG2DL expression on various healthy, non-cancerous/infected tissues, and these may serve as off-tumor ligand sources for NKG2D-CAR-T cells *in vivo*. In humans and mice, several types of haematopoietic cells have been described to express NKG2DL, including monocytes, dendritic cells, and macrophages [300]. At a transcriptional level, several NKG2DL are expressed across diverse tissues such as the spleen, lungs, gut and bronchial epithelia, cardiac and skeletal muscle, as well as the skin [300-303]. Data from others suggest epithelial, endothelial and antigen presenting cells may constitutively express NKG2DL such as ULBPs [32]. The confirmation of NKG2DL expression at the protein level is complicated by an absence of data in the literature as well as limited validated reagents for assaying surface expression of NKG2DL [300,301]. There are some known differences that may explain our observed strain-specific differences in NKG2D-CAR-T cell toxicity between BALB/c and C57BL/6 mice. For example, there are known expression differences in the NKG2DLigands H60a and RAE-1 between BALB/c and C57BL/6 mice [301,304]. The lack of solid understanding of the expression patterns of NKG2DL in humans (or mice), coupled with our data

indicating the potential for toxicity, contradicts the use of NKG2D-CAR-T cells for therapeutic applications in humans without considerable care and monitoring.

Endogenous NKG2D receptors exist in the presence of these non-target NKG2DLs without inducing toxicity. We hypothesize that inherent differences between these receptors and NKG2D-CARs explain our toxicity observations. NKG2D-CARs bind NKG2DL, which induce T cell activation and cytolytic functions[257]. Similarly, in NK cells, NKG2D acts as a positive signal to induce cytolytic functions[34,305]. However, in NK cells these positive signals are also balanced by inhibitory signals from other NK cell surface receptors that act in concert to determine the fate of the target cell[34]. Under homeostatic conditions, low levels of NKG2DL, and concomitant NKG2D signaling would occur in concert with inhibitory signals, such as the presence of MHC-1, preventing NK cell reactivity[24]. Removing the contributions of the inhibitory signals, as in NKG2D-based CARs, effectively takes the brakes off NKG2D-mediated cytotoxicity. Coupling this with a highly expressed, highly activating chimeric NKG2D receptor can therefore have potentially serious consequences, as is epitomized by our toxicity data with NKz10-CAR-T cells.

One of our most striking observations was the varied manifestation of toxicity between the two different strains of mice tested. Whereas treatment of BALB/c mice with both NKz10- and NK28z-CAR-T cells resulted in decreases in both core body temperature and weight loss, these decreases were diminished in their C57BL/6 counterparts (**Fig. 3.4**). Contrastingly, NKz-CAR-T cell treated

mice showed no physical signs of illness, and only very slight changes in weight and temperature in either mouse strain (**Fig. 3.4**). In fact, the NKz-CAR only began to show adverse effects when combined with cyclophosphamide pre-treatment, resulting in considerable morbidity and mortality in BALB/c mice (**Fig. 3.5**). In contrast, when NKz-CAR-T cells were adoptively transferred into CTX pre-treated C57BL/6 mice, only mild weight loss or temperature changes were observed (**Fig. 3.5**). These strain-specific differences may help to explain why our observations of NKG2D-CAR-T cell mediated toxicities are the first reported, despite an extensive history of NKG2D-CARs in the literature. Our findings are consistent with previous reports describing NKz-CAR-T cells as a tumor therapy in the absence of overt toxicity using C57BL/6 mice, even when combined with CTX[257,258,260,289]. In contrast, the NKz10-CAR, which demonstrated the most severe toxicity in our studies, has only been previously tested on human NK cells using immunodeficient animals, which precludes the ability to assess toxicity due to a lack of cross-reactivity[262,306]. The NKG2D CARs fused to CD28/CD3ζ, such as that used in our study, or 4-1BB/CD3ζ, have thus far only been tested using *in vitro* assays, and so possible toxicities induced by this type of receptors have yet to be evaluated[263,264]. Our data shows that NK28z has the potential to cause severe toxicities in BALB/c mice, while not in C57BL/6 mice, and that the severity of this toxicity can be increased by CTX pre-conditioning. In summation, we can conclude that NKG2D-CAR-T cells are capable of inducing toxicity that is variable in severity depending on mouse strain and the pre-conditioning treatment

used prior to ACT. As such, our findings suggest that pre-clinical studies in C57BL/6 mice may underestimate the toxicity associated with NKG2D-CAR-T cells.

While a hierarchy in T cell surface expression of our NKG2D-CARs (NKz < NK28z < NKz10; **Fig. 3.1 a-b**) was consistent between T cells from BALB/c and C57BL/6 donors, we also observed startling differences when comparing CAR-T cell functionality between mouse strains. While all three NKG2D-CARs exhibited similar *in vitro* functionality in BALB/c T cells, the CARs displayed reduced overall functionality, including both capacity to induce cytokine production and cytotoxicity when expressed in C57BL/6 T cells (**Fig. 3.3**). In both cases, functional abilities did not appear to correlate with the level of receptor surface expression. These studies reveal previously unappreciated strain-specific differences in CAR-T cell functionality following NKG2D-CAR engineering, indicating an important role for the recipient T cells in CAR function.

Our data suggests that measuring CAR-T cell functionality *in vitro* does not equate to functionality *in vivo*. All three of our NKG2D-CAR constructs displayed equivalent frequencies of cytokine production, as well as killing of tumor targets *in vitro* when using BALB/c T cells. Following adoptive transfer however, we observed a clear hierarchy in the severity of toxicity induced by the different NKG2D-CAR-T cells. Interestingly, the intensity of NKG2D-staining on engineered T cells did correspond to the level of toxicity observed, with NKz showing the least, NK28z an intermediate level, and NKz10 showing the highest

in both NKG2D mean fluorescence intensity, as well as toxicity in BALB/c mice. The same comparison can be made between surface expression and toxicity in C57BL/6 T cells. The NKz10 proved the only CAR to induce toxicity in C57BL/6 mice, showing the highest level of surface expression of the three NKG2D-CARs. This hierarchical toxicity was also observed in the magnitude of serum cytokine elevations in BALB/c mice. The high levels of inflammatory cytokines likely contributed to systemic toxicity, similar to those observed in some clinical trials of T cell therapies [90]. This data suggests that the level of NKG2D-CAR surface expression on each cell may be predictive of their *in vivo* functionality.

An important commonality between NKG2D-CAR-T cell treated BALB/c and C57BL/6 mice was the exacerbation of observed toxicities in the context of chemotherapeutic, specifically CTX, pre-treatment (**Figs. 3.4 and 3.5**). It has been shown previously that NKG2DL are upregulated during genotoxic stress induced by DNA damage[307,308]. As CTX is a known DNA-damaging agent, we speculate that CTX treatment results in upregulation of NKG2DL *in vivo*[309,310]. This is further corroborated by data suggesting that a single dose of cyclophosphamide is sufficient to induce oxidative stress in the lungs of mice[311]. As oxidative stress is known to induce upregulation of NKG2DL[32,302,308], this may also explain why CTX pre-conditioning exacerbated the off-tumor cytotoxicity of NKG2D-CAR-T cells and why pathological studies determined the lungs of NKG2D-CAR-T cell treated mice showed lethal levels of inflammation. Alternative methods of pre-conditioning lymphodepletion may not alleviate the CTX-driven toxicities

observed in our study, as previous studies have found expression of the NKG2DLs Rae-1 and H60a to be elevated on BALB/c bone marrow cells following irradiation[304]. This poses a significant concern for using NKG2D-CAR-T cells in human trials, as patients may have treatment histories that include chemotherapeutic and/or radiation therapies. In addition, the bulk of adoptive transfer therapies conducted to date have utilized some form of lymphodepletion prior to ACT, as it has been shown to increase antitumor efficacy[139,179,200,312]. Our data indicates that combining this pre-conditioning with NKG2D-CAR-T cell treatment could have toxic consequences.

Ultimately, our data shows that NKG2D-based CARs have the potential to induce significant toxicities *in vivo*, especially if delivered subsequent to lymphodepletion regimens. NKz-CAR-T cells showed the lowest levels of observed toxicity in both BALB/c and C57BL/6 hosts. However, given that mortality was observed in some CTX pre-treated BALB/c mice treated with NKz-CAR-T cells, clinical translation should be undertaken with extreme caution if not avoided. NKz10-CAR-T cells showed evidence of toxicity even in the absence of CTX pre-treatment in both BALB/c and C57BL/6 hosts. This highlights potential drawbacks to driving high levels of CAR surface expression, which can allow CAR-T cells to respond well to even low levels of antigen *in vivo*. Our study accentuates the need to identify tumor-restricted antigens, or antigens with limited expression off-tumor (on non-vital organs), for targeting with CAR-T cell therapy to avoid the potential for off-tumor toxicity.

— CHAPTER 4 —

Dual-specific CAR-engineered T cells combined with TCR boosting by oncolytic rhabdovirus

Introduction

Upon adoptive transfer, CAR-T cells must be able to survive both within the host and within the tumor environment. The ability of adoptively transferred cells to engraft and persist long-term has become one of the goals of ACT therapy design, as successful trials have shown dramatic ability of transferred cells to persist for years following treatment[179,297,313]. The signaling elements of a CAR attempt to recapitulate TCR signaling by incorporating the TCR-CD3ζ chain as well as signaling domains from costimulatory receptors like CD28 and 4-BB[167,172]. Despite this, activation of T cells through their native TCR remains the most efficient and complete method of T cell activation.

It has been noted that CAR-engineered T cells show reduced proliferation in response to CAR activation when compared to the proliferation induced by TCR activation on the same cells[314,315]. As T cell proliferation following ACT has been implicated in contributing to the success of ACT[316,317], this has important consequences for the success of CAR-T cell therapies. Indeed, many current ACT protocols aim to enhance T cell proliferation *in vivo* through the provision of the pro-proliferative cytokine IL-2[138,317,318]. IL-2 therapy itself has significant toxicities associated with its use, and so it would be ideal to elicit T cell proliferation through alternate methods[319]. CAR-engineered T cells do retain their responsiveness to TCR stimulus, and thus can be incited to proliferate via TCR activation. Indeed, all CAR-T cells are actually "dual-specific" as they can respond to antigen stimulation via their TCR and their CAR [314,315,320,321].

Exploiting TCR signaling in CAR-T cells has been achieved by selectively culturing T cells that recognize viruses that lead to chronic infection in humans, such as EBV, CMV and adenovirus[321,322]. In particular, CAR-engineered, EBV-specific T cells can be found at higher levels than CAR-engineered bulk T cells following adoptive transfer; an observation that lead researchers to conclude that the EBV-specific T cells displayed better engraftment as a result of TCR stimulation by EBV antigen *in vivo*[323]. These EBV-reactive, CAR-engineered T cells retained their ability to proliferate in response to their TCR, destroy virally infected cells, as well as recognize and lyse tumor cells[321]. Clinically, complete regression of neuroblastoma was observed in patients treated with EBV-reactive dual-specific T cells engineered to recognize GD2 neuroblastoma antigens, with T cell persistence associated with positive clinical outcomes[324]. Competition experiments were performed by infusing patients with equal numbers of CAR-engineered bulk T cells and CAR-engineered EBV-specific T cells. While the EBV-specific T cells displayed greater engraftment over the first 6 weeks, engraftment beyond this period did not appear to be influenced by the TCR[323,324].

It is attractive to consider enhancing CAR-T cell survival and proliferation by providing additional stimulus through their endogenous TCR using a vaccination-like strategy. Attenuated rhabdoviruses have proven to be excellent T cell boosting agents with the added benefit of exerting their own antitumor functionality through viral oncolysis[204,235,238]. It is therefore intriguing to consider a therapy that combines T cells that recognize antigen carried within the

rhabdovirus with CAR engineering to create CAR-T cells that can recognize the tumor through their CAR and be boosted by the virus through their TCR (**Fig. 4.1)**. Our group has shown that an attenuated strain of VSV can selectively replicate within tumor tissues as well as drive significant T cell responses to antigens carried within the virus[235,237,286]. Infection of the tumor by oncolytic rhabdovirus also results in production of multiple pro-inflammatory molecules, further stimulating antitumor immunity[325,326]. Combining CAR-T cell therapy with oncolytic rhabdovirus boosting aims to capitalize on these viral properties.

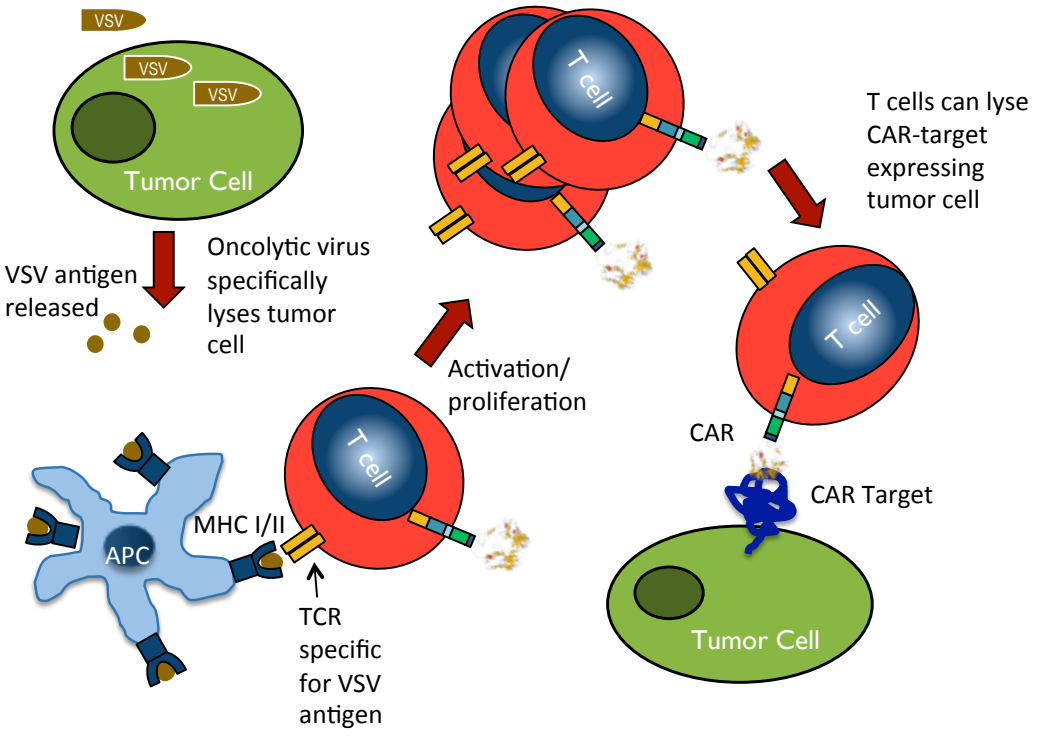

Figure 4.1: Dual-specific T cell activation through both TCR and CAR. Schematic illustrating the predicted activation and function of dual-specific CAR-T cells following adoptive transfer.

In this chapter, we have investigated the combination of CAR-engineered T cells with recombinant VSV capable of boosting the T cells through their endogenous TCR. We hypothesized that combining CAR-engineered transgenic T cells with oncolytic virotherapy would result in enhanced T cell engraftment, expansion, and ultimately antitumor activity. Through use of *in vivo* imaging techniques, we have identified the requirement for careful selection of pre-conditioning lymphodepletion regimes, as irradiation abrogates the boost effect observed when utilizing chemotherapy. The combination of cyclophosphamide pre-treatment, adoptive transfer of dual-specific CD8+ or CD4+ T cells and OV-stimulated boosting proved highly effective at driving T cell expansion and engraftment *in vivo*. Despite this, combination of these therapies was unable to increase the antitumor efficacy of either therapy independently.

Results

Dual-specific T cells respond to both TCR and CAR stimulation

To test the concept of boosting dual-specific CAR-T cells with oncolytic VSV, we employed murine transgenic T cells specific for known LCMV antigens. For evaluating CD8+ T cell responses, we utilized splenocytes from P14 mice that carry a transgenic TCR specific for the MHC-I-restricted LCMV peptide GP33-43[327]. To evaluate CD4+ T cells as dual-specific T cells, we utilized splenocytes derived from SMARTA transgenic mice that express a transgenic TCR specific for the MHC-II-restricted LCMV peptide GP61-80[328]. Transgenic murine T cells

activated by stimulation with their cognate peptide ligands were readily engineered with CAR-containing retroviruses, resulting in up to 80% of cells expressing the HER2-CAR on either P14 CD8+ T cells or SMARTA CD4+ T cells (**Fig. 4.2a**). We tested the ability of these dual-specific T cells to respond to antigen through either their TCR or the HER2-CAR by stimulating with either their cognate peptide or HER2-Fc respectively. Dual-specific CD8+ T cells showed strong cytokine responses induced by either CAR or TCR (LCMV peptide GP33-43) stimulation, with 17-22% of cells producing both IFNγ and TNFα respectively (**Fig. 4.2b**). However, TCR triggering seemed to activate a subpopulation of cells that produced IFNγ but not TNFα (**Fig. 4.2b**, upper left-hand quadrant of density plots), whereas the CAR did not seem to activate cytokine production in this subpopulation. In contrast, dual-specific CD4+ T cells showed similar cytokine responses induced by either CAR or TCR (LCMV peptide GP61-80) stimulation (**Fig. 4.2c**). Overall, while CAR and TCR stimulation seem to induce similar cytokine production in CD4+ T cells, TCR stimulation seems to trigger a broader range of CD8+ T cells than the CAR.

