Overcoming Challenges in Adoptive Cell Therapies with Dual-Specific T-cells and Oncolytic Viral Boosting

By

Donald James Bastin, HBSc (Co-op)

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements for the Degree

Master of Science

McMaster University

Hamilton, Ontario

Abstract

The adoptive transfer of cancer-specific T-cells has demonstrated success as a novel treatment strategy in some hematological malignancies but this approach has not yet achieved widespread curative potential in the majority of tumors. To circumvent many of the limitations currently facing adoptive cell therapies, our lab has recently developed a combination therapy involving the *in vivo* boosting of adoptively transferred tumor-specific memory T-cells with an oncolytic viral vaccine. While this represents a demonstrably powerful approach in preclinical models of cancer it is limited by its targeting of a single antigen. Therapeutic resistance is a common concern when targeting a single antigen or pathway and an ideal therapy would include built-in mechanisms to address the heterogeneity and mutability that is inherent to cancer. Thus the focus of this research involved the development of a strategy to target therapeutic resistance in the context of the adoptive cell transfer with oncolytic viral boost regimen.

In order to address the single antigen limitations, the engineering of tumor-specific T-cells with a targeting capacity for a second antigen is described. In addition to their endogenous tumor target it is shown that these cells have specificity for and can kill cells expressing ligands for the natural killer group 2 member D receptor which are commonly upregulated on both cancer cells and components of the tumor microenvironment. Indeed it is demonstrated in an *in vivo* model of relapse that T-cells capable of targeting both antigens produce more consistent and prolonged remissions than those with only their endogenous targeting capacity. Furthermore pharmacological strategies for the enhancement of engineered T cell survival and efficacy are also described. Finally the early development of a chimeric tumor model to further characterize the potential of dual-specific T-cells to address tumor heterogeneity is presented.

Acknowledgements

I would like to take this opportunity to thank my supervisor Dr. Yonghong Wan for his continuous support and guidance over the course of my studies. His willingness to meet and provide input at virtually any time was greatly appreciated. I further appreciated his supportiveness and encouragement both in terms of pursuing research directions that I personally found interesting and with respect to my future career goals. I would also like to thank the Wan lab as a whole. I greatly appreciated all of their input at lab meetings and throughout the course of day to day discussions. In particular Dr. Scott Walsh was instrumental both in terms of teaching me new techniques in the lab and troubleshooting. He was also a great source of motivation for me and the lab as a whole.

Thank you to my other committee members Drs. Martin Stämpfli and Jonathan Bramson for lending their expertise at our meetings and when we ran into each other in the halls. They were both encouraging and friendly and made me feel like they had my back. I would further like to thank Dr. Bramson and his lab for the resources and training with which they provided me. Joni Hammill was especially helpful in lending her experience and time for troubleshooting and greatly contributed to my ability to carry this work out efficiently. The Bramson lab as a whole made me feel welcome and included me in a variety of their extracurricular activities. As an extension of this I also greatly appreciated the friendliness of my fellow graduate students as well as the faculty and staff within the McMaster Immunology Research Center. I am also grateful to the staff in the Central Animal Facility. Specifically, Robin Howe devoted a large amount of time to caring for my mice and was always a friendly face I could look forward to seeing during long days in the CAF.

While I am indebted to all those who worked with me directly during my time at McMaster I am also thankful to those outside of school who supported me over the course of my studies. First and foremost to my parents back home who were always willing to listen and provide optimism when experiments failed and whose willingness to bend over backwards for their family and friends is truly inspirational. To my grandparents, brother and extended family here in southern Ontario for the many Sunday night dinners and afternoon visits which helped to keep me sane during the more stressful times. To all my friends back home and new friends here for making this journey one to remember. Also to my fellow swimmers and everyone in the Golden Horseshoe Aquatic Club for the all the practices that never failed to put a smile on my face.

Lastly I would like to thank Chris LeBrun for all the motivation and coaching he gave me ever since I could walk. Chris was an unwaveringly positive influence throughout my life and was instrumental in my decision to pursue a Masters degree. Throughout his battle with leukemia Chris hung to the mantra of “positive, optimistic, realistic”, words which continue to inspire me in all endeavours I undertake. Remembering that we do this work in hopes of helping people like you reminds me of how lucky I am to have had the opportunity to be part of such research.

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List of Abbreviations

ACK - Ammonium-Chloride-Potassium
ACT – Adoptive Cell Therapy
AICD – Activation Induced Cell Death
ALL – Acute Lymphoblastic Leukemia
APC – Antigen Presenting Cell
AREB – Animal Research Ethics Board
ATP – Adenosine Triphosphate
BCR-ABL – Breakpoint Cluster Region – Abelson Murine Leukemia
BiTE – Bispecific T-cell Engager
CAF – Central Animal Facility
CAsF – Cancer Associated Fibroblast
CAR – Chimeric Antigen Receptor
CD – Cluster of Differentiation
CML – Chronic Myelogenous Leukemia
CRS – Cytokine Release Syndrome
CTLA-4 – Cytotoxicy T-Lymphocyte Associated Protein 4
DAMP – Damage Associated Molecular Pattern
DC – Dendritic Cell
DCT – Dopachrome Tautomerase
DMEM – Dubelco's Modified Eagle Medium
DNAM-1 – DNAX Accessory Molecule 1
FACS – Fluorescence Assisted Cell Sorting
FBS – Fetal Bovine Serum
FDA – Food and Drug Administration
FoxP3 – Forkhead Box P3
hDCT – Human Dopacrhome Tautomerase
HEV – High Endothelial Venule
HLA – Human Leukocyte Antigen
ICS – Intracellular Cytokine Staining
ID - Intradermal
IDO - Indoleamine 2,3-dioxygenase
IFN - Interferon
Il – Interleukin
IP – Intraperitoneal
IV – Intravenous
IVIS – In Vivo Imaging System
LB – Lysogeny Broth
MAGE-A3 – Melanoma Associated Antigen 3
MDSCs – Myeloid – Derived Suppressor Cells
mERK – Mutant ERK
MHC – Major Histocompatibility Complex
MMP – Matrix Metalloproteinase
mTOR – Mammalian Target of Rapamycin
NEAA – Non-Essential Amino Acids

NK – Natural Killer
NKG2D – Natural Killer Group 2 Member D (receptor)
NKG2DL – Natural Killer Group 2 Member D Ligand
NO – Nitric Oxide
NRG - NOD.Cg-Rag1^{tm1Mom} Il2rg^{tm1Wjl}
NSAID – Nonsteroidal Anti-Inflammatory Drug
OV – Oncolytic Virus
OVA - Ovalbumin
OVV – Oncolytic Viral Vaccine
PAMP – Pathogen Associated Molecular Pattern
PBS – Phosphate Buffered Saline
PD-1 – Programmed Cell Death Protein 1
PD-L1 – Programmed Death Ligand 1
PFU – Plaque Forming Unit
P/S – Penicillin / Streptomycin
RAG – Recombination Activating Gene
RPMI – Rosalind Park Memorial Institute Medium
ScFv – Single Chain Variable Fragment
sf – Serum Free
TA – Tumor Antigen (encompasses TSAs and TAAs)
TAA – Tumor Associated Antigen
T_{CM} – Central Memory T-cell
TCR – T-cell Receptor
T_{Eff} – Effector T-cell
TGF-β – Transforming Growth Factor Beta
TIL – Tumor Infiltrating Lymphocyte
TME – Tumor Microenvironment
TNF-α - Tumor Necrosis Factor Alpha
Treg – Regulatory T-cell
TSA – Tumor Specific Antigen
VEGF – Vascular Endothelial Growth Factor
VSV – Vesicular Stomatitis Virus

Chapter 1

Introduction

1. Introduction

1.1 Cancer

1.1.1 Cancer Biology

Cancer represents a diverse collection of diseases which share the common defining characteristic of dysregulated cell proliferation and persistence[1,2]. The health of a multicellular organism relies on the cooperation of its constituent cell populations and tight control over their replication and lifespans[3]. Thus when individual cells acquire mutations which enable them to successfully expand and survive outside the confines normally allotted to them, their outgrowth is destructive to the organism[1]. Broadly speaking it is this phenomenon which underlies cancers and collectively these maladies represent a major cause of death worldwide. In 2016, roughly 202,400 Canadians developed and 78,800 died from cancer and it remains a significant burden to healthcare systems in Canada and globally[4].