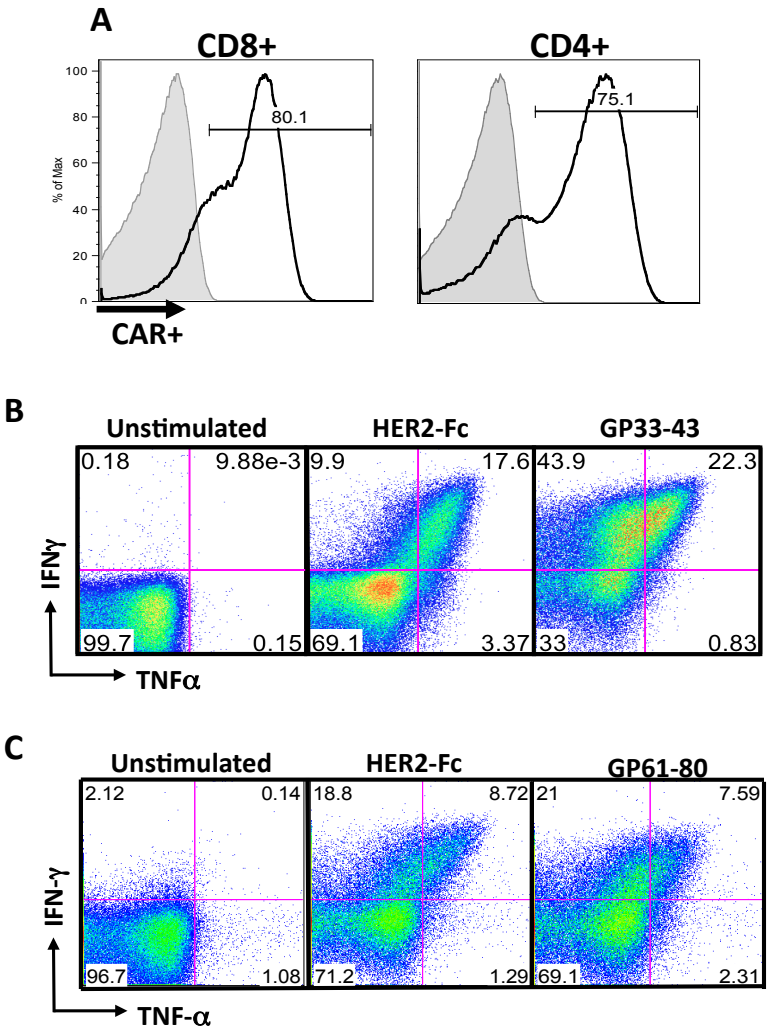

Figure 4.2: Dual-specific T cells show greater response to TCR stimulation than CAR stimulation. P14 (CD8+ transgenic) or SMARTA (CD4+ transgenic) splenocytes were engineered to express the HER2-CAR. a) CAR expression was analyzed by staining with HER2-Fc chimera, with detection using goat anti-human IgG-PE, and analyzed by flow cytometry. Shaded peaks indicate secondary-only controls. (b-c) Dual-specific T cells were stimulated with either vehicle control (unstimulated), plate-bound HER2-Fc, or (b) GP33-43 (P14 dual-specific T cells) or (c) GP61-80 (SMARTA dual-specific T cells) for 4 hours, with cytokine response visualized by flow cytometry.

The method and use of pre-conditioning impacts engraftment and the boosting of dual-specific T cells

Several studies have demonstrated the benefits of preparing a patient for ACT using a lymphodepleting regime such as total body irradiation or chemotherapy[137,139,329]. As VSV is known to induce lymphopenia that peaks 24-48hrs post-administration[330], we hypothesized that OV administration prior to ACT would enhance CAR-T cell engraftment. To test this question, mice bearing MC38-HER2 tumors were infused with VSVΔM51 either 24 or 48hrs prior to ACT of HER2-CAR-T cells, and T cell engraftment was monitored via IVIS imaging. We found very little luciferase signal within the mice following adoptive transfer and the signal that was evident was restricted to the spleen for 24 hours post ACT and disappeared soon after (**Fig. 4.3a-b**). The timing of VSV administration did not impact the CAR-T cell engraftment, as we observed comparable signal whether the VSV was administered 24hr or 48hr prior to ACT (**Fig. 4.3a-b**). Neither treatment affected tumor growth in these mice (**Fig. 4.3c**). From these experiments, we determined that VSV-induced lymphopenia alone was insufficient to allow for successful engraftment of CAR-T cells.

We next questioned whether boosting the T cells through their TCR would promote T cell engraftment following ACT. To boost the dual-specific T cells, we employed the VSVΔM51-GP33/61 vector. This virus contains the LCMV epitopes recognized by both P14 and SMARTA transgenic T cells, and thus can provide a TCR boost to dual-specific T cells generated from either cell type. As

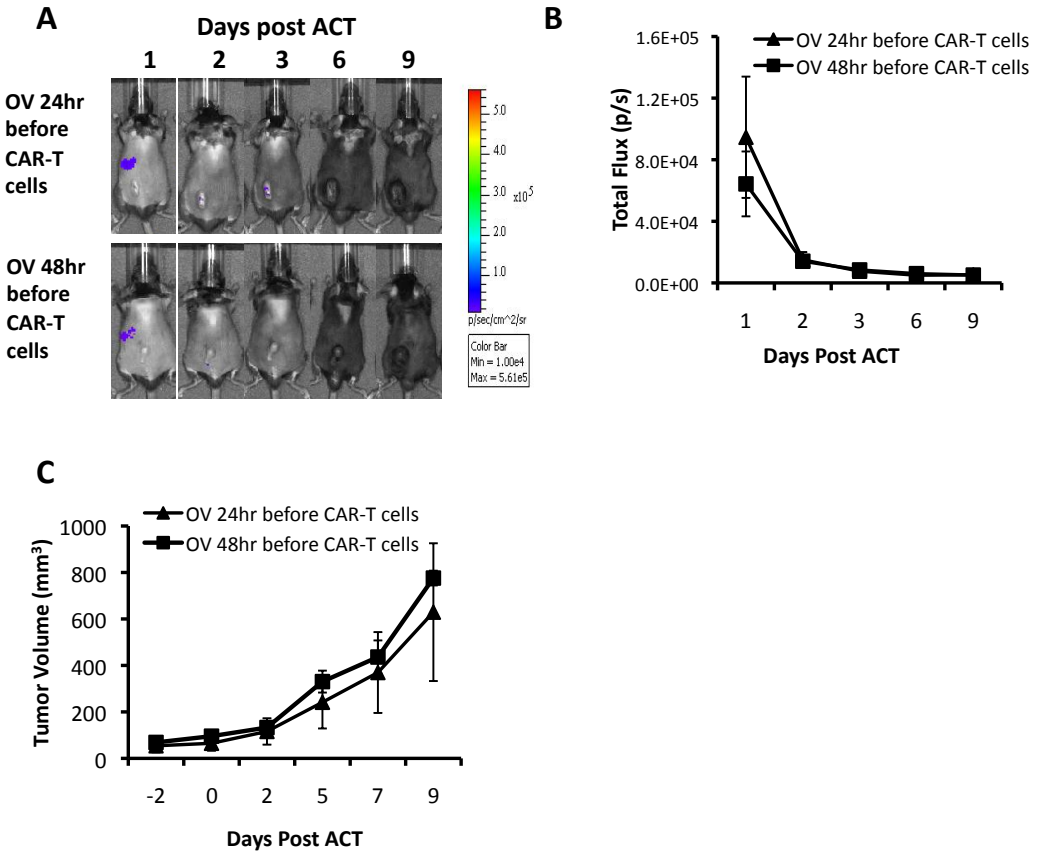

Figure 4.3: VSV-induced lymphopenia is insufficient to allow CAR-T cells to engraft. Splenocytes were engineered with the HER2-CAR-effLuc retrovirus, and adoptively transferred into mice that had received $2x10^9$ PFU VSVΔM51 24 or 48 hours prior to ACT. (a) T cells were visualized using IVIS imaging, with total flux (b) calculated using LivingImage software (N=3). (c) Tumor growth was monitored by digital caliper measurement. Error bars indicate ± SEM.

VSVΔM51 is known to have a rapid induction of gene expression[204], we chose to deliver the virus 6 hours post ACT to allow the T cells time to migrate towards tissues where the antigen may be presented such as the spleen**.** We combined administration of dual-specific P14 T cells with VSVΔM51-GP33/61 to tumor-bearing mice, and evaluated T cell proliferation and engraftment by *in vivo* imaging using the IVIS imager. Interestingly, the boost did augment the T cell engraftment; however, the dual-specific T cells appeared to be retained in the spleen for the first three days post ACT and showed limited migration and persistence within the tumor (**Fig. 4.4**). This treatment also had no impact on the growth of the tumors, indicating these treatments alone were insufficient to promote long-lived engraftment or antitumor immunity (*data not shown*).

We next evaluated the impact of sublethal irradiation prior to adoptive transfer as an alternate lymphodepletion method to enhance engraftment[137,139,331]. Animals were irradiated with 500 rads of whole body irradiation. Twenty-four hours later, dual-specific T cells were infused intravenously followed by two doses of $2x10^9$ PFU VSVΔM51-GP33/61 6 and 48 hours later. While the sublethal irradiation enhanced T cell engraftment, the OV boost did not seem to impact the engraftment (**Fig. 4.5a-b**). We did observe antitumor responses induced by both dual-specific T cells alone as well as the combination therapy (**Fig. 4.5c**). However, during concurrent experiments we noticed that irradiation alone could produce regression of MC38-HER2 tumors, making this model a poor

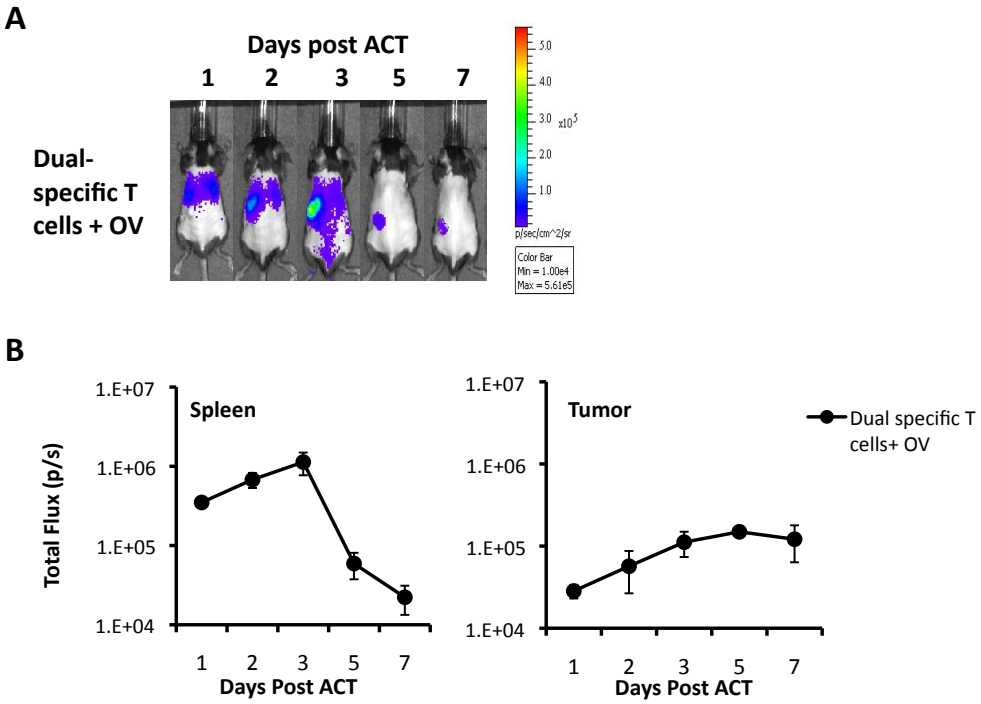

Figure 4.4: Dual-specific T cells show transient engraftment following boosting. P14 splenocytes were engineered with the HER2-CAR retrovirus, and adoptively transferred into mice bearing MC38-HER2 tumors. This was followed 6hr later by administration of $2x10^9$ PFU VSVΔM51-GP33/61, after which animals were imaged using IVIS. (a) Representative animal images showing distribution of Dual-specific T cells *in vivo*. (b) Quantification of dual-specific T cell luciferase signal from spleen and tumor (N=3-4). * $P<0.05$, ** $P< 0.01$.

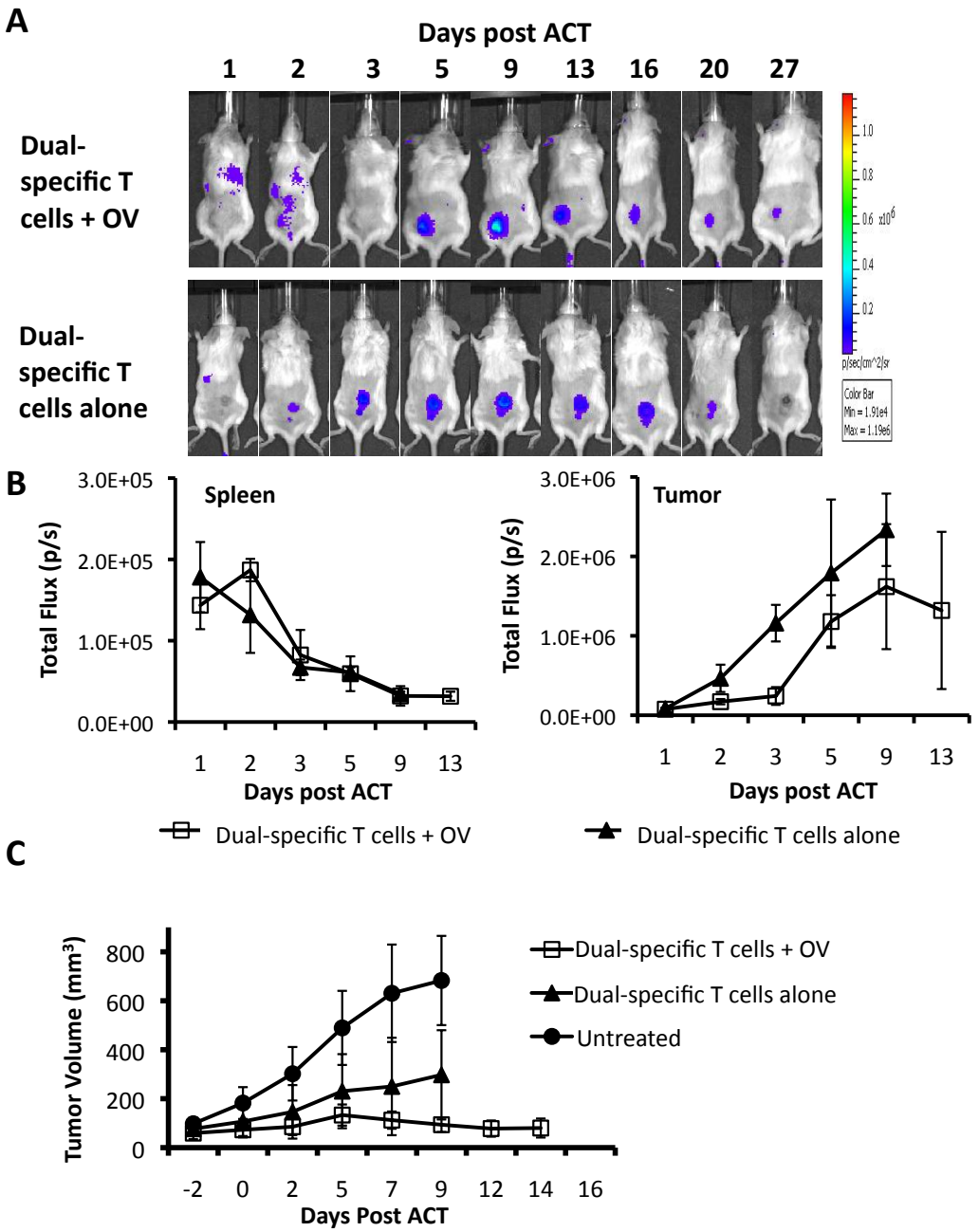

Figure 4.5: Pre-conditioning with sublethal irradiation allows dual-specific T cells to persist, without OV-induced boosting. P14 splenocytes were engineered with the HER2-CAR-effLuc retrovirus, and adoptively transferred into mice that had been exposed to sub-lethal irradiation (500 rads) 24 hours prior. This was followed by administration of $2x10^9$ PFU VSVΔM51-GP33/61, after which animals were imaged using IVIS. (a) Representative animal images showing distribution of Dual-specific T cells *in vivo*. (b) Quantification of dual-specific T cell luciferase signal from spleen and tumor (N=3-4). * $P<0.05$, ** $P< 0.01$. (c) Tumor growth as measured by digital calipers. (N=3) * $P<0.05$.

choice for therapeutic studies. Thus, while we could develop understanding about T cell engraftment and migration, we could not make conclusions about the functionality of our CAR-engineered T cells.

We reasoned that the lack of boosting following VSVΔM51-GP33/61 administration in the irradiated mice was due to a lack of antigen presentation. Therefore, we evaluated the utility of cyclophosphamide (CTX) as a method for pre-conditioning animals prior to ACT. A titration experiment was performed where mice received different amounts of CTX prior to ACT with CAR-engineered P14 T cells followed by VSVΔM51-GP33/61 boost. A single dose of 150mg/kg CTX administered 24 hours prior to ACT enabled robust T cell engraftment of the tumors of recipient animals following the VSV boost and was explored further (**Fig. 4.6a,** middle row; **b**). CTX pre-treatment was found to enhance both engraftment and boosting CAR-engineered P14 and CAR-engineered SMARTA cells (**Fig. 4.7**). We observed significant differences in CD8+ T cell engraftment and expansion in both the tumor site (**Fig. 4.7a**) and total body flux signal (**Fig. 4.7b**) in animals receiving CTX pre-conditioning. Dual-specific CD8+ T cells boosted with VSVΔM51-GP33/61 showed a greater than ten-fold increase in total body flux 6 days post ACT as compared to animals lacking the boost (**Fig. 4.7b**). Interestingly, dual-specific CD4+ T cells showed an even more dramatic proliferative response to VSVΔM51-GP33/61, reaching a total body flux peak almost 100-fold higher than those not boosted (**Fig. 4.7d-e**).

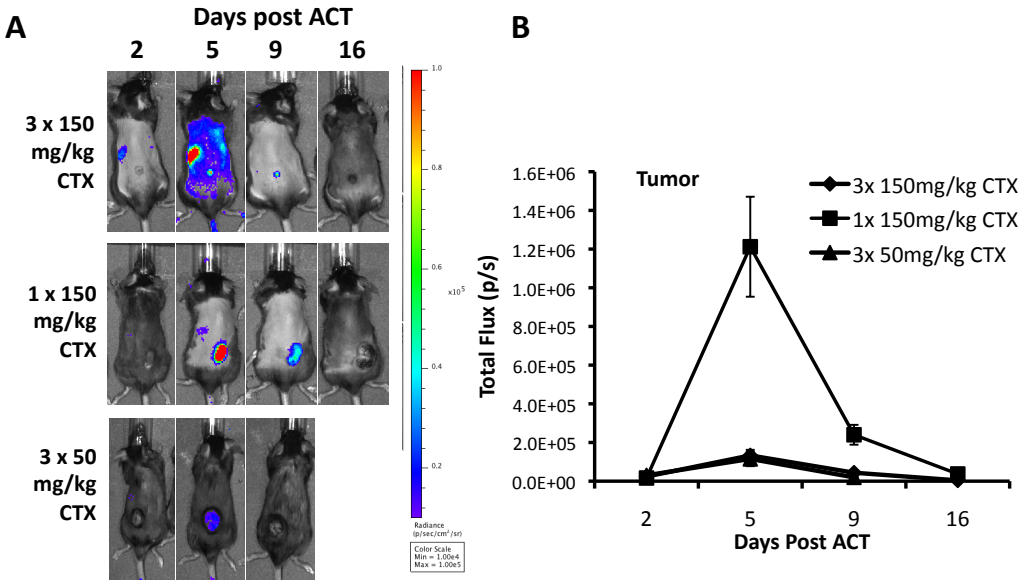

Figure 4.6: Cyclophosphamide as a pre-conditioning agent. P14 splenocytes were engineered with the HER2-CAR-effLuc retrovirus, and adoptively transferred into MC38-HER2-tumor bearing animals pre-treated with either a single dose of 150mg/kg CTX 24 hours prior, or three doses of either 150mg/kg or 50mg/kg CTX given 24hrs apart with the last dose given 24 hrs pre-ACT. This was followed by administration of $2x10^9$ PFU VSVΔM51-GP33/61, after which animals were imaged using IVIS. (a) Representative animal images showing distribution of dual-specific T cells *in vivo*. (b) Quantification of dual-specific T cell luciferase signal from spleen and tumor (N=3-4).

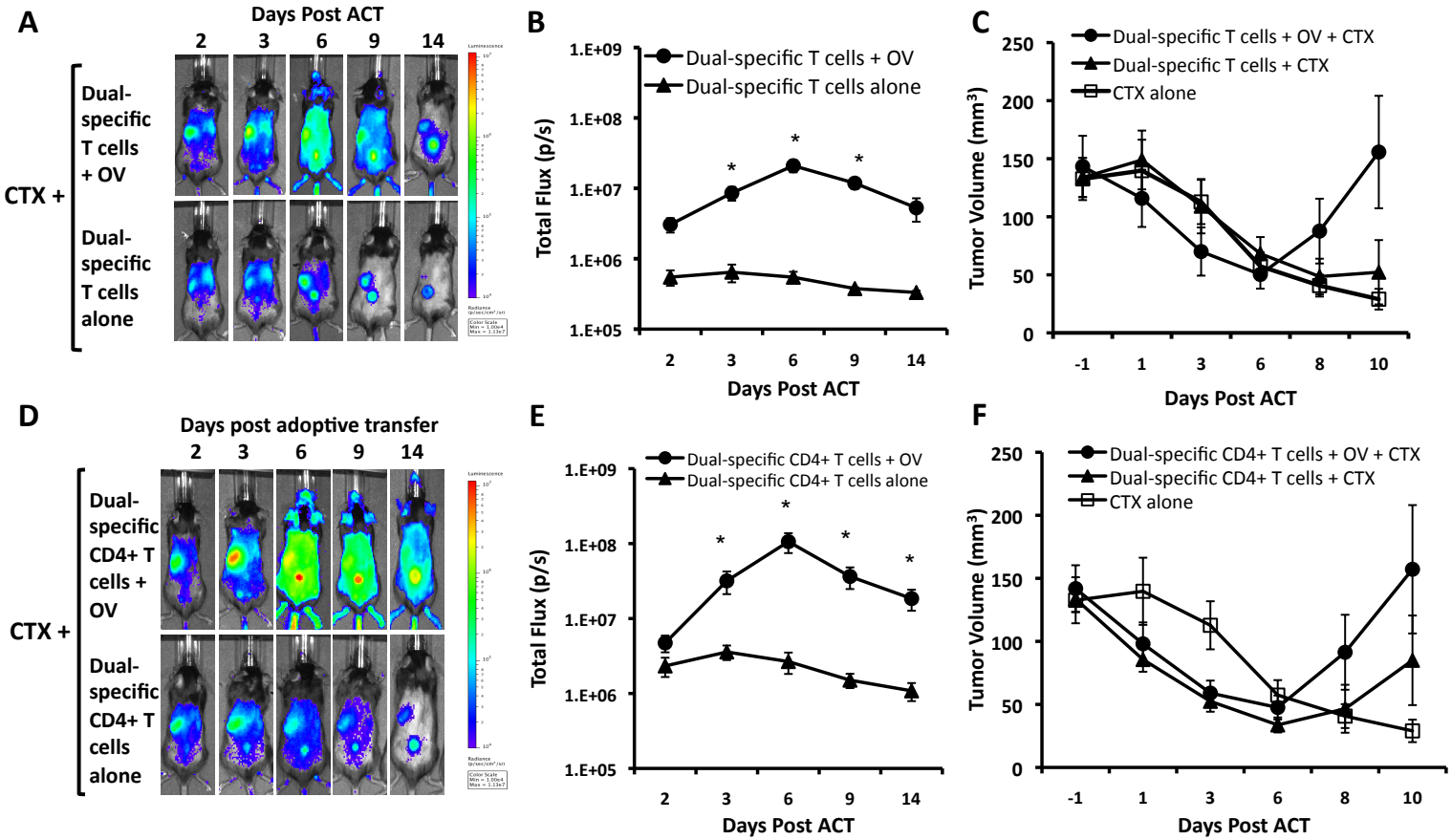
A
Days Post ACT
2
3
6
9
14
CTX +
Dual-specific T cells + OV
Dual-specific T cells alone
B
Dual-specific T cells + OV
Dual-specific T cells alone
Total Flux (p/s)
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
Days Post ACT
C
Dual-specific T cells + OV + CTX
Dual-specific T cells + CTX
CTX alone
Tumor Volume (mm³)
250
200
150
100
50
0
-1
1
3
6
8
10
Days Post ACT
D
Days post adoptive transfer
CTX +
Dual-specific CD4+ T cells + OV
Dual-specific CD4+ T cells alone
E
Dual-specific CD4+ T cells + OV
Dual-specific CD4+ T cells alone
Total Flux (p/s)
Days Post ACT
F
Dual-specific CD4+ T cells + OV + CTX
Dual-specific CD4+ T cells + CTX
CTX alone
Tumor Volume (mm³)
Days Post ACT

Figure 4.7: Pre-conditioning chemotherapy significantly enhances OV-boosting of dual-specific T cells. (a-c) P14 splenocytes were engineered with the HER2-CARand adoptively transferred into mice bearing MC38-HER2 tumors treated with 150mg/kg CTX 24 hours prior to ACT. T cells were boosted with $2x10^9$ PFU VSVΔM51-GP33-43 where indicated, after which animals were imaged using the IVIS imager. (a) Representative animal images showing signal from dual-specific CD8+ T cells *in vivo*. (b) Quantification of the luciferase signal (N= 4-5), *P<0.05. (c) Tumor growth of the mice was monitored by digital caliper measurement. (d-f) Wild type MC38-HER2 tumor bearing mice were treated with 150mg/kg CTX, and 24 hours later received SMARTA T cells bearing the HER2-CAR. Animals were boosted with $2x10^9$ PFU VSVΔM51-GP33/61 where indicated. (d) Representative animal images showing distribution of dual-specific CD4+ T cells *in vivo*. (d) Quantification of dual-specific T cell luciferase signal (N=4-5), * P<0.05. (f) Tumor measurements were taken via digital calipers.

Unfortunately, like radiation, CTX treatment alone induced regression of the MC38-HER2 tumors, similar to sublethal irradiation, and thus we cannot use this model for therapeutic studies (**Fig. 4.7c, f**).

Despite being unable to assess effectiveness of the combination therapy, we were able to glean information regarding pre-conditioning regimes and CAR-T cell boosting. While neither naïve nor sublethally irradiated animals showed a dramatic boost response in response to VSV, animals receiving CTX pre-conditioning were capable of significantly boosting the expansion of adoptively transferred T cells *in vivo*. The differences in CAR-T cell boosting ability found between irradiated or CTX-treated animals likely stems from the substantial differences in lymphopenia caused by each treatment. We followed lymphocyte frequencies in the peripheral blood of mice following each pre-conditioning method, ACT and OV-boost, and found that sublethal irradiation induced the most robust, long-lived lymphopenia (**Fig. 4.8a**, triangles). The effects of CTX were less dramatic, and resolve more quickly (**Fig. 4.8a**, squares). Provision of VSV alone resulted in the lowest level of lymphopenia, and also resolved quickly (**Fig. 4.8a**, circles). Interestingly, sublethal irradiation did allow for CD4+ and CD8+ T cell expansion and reconstitution quickly, expanding by 6 days post ACT (**Fig. 4.8b, c**), while B cells did not reconstitute within the first 10 days following ACT (**Fig. 4.8d**). All groups showed reductions in the overall proportion of B cells within the lymphocyte population over time, likely corresponding to the

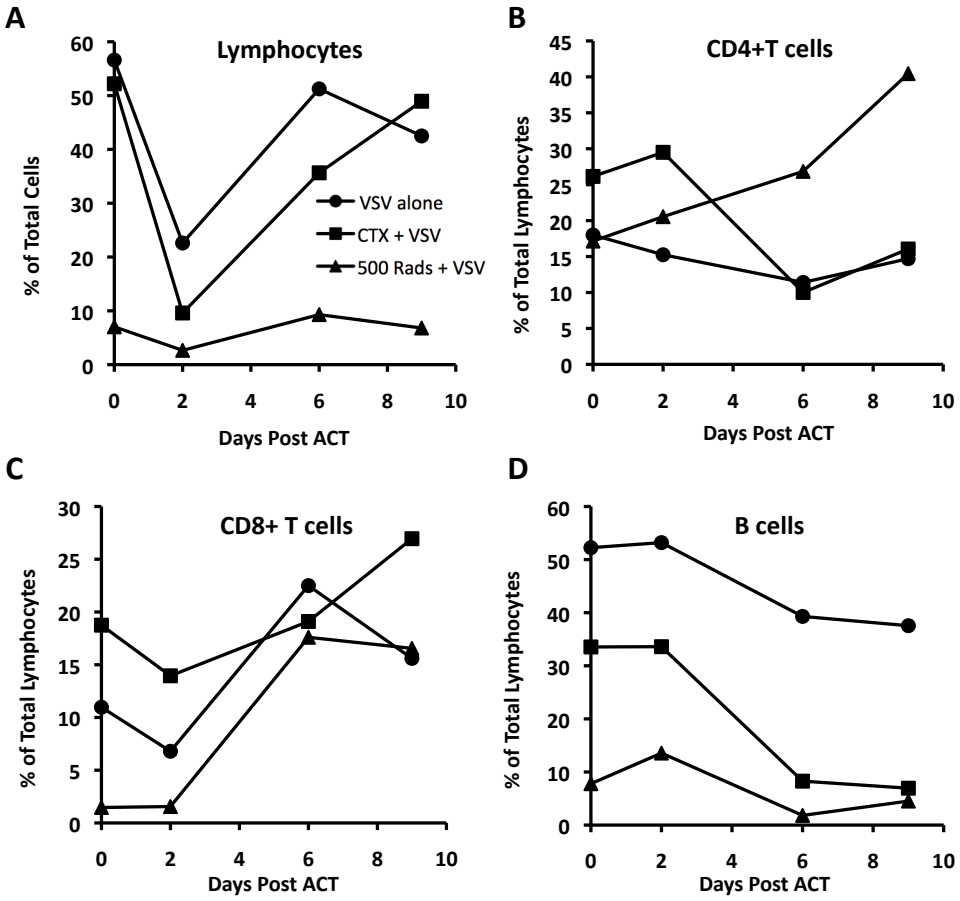

Figure 4.8: Pre-conditioning methods induce varying levels of lymphopenia. (a) The frequency of peripheral lymphocytes was determined by FSC and SSC profile of blood samples from two mice per group from mice bearing MC38-HER2 tumors receiving either no pre-conditioning ("VSV alone", circles), 150mg/kg CTX (squares) or sublethal irradiation (triangles) prior to ACT of HER2-CAR T cells and boosted with $2x10^9$ PFU VSVΔM51-GP33-61. (b) CD4+ and (c) CD8+ T cells stained with respective co-receptor antibodies. (d) B cells evaluated by B220+ staining of lymphocytes.

increasing frequencies of CD4+ and CD8+ T cells across the same time period (**Fig. 4.8b-d**).