While the past century has seen unprecedented advances in both the treatment and molecular understanding of this disease, cancer remains a major cause of morbidity and mortality. The difficulty in treating this illness lies in the fact that is, in reality, a heterogeneous collection of diseases rather than a single entity[5]. Indeed there is now abundant evidence that cancer cells in general possess a "mutator phenotype"[6,7]. That is to say that cancers are typified by loss of regulatory safeguards on DNA replication. Such failure of genomic maintenance machinery allows for drastically accelerated mutations and endows the cancer cells an adaptive advantage in the face of Darwinian selection with their non-malignant counterparts[7,8]. Thus patients diagnosed with the same type of cancer can in reality harbour diseases with hugely different mutational landscapes and characteristics[9]. This increased mutability also underlies the development of drug

resistance as malignant cells that can rapidly mutate are able to adapt and overcome the selective pressures imposed by a variety of therapies[5,10,11].

Cancers display a further level of complexity with respect to the microenvironment in which they exist. Indeed it is now well established that rather than subsisting in isolation, cancers engage and reprogram "normal" cells in their surroundings to become actively tumor promoting[12]. These corrupted cells become instrumental to the progression of disease and a variety of the hallmark behaviours of cancer as a whole[12]. For example, through the release of TGF-β and other signalling molecules, cancer cells reprogram resting normal fibroblasts into activated cancer associated fibroblasts (CAsFs)[13,14]. CAsFs then respond with signals which increase cancer cell proliferation, metastasis and therapeutic resistance[15]. Other cell types including immune cells and cells of the endothelium have similarly been shown to, under the influence of malignant cells, become drivers of multiple aspects of tumor progression. For this reason, tumors have been depicted as organs in which multiple cell types interact and cooperate to form a larger, more complex structure[12]. Thus, addressing the tumor microenvironment represents an additional challenge to developing cancer therapies.

1.1.2 Standard of Care Therapies

The first true treatments for neoplastic disease were surgical. Records dating as far back as 2500 B.C. contain what appears to be the earliest description of the disease but suggest that treatments beyond palliating to be futile[16,17]. While surgical strategies were practiced to varying degrees following and possibly prior to this time, the advent of anesthesia and antisepsis in 1846 and 1867 respectively revolutionized the practice. This led to an explosion of increasingly aggressive "radical" surgeries which sought to purge patients of disseminated disease[16,18].

Although surgery provided an invaluable tool for the treatment of localized tumors, subsequent work has shown that the radical surgeries that followed provided little benefit compared to more conservative localized tumor removal. Distantly metastasized disease would be extremely difficult to eradicate by surgery alone, regardless of how aggressive the operation and therefore additional strategies were needed[16,18,19].

The discovery of X-rays by Roentgen in 1895 and radiation by Marie and Pierre Currie in 1898 provided a novel form of treatment for neoplastic diseases which was met with considerable excitement[16,20]. Because of the cytopathic effect of these forms of energy on cells, particularly rapidly dividing cells, they were quickly assimilated as cancer therapeutics[16,20,21]. Strategies were rapidly developed to increase their energy and target deeper tissues as well as better localize their effects to the tumor. While radiotherapy remains an invaluable tool in the oncologists' arsenal it, like surgery, struggles with addressing metastasis[16,20,21]. For this reason, a systemic strategy was sought[16].

The advent of chemotherapy came about through several lines of inquiry. Inspired by the growth promoting effect of folic acid in childhood leukemia Farber e*t al.,* in 1948, reported the use of folic acid analogues to produce transient remissions in this disease[22]. At the time such a finding was unprecedented as there was essentially no treatment options for such cases[16]. Also in the 1940s, results were published using nitrogen mustards in cancers of the blood based on observations of their efficacy in depleting the hematological system made during the second world war[23,24]. This together with the success of antibiotics in treating bacterial infections and the development of improved mouse models spawned an ongoing search for chemical compounds with anticancer activity[24].

The three aforementioned strategies of surgery, radiation and chemotherapy have become known as the traditional "pillars" of oncology and represent a large part of the standard of care for cancer today[25]. Standard of care frequently relies on combinations of such strategies to achieve the optimal results. For example, the use of localized therapies (surgical or radiological) to debulk the primary tumor alongside adjuvant therapies (such as chemotherapy) which target micrometastasis have drastically improved the long term survival in many forms of breast cancer[26]. Recent advances in the molecular understanding of cancer have allowed for the development of "targeted" or "precision" therapies which target pathways specifically altered in malignancies. The goal of such strategies is to achieve selective killing of malignant cells and thereby reduce systemic toxicities associated with traditional chemotherapy. This has been called a fourth pillar of oncology[25]. One of the first such therapies targeted the ATP-binding site of the protein generated from the BCR-ABL fusion known as the Philadelphia Chromosome in patients with Chronic Myelogenous Leukemia (CML)[24,27,28]. As genomic and molecular technology rapidly progresses, the drive to characterize a patients' cancer is becoming a reality allowing for the selection of the optimal treatment tailored to that specific tumor. Thus, "personalized medicine" as it is known is increasingly allowing even greater levels of precision of therapy on an individual basis[29].

Despite incredible progress in the treatment and molecular understanding of cancer, particularly over the past century, this disease has remained a major cause of death and suffering. While the standard of care has drastically evolved, cancer therapies by and large remain highly toxic to healthy cells as well as their cancerous targets. While targeted therapies have been able to mitigate some of these effects, cancer by its very nature is able to mutate and adapt to a wide variety of selective pressures. Thus in addition to further delving in to the biological

underpinnings of this disease and further expansion on current therapies, new strategies are also needed. As such, the field of biological therapies is rapidly evolving. In particular the past decade has seen a revolution in the domain of immunotherapies which are poised to become the fifth pillar of oncology[25]. Thus the remainder of work presented in this report will focus on the development and improvement of immunotherapeutic strategies as a novel means of addressing the challenges presented by neoplastic disease.

1.2 The Immune Response to Cancer

1.2.1 Cancer and the Immune System

The relationship between cancer and the immune system is complex and highly context dependent. On one hand it is now widely accepted that the immune system has the capacity to identify and eliminate pre-neoplastic and malignant cells and thereby offer protection against tumors[30,31]. Conversely, infiltrating immune cells are also recognized as important and sometimes instrumental players in the tumor microenvironment and can facilitate a variety of aspects of neoplastic progression[12,32,33]. To reconcile these opposing views of the interplay between the immune system and a growing tumor consideration of context is essential. In simplified terms chronic inflammation will play a role biased towards tumor progression through mechanisms such a tissue remodeling, provision of growth factors and inhibition of anti-tumor immune responses. An acute, typically adaptive, response on the other hand can have a tumoricidal role through direct and cytokine mediated cytotoxicity[32,33].