Dual-specific CD4+ T cells show strong boosting ability *in vivo*, but lose antitumor functionality when combined with OV boost

We have shown previously that the murine B16F10 melanoma model is resistant to irradiation and CTX[94,332]. In addition, this tumor model can be targeted using an anti-VEGFR2-CAR[256,333]. Based on our observations that dual-specific CD4+ T cells display such robust expansion following OV boost and our previous reports that CD4+ T cells can mediate rejection of B16 tumors[95,275]and similar reports from others[334], we chose to test the therapeutic activity VEGFR2-CAR SMARTA T cells combined with VSVΔM51-GP33/61 in the B16 model. SMARTA T cells were engineered to express either the VEGFR2-CAR or the irrelevant HER2-CAR. Mice bearing B16F10 tumors were treated with CTX prior to ACT with the indicated dual-specific CD4+ T cells followed by VSVΔM51-GP33/61. T cell engraftment and expansion was monitored through *in vivo* imaging of luciferase activity. T cells were also monitored in the blood of mice by flow cytometry. In accordance with our previous observations, inclusion of the OV boost induced a significant increase in both the total imaging flux signal and frequency of circulating tumor-reactive dual-specific T cells (**Fig. 4.9a-b**). By imaging, the boosted T cells showed an expansion of over 10-fold compared to animals not receiving the virus boost (**Fig. 4.9b**). In the blood, boosted dual-

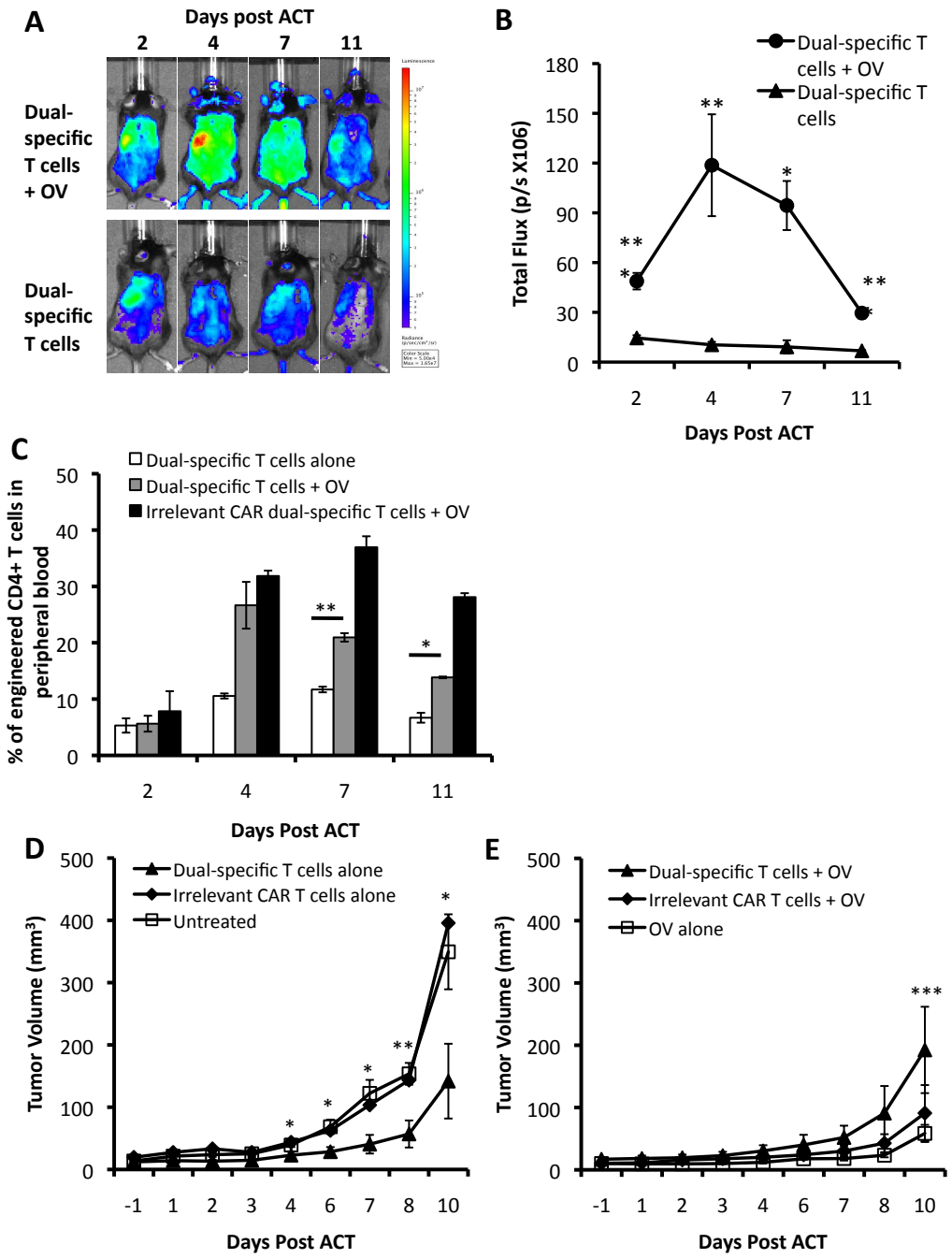
A
Days post ACT
2
4
7
11
Dual-specific T cells + OV
Dual-specific T cells
B
Dual-specific T cells + OV
Dual-specific T cells
Total Flux (p/s X106)
Days Post ACT
C
Dual-specific T cells alone
Dual-specific T cells + OV
Irrelevant CAR dual-specific T cells + OV
% of engineered CD4+ T cells in peripheral blood
Days Post ACT
D
Dual-specific T cells alone
Irrelevant CAR T cells alone
Untreated
Tumor Volume (mm³)
Days Post ACT
E
Dual-specific T cells + OV
Irrelevant CAR T cells + OV
OV alone
Tumor Volume (mm³)
Days Post ACT

Figure 4.9: Dual-specific CD4+ T cells show enhanced boosting capacity, but loss of anti-tumor immunity when combined with OV. SMARTA splenocytes were engineered to express either the VEGFR2-CAR or HER-2-CAR, and adoptively transferred into into C57BL/6 mice bearing 5-day B16F10 tumors 24 hours after pre-conditioning with 150mg/kg CTX. This was followed by administration of $2x10^9$ PFU VSVΔM51-GP33/61 where indicated ("+OV"). (a-b) T cells were visualized via IVIS imaging following luciferin administration, and quantified using LivingImage. (c) Peripheral blood samples were taken at the indicated time points, and adoptively transferred T cells detected by the frequency of Thy1.1+ CD4+ T cells via flow cytometry. (d-e) Tumor growth was monitored by digital caliper measurement. All graphs are presented as mean ± SEM, N=4, * $P<0.05$, ** $P< 0.01$, *** $P<0.001$.

specific T cells showed approximately two-fold higher frequencies over those not receiving OV boost (**Fig. 4.9c**). This expansion was also observed for the control irrelevant CAR-T cells, indicating the expansion was CAR-independent (**Fig. 4.9c**).

Based on the observation that the dual-specific T cells were expanding dramatically compared to their counterparts not receiving a boost, we anticipated this would result in enhanced antitumor immunity. We found that tumor-reactive dual-specific CD4+ T cells were capable of significantly slowing the progression of B16F10 tumors in comparison with untreated or irrelevant CAR-treated controls, leading to enhanced survival (**Fig. 4.9d**). We also observed an enhancement of the oncolytic effect of VSV when mice were pre-treated with CTX (**Fig. 4.9e**, open squares). However, when the dual-specific CD4+ T cells were combined with VSVΔM51-GP33/61, we could not improve upon the antitumor response of the VSV alone, and at later time points showed a significant loss of the VSV-induced antitumor response (**Fig. 4.9e**, closed triangles).

To understand these combination effects, we examined the functions of CAR-T cells in the periphery and in the tumor following ACT. Groups of mice received either irrelevant HER2-CAR or VEGFR2-CAR dual-specific CD4+ T cells, with or without VSVΔM51-GP33/61. The use of the HER2-CAR allows us the ability to analyze CAR reactivity post-ACT in the absence of *in vivo* CAR recognition, and evaluate how the viral boost impacts CAR function. Our data revealed that both CAR-T cells could be expanded in the periphery through their TCR by the

VSV boost (**Fig. 4.9c**), and we anticipated that the boost would increase the functionality of the dual-specific T cells. Both HER2-CAR and VEGFR2-CAR CD4+ T cells were functional prior to ACT, with 38% and 31% of respective cells producing both IFNγ and TNFα (**Fig. 4.10a**). Following ACT, we harvested spleens to analyze CAR functionality with and without boost, as well as harvested tumors for RNA/qPCR analysis of the activity and accumulation of our transferred T cells. We stimulated isolated splenocytes with either GP61 peptide (to analyze TCR functionality) or plate-bound HER2-Fc/VEGFR2-Fc proteins (to analyze CAR reactivity) and stained for production of IFNγ and TNFα. We found no differences between animals receiving dual-specific HER2-CAR or VEGFR2-CAR-bearing T cells in their ability to respond to GP61 peptide (**Fig. 4.10b**, right). Both groups of mice showed substantially higher cytokine production after VSV boost, with responses peaking at day 6 (**Fig. 4.10b**, right), which corroborates the imaging and blood phenotype data showing the productive boosting of transferred T cells. We were able to detect HER2-reactive T cells from mice receiving HER2-CAR T cells both with and without boost, with the frequency of HER2-reactive cells also increasing following the OV boost, indicating that the boost itself did not impact CAR function (**Fig. 4.10b**, centre). We were unable to detect any reactivity through the VEGFR2-CAR at any of the time points tested (**Fig. 4.10b**, left).

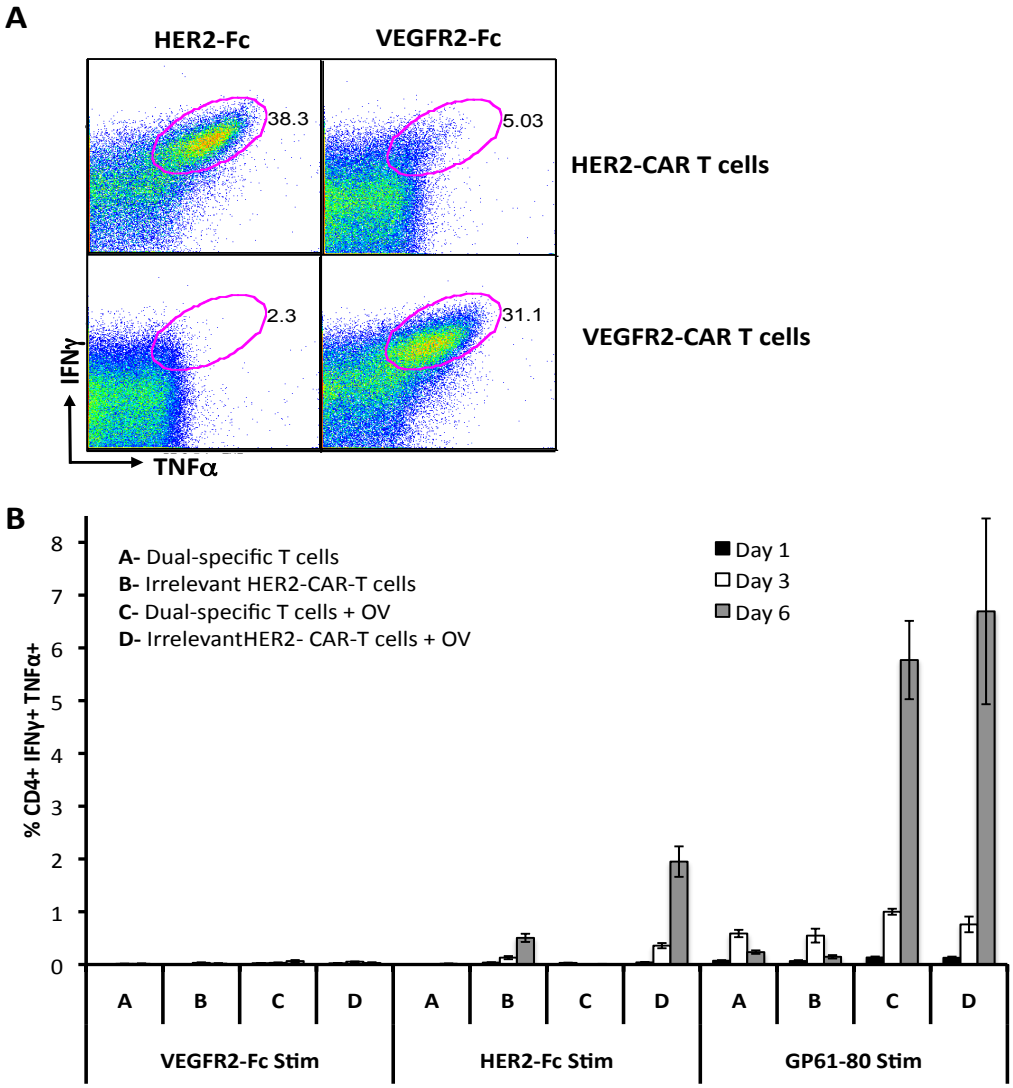

Figure 4.10: Tumor-reactive dual-specific T cells display loss of *ex vivo* CAR functionality, while retaining TCR reactivity. MC38-HER2 tumor bearing animals were pre-conditioned with 150mg/kg CTX 24 hours prior to receiving dual-specific CD4+ T cells or irrelevant CAR-engineered SMARTA T cells (bearing the HER2-CAR), with or without OV boost. (a) Functional analysis of the HER2-CAR or VEGFR2-CAR T cells prior to ACT. Cells were stimulated with HER2-Fc or VEGFR2-Fc for 4 hours and cytokine production evaluated by flow cytometry. (b) Animals were sacrificed at the indicated time points, and splenocytes stimulated with either GP61-80 peptide or plate-bound HER2-Fc or VEGFR2-Fc for 4 hours. Cytokines were visualized by flow cytometry. All graphs are presented as mean ± SEM, N=4.

To understand the impact the combination therapy was having within the tumor, we performed qPCR on isolated tumor cDNA to analyze frequencies of T cells within the tumors, as well as identify levels of immune activation within the tumor over time by IFNγ production. We could not measure any evidence of T cell infiltration into the tumors using sensitive qRT-PCR measuring TCRα and luciferase. We were also unable to detect IFNγ transcription in any of the tumors analyzed. Consistent with these results, we observed voids in the imaging flux signal where tumors were present (**Fig. 4.9a**). These data combined suggests that the T cells did not infiltrate the tumor.

Discussion

In this chapter, we have investigated the concept of pairing CAR-engineered T cells with oncolytic viruses capable of boosting the T cells through their endogenous TCR. Our findings indicate that dual-specific CD4+ or CD8+ T cells are amenable to boosting *in vivo*, although they may not retain functionality through both receptors following this boost. However, our evaluation of *in vivo* antitumor efficacy of dual-specific T cells combined with OV boosting was met with several challenges. The MC38-HER2 model proved to be inappropriate for our therapeutic studies as the tumor regressed following sublethal irradiation and CTX treatment independent of ACT. We also found that the dual-specific T cells did not infiltrate the B16F10 melanoma following the boost, making this model inappropriate for therapeutic studies as well. We evaluated a number of other

tumor models that could be established in mice with the H-2^b haplotype, including E0771, parental MC38 and 4T1.2, but these models were not found to be any more appropriate than the models already described in this chapter. Thus, we were unable to develop a model to examine therapeutic activity because our tumor lines underwent spontaneous regression following treatment with our conditioning regimens.

Despite the inability to completely assess functionality of this combination therapy *in vivo*, our studies have nonetheless revealed important considerations for future studies. In particular, our data indicate that the method of pre-conditioning plays an important role in dictating the responsiveness of adoptively transferred T cells to viral boosting. Sublethal irradiation induces significantly greater lymphopenia than either CTX or VSV (**Fig. 4.8a**). This likely results in a larger reduction in the APC population, as the patterns of lymphodepletion observed after sublethal irradiation mirror those reported previously when using lethal irradiation[332]. The level of lymphodepletion induced by CTX treatment appears to achieve the right balance between preservation of APCs for boosting, as well as space and homeostatic cytokine liberation for the adoptively transferred T cells[161,331]. This has important implications for combination therapies such as those described here, as data from others suggests that higher intensity lymphodepletion enhances the therapeutic efficacy of adoptively transferred T cells[138,139].

Even though we observed spontaneous regression of some tumors following radiation and CTX, we were surprised that the CAR-T cell therapies did not lead to a more rapid regression or complete regression in a greater number of mice. There are several possible explanations for the lack of antitumor effect observed by the CAR-T cells. The imaging data could be misleading as to the number of T cells actually present within the tumors. The enhanced luciferase construct in our engineered T cells has previously been evaluated to determine the relative flux signal compared to the cell numbers present after injection[276]. Using the calculations provided by this group would suggest the numbers of T cells detected in the tumors in our *in vivo* studies to be ~30,000 cells at the peak of expansion. As we initiated tumor growth with at least 10^5 cells, at the time of treatment there would have been a markedly low T cell to tumor cell ratio within the tumor.

The timing of administration of the ACT and OV-boost may also have impacted the antitumor benefit afforded by the combination therapy. All of our imaging data indicates the CAR-T cells take several days to boost and traffic throughout the host, with maximal signals observed around 6 days post ACT. However, VSV has been shown to induce a rapid vascular shutdown following tumor infection, resulting in a loss of tumor perfusion within 24 hours of treatment[326]. This would prevent CAR-T cells from successfully accessing the tumor, especially if they take an additional few days to migrate to the tumor. We could not detect any measurable T cell infiltrate or signs of immune activation

between 1 to 6 days post ACT, suggesting the T cells are incapable of penetrating the tumor. It is possible that any observed signal around the tumors via imaging is derived from the skin or areas adjacent to the tumor, as we have observed increased vasculature surrounding the tumors *in vivo*. As the cells are circulating throughout the periphery, this could contribute to the increased flux signal near the tumor site. Finally, it is possible that our CAR-T cells simply are not functional *in vivo*. We have attempted several different methods to detect signs of T cell activation within the tumor, including administering CAR-T cells intratumorally, with no evidence of T cell responses. While our CAR-T cells have demonstrated clear *in vitro* cytokine production and killing ability, it is possible that this fails to translate to *in vivo* functionality.

Our results demonstrate some of the intricacies and difficulties of combination therapies, requiring significant optimization of dosage, timing and ordering of the individual components. Future combination trials should carefully evaluate the impacts of each component on the trafficking and functionality of the adoptively transferred cells, keeping in mind the goal of enhancing T cell efficacy. The use of appropriate animal models is also an important component, as our studies indicated potential for significant differences in even T cell migration patterns between different tumor models. Importantly, careful consideration of the combinations of pre-conditioning, T cell types used, and contents of the boosting vector may aid in developing the optimal combination of ACT and OV boosting.

— CHAPTER 5 —

CAR-engineered T cells as oncolytic virus carriers

Introduction

Oncolytic viruses (OVs) are capable of selectively infecting, replicating in, and killing tumor cells, while avoiding healthy tissues[229]. In addition, these viruses have been shown to induce robust immune responses, potentiating the antitumor response within a host [204,235]. Vesicular Stomatitis Virus (VSV) has been found to bear these properties [204,207]. Mutations in the M protein (VSVΔM51) enhance the interferon-sensitivity of this virus, significantly increasing both its safety and its tumor tropism [204,205,207]. Vaccinia virus (VV) has also been tested extensively in pre-clinical models and clinical trials where systemic treatment with the virus was shown to be safe[227,228]. We are particularly interested in a recombinant VV containing deletions of the thymidine kinase and viral growth factor genes, resulting in a "double-deleted" vaccina virus (vvDD)[270]. This recombinant virus shows enhanced tumor tropism, with limited replication within resting cells[270].

To this point, clinical trials of systemic VV have employed high doses of virus, ranging from $1x10^5$ to $3x10^7$ PFU/kg per patient[227,229]. The use of VSV in clinical trials has been limited thus far, though animal studies typically employ doses greater than $5x10^8$ PFU per mouse, suggesting human dosages would also be quite high[204,207,286,335]. It is speculated that such high doses are required when delivering the virus intravenously because multiple blood-borne defense mechanism can eliminate the virus, such as complement, antibodies, and immune cells, so the dose must saturate these defense mechanisms to enable delivery of

virus to the tumor[336].

Adoptive cell transfer (ACT) therapies have emerged as effective treatments for certain types of cancer, including the use of tumor infiltrating lymphocytes for melanoma and engineered T cells for hematological malignancies[138,179,181,337,338]. As evidenced by the successes in ACT studies, adoptive transfer of T cells results in T cells migrating to the tumor site in order to perform their antitumor functions. Interestingly, OVs have been found to naturally associate with circulating lymphocytes such as B cells[267]. It is therefore attractive to consider loading lymphocytes with OVs prior to adoptive therapy. In this way, the adoptively transferred T cells loaded with OVs should be capable of delivering the OV to the tumor site. Indeed, previous reports have shown that transgenic murine T cells can be used to deliver OVs to established tumors, and that this combination can result in tumor rejection[339,340]. Loading VSV onto T cells protects the virus from neutralizing antibodies, while retaining its antitumor efficacy[341,342]. Similarly, VV can be effectively carried and deposited within tumors using cytokine-induced killer (CIK) cells, leading again to antitumor efficacy[343,344]. With the promising results observed in clinical trials of adoptive transfer of T cells engineered with chimeric antigen receptors (CARs), we were interested in determining whether CAR-engineered T cells could be loaded with OV and maintain their anti-tumor function; effectively creating dual-pronged anti-tumor agent. In this manuscript, we demonstrate that both VSVΔM51 and vvDD can be successfully loaded murine and human CAR-T cells without affecting CAR expression, viability or

functionality. Our data further shows that OV-loaded CAR-T cells are capable of depositing virus onto tumor targets, and that this combination has the potential to enhance the efficacy of each of the two approaches. These data provide the basis for combining these two therapies for future therapeutic applications.

Results

OV-loading of CAR-T cells does not impact CAR expression

We first sought to determine the feasibility of combining CAR-T cells with OV-loading as well as determine the optimal viral MOI for use in our studies. Murine T cells engineered with a CAR-'ve control retrovirus (to avoid potential effects of the CAR) were loaded with either VSVΔM51-GFP or vvDD-GFP at MOI=0.3, 1, and 3 (**Fig. 5.1a**). Our preliminary experiments found that loading CAR-T cells with an MOI of 3 resulted in the highest level of both VSVΔM51-GFP and vvDD-GFP deposition on tumor targets over the lower MOI described above, as well as increased reproducibility between replicates (**Fig. 5.1a**). We observed the same outcome when testing human T cells engineered with a CAR-'ve lentivirus, with MOI=3 showing the highest virus deposition (**Fig. 5.1b**). Based on these results, all subsequent experiments utilized this MOI for all T cells and viruses.

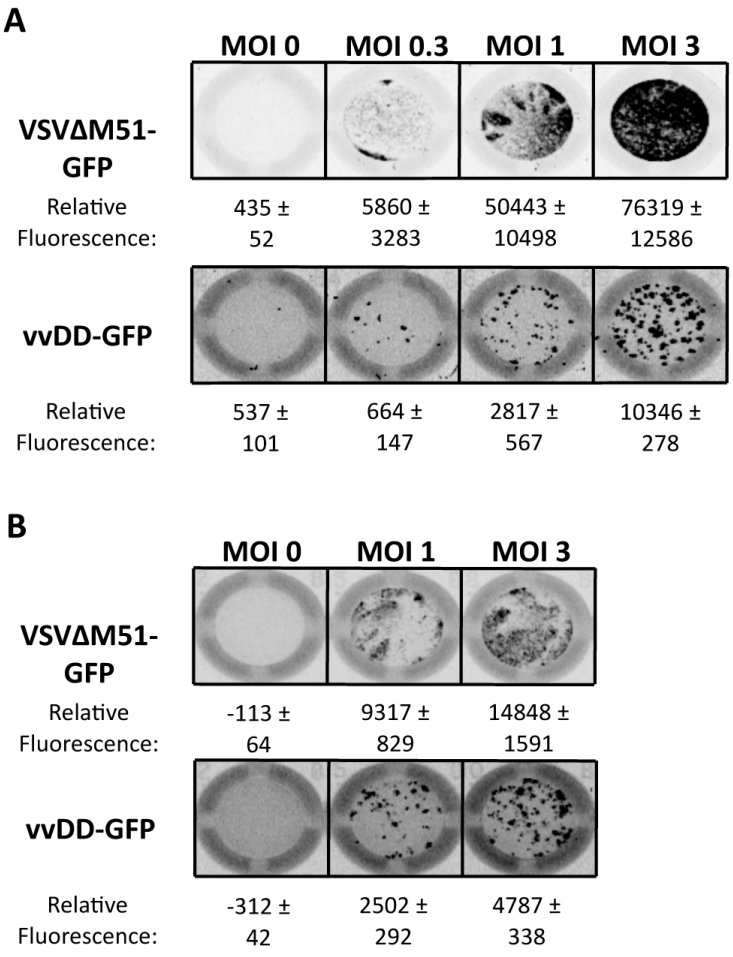

Figure 5.1: Dose titration of OV-loading. (a) Murine T cells or (b) human T cells engineered with CAR-negative (CAR-'ve) vectors were loaded with the indicated MOI of either VSVΔM51-GFP or vvDD-GFP, washed, and incubated with D2F2 tumor cells to test OV deposition. Relative fluorescence determined by Image Quant software and is presented as mean ± SEM from at least 2-3 replicates, normalized to tumor-only wells.

We next looked to test whether OV-loading had any effect on the T cells. Engineered T cells were loaded with an MOI=3 of either VSVΔM51-GFP or vvDD-GFP. Following washing, cells were incubated overnight and analyzed for virus replication, or changes in CAR expression or functionality. We examined cells for virus infection via GFP production by flow cytometry, and found very minimal infection of murine T cells by either VSVΔM51-GFP or vvDD-GFP in murine CAR-T cells (**Fig. 5.2a-b**). While GFP+ cells did reach statistical significance after loading with vvDD-GFP, in all cases, GFP expression was observed in <1% of the cells (**Fig. 5.2b**). We went on to characterize the impact of OV-loading on CAR expression, and found there to be no difference in the level of CAR surface expression after loading with either OV (**Fig. 5.2c**).

We next tested the ability of human CAR-T cells to be loaded with OV. We engineered human T cells with lentiviruses containing the human HER2-CAR cDNA (or a CAR-'ve control) and loaded them with VSVΔM51-GFP or vvDD-GFP. Interestingly, we observed significantly higher levels of vvDD-GFP replication in both CAR-'ve and HER2-CAR-T cells, reaching above 8% GFP+ cells (**Fig. 5.2d-e**). In contrast, VSVΔM51-GFP-loaded cells showed below 2% GFP+ cells (**Fig. 5.2d-e**). The increase in virus replication in human CAR-T cells did not alter T cell viability, with cells maintaining greater than 80% viability regardless of OV-loading. In addition, akin to the murine T cells, OV-loading did not cause any changes in CAR expression on human T cells (**Fig. 5.2f**). This data suggests that OV-loading does not impact CAR surface expression.

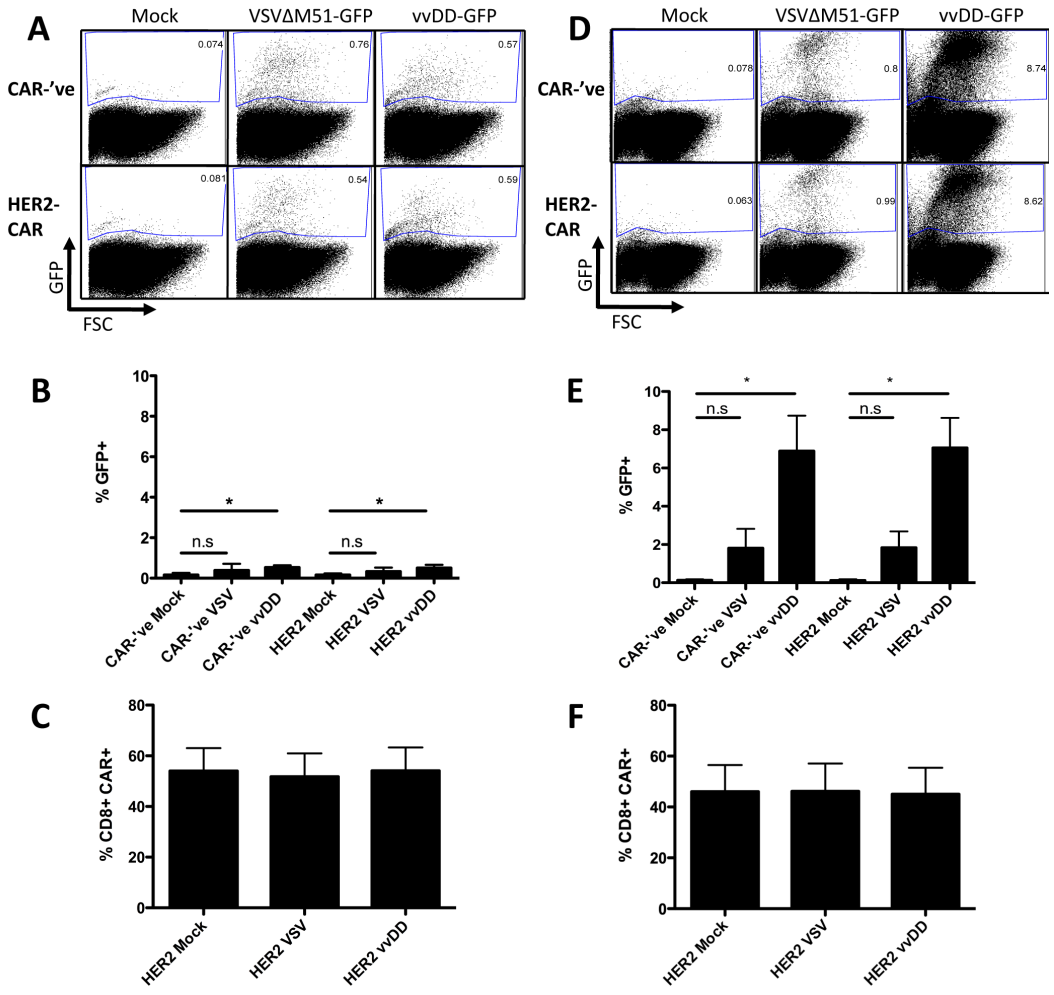

Figure 5.2: OV-loading does not impact CAR expression. (a-c) Murine T cells or (d-f) human T cells engineered with either CAR-'ve or HER2-CAR vectors were loaded with VSVΔM51-GFP or vvDD-GFP, washed, and incubated overnight before analysis. (a-b) Murine T cells show minimal VSVΔM51-GFP or vvDD-GFP replication after loading, as measured by GFP+ flow cytometry signal. Data are presented as representative plots or +/- SEM from 3 independent experiments. *P<0.05, n.s = not significant. (c) CAR expression was evaluated after OV-loading via staining with HER2-Fc Chimera and visualized by flow cytometry. Results are presented as mean +/- SEM from 3 independent experiments. (d-e) Human T cells show low levels of VSVΔM51-GFP or vvDD-GFP replication after loading. Data are presented as representative plots or +/- SEM from 2 independent experiments. *P<0.05; n.s = not significant. (f) CAR expression was unaffected by OV-loading, evaluated as described above. Results are presented as mean +/- SEM from 2 independent experiments.

CAR-T cells show no functional impairments following OV-loading

We next sought to determine whether CAR-T cell functionality was affected by loading with OV. We loaded murine CAR-T cells with OV as described, and stimulated with HER2-Fc for 4 hours, followed by flow cytometry staining for cytokine production. Loading of either VSV or vvDD onto murine T cells resulted in minimal decreases in overall cytokine production that was not significant (**Fig. 5.3a**). All T cells were capable of producing both IFNγ and TNFα following CAR stimulation (**Fig. 5.3a**). To evaluate the ability of CAR-T cells to kill target cells with and without OV-loading, varying ratios of T cells to tumor targets were co-cultured for 6 hours, minimizing the potential for virus-mediated killing within the short incubation period. Indeed, OV-loading did not alter the CAR-T cell's ability to selectively kill their HER2+ tumor targets (D2F2/E2) while sparing the HER2-'ve D2F2 cells (**Fig. 5.3b**). Similarly, OV-loading of human CAR-T cells also did not impact their ability to produce cytokine in response to CAR stimulation (**Fig. 5.3c**) or their ability to specifically kill HER2+ tumor targets (**Fig. 5.3d**). Taken together, our data suggests that engineered T cells can be loaded with OV without causing any impairment to the CAR-T cells.

CAR-T cells can successfully transfer OVs to tumor cells

To determine if OV-loaded CAR-T cells could effectively deposit virus on tumor targets, we co-cultured CAR-'ve or HER2-CAR-T cells loaded with either

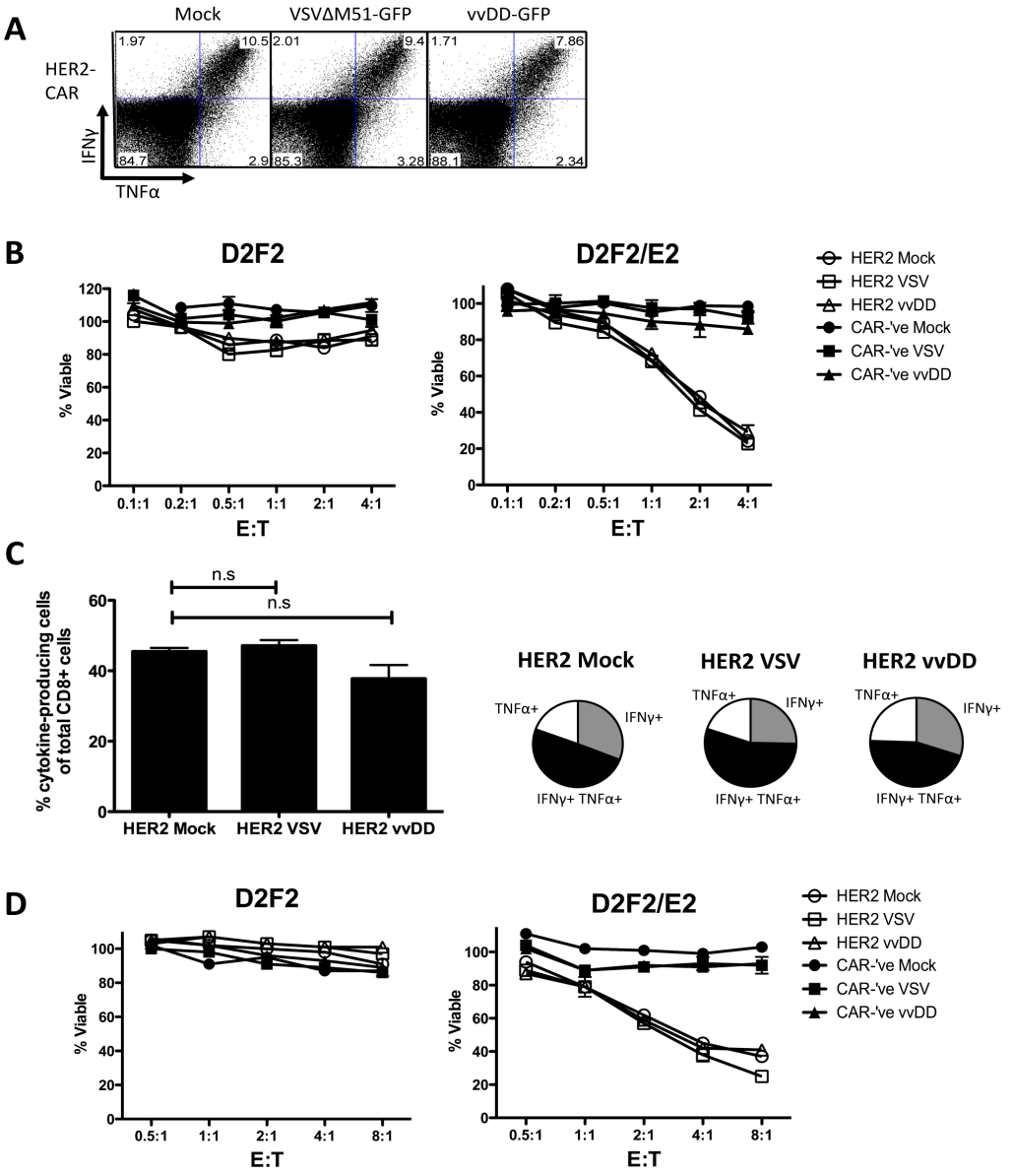

Figure 5.3: OV-loading does not impact CAR-T cell function. (a-b) Murine T cells or (c-d) human T cells engineered with CAR-'ve or HER2-CAR vectors were loaded with VSVΔM51-GFP or vvDD-GFP, washed, and incubated overnight before functional testing. (a, c) OV-loaded T cells were stimulated for 4 hours with plate-bound HER2-Fc in the presence of brefeldin A. Cytokine production was equivalent between mock or OV-loaded T cells. (a) Data are representative flow plots of 2 independent experiments, or (c) pooled from at least two independent experiments. (b, d) Mock or OV-loaded CAR-T cells were co-cultured with D2F2 or D2F2/E2 tumor cells for 6 hours. After washing off T cells, tumor cell viability was assessed via alamarBlue assay. Data are representative of 2 independent experiments for each murine or human T cells.