1.2.2 Tumor Promoting Roles of Infiltrating Immune Cells

Epidemiological studies have shown that individuals with chronic inflammatory disorders display higher rates of cancer and that long term use of non-steroidal anti-inflammatory drugs

(NSAIDs) reduces this risk[34,35]. This is illustrative of the capacity of immune cells to promote tumor development. The mechanisms by which chronic inflammation contributes to carcinogenesis can be thought of as aberrations of processes employed in wound healing. Following an acute response, the immune system employs a number of mechanisms to achieve tissue repair. These processes are normally regulated in a temporal fashion however their continued activation can result in conditions favourable to malignancy[8]. For example, matrix metalloproteinases (MMPs) derived from myeloid cells have been implicated in vascularization of incipient tumors. In particular, macrophages in the tumor microenvironment have been shown to release MMP-9 which contributes to angiogenesis through the liberation of vascular endothelial growth factor (VEGF)[32,36,37]. Proinflammatory cytokines including IL-6 and TNF-α have been shown to have a plethora of roles in cancer development from delivering survival signals to cancer cells to the promotion of metastasis[32,38–40]. The contributions of immune cells in the context of chronic inflammation to cancer development and progression have been reviewed in great depth elsewhere[12,32,41].

In addition to the direct growth-promoting roles of immune cells on the tumor and its microenvironment, regulatory immune cells play an important role in suppressing anti-tumor immunity both locally and systemically. Myeloid derived suppressor cells (MDSCs) and regulatory T-cells (T_{Reg}s) are key players in this domain[42]. MDSCs represent a diverse population of typically poorly differentiated cells of myeloid origin defined by their ability to impair immune responses[43,44]. This phenotype is frequently the result of signalling programs initiated by the tumor[42,44]. MDSCs carry out their suppressive functions through direct and indirect mechanisms including the production of T-cell antagonizing compounds such as nitric oxide (NO), depletion of nutrients critical to T-cell function and expression of T-cell inhibitor

receptors such as programmed death-1 (PD-1)[44–47]. MDSC signalling[48] and a plethora of other mechanisms[49] can also play a role in the induction of Tregs. These are immunosuppressive CD4+ T-cells defined by the expression of the forkhead box P3 (FoxP3) transcription factor. Tregs exert their immunosuppressive effects through a variety of mechanisms including consumption of interlukin-2 (Il-2), antagonizing of antigen presenting cells (APCs) and production of immunosuppressive cytokines[50]. Immunosuppression in cancer patients can often extend beyond the tumor to achieve a systemic impairment of immune response[51].

1.2.3 The Immune Response Against Cancer

While aspects of the immune system can be usurped to foster cancer, a variety of cancer-protective mechanisms of the immune system have been observed. Indeed cancer cells can express mutant antigens and a variety of stress signals which distinguish them from healthy self to cells of the innate and adaptive immune system[52,53]. While targets of innate cells such as ligands for the natural killer group 2 member D (NKG2D) receptor[54] and DNAM-1[55] have been shown to be upregulated on various tumors[53], the role of CD8+ T-cell recognition of mutant antigens has typically been emphasized[56].

CD8+ T-cells mediate direct killing of cells expressing foreign epitopes presented on major histocompatibility complex (MHC) class I molecules. Recognition of peptide-MHC complexes occurs through the T-cell receptor (TCR). TCR gene rearrangement allows the adaptive immune system to generate T-cells with the capacity to recognize a virtually limitless array of peptides[57]. With such diversity it is not surprising that T-cells recognizing tumor antigens (TAs) can readily be produced. Following recognition, CD8+ T-cells possess a variety of mechanisms to elicit cytotoxicity against tumor or virally infected cells. These include direct killing pathways such as

those mediated by Fas/FasL interaction[58], cytotoxic molecules such as perforin/granzyme[59] as well as cytokine-based strategies including secretion of interferon (IFN)-γ which counteracts angiogenesis[60].

To generate a CD8+ T-cell response against cancer professional antigen presenting cells (APCs), typically dendritic cells (DCs), must phagocytose a TA. With the appropriate maturation signals these DCs will carry the antigen to secondary lymphoid organs where they will perform cross presentation in the context of costimulatory signals to activate naïve CD8+ T-cells. In the optimal scenario activated T-cells will give rise to both cells with an effector phenotype, which will traffic to the tumor and mediate killing, as well as memory T-cells, which maintain long term protection[30]. This process is however, susceptible to inhibition by the growing tumor[30].

There exist a variety of potential categories of TAs. Neoantigens are the result of mutations in the neoplasm and represent cancer specific targets. Tissue differentiation antigens are expressed on the tumor as well as its tissue of origin although possibly at different levels. Cancer/testis antigens are peptides normally expressed only by the testis or germline tissues that tumors also activate expression of. Additionally cancers of viral origin may retain expression of a targetable viral antigen[61]. Traditionally neoantigens and viral proteins have been termed tumor specific antigens (TSAs) as their expression should be restricted to malignant cells while other groups of antigens that are differentially expressed on tumor tissues are referred to as tumor associated antigens (TAAs). Since neoantigens are derived from self while TAAs are self-antigens, T-cells responding to these epitopes are susceptible to mechanisms of central and peripheral tolerance which are designed to limit autoreactive cells so as to prevent autoimmunity[62]. Though this presents a challenge in mounting a T-cell response against cancer

cells, anti-tumor CD8+ T-cell responses have been documented both naturally[52] and following traditional therapeutic strategies such as chemotherapy[63].

1.2.4 Cancer Immunoediting

Given the capacity of the immune system to recognize and destroy malignant cells, the fact that cancer even develops may be seen as somewhat perplexing. Indeed Burnet and Thomas proposed as early as the 1950s that a primary function of the immune system involves protection from neoplasms which was later formalized into the concept of cancer immunosurveillance[64,65]. Nevertheless, cancer does indeed develop. Thus the theory of cancer immunoediting was subsequently established in order to explain the occurrence of cancer in a relatively immunocompetent host[31,66]. In its current version this theory holds that the development of cancer occurs in three stages. Initially, incipient malignant cells are recognized and eradicated (elimination stage). However in a case that will eventually develop to malignancy, the immune system and cancer eventually reach a balance stage known as equilibrium. In this phase the cancer does not grow out but also is not eliminated by the immune system. At this point editing occurs in which the selective pressure of the immune system shapes a tumor with reduced immunogenicity that is able to evade or suppress an immune response. This allows for the escape phase wherein the balance is tipped in favour of the tumor which results in clinical disease[31,65,67].

Compelling evidence is gradually mounting for each of these stages individually and as a whole (reviewed in 31), an in depth discussion of which is beyond the scope of this dissertation. This model however has provided a crucial understanding of what an established neoplasm has accomplished from an immunological standpoint. That is to say that clinical disease occurs when

a tumor has become “invisible” to the immune system, has sufficiently dampened an anti-tumor response or achieved some combination of these mechanisms[31,68].

Tumors can become hidden from the adaptive immune system by a variety of strategies. Loss of MHC (human leukocyte antigen, HLA in humans) has been recognised[69] as well as defects in upstream components critical to antigen processing and presentation[70]. Elegant studies have also implied that highly immunogenic epitopes in nascent tumors are lost during immunoediting[56] Furthermore tumor cells can become unresponsive to factors such as IFN-γ[68,71]. In addition to the immunosuppressive strategies orchestrated by MDSCs and Tregs detailed above, tumors can exert a multitude of immunosuppressive signals. Ligands for PD-1 have been observed on a variety of primary human cancer isolates, allowing for the outgrowth of more immunogenic neoplasms[72]. Expression of a variety of other T-cell inhibitory mediators including indoleamin 2,3-dioxygenase (IDO)[73] and galectin-1[74] by tumors has also been described. Tumor-mediated immunosuppression is not limited to mechanisms directed at inhibiting T-cell responses. For example, tumors have been shown to interfere with the activity of NK cells to ensure their own survival[75]. Thus an established tumor typically represents a situation in which the balance between tumor and immune system has been skewed to favour the tumor[31,65,67].