VSVΔM51-GFP or vvDD-GFP at a 1:1 ratio with D2F2 breast tumor targets, which do not carry the target for the CAR (human HER-2) and thus will not be affected by CAR signaling. After 24 hours, virus replication was evaluated as GFP-fluorescence using the Typhoon Imager (**Fig. 5.4**). We observed that both CAR-'ve and HER2-CAR-T cells could successfully transfer VSVΔM51-GFP to tumor targets with the same level of efficiency (**Fig. 5.4a, c**). This was true for both murine and human CAR-T cells, which displayed similar patterns of replication within the monolayer (**Fig. 5.4a,c left panels**). These replication patterns indicated that the virus was infecting specific foci and spreading throughout the monolayer (**Fig. 5.4**). This furthers the notion that the virus itself is replicating within the tumor cells as opposed to simply infecting cells at the 1:1 culture ratio.

OVs deposited by either CAR-'ve or HER2-CAR-T cells revealed similar patterns of replication within the wells, again showing specific foci of infection within the cell monolayer (**Fig. 4.4b, d, left panels**). Similar patterns of infection/replication within the target cells were observed regardless of whether CAR-'ve or CAR+ cells were used, indicating that replication is in general similar between these cells. Overall, our data suggests that CAR-T cells can function as effective vehicles for OV-transport.

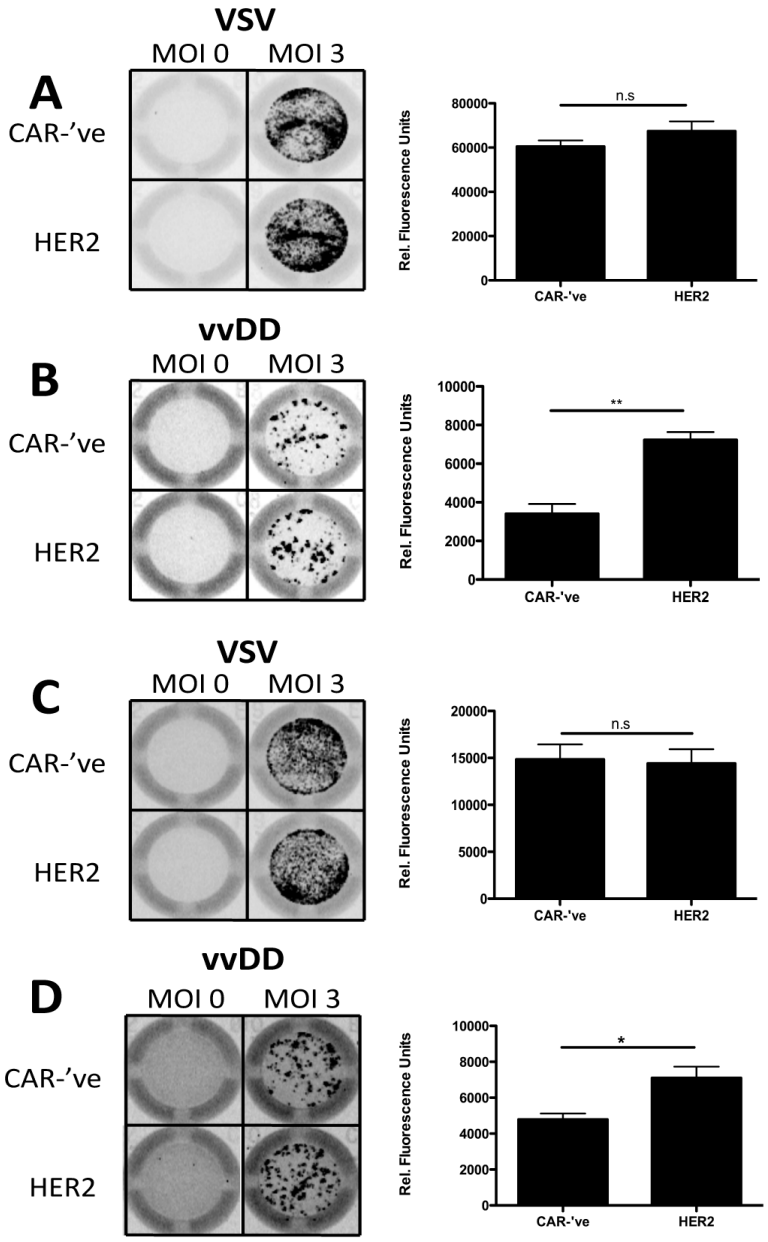

Figure 5.4: OV-loaded CAR-T cells can deposit OV onto tumor targets. Murine (a-b) or human (c-d) CAR-'ve or HER2-CAR T cells were loaded with MOI of 0 or 3 of (a, c) VSVΔM51-GFP or (b, d) vvDD-GFP and co-cultured with D2F2 tumor targets for 24 hours. Virus replication was visualized using the Typhoon Imager to detect GFP signal (left panels). The level of virus replication was quantified using ImageQuant (right panels). Quantification is expressed as mean ± SEM, *P<0.05, **P<0.01, n.s= not significant. Data is representative of 2-3 independent experiments performed in triplicate for each murine and human cells, normalized to tumor-only wells.

Cognate interaction between CAR-T cell and tumor does not impact ability to deposit OV

As CAR-T cells produce cytokines such as IFN-γ following CAR ligation, which could impact virus replication, we wanted to determine whether CAR activation would negatively impact OV-loading of tumor cells. We co-cultured VSVΔM51-GFP or vvDD-GFP loaded CAR-'ve or HER2-CAR-T cells at a 1:1 ratio with the HER2+ D2F2/E2 cell line for 24 hours, and evaluated virus replication by GFP production. We observed no differences in either virus' ability to replicate in the presence of cognate interaction between the CAR and the tumor target using either murine or human CAR-T cells (**Fig 5.5**). While there appeared to be a trend towards a decreased virus load in the HER2-CAR-T cell groups (**Fig. 5.5**), the effect of HER2-CAR-mediated killing of target cells must also be considered, as this reduces the number of target cells available to be infected. Taken together, our data shows that CAR ligation does not impede the ability of OV-loaded T cells to deposit virus or for the virus to replicate in tumor targets.

OV-Loading of CAR-T cells can enhance tumor cell killing

To evaluate the ability of OV-loaded CAR-T cells to enhance the tumor killing relative to CAR-T cells alone, we evaluated *in vitro* killing of three different HER-2-positive tumor cell lines. The three lines expressed HER2 to varying degrees (**Fig. 5.6a**), with A549 showing the lowest level of HER-2 expression, D2F2/E2 displaying the highest level of expression and T47D

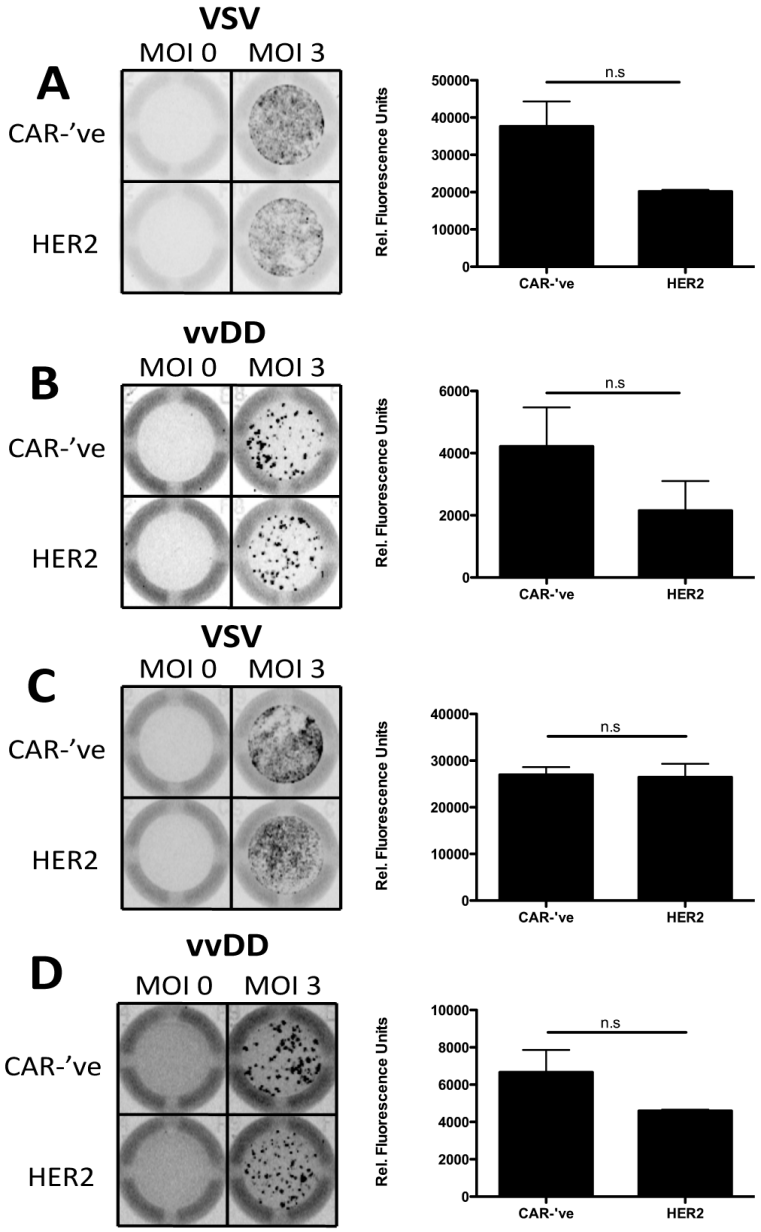

Figure 5.5: Cognate interaction does not impair CAR-T cell ability to transfer OV. Murine (a-b) or human (c-d) CAR-'ve or HER2-CAR T cells were loaded with MOI of 0 or 3 of (a, c) VSVΔM51-GFP or (b, d) vvDD-GFP and co-cultured with D2F2/E2 tumor targets for 24 hours. Virus replication was visualized using the Typhoon to detect GFP signal (left panels). Virus replication was quantified using ImageQuant (right panels). Quantification is expressed as mean ± SEM, *P<0.05, **P<0.01, n.s= not significant. Data is representative of 2-3 independent experiments performed in triplicate for each murine and human cells, normalized to tumor-only wells.

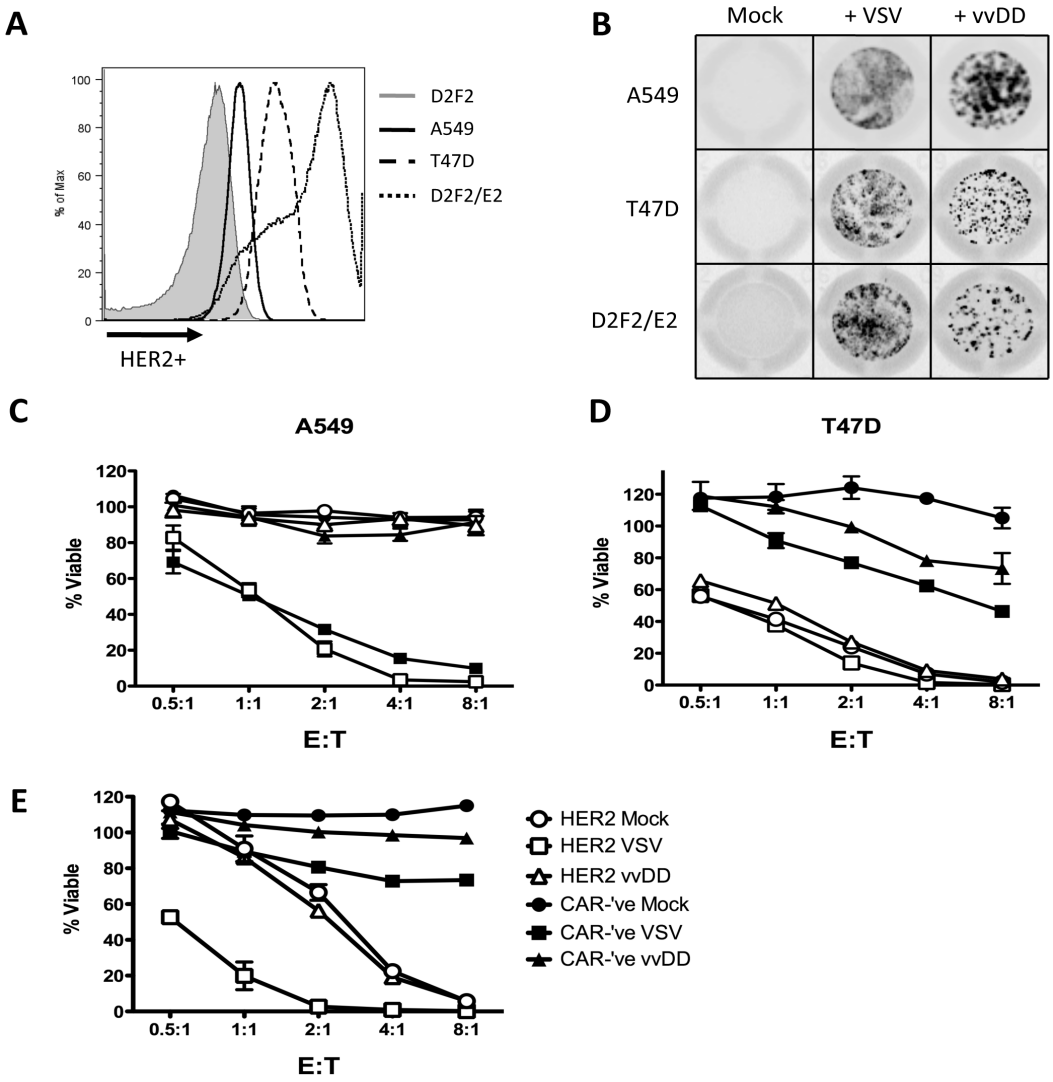

Figure 5.6: OV-loaded CAR-T cells can enhance killing of tumor targets. Human HER2- CAR-T cells (or CAR-'ve controls) loaded with an MOI of 3 of either VSVΔM51-GFP or vvDD-GFP were co-cultured at varying effector to target ratios (E:T) with tumor targets. (a) Murine (D2F2 and D2F2/E2) and human (A549, T47D) tumor cell lines were stained for HER2 expression using anti-human HER2, followed by anti-human IgG-PE and visualized by flow cytometry. (b) A549, T47D and D2F2/E2 show differing susceptibilities to VSV or vvDD replication. Wells were imaged using a Typhoon imager after 24 hours of co-culture at the 1:1 effector to target ratio with OV-loaded CAR-'ve cells as described above. Wells are representative of at least 2-3 independent experiments performed in triplicate. OV-loaded T cells were co-cultured with (c) A549, (d) T47D or (e) D2F2/E2 tumor cells overnight. After washing off the T cells, tumor cell viability was determined via alamarBlue assay. Data is representative of 2-3 independent experiments, presented as mean ± SEM of triplicate wells.

revealing an intermediate level of expression. These cell lines are also susceptible to both VSVΔM51-GFP and vvDD-GFP replication, as we could readily detect virus replication in these cells following co-culture with OV-loaded CAR-T cells (**Fig. 5.6b**). The three lines display similar levels of susceptibility to VSVΔM51-GFP infection following deposition by OV-loaded CAR-T cells (**Fig. 5.6b, middle column**). In contrast, there appeared to be greater difference in susceptibility to infection following deposition of vvDD by the CAR-T cells (**Fig.** 5.**6b, right column**), as the A549 cells seem to support greater vvDD replication than the other lines. The 3 cell lines displayed differential sensitivity to killing by CAR-T cells where the A549 cells were relatively resistant to killing, the T47D cells were most sensitive to killing and the D2F2/E2 cells displayed intermediate sensitivity (**Fig. 5.6c-e, open circles**). The combined differences in sensitivity to CAR-mediated killing and OV infection provides a good spectrum of tumor cells in which to evaluate the efficacy of combining CAR-T cells and OV-loading. The A549 lung adenocarcinoma proved very sensitive to VSV-mediated oncolysis following deposition by T cells, but did not reveal any combinatorial effects of the CAR-T cells and the OVs (**Fig. 5.6c**). Deposition of vvDD-GFP did not affect the viability of the A549 cells despite the observed virus replication (**Fig. 5.6b, upper right squares and Fig. 5.6c, triangles**). While the T47D line demonstrated sensitivity to killing by both VSV and vvDD deposited by CAR-‘ve T cells, the robust killing by the CAR-T cells obscured the benefit of any combinatorial effects (**Fig. 5.6d**). We observed mild viral oncolysis of D2F2/E2 breast tumor

cells following deposition VSVΔM51-GFP by CAR-'ve T cells (**Fig. 5.6e, closed squares**). As described earlier, the D2F2/E2 line was sensitive to killing by HER2-CAR-T cells and this killing was not affected by loading with vvDD. Interestingly, we did observe a marked combinatorial killing effect of the HER2-CAR-T cells loaded with VSVΔM51-GFP (**Fig. 5.6e, open squares**). Overall, our data shows that OV-loading of CAR-T cells not impair the functionality of the CAR-T cells alone and that the addition of the virus enables efficient killing of targets that might otherwise be resistant to CAR-T cell therapy. Moreover, we observed a combinatorial effect on a cell line (D2F2/E2) that was only moderately sensitive to either method alone.

Virus from OV-loaded T cells replicates rapidly upon transfer to tumor targets

To get a better understanding of the degree of virus replication following deposition by T cells, we quantified the virus attached to the input T cells and then measured the virus titers in the supernatant of the infected tumor targets 24 hours following deposition by T cells. Interestingly, we found that VSVΔM51-GFP loading resulted in very low PFU of virus remaining associated with the CAR-T cells, with 20-30 PFU per $1.25x10^5$ cells from CAR-'ve or Her-2 CAR-T cells respectively (**Table 5.1**). In contrast, vvDD-GFP loading resulted in significantly higher virus load associated with the T cells, with $0.5\text{-}1.2x10^4$ PFU per $1.25x10^5$ cells (**Table 5.1**). To evaluate virus replication after co-culture with tumor targets, we added $1.25x10^5$ OV-loaded T cells to $1.25x10^5$ D2F2 tumor

Table 5.1: Virus titrations from loaded CAR-T cells. T cells were loaded with either VSVΔM51-GFP or vvDD-GFP at an MOI of 3. T cells were collected post-wash for "input virus" titration. OV-loaded T cells were co-cultured at a 1:1 ratio with D2F2 tumor cells for 24 hours. Supernatants were collected and virus titrated as "virus output". VSVΔM51-GFP was titrated using agarose overlays on Vero cells, while vvDD-GFP was titrated using CV-1 cells and visualized with crystal violet staining. Error is expressed as ± SEM.

Virus Titer (PFU)

	VSV		**vvDD**	
	Input Virus (from 1.25 x10^5 T cells)	**Virus Output** (following 24h co-culture)	**Input Virus** (from 1.25 x10^5 T cells)	**Virus Output** (following 24h co-culture)
CAR-'ve T cells	30.02 (±3.46)	$2.03x10^7$ ($±5.63x10^6$)	$1.19x10^4$ ($±4.83x10^3$)	$8.88x10^5$ ($±1.39x10^5$)
Her2-CAR-T cells	20.42 (±3.37)	$1.74x10^7$ ($±4.21x10^6$)	$5.51x10^3$ ($±4.38x10^2$)	$8.38x10^5$ ($±2.98x10^4$)

cells. After 24 hours we harvested supernatants and titrated the resulting virus. After co-culture of VSV-loaded CAR-T cells, we observed a dramatic amplification of the virus, resulting in up to $2x10^7$ PFU (**Table 5.1**). This corresponds to a greater than $7.5x10^5$-fold increase in viral titer, stemming from an effective MOI of 0.0002. There was no significant difference between cultures where the virus was deposited by CAR-'ve or HER2-CAR-T cells (**Table 5.1**). We performed the same experiment with T cells loaded with vvDD achieving MOIs of 0.04 and 0.1 for HER2-CAR and CAR-'ve-T cells respectively. Virus titers in the culture supernatant reached upwards of $8.88x10^5$ PFU24 hours following virus deposition (**Table 5.1**). This corresponds to an average 114-fold increase in virus load across T cell types. Again, there was no significant difference between CAR-'ve and HER2-CAR-T cell-derived vvDD-GFP virus titer. Taken together, our data shows that OV-loading of CAR-T cells can be a viable combination, as these T cells can effectively transfer virus to tumor targets, which can serve to enhance the antitumor efficacy of each of these therapies.

Discussion

Cancer immunotherapy is a rapidly expanding field, with significant advances using T cells, viruses, antibodies, alone or in combination, as therapeutics. While ACT has shown significant promise in treating hematological malignancies, solid tumors continue to show lower levels of response [129]. The use of oncolytic viruses as a stand-alone therapy has emerged as an additional

promising treatment, with examples such as T-VEC showing durable complete or partial clinical responses in a recent Phase III study[229]. However, many of these OV therapies rely on either intratumoral administration, which is difficult in the case of metastatic disease, or very high virus titers for systemic administration, which increases the risk of off-target effects[214,226,227,229,345]. As such, there is considerable room for improvement of each of these therapies. The concept of combining T cells with OV-loading has been addressed in a limited number of reports employing either transgenic murine T cells or human CIKs [339,340,343,346]. These studies showed that activated murine T cells were capable of carrying and depositing VSV onto tumor targets, showing an enhanced efficacy over either used as a monotherapy[339]. In addition, both mouse and human CIKs provided successful transport of vvDD, resulting in improved antitumor efficacy[343,346]. Engineering T cells to recognize surface-expressed tumor antigens using CARs avoids the MHC restriction encountered by TCR-activated T cells[165]. This engineering process allows for precise targeting of known target antigens, and facilitates re-targeting of bulk T cell populations[165]. Our study demonstrates the feasibility of loading CAR-engineered T cells with OV for use as a combination therapy.

Loading CAR-T cells with either VSV or vvDD did not result in any phenotypic or functional changes to human or murine CAR-T cells, indicating that the loading of these viruses is a relatively innocuous process. Additionally, the ability of OV-loaded CAR-T cells to transfer their virus load to tumor targets

was not impaired by recognition of the tumor by the CAR. This is an important consideration, as combination therapies must not interfere with the effects of each treatment used on its own. The combination of OV with CAR-T cells can provide complementary benefits to the killing capacity of each component. Virus replication within the tumor and lysis of tumor cells induces inflammation, which serves to drive endogenous antitumor immunity[347]. In a mouse model of ACT, combining local VSV delivery with tumor-reactive T cells served to maintain the activation status of adoptively transferred cells[347,348]. By packaging the OV onto a T cell carrier, the OV is protected from immune recognition in the blood stream, and transported to the tumor[341,342]. Thus these therapies possess complementary properties.

One potential concern for combining CAR-T cells with OV is that several OV, including VSV and vvDD, have been shown to induce vascular shut down within the tumor during oncolysis[228,325]. This has the potential to prevent CAR-T cells from infiltrating the tumor, which would limit the efficacy of the CAR-T cells. By loading the OV onto CAR-T cells, the virus and T cells would both be present within the tumor together, limiting the likelihood of one restricting access for the other. In order to better drive OV-loaded CAR-T cell trafficking to the tumor, this therapy could be further combined with pre-conditioning irradiation or chemotherapy that have been shown to significantly enhance engraftment of adoptively transferred T cells[137-139]. These therapies have also been shown to enhance antitumor efficacy and reduce neutralizing antibody generation when

using OVs, and so may serve to enhance both arms of this combination[349].

Our data supports the use of OV-loaded CAR-T cells to treat a variety of different tumors. In particular, our data shows the effects of treating tumors with varying sensitivities to either OV or CAR-T cell mediated killing. CAR-T cells alone had minimal effect on A549 lung carcinoma cells, however T cell-transported VSVΔM51 proved effective at eliminating these cells (**Fig. 5.5c**). Treatment of T47D cells showed maximal killing with HER2-CAR-T cells, without a measurable effect of the OVs (**Fig. 5.6d**). However, as these viruses can disrupt tumor vasculature as well as drive endogenous immune responses in addition to oncolysis, these could still be of use for clinical treatment of tumors with varying sensitivity to CAR-T cells. Finally, when utilizing OV-loaded CAR-T cells to treat D2F2/E2 cells, we observed a significant enhancement of tumor cell killing when combining VSV and HER2-CAR-T cells over each treatment independently (**Fig. 5.6e**). These data are particularly promising, as they suggests that OV-loaded CAR-T cells may be used to treat heterogeneous tumors. Tumor cells that are resistant to CAR-T cell mediated killing may be effectively killed by oncolysis, while those with some degree of susceptibility to each treatment independently can be killed readily by the combination treatment. Additionally, neither treatment appears to impair the antitumor functionality of the other, showing that these two therapies are indeed compatible. Combining these treatments could provide protection against antigen-loss variants, as the OV are capable of driving endogenous immune responses to additional tumor antigens,

extending the antitumor response[215]. As the effective MOI of viruses derived from OV-loaded CAR-T cells is very low, our data suggests that pre-loading onto tumor-reactive T cells can significantly enhance OV delivery to the tumor. This therefore presents a multi-pronged antitumor approach that allows for significantly lower OV dosages than if the two therapies were used concurrently.

We failed to distinguish an advantage to using vvDD-loaded CAR-T cells over the use of CAR-T cells alone in our studies. We speculate this corresponds to limitations of *in vitro* assays, where the CAR-T cells are capable of killing tumor targets faster than vvDD can. Our data showed that even in the absence of observable cytolysis, vvDD-GFP replicated readily in all three tumor lines, albeit to varying degrees (**Fig. 5.6b**). The combination of vvDD loaded onto CAR-T cells may prove a more effective combination in the context of an established tumor. This way, the virus will be able to infect and kill tumor cells unaffected by CAR-T cell treatment, as well target the tumor vasculature, causing tumor destruction through both direct and indirect means [228]. Importantly, loading of CAR-T cells with vvDD did not negatively impact the functionality of the CAR-T cells, and so could provide benefit as a combination therapy.

Additional advantages of packaging OV onto CAR-T cells are that OVs have the unique capabilities of encoding additional genes within them that can be used to enhance the activity of the T cells. The functionality and survival of CAR-T cells can be enhanced through provision of cytokines such as IL-15 or IL-12 within the OV, allowing for production of these cytokines within the tumor

microenvironment[286,350]. The OV could encode a target antigen recognized by the endogenous T cell receptor on the CAR-T cell, allowing for boosting of the transferred T cells[321,351]. Alternatively, the OV could code for either siRNA or miRNA that could downregulate ligands for immunosuppressive T cell receptors such as CTLA-4 or PD-L1, as blockade of these pathways has been shown to enhance ACT success[118,352]. Thus there are numerous possibilities for further combination therapies using OV-loaded CAR-T cells.

Overall, our studies combining OV-loading with CAR-T cell transfer provide the proof-of-principle that this combination is both feasible and effective at enhancing antitumor responses. This lays the groundwork to test various other OVs, as well as the modifications of the OV transgenes to drive enhanced T cell function. Importantly, our data aids in developing an understanding of the interplay between CAR-T cells and OVs when used as a combination therapy.

— CHAPTER 6 —

Discussion

Final Discussion

In this final chapter, I will briefly summarize my research findings as described in chapters 3-5. In light of these data, the discussion will focus on key concepts that have come to light over the course of this research. Finally, I will discuss strategies to improve adoptive transfer therapeutics in the context of combination therapies and the involvement of oncolytic virus treatments.

1.0 Summary of research findings

The research conducted throughout my PhD studies has focused on developing cancer immunotherapy platforms using CAR-T cells, with an emphasis on combining these with oncolytic virotherapy. The results presented in **Chapter 3** revealed previously unreported toxicities when using CARs to target NKG2DL. Serum cytokine analysis revealed a broad cytokine storm suggesting that multiple cell types were triggered by the CAR-T cells. The severity of these toxicities was influenced by a number of factors, including the specific CAR configuration, the strain of mouse, and the administration of cyclophosphamide prior to adoptive transfer. Our data uncovered a distinct hierarchy of toxicity that mirrored the level of CAR surface expression despite no obvious functional differences *in vitro* between T cells engineered with the various CAR constructs. These results suggest that *in vitro* functional assays may not be predictive of *in vivo* responsiveness or toxicity while the level of receptor surface expression may prove more indicative, at least in the context of the NKG2D CARs. In addition,

we observed dramatic strain-specific differences in CAR expression as well as toxicity, suggesting that both the donor cells as well as host ligand expression may play a role in NKG2D-CAR-mediated toxicity. Overall, these findings expose potentially hazardous outcomes of NKG2D-CAR-T cell therapy, which argue against further development of these CARs for ACT.

The work detailed in **Chapter 4** evaluated the concept of boosting CAR-T cells through their endogenous TCR using an oncolytic vaccine. Our data indicated that dual-specific CAR-T cells can be boosted *in vivo* through TCR stimulation provided by oncolytic VSV. However, pre-conditioning lymphodepletion was required to achieve T cell engraftment following ACT. The selection of pre-conditioning method was determined to be critical for enabling T cell boosting by the oncolytic vaccine. Pre-conditioning with sublethal irradiation allowed for long-term T cell engraftment, however we were unable to observe any benefit afforded by the oncolytic vaccine boost. Conversely, pre-treatment with CTX enabled both T cell engraftment as well as robust boosting responses induced by VSV vectors containing the TCR-recognized antigen. Despite testing a multitude of tumor models, we were unable to find model that was not cured by the conditioning regimen (i.e. sublethal irradiation or CTX). Therefore, given the importance of conditioning regimen to CAR-T cell engraftment, we could not test the primary hypothesis of this chapter. This experience highlights the true limitation of animal models as simple treatments (ie. a single dose of chemotherapy) can produce complete tumor regression; a circumstance that does

not occur with human tumors. Nevertheless, the results of this chapter revealed important aspects of the responsiveness of the dual-specific T cells and provided important knowledge regarding preferred methods to condition the host prior to adoptive transfer. This new knowledge will be quite valuable as these technologies develop in the clinical setting.

In **Chapter 5**, we investigated loading CAR-T cells with OV based on the hypothesis that engineered T cells could function as delivery vehicles for the virus. Loading engineered T cells with VSV or VV did not influence CAR expression, cytokine production, or killing capacity of either murine or human T cells, which suggests that the loading process itself is relatively inert. We found that not only could CAR-T cells successfully deposit OV onto tumor targets, but that this process was unaffected by CAR-mediated tumor cell recognition. In fact, combining CAR-mediated killing and OV-induced tumor cell destruction showed the potential for complementary killing, enhancing the effectiveness of each therapy used independently. Of particular significance, we determined that the effective MOI of virus loaded onto CAR-T cells was extremely low. This combined with the ability of OVs to be deposited onto and rapidly replicate within tumor targets supports the notion that CAR-T cells represent an effective method for delivering even low doses of virus to tumors. Extrapolating these observations to the clinical setting, loading of OVs onto T cells prior to treatment could significantly reduce the amount of virus required for treatment.

2.0 Biological implications

2.1 Targeting self-antigens with CAR-T cells

For the majority of cancers, a lack of tumor-restricted antigens is an unfortunate reality. As a result, therapeutic strategies employing engineered T cells typically target antigens that are overexpressed or upregulated on tumor cells, but may also show some level of expression on healthy tissues[299]. It is therefore not surprising that the majority of adverse events associated with adoptive T cell therapy have been "on-target, off-tumor" toxicities where the transferred T cells attack cells outside of the tumor that are required for normal physiology[181,186,196,197,201]. While the use of CARs circumvents the endogenous central tolerance mechanisms designed to prevent T cells from recognizing self-antigens, this also removes tolerogenic processes that could protect healthy tissues from CAR-T cell attack. To avoid unexpected toxicities, early CAR antigen selection was driven by pre-existing monoclonal antibodies known to have an acceptable safety profile when used in humans[299]. However, the avidity of surface-expressed CARs is significantly higher than soluble antibody. Thus, a careful analysis of potential toxicities must be considered[299]. For example, HER2-antibodies showed acceptable safety profiles in human studies, yet HER2-CAR-T cells can be very toxic, illustrating the need for careful design of human trials[201,299].