1.3 Cancer Immunotherapy

1.3.1 The Fifth Pillar

As the field of cancer immunology has progressed, opportunities to employ this knowledge in developing novel therapeutic regimens have become a reality. Broadly speaking cancer immunotherapy seeks to employ the immune system as a means of providing long term, specific protection against neoplastic disease[76]. This encompasses a variety of strategies which aim to

take advantage of the immune system's capacity to recognize and destroy cancerous cells. These strategies then seek to reverse evasive or suppressive mechanisms established by the tumor in order to tip the balance from tumor escape to elimination by the immune system[77]. With early success in clinical trials and a variety of approaches on the rise, these strategies have been called a new pillar in the treatment of cancer[25,78].

The first FDA approved agents aimed at stimulating the immune system for the treatment of cancer fall under the class of checkpoint inhibitors. These represent drugs which target negative regulatory pathways such as PD-1 which under normal circumstances prevent an over exuberant immune response and limit autoimmunity[79]. The first such pathway to be targeted was signalling though cytotoxic T-lymphocyte associated protein 4 (CTLA-4). CTLA-4 is upregulated on T-cells following activation and competes with greater affinity than CD28 for the costimulatory molecule B7 during antigen presentation but transmits an inhibitory signal to the T-cell in order to limit proliferation[80]. Because of its negative role in regulating immune responses, blocking of CTLA-4 using antibodies was investigated as a therapeutic strategy in murine models of cancer. These pioneering works showed early success but also demonstrated the induction of autoimmunity in the form of depigmentation in the skin of mice treated in melanoma models[81,82]. Nevertheless clinical trials were subsequently conducted and demonstrated extensions in survival with accompanying autoimmunity in a clinical setting[83,84]. These successes subsequently fueled research into blockade of PD-1 and its ligand, PD-L1 which also yielded promising results[79]. FDA approvals for drugs targeting both checkpoints were initially obtained for melanoma[79] with approvals for additional indications continuing[85].

Building on the success of checkpoint blockade, there has been an explosion of other strategies aimed at eliciting a therapeutic immune response against cancer. Vaccination strategies

including peptide, nucleic acid, viral or other delivery vectors, dendritic cell and even lysed tumor cell vaccines are at varying stages of investiagion[86]. Considerable success has been observed in clinical trials seeking to bypass the need for vaccination by adoptively transferring T-cells which are naturally or have been genetically modified for tumor specificity[87]. Using immunostimulatory drugs as a means of enhancing the success of conventional strategies such as radiotherapy represents another strategy which is garnering interest[88]. Thus a further strength of immunotherapeutic approaches are their potential to synergize both with each other and with traditional therapies[89,90].

1.3.2 Adoptive Cell Transfer

Adoptive cell transfer or adoptive cell therapy (ACT) describes a number of different approaches which employ the administration of *ex-vivo* cultured tumor-specific CD8+ T-cells to mediate therapeutic tumor regression. The idea that T-cells have the potential to destroy a tumor but are typically impaired in the context of neoplastic disease represents the underlying rationale for ACT. Thus by expanding and educating the T-cells *ex-vivo* these protocols seek to overcome such negative regulatory mechanisms[91–93].

Adoptive cell therapies differ both in the source of tumor-specific T-cells and the culturing protocols used to grow them. Initially, ACT typically employed cultured tumor infiltrating lymphocytes (TILs). In these protocols, the tumor itself would serve as a source of cancer-specific T-cells. Such strategies offer the advantage of not requiring knowledge of the actual target antigen but are limited by the technical challenge and cost of growing large numbers of tumor-specific T-cells on a patient by patient basis[94]. To overcome these challenges, strategies to genetically modify bulk T-cells were developed. These protocols can employ transgenic TCRs or

chimeric antigen receptors (CARs, discussed in a subsequent section) which allow for the re-targeting of T-cells to a TA selected by the physician. Although this allows for a much larger starting pool of tumor-specific T-cells it requires the identification of antigens shared by a large proportion of tumors which is not a trivial endeavour[95]. Another approach to ACT involves the enrichment of tumor specific T-cells from the peripheral blood of a patient. In these protocols, cancer-reactive T-cells are isolated from blood products by means such as artificial antigen presenting cells. A significant challenge in these protocols is also the low starting frequency of tumor-specific T-cells[91,96].

Another consideration in carrying out ACT protocols involves the means by which cells are cultured prior to transfer. Traditional protocols employed Il-2 to drive T-cell proliferation and differentiation, typically resulting in T-cells with an effector phenotype. While these cells are highly cytolytic, their persistence is limited by terminal differentiation. To this end a large body of evidence now supports the use of cytokine cocktails to elicit a less differentiated memory or naïve phenotype[97–100]. Cells cultured in this way can be defined by improved persistence. However these protocols are subject to their own challenges as it becomes difficult to culture sufficiently large numbers of T-cells for ACT while simultaneously limiting their differentiation[97].

Clinical evidence to this point has demonstrated the potentially curative potential of ACT in hematological malignancies[101]. Indeed in one study transfer of CD8+ T-cells targeting CD19 was successful in achieving complete regression in 90% of patients with B-cell derived acute lymphoblastic leukemia (ALL) who were refractory to other treatments[102]. While impressive results have been obtained in other trials treating liquid tumors, solid tumors have by in large remained a challenge for a number of reasons[103]. These difficulties are not necessarily unique to

solid malignancies but can be accentuated in these circumstances. Indeed the persistence of transferred cells and culturing of high enough number of cells represents a significant limitation. Furthermore heterogeneity within the tumor and its microenvironment present a challenge both from the standpoint of achieving sufficient T-cell infiltration and in targeting a population of cancer cells that can be antigenically diverse[103,104]. The induction of autoimmune disease as well as the development of immune pathology have also limited many trials[105,106].

ACT and immunotherapies in general have demonstrated several distinct types of toxicities[105,106]. Autoimmunity can be induced both through off target cross reactivity of transferred cells as well as by on target-off tumor targeting of TAAs expressed on non-tumor tissues. For example in a trial employing T-cells bearing an affinity enhanced TCR targeting the cancer-testis antigen MAGE-A3, patients succumbed to cardiogenic shock resulting from unpredicted recognition of the protein titin on cardiomyocytes[107]. Conversely targeting of melanocyte antigens identified an *in vivo* screen for the treatment of melanoma resulted in killing of melanocytes in the skin, eye and ear in a separate trial[108]. Furthermore, toxicities resulting from the sheer magnitude of the immune response achieved in such protocols have also been observed. Cytokine release syndrome (CRS) as it is known describes a situation in which the plethora of cytokines produced from highly activated transferred cells can cause a variety of systemic toxicities such as fever, hypotension and vasodilation to name a few. The range of effects is highly variable and likely not attributable to any single cytokine but the situation can, in some cases, be fatal[106].

It is becoming evident that while ACT has shown tremendous promise there remains a multitude of issues to address if it were to become a frontline therapy for a variety of malignancies. It is possible that the combination of ACT with other immunotherapeutic

strategies may prove useful in compensating for the limitations of either therapy individually[90,109]. Thus the focus of this dissertation will involve the use of oncolytic viral vaccines to modulate adoptively transferred cells *in vivo* as a means of synergistically enhancing the efficacy of either therapy.

1.3.3 Oncolytic Viruses

Oncolytic viruses (OVs) represent a novel form of cancer therapy, the immunotherapeutic applications of which have only recently taken center stage[90,110]. Broadly speaking, OVs are infectious agents with a preferential tropism for tumors or cancer cells. While many viruses naturally exhibit a bias for neoplasia this has been further accentuated in recent decades through genetic engineering[111]. This natural or designed selectivity can be rationalized by comparing the environment established by malignancies to that which is considered optimal for a viral infection. Indeed tumors provide an environment rich in building blocks and susceptible cells, typically with some level of immune-dampening and cellular resistance to apoptosis which is conducive to viral infection[112,113].

Early clinical trials with OVs demonstrated that the cancer-selective lytic ability was not sufficient to mediate complete cures[114]. However the anti-tumor immune response that was elicited represented a powerful means of achieving some levels of tumor destruction[115,116]. This immune response is attributable to a variety of factors, the relative importance of which will depend on the specific virus in question. In general, because of their pathogenic nature OVs will provide the immune system with pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) in the context of a tumor[117]. This can induce DC maturation which promotes the priming of an adaptive immune response against antigens taken

up in the tumor microenvironment[118]. Furthermore detection of OVs in the tumor microenvironment can redefine the inflammatory milieu within the tumor from one that is suppressive to one that is immunostimulatory through a variety of signals[119,120]. Indeed the cytokines and chemokines produced upon OV infection have been shown to recruit a variety of immune cells to the tumor[120–122]. Additionally, tumor infection and lysis can promote the cross presentation of tumor antigens thus enhancing the breadth of immune response which can be mounted against the tumor[123,124]. Collectively these effects have been described as *in situ* vaccination in that they can orchestrate an adaptive immune response against a tumor[113].

1.3.4 Oncolytic Viral Vaccines and The Prime Boost

Following recognition of the importance of the OV-mediated antitumor immune response, a variety of strategies to enhance this activity were subsequently pursued[90,113]. In order to ensure an anti-tumor response and direct it against a defined tumor antigen, one strategy involved encoding this TA into the oncolytic viral backbone. This novel agent, termed an oncolytic viral vaccine (OVV), has indeed been shown to be capable of inducing such a response[125]. A drawback to such an approach however is that while a TA is a self or self-derived antigen, antigens in the virus are foreign and thus the anti-viral response typically eclipses the anti-tumor response when vaccinating with a single OVV[126].

In order to circumvent this biological limitation and achieve a comparatively more intense anti-tumor response, a heterologous prime-boost response has been developped[127–129]. In this strategy, two distinct viruses encoding a common TA are employed sequentially. A priming vector is initially given to prime an antitumor immune response. Following a variable time period (4-12 days depending on the protocol employed), a boosting OVV encoding the same TA

is given. Since the anti-TA immune response has already been primed the response elicited to the TA upon administration of the boosting vector is a secondary response. However since this is the first time the immune system is exposed to the boosting vector, the antiviral response is a primary one. Since a secondary response will almost invariably eclipse a primary response the relative potency of the antiviral and anti-tumor immune responses is shifted in favor of an anti-TA response[90,127–129].

While it should be possible to employ a variety of OVV vectors as boosting agents in this protocol, the rhabdoviruses vesicular stomatitis virus (VSV) and Maraba virus have demonstrated unique attributes making them particularly amenable to this protocol. These rhabdoviral vectors achieve substantially greater immune responses when boosting is performed intravenously as opposed to other routes including intranasal and intramuscular administration[129]. This along with the observation that boosting could be carried out with surprisingly short intervals between prime and boost[129] prompted mechanistic studies into the unique biology underlying these viruses as boosting vectors. With many vectors, boosting at 4 days following priming would result in detriment to the efficacy of the boost as effector T-cells (T_{Eff}) generated during the priming phase would recognize and kill migratory APCs infected with OVV based on their presentation of the virally-encoded TA[90,130]. Interestingly, VSV and Maraba virus have been shown to display a unique tropism for B-cells located in the follicular region of the spleen. These B-cells are then capable of acting as a continued source of antigen for DCs in the follicle to present to memory T-cells (T_{CM}), thus driving an immune response. Since antigen presentation occurs primarily in the follicles, into which T_{Eff} cannot traffic, this process is protected from feedback inhibition and rapid boosting of large magnitude is achievable[90,131]. Building on our group's experience with rhabdoviruses in the prime-boost protocol we have

explored their potential as boosting agents for adoptively transferred T-cells as a means of combining the benefits of both of these approaches.

1.4 Adoptive Cell Transfer with Oncolytic Viral Boosting

1.4.1 ACT with OVV: A Variation on the Prime-Boost Strategy

The current direction being explored in our lab involves the use of OVVs to boost adoptively transferred tumor-specific T-cells *in vivo*. The boosting capacity of OVVs extends beyond viral vaccine primed TA-specific T-cells as evidenced by studies employing OVVs as boosting agents for DNA vaccines[90,132]. Following this rationale, work previously done in our lab reasoned that an OVV-specific for a TA targeted by adoptively transferred cells of a memory phenotype would efficiently boost these cells *in vivo* (see **figure 1A**)[90]. From a conceptual standpoint this approach offers a number of advantages over either therapy alone. Firstly because the cells used are defined by a memory phenotype they should be able to persist long term in a patient treated with such therapy and should also be ideal substrates for boosting. This would overcome any variability in the efficiencies of priming achieved in the traditional prime-boost protocols. Due to the fact that the bulk of expansion occurs *in vivo* as a result of OVV-boosting a smaller number of T-cells could be adoptively transferred initially, reducing challenges surrounding culturing of sufficient cells. Furthermore, any infection of the tumor by the OVV could facilitate the recruitment and infiltration of boosted cells into malignant tissue. By administering cells of a less activated memory phenotype and activating these cells through vaccination, initial toxicities associated with the administration of highly activated effectors may be reduced.

1.4.2 Antigen Negative Relapse in ACT+OVV

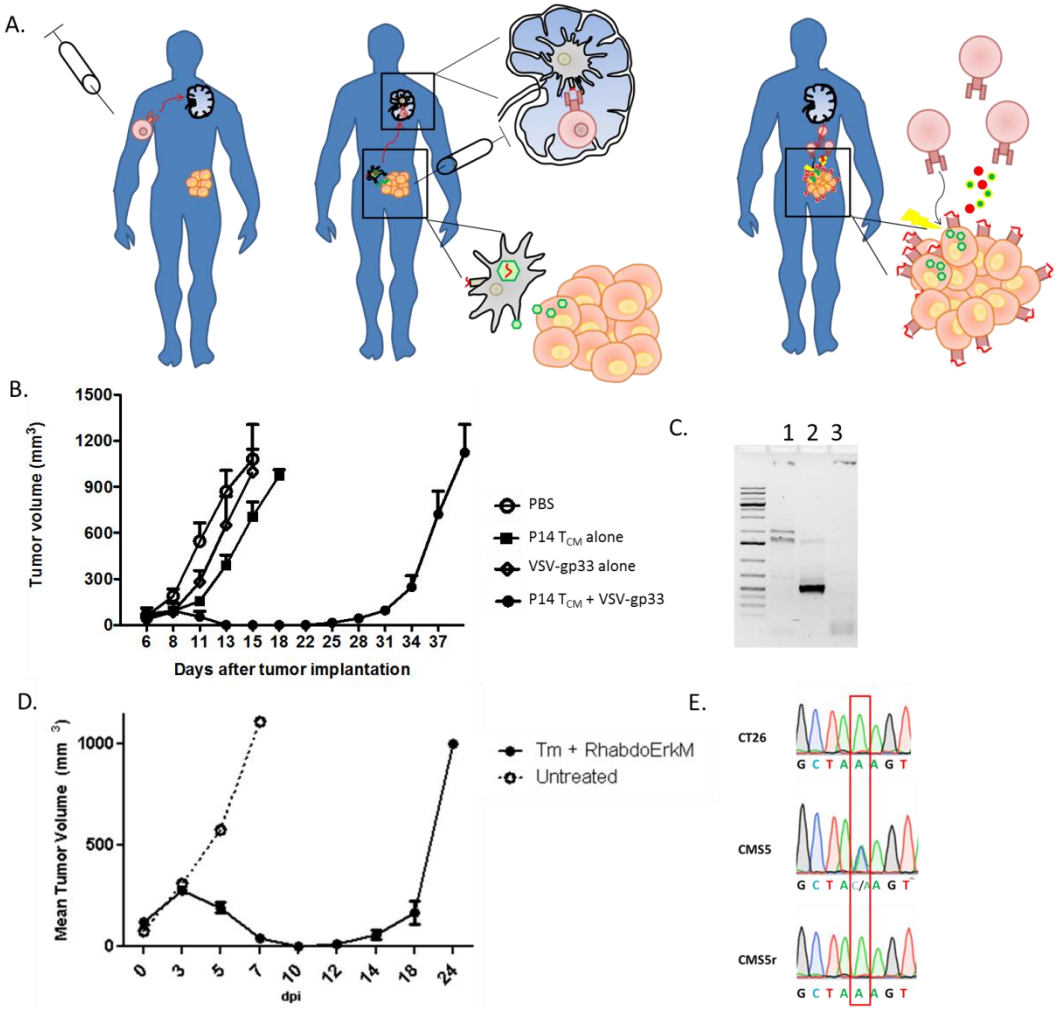

Figure 1. Illustration of the adoptive cell transfer (ACT) with oncolytic viral vaccine (OVV) boosting protocol and preliminary data showing initial tumor regression followed by antigen negative relapse. A. In the ACT + OVV protocol, tumor-specific T-cells defined by a memory-like phenotype are initially administered to the patient and home to secondary lymphoid organs (a). Subsequently an OVV expressing the target of the T-cells is administered and causes presentation of the cognate antigen to the adoptively transferred cells (b). Antigen presentation drives proliferation and generation of effector T-cells which, aided by oncolytic infection of the neoplasm, traffic to the tumor and mediate cytotoxic effect (c). **B.** C57Bl/6 mice bearing B16 melanoma tumors expressing gp33 were treated with VSVgp33, P14 memory-like T-cells, a combination of P14 cells followed by VSVgp33 or PBS control. T-cells were given seven days post tumor implantation while virus was given on day 8.Tumor volumes were recorded throughout.(Tumor D0, T-cells D7, Virus D8) **C.** PCR using primers for the gp33 transgene and genomic DNA from (1) parental B16 cells which do not express gp33, cultured B16gp33 cells or cells isolated from relapsed B16gp33 tumors. **D.** NRG mice bearing CMS-5 tumors established for one week were treated with memory cultured DUC18 cells followed by MRB expressing mERK the following day (Tumor D -7, T-cells D -1, Virus D0). Control groups were left untreated and tumor volumes were recorded until endpoint. **E.** Sequencing of the ERK gene in the CT26 cell line (which does not carry a mutant ERK), the CMS-5 cell line (which heterozygously expresses a point mutation in ERK) and cells isolated from relapsing CMS-5 tumors in NSG mice. Panels B and C were based on work performed by Andrew Nguyen. Panel D was based on work carried out by Lan Chen and panel E was obtained by Scott Walsh.

One concern with therapies targeting a single antigen is the escape of malignant cell which lose or never express the target antigen as a result of the heterogeneity and mutability of cancer[133]. Indeed this phenomenon has been observed both in preclinical and clinical ACT protocols and results in relapse of disease that was ostensibly under control[134–136]. We have observed this phenomenon in various models employing our ACT + OVV approach. P14 mice represent a transgenic strain on a C57Bl/6 background with T-cells showing exclusive specificity for the LCMV gp33 epitope (gp33). In wild type C57Bl/6 mice bearing B16F10 melanoma tumors expressing gp33 treated with P14 cells cultured for a memory phenotype and boosted with VSV encoding gp33, transient tumor control is observed prior to aggressive relapse (**figure 1B**). Screening for the gp33 gene by PCR demonstrates that while parental tumors express this epitope, relapsing tumors do not (**figure 1C**) implying antigen negative escape. In a different model, cells of the CMS-5 fibrosarcoma line (BALB/c background) heterozygously express a mutant ERK peptide (mERK) which serves as a target for transgenic T-cells from DUC18 mice. When these tumors are established in immunodeficient NRG mice, initial regression is also observed and this is followed by relapse (**figure 1D**). Sequencing of tumors isolated from relapsing mice reveals that like the CT26 cell line (which does not express mERK) but unlike the CMS-5 parental tumors, CMS-5 cells isolated from relapsing tumors do not express the mERK peptide (**figure 1E**). Thus the combination of ACT with a boosting OVV provides an attractive therapeutic platform. However, this therapy is limited in its targeting of a single antigen and achieving complete cures with such a protocol may require built in mechanisms to address the heterogeneity and adaptability inherent to cancer, especially in clinical tumors.

1.5 Dual Specific T-cells in ACT + OVV Combination

1.5.1 Dual-Specific T-cells as a Strategy to Overcome Antigen Negative Relapse

A variety of strategies have been explored to mitigate antigen-negative escape in adoptive cell therapies. In a preclinical study targeting chicken ovalbumin (OVA)-expressing B16F10 melanoma tumors (B16-OVA), cotransfer of OT-1 T-cells specific for OVA alongside Pmel cells which target the B16F10 antigen gp100 was superior than either cell type administered as a monotherapy[137]. Another group showed that T-cells engineered to express CARs targeting two antigens mediated superior effects than targeting either single antigen in a glioblastoma model[138]. Indeed targeting multiple antigens has been suggested as a means of mitigating antigen loss escape observed in trials with CD19 CARs for B-cell leukemias[139].

In the context of the ACT + OVV approach developed in our group targeting a second antigen is desirable as a strategy to circumvent relapse or deal with antigenically heterogeneous tumors. In order to take advantage of the boosting and intratumoral trafficking previously demonstrated in our regimen, we proposed the genetic engineering of the transferred tumor cells to target a second antigen rather than administering a second cell population. Thus the generation and application of T-cells with a native and engineered specificity for distinct TAs in the context of our ACT + OVV protocol is described herein. These cells are functionally dual-specific as they display targeting capacity for two antigens. While previous works have employed dual-specific T-cells in order to achieve boosting of tumor-specific T-cells in response to vaccination or latent viral infection, these cells were not specific for two TAs[140,141].

1.5.2 Chimeric Antigen Receptors as a Means of Targeting a Second Antigen

A variety of strategies to elicit T-cell activation in response to non-native targets have been explored in the field of cancer immunotherapy. These include administration of bispecific T-cell engagers (BiTEs)[142] and engineering with either transgenic TCRs or CARs[95]. The ability to

target surface antigens in an MHC-independent manner conferred by CARs was particularly attractive for this work as a means of targeting a second TAA in a distinct way from the endogenous TCR. Briefly, chimeric antigen receptors are synthetic constructs that are minimally composed of an extracellular targeting domain (usually an antibody single chain variable fragment, ScFv) attached to intracellular the CD3ζ domain which mediates T-cell activation. While this minimal design represents an important step on the path towards the CARs employed today, targeting domains linked to CD3ζ alone did not mediate reliable T-cell activation or persistence *in vivo*. In an effort to confer a signal more akin to normal T-cell activation in the context of CARs, second generation CARs were developed which include a costimulatory domain such as CD28 or 41-BB. T-cells engineered with these CARs display vastly improved antitumor effect and persistence and are widely employed in current clinical trials. Third generation CARs with additional costimulatory domains have also been developed but these constructs have not attained widespread popularity at this point[143].

1.5.3 Selection of a Second Target for Dual-Specific T-cells

The approach described herein is unique in that the targeting of two tumor antigens is accomplished by the same T-cell population and this population is also boostable by our OVV. A critical consideration in the development of such a strategy is the selection of an appropriate second antigen. In the CMS-5 model which is a transplantable murine tumor employed in our lab, DUC18 transgenic T-cells display endogenous specificity for the TSA mERK. We postulated that an ideal second target would be widely expressed on cancer cells themselves as well as components of the tumor microenvironment. The use of a CAR to confer a second specificity allows that the second antigen be non-MHC restricted and thus not susceptible to MHC downregulation.

Ligands for the NKG2D receptor represent such a broadly tumor and TME-specific target. NKG2D ligands are stress-induced proteins frequently upregulated on a variety of human and murine tumors[144,145]. While the NKG2D receptor is sufficient to activate NK cells, it performs a costimulatory function on T-cells and alone cannot mediate full activation of T-cells[144]. T-cells engineered with a CAR targeting NKG2DL have demonstrated efficacy in preclinical models of cancer[144–150] and are currently being employed in a phase I clinical trial (NCT02203825). Additionally, NKG2D ligand expression has been shown to be upregulated on components of the tumor microenvironment such as tumor vasculature[151] and immunosuppressive cells[152] both of which have been shown to be targeted by NKG2D CAR T-cells. Thus targeting of NKG2D ligands as a second antigen in our ACT + OVV approach could provide added benefit and contribute to durable remissions through multiple pathways.

One concern with targeting NKG2DL in immunotherapies would be its expression on non-tumor tissues. Indeed VanSeggelen et al. demonstrated in 2015 that targeting NKG2D ligands with CAR T-cells resulted in both on-target-off-tumor pathology in the lung as well as severe cytokine-induced toxicities in murine models[153]. Dose dependent toxicities were also observed in a study by Sentmen et al[154]. While this is a potential drawback to employing NKG2DL as a second target in our ACT + OVV approach, it also presents an opportunity to evaluate the safety of this approach. Both studies demonstrating toxicity employed large doses of highly activated, effector CAR T-cells activated *ex vivo* with Il-2[153,154]. We believe that the lower doses of T-cells administered in our therapies as well as their activation *in vivo* (as opposed to administration in a highly activated status) will reduce cytokine-mediated toxicity. While the focus of this work will be on the ability of dual-specific cells to prevent relapse, this may also provide indirect evidence

for the enhanced safety of our ACT + OVV approach compared with conventional ACT protocols.

1.6 Hypothesis, Goals and Synopsis

With this project we sought to build on the powerful ACT + OVV platform developed in our lab. We have demonstrated that the combination of ACT and OVV can indeed provide a means of circumventing issues associated with T-cell persistence and achieving sufficient numbers for adoptive transfer. While this can produce durable cures in some models, both clinical and preclinical work has demonstrated the limitations inherent to therapies directed at only one antigen. ***It was thus hypothesized that the efficacy of the ACT + OVV approach could be further improved by incorporating a means of dual-antigen into transferred cells to directly address tumor heterogeneity.*** In this regard the goals of this work were threefold. Firstly we sought to establish a means of conferring a second antigen specificity onto T-cells that were already tumor specific while retaining their ability to respond to OVV-boosting. Subsequently we sought to characterize these dual-specific cells both *in vitro* and *in vivo* in terms of responsiveness to targets bearing both or only one of their target antigens. Finally we explored the efficacy of these modified cells in achieving their primary goal of eliminating antigen-negative variants in tumor models designed to address relapse and intratumoral heterogeneity.

To this end, this dissertation has been structured to describe both the *in vitro* development and characterization of these dual-specific cells followed by investigations into their utility *in vivo*. In the *in vitro* section, the challenges associated with engineering murine T-cells with a chimeric antigen receptor while retaining their memory phenotype and are described along with strategies developed to circumvent these restrictions. Additionally evidence is provided for the

functionality of the engineered cells in responding to a second antigen. In the *in vivo* section, the development of assays to establish the killing capacity of these cells through their engineered CAR in the context of the ACT + OVV regimen is described. Challenges to the use of these cells as well as limitations with regard to the selected second target, ligands for the NKG2D receptor are also described. Evidence is provided for their ability to prolong remission in a relapse model of cancer and the initial development of a chimeric tumor system in which to further assay their impact is described. Finally preliminary data investigating pharmacological strategies aimed at enhancing the effects of these dual-specific T-cells and overcoming limitations identified in the early stages of their characterization is provided.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1 Tumor Cell Culture

Cell lines were purchased from ATCC with the following exceptions. RenCa cells were a kind gift from Dr. Carloina Ilkow (Ottawa Hopsital Research Institute, Ottawa, Ontario). 4T1.2 and PLAT-E cells were obtained from Dr. Jonathan Bramson (McMaster Immunology Research Center, Hamilton, Ontario). CMS-5 relapse cells (CMS-5r) were isolated by Scott Walsh from relapsing CMS-5 tumors in NRG mice treated with DUC18 cells boosted by a Maraba MG1 virus vector expressing the mutant ERK peptide target of the DUC18 cells.

All cells were maintained in incubators (FormaScientific) at 37˚C and 5% CO_2. CMS-5, a murine fibrosarcoma expressing a mutant ERK peptide (mERK)[155] were grown in Dubelco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100mg/mL streptomycin and 2mM L-glutamine. CMS-5r cells were cultured in the same media. RenCa cells (murine renal carcinoma) were grown in Rosalind Park Memorial Institute (RPMI) media with 10% (FBS), 100 U/mL penicillin, 100mg/mL streptomycin, 2mM L-glutamine, 0.01mM non-essential amino acids (NEAA) and 1mM sodium pyruvate. 4T1.2 (murine breast carcinoma) cells were grown in RPMI with 10% FBS and 100U/mL penicillin/100mg/mL streptomycin. ID8 cells (murine ovarian carcinoma) were grown in RPMI with 10% (FBS), 100 U/mL penicillin, 100mg/mL streptomycin, 2mM L-glutamine, 0.01mM non-essential amino acids (NEAA), 1mM sodium pyruvate, 0.1% beta-mercaptoethanol and 10mM HEPES. CT26 cells were cultured in AlphaMEM media supplemented with 10% FBS, 100 U/mL penicillin, 100mg/mL streptomycin and 2mM L-glutamine. B16 and B16gp33 cells (murine melanoma) were cultured in MEMF11 media supplemented with with 10% FBS, 100 U/mL penicillin, 100mg/mL streptomycin, 2mM L-glutamine, 0.01mM NEAA, 0.1% beta-mercaptoethanol and

1mM sodium pyruvate.Cells were maintained for 1 week prior to injections and for a maximum of 2 weeks for use *in vitro*. Cell culture reagents were purchased from Gibco.

2.2 Isolation and culture of Transgenic T-cells

Transgenic T-cells were grown from DUC18 and P14 mice. Colonies were maintained in ultraclean rooms at the Central Animal Facility (CAF) at McMaster University. The DUC18 strain was provided by Dr. Lyse Norian (University of Iowa, Iowa) while the P14 strain was obtained from Taconic Breeding Laboratories (Germantown, NY). Mice were sacrificed at 6 weeks old (or older) and splenectomised. Fresh spleens were cut and squeezed using microscope slides to extract splenocytes in Hank's buffer. The resulting solution was centrifuged at 1500xg for 5 minutes and resuspended in ACK lysis buffer (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1mM Na_2EDTA, pH 7.2-7.4) for 5 minutes to lyse red blood cells. The lysis solution was subsequently diluted in Hank's buffer an centrifuged. Splenocytes were resuspended in T-cell media (RPMI supplemented with 10% FBS, 100 U/mL penicillin, 100mg/mL streptomycin, 2mM L-glutamine and 0.1% beta-mercaptoethanol) and plated in 1mL of cytokine supplemented T-cell media in a 24 well plate at densities of 2E6 cells / well (DUC18) or 1E6 cells/well (P14). Activation was performed using cognate peptides unless otherwise specified. mERK (QYIHSANVL)[156] and gp33 (KAVYNFATM) peptides were obtained from Biomer Technologies (Pleasanton, California). Peptides were used at concentrations of 0.1ug/mL in culture. In instances of antibody activation, αCD3 and αCD28 antibodies were obtained from Dr. Jonathan Bramson (McMaster University, Hamilton, Ontario) and used at concentrations of 0.1ug/mL in culture. To obtain a memory phenotype cytokines used were recombinant human Il-21 (rhIL-21) and rhIL-15 at concentrations of 10ng/mL alongside the mTOR inhibitor rapamycin at 20ng/mL. To obtain an effector phenotype cells were cultured with rhIl-2 at 30ng/mL. Memory cultures were expanded

2.5X every 2 days with fresh cytokine supplemented media (no additional peptide added). Effector cultures were expanded 10X on day 2 or 3 with rhIl-2 supplemented media. All T-cells were used within one week of spleen isolation. Cytokines and rapamycin were obtained from BioLegend (San Diego, California)

2.3 Production of Retroviruses and Transduction

Murine T-cells were transduced using an NKG2D-CAR virus which was a kind gift of Dr. Jonathan Bramson. The virus (NKz10), described by VanSeggelen et al in 2015[153], encodes a CAR with a targeting domain for murine NKG2D ligands linked to the murine CD3ζ chain and the DAP10 adaptor protein through which NKG2D signaling naturally occurs. This construct was shown to be the most potent of three different NKG2D CARs based on induction of toxicity in murine models[153]. The viral genome plasmid and pCL-ECO packaging plasmid[157] were grown in GT116 *E coli* cells (a kind gift from Dr. Sarah Wootton) in standard lysogeny broth (LB) with 100ug/mL ampicillin (Bioshop, Burlington, Ontario). Plasmids were isolated using a maxiprep kit from Invitrogen (Grand Island, NY) according to manufacturer's protocol.

Retroviruses were produced in PLAT-E cells. PLAT-E cells were maintained in DMEM with 10% FBS, 100 U/mL penicillin, 100mg/mL streptomycin, 10mM HEPES and 2mM L-glutamine. For transfection, media was replaced with media containing 50mg/mL Normocin instead of penicillin and streptomycin. Cells were plated in T75 flasks one day prior to transfection at roughly 5E6 cells/flask. Transfection was carried out with lipofectamine 2000 (Invitrogen; Grand Island, NY). For each flask, 10ug of each plasmid were mixed in 2.5mL of serum free DMEM (sfDMEM) separately from 45uL of lipofectamine in an additional 2.5mL of sfDMEM. These solutions were left separately for 5 minutes and DNA solution was subsequently added to

lipofectamine solution, mixed and left for 20 minutes. This solution was then added to PLAT-E cells and allowed to transfect overnight. The following morning, media was changed to 10mL fresh transfection media supplemented with 1mM sodium butyrate (Sigma-Aldrich, Oakville, ON) and left for a day before harvest. Retroviruses were harvested via 5 minute centrifugation of PLAT-E supernatants at 1500xg to remove any detached PLAT-E cells. Supernatants were subsequently filtered through a 0.45um filter (Pall Life Sciences; Port Washington, NY). Filtered supernatants were concentrated roughly 30X (i.e. media from 3 T75 plates was concentrated to 1mL) using an Amicon Ultra 15mL 30K filter (EMD Millipore; Etiobicoke, ON) spun at 3000xg. Viruses were either used immediately or frozen once before use. In some instances fresh transfection media was added to PLAT-Es immediately after the first harvest to produce a second batch of retrovirus as this supernatant was also demonstrated to produce comparable transductions.

For transduction, concentrated virus supernatants were supplemented with 20ug/mL lipofectamine 2000 (Invitrogen; Grand Island, NY) and 16ug/mL polybrene (ABM; Richmond, BC). Unless otherwise specified, transduction was performed prior to T-cell activation using 100uL of retrovirus solution. Viruses were spinfected onto T-cells in 400uL T-cell media (no cytokines or peptide) in 24 well plates via centrifugation at 1500xg for a minimum of 10 minutes. Subsequently, plates were placed in a 37˚C cell incubator for 2 hours followed by addition of 500uL of T-cell media supplemented with 2X cytokine and peptide concentrations (to reach a final volume of 1mL per well with 1X cytokines and peptide).

2.4 *In vitro* Cytotoxicity Assays

For *in vitro* killing assays, tumor cells were plated at 6000 cells/well in a 96 well plate in 100uL of their corresponding media and left for 24 hours. Subsequently T-cells were added in 100uL of T-cell media at the indicated ratios assuming targets doubled over the incubation period. Co-incubation was done for 18 hours after which time all media was removed. Remaining cells were washed 3 times with warm phosphate buffered saline (PBS). For determining cell viability 100uL of tumor cell media containing 0.025mg/mL of resazurin salt was placed on the cells and left for 2-3 hours (until control well with no effectors visibly turned pink). Viability readings were calculated based on the absorbance of reduced rezasurin (resorufin, 560nm excitation/590nm emission)[158]. Each ratio was done in triplicate and normalized to a target-alone control. Trends displayed are consistent with a minimum of 3 technical replicates per cell line assayed.

2.5 Flow Cytometry and Surface Staining

All flow cytometry was performed using a BD LSR II flow cytometer and data was analyzed using FlowJo software. For surface staining, samples were pelleted in a 96 well dish and resuspended in 25uL FACS buffer (PBS with 0.5% bovine serum albumin) with FC block (BD, 1:200) and incubated at 4˚C for 15 minutes. 25uL of 2X concentrated surface staining antibodies in FACS was subsequently added directly to the blocking solution. The following antibodies were used throughout this work and the indicated dilution represents that which is required for a 1X solution: NKG2D (APC, 1:100), CD8α (Pacific Blue, 1:200 and PE, 1:400), CD62L (AlexaFluoro700, 1:400), CD44 (FITC, 1:400) and Thy1.1 (FITC, 1:1000 or PE, 1:400). Viability markers used were either 7-AAD or fixable viability dyes FVD510 or FVD575. For 7-AAD staining, surface stained samples were washed with FACS buffer and subsequently resuspended in FACS buffer with 7-AAD at 1:200 immediately prior to filtration and running on

the flow cytometer. For fixable viability dyes, cells were washed twice in PBS and stained with dye diluted 1:1000 in PBS (25uL/well) for 10 minutes at room temperature with protection from light. Cells were subsequently washed and resuspended in FACS buffer and filtered for running. All antibodies were purchased from BD Pharmingen (Missaussauga, ON) with the exception of Thy1.1 (FITC) and IFNγ (Pacific Blue) which were from eBioscience/Affymetrix (Santa Clara, California) and anti