Understanding of target ligand expression patterns on healthy and tumor cells remains a key objective for future CAR (and TCR)-engineered T cell therapies. There are currently mixed reports on the expression of NKG2DL on healthy tissues, where some groups report that these are widely expressed on a multitude of tissues, and others advocate targeting these antigens for cancer immunotherapy due to their lack of high expression on healthy tissues[288,300,302,353]. Our data reveals previously unappreciated toxicity when targeting NKG2DL with CAR-T cells, mediated by inflammation in the lungs and a systemic cytokine storm. Similarly, treatment of renal cell carcinoma patients with CAR-T cells targeting carboxy-anhydrase-IX (CAIX) revealed liver toxicities that resulted in suspension of the trial[354,355]. The toxicity of the CAIX-CAR was attributed to on-target effects on CAIX-positive cells within the bile duct epithelium[354]. Blocking the CAIX antigen in the liver using a monoclonal antibody prevented the liver toxicity and was anticipated to allow for treatment with higher doses of T cells[354]. As initial studies observed no clinical responses to the lower doses of CAIX-CAR-T cells, blocking the toxicity and treating with higher doses of cells may allow for measurable antitumor efficacy[354]. These data stress the importance of understanding antigen expression on healthy tissues when developing CAR-T cells for cancer therapy.

Careful selection of tumor antigens can aid in the prevention of off-tumor serious adverse effects of CAR-T cells. The best-studied example of this are the clinical trials of CD19-reactive CAR-T cell therapy, where the CAR-T cells often

eliminate all B cells, both healthy and malignant[179,181,182,184,186,199,200]. The ablation of healthy B cells requires patients to receive immunoglobulin therapy to protect against infections, which is a manageable toxicity. Further, most of the patients experiencing B cell aplasia have also achieved full, complete remission of their cancer[181,186,200]. Thus, this toxicity is considered to be both manageable and acceptable in the context of the therapeutic outcome. As another example, CARs targeting mesothelin are currently under development[356]. Mesothelin is highly expressed on mesothelioma, pancreatic and ovarian cancers, while it is generally considered to be absent from vital organs[356]. Preliminary data using an anti-mesothelin CAR has reported induction of antitumor immunity in the absence of toxicity, suggesting this CAR to be safe for human use[357]. These data demonstrate that it is possible to select antigens that are overexpressed by the tumor and are also expressed in non-vital tissues, which may provide an acceptable level of off-tumor toxicity while still eliminating cancers.

2.2 Of mice or men?

It is provocative to consider whether the data generated in this thesis, and other murine models, will actually translate to human T cells and human studies. While we observed drastic toxicities using NKG2D-CAR T cells, particularly following CTX treatment, the lack of understanding of NKG2DL expression in humans and mice leads to the question of whether similar results should be expected in human trials. Delivery of syngeneic TCR-engineered T cells to

lymphodepleted mice produced lethal GVHD[194]; however, studies from the National Cancer Institute treating over 100 patients with TCR-transduced T cells after lymphodepleting chemotherapy found no evidence of GVHD in any of the patients treated[358]. A fair comparison of these different observations is complicated not only by the difference in species but also the difference in treatment protocols. For example, the murine study above used a TCR recognizing a completely foreign antigen paired with total body irradiation, whereas the human studies used tumor-targeted TCRs in combination with chemotherapy[194]. Thus, although the mouse experiments highlighted an important potential toxicity, further investigation in humans is required before we can establish whether this toxicity is truly a concern. There have also been circumstances of toxicities in humans that were not predicted by murine studies. For example, the observed toxicity induced through use of the third generation HER2-CAR in a human trial was not predicted in murine xenograft models[201,359]. Likewise, extensive murine studies using TCR-engineered T cells recognizing MAGE-A3 failed to predict the cardiac and neurological toxicities observed in human patients[196,197,360]. In all of these cases, the preclinical murine studies used a host that lacked endogenous expression of the target antigens (human HER-2 and human MAGE proteins), which prevents true testing of on-target/off-tumor toxicity. The consequences of targeting antigens considered to be potential "universal target antigens" have been highlighted in a recent study of targeting fibroblast activation protein (FAP) on tumor stromal fibroblasts[361,362]. FAP has

been reported to be selectively expressed on tumor associated fibroblasts, and as depletion of FAP can abrogate tumor growth, FAP was considered a strong candidate for CAR T cell therapy[361,363]. However, in syngeneic mouse models, FAP-reactive CAR-T cells were found to produce lethal bone marrow toxicities in two different mouse strains[361]. It was further determined that FAP-CAR-T cells could recognize both murine and human bone marrow stromal cells, indicating this toxicity may also be a concern for human trials[361]. These results reinforce the need to employ fully syngeneic systems and receptors for endogenous targets to obtain meaningful toxicity data from preclinical murine models. While the presence of toxicity in murine models may not predict toxicity in human patients, these models are powerful tools for identifying potentially dangerous on-target/off-tumor effects and should be used to inform clinical trial design.

Our data using NKG2D-CAR T cells suggests that the *in vitro* functionality of CAR-T cells is poorly predictive of their *in vivo* activity. There is a current lack of understanding of how best to predict *in vivo* functionality of CAR-T cells prior to ACT. In studies of TCR-engineered T cells, the avidity of the TCR was shown to predict the outcome in murine infectious disease models[364]. In the case of CAR-T cells, the affinity of the scFv was directly correlated to the antitumor activity in xenograft models[365]. In order to successfully predict *in vivo* function of engineered T cells, greater understanding of the relationship between phenotypic and functional profiles will be required.

In the context of loading CAR-T cells with OV, there appear to be limited differences between murine and human T cells. T cells from both species were readily loaded with OV, showed low levels of viral infection of the T cells, and could successfully transfer virus to their target cells. These data suggest that while there may be inherent biological differences between murine and human T cells[366], these differences should not affect their ability to function as virus carriers.

Taken together, murine studies remain a valuable tool for answering biological questions such as the interactions between host, T cell and virus, identifying the potential for toxicity, and for understanding the processes that contribute T cell migration and tumor engraftment following adoptive transfer. However, the ultimate answers with regard to safety and efficacy will only be gained from well-conducted human clinical trials.

3.0 Improving CAR-T cell therapies

To this point, this discussion has focused on the successes and challenges faced by CAR-T cell therapy for cancer. In this section, I would like to discuss future strategies to enhance the therapeutic index through augmenting antitumor activity while reducing toxicities.

3.1 Improving CAR-T cell safety

As described earlier, the greatest toxicities seem to arise from on-target/off-tumor effects where vital tissues are destroyed by CAR-T cells. There have been several different methods described for selectively eliminating adoptively transferred cells in the event of toxicity. Engineering T lymphocytes to express the HSV-TK gene renders the T cells sensitive to ganciclovir treatment[367]. This method has been employed to deplete allogeneic donor T cells following HSCT to prevent GVHD[367]. However, the introduction of the HSV-TK gene has also proven to be immunogenic, resulting in the elimination of the transferred cells in the absence of ganciclovir[368]. Another strategy has engineered T cells to express a truncated variant of EGFR, which could not deliver the EGFR signal but did display the epitope target of cetuximab. Using this method, CAR-T cells expressing the truncated EGFR could be selectively depleted using the commercially available cetuximab antibody[369]. An interesting method that has been recently validated in the clinic involves the expression of an inducible suicide gene consisting of a modified caspase 9 protein (icasp9) that can be dimerized and activated by a synthetic drug, resulting in the rapid death of the engineered T cell[370]. This strategy was validated in pediatric patients receiving donor lymphocyte infusions following HSCT, where patients who developed GVHD were treated with the dimerizing drug and showed over 90% depletion of the engineered T cells within 30 minutes of treatment[370]. In the setting of CAR-T

cell therapy, the icasp9 suicide gene system will be evaluated in an upcoming clinical trial of a third generation anti-GD2-CAR[299].

While the above-mentioned strategies are useful methods to deplete pathogenic CAR-T cells, they do not address the larger problem of on-target/off-tumor reactivity. Several groups have shown that dissociation of CAR signaling domains through targeting two distinct tumor antigens enhances tumor cell specificity[371-373]. The first CAR targets one tumor antigen, and delivers a sub-optimal CD3ζ signal, while the second CAR recognizing a separate tumor antigen delivers the costimulatory signal[371-373]. Thus, full activation of the CAR-T cell only occurs upon recognition of two targets on the tumor cell, reducing the likelihood of off-tumor toxicities due to the expression of single targets on non-tumor tissue [371-373]. A similar concept using a dual-CAR system maintains the original, second-generation CAR, but adds a an "inhibitory CAR", or iCAR, to the engineered cells [374]. The iCAR bears a targeting element specific for a self-antigen that may be co-expressed with the activating CAR target on healthy cells, but would be absent on tumor cells[374]. Upon recognition of both antigens, the T cell would receive activating and costimulatory signals through their normal CAR, and inhibitory signals via either PD-1 or CTLA-4 signaling domains within the iCAR[374]. Ligation of the iCAR significantly reduces the level of activation-induced cytokine production and proliferation triggered by activating CAR signaling[374]. An added advantage to this method is that the iCAR inhibitory functions are both temporary and reversible, allowing the CAR to exert anti-tumor

functions upon stimulation by target cells lacking the iCAR target[374]. However, this concept does suffer from the same limitations as single antigen CAR-targeting, with the requirement for identification of a tumor-excluded antigen to target with the iCAR. Nonetheless, methods for reducing toxicity of CAR-T cells via enhancing tumor selectivity will undoubtedly further the success of CAR-T cell therapy.

3.2 Enhance the long-term proliferation, survival and persistence of CAR-T cells

While the use of lymphodepleting regimes has shown benefit to adoptive transfer therapies, our studies have revealed circumstances where the preconditioning regime can limit the level of T cell stimulation (presumably by affecting APC numbers or function) and exacerbate toxicity. It is interesting to consider other methods of enhancing T cell proliferation and engraftment following ACT that could bypass the need for lymphodepletion. For example, T cells derived from central memory precursors have been shown to engraft and persist at significantly higher levels than those derived from effector memory cells, even in the absence of lymphodepletion[161,375]. Indeed, the differentiation status of the T cells used for ACT correlates with therapeutic outcome in both pre-clinical and clinical studies. The most effective T cells were the least differentiated and maintained the highest proliferative capacity[134,376,377]. Using a defined composition of naïve and memory CD4+ and CD8+ T cells has shown

potent antitumor responses in xenograft models[378]. The use of defined cell proportions and types has progressed to a Phase I/II trial, in which patients receive central-memory-derived CD4+ and CD8+ CAR-T cells specific for CD19[378]. While this trial has only recently opened, preliminary results presented at the 2013 Society for Immunotherapy of Cancer Annual Meeting described complete responses in 3 out 4 patients and a partial response in the fourth patient. As such, the use of specific types of T cells may serve to enhance the overall survival and efficacy of CAR-T cells following ACT.

The use of memory T cells can also facilitate the use of dual-specific T cells as therapeutics. Previous studies using EBV-specific T cells engineered to express a GD2-specific CAR observed selective survival benefits of those CAR-T cells recognizing EBV antigens where the recipient was EBV+ (and thus presumed to have endogenous antigen expression) [323,324]. However this concept has not been paired with the provision of a bolus of virus intended to serve as a booster. Based on my results, it would be expected that combining CAR-engineered memory-derived antigen-specific T cells with an oncolytic vaccine boost should provide a more dramatic effect on T cell proliferation, persistence and antitumor immunity than was observed using effector CAR-T cells in our studies.

Alternatively, CAR-T cells can be modified to include immunostimululatory cytokines to enhance T cell survival and proliferation. CAR-T cells have been modified to express IL-15, which promotes T cell

proliferation as well as reverses the effects of Treg-mediated suppression[299,379]. In comparison to unmodified CD19-CAR-T cells, those expressing IL-15 showed a substantially greater *in vivo* expansion, reduced expression of suppressive markers such as PD-1, and overall improved antitumor efficacy in murine xenograft models[380]. The expression of IL-15 in the engineered cells was controlled by CAR engagement, providing a reduced risk of antigen-independent proliferation, as well as provides the survival and proliferative signals within the tumor environment[380].

3.3 Modulating the tumor microenvironment to enhance CAR-T cell and OV therapy

The tumor microenvironment remains one of the largest hurdles to successful immunotherapy, as it is involved in rapidly shutting down the antitumor immune response. Thus even with potent CAR-T cell activation, it is possible that the tumor will be able to adapt in such a way so as to prevent clearance of the tumor. While our attempts to circumvent this using dual-specific CAR-T cells combined with oncolytic vaccines encountered a number of difficulties, future studies combining CAR-T cells with OV therapy may benefit from additional immunostimulatory elements.

The use of lymphodepleting chemotherapy has been clearly shown to aid in the engraftment and persistence of adoptively transferred T cells by the work described in this thesis as well as by others as discussed in previous sections.

Chemotherapy has also proven effective at enhancing the effectiveness of OV therapy. In particular, CTX pre-treatment resulted in a significant increase in viral replication and oncolysis using a number of different viruses in treating animal models of glioma[381,382]. We observed a similar benefit of CTX on VSV-mediated suppression of B16 melanoma (**Chapter 4, Fig. 8e**). As pre-conditioning lymphodepletion agents such as CTX can enhance the effectiveness of CAR-T cells and OV therapies independently, there is potential for all three to act in concert to achieve antitumor function. This will however depend on dosage and timing evaluations for each of the components. These may be more appropriate to test in the context of OV-loaded CAR-T cells, as the T cells would benefit from the availability of cytokines for growth, and the deposited virus would have the benefit of reduced immunological viral clearance.

Including immunostimulatory cytokines within the CAR vector can also function to modulate the tumor microenvironment. The inclusion of IL-12 in engineered T cells for ACT has been shown to effectively re-program suppressive bone marrow-derived stromal cells within the tumor to stimulate T cell activation[383]. IL-12 encoded within the CAR vector has also been shown to circumvent the requirement of chemotherapeutic pre-treatment in treating murine CD19+ tumors[384]. These cytokine vectors can be designed to be expressed only upon CAR engagement, again restricting production of the cytokine to the local tumor environment[385]. Cytokines could also be encoded within an oncolytic viral vector, which would restrict production to the tumor environment, and activate

both adoptively transferred cells and the host's endogenous immune system. In particular, through activation of the innate immune system through IL-12 release, the combination of CAR-T cells and oncolytic vaccines may benefit through the enhanced antigen presentation of viral-associated antigens as a mechanism to drive boosting of dual-specific T cells. In the absence of a viral-antigen specific TCR, the combination of CAR-T cells loaded with an OV carrying cytokine payloads such as IL-12 would also allow for rapid cytokine production and dispersal throughout the tumor as the virus lyses the target cell and spreads to neighboring tumor cells.

Given the successes in using immunological checkpoint blockade or adoptive transfer of T cells, it is unsurprising that trials are currently investigating combining these two treatments. A current Phase II trial will combine adoptive transfer of antigen-specific CD8+ T cells with anti-CTLA-4 therapy in melanoma patients[129]. In pre-clinical models, the antitumor functionality of CAR-T cells was significantly enhanced when combined with PD-1 blockade[386]. In the context of OV therapy, combining intratumoral OV administration and systemic CTLA-4 blockade was able to induce the regression of both injected and distant tumors, further displaying the success combination therapies may have[387]. It is provocative to consider the potency of therapeutic regimens that will employ CAR-T cells, OVs and checkpoint blockade, a combination that will be inevitably examined clinically once the toxicity profiles of the individual drugs are better understood.

4.0 Concluding Remarks

Cancer immunotherapies have attained significant clinical success in recent years. However, it is clear that the next generation of immunotherapy will require a multi-faceted approach. T cells have proven antitumor activity, which can be exploited through the use of adoptive transfer therapies. Promoting T cell engraftment, proliferation and long-term persistence of transferred T cells is essential for successful T cell-mediated antitumor immunity. As such, devising methods for enhancing these properties will continue to be investigated.

The use of OVs in conjunction with CAR-T cell therapy may provide a unique platform for ACT. Our data has shown that oncolytic vaccines can be used to potently boost CAR-T cell proliferation *in vivo.* In addition, CAR-T cells can be used as effective carriers of OV, allowing for viral deposition and replication within target tumor cells. Future studies should focus on using the combination of OV and CAR-T cells to drive T cell proliferation and persistence following ACT, while also allowing for OV replication within the tumor.

The specific design of CARs used to engineer T cells may impact receptor expression and subsequent functionality. However, choice of target antigen and the relationship between *in vitro* and *in vivo* T cell function remains a priority. Our data revealed that targeting an antigen expressed on vital organs with a highly activating CAR can result in severe toxicity. It is important to remember that highly effective cancer immunotherapeutic approaches will likely have potential

to induce concomitant autoimmunity. Thus, strategies for both predicting and mitigating these on-target, off-tumor effects should be thoroughly studied.

Taken as a whole, the research presented in this thesis has provided us with greater understanding of the interplay between CAR-T cells and OVs, as well as the impact that current standard therapies such as chemotherapy may have when used in conjunction with these immunotherapies. Further understanding of how the functionality of immunotherapies changes when used in combination with other approaches will ultimately inform specific combinations for use in cancer therapy. Overall, advancements in combination therapies have dramatic potential to increase the successfulness of cancer immunotherapy in the clinic.

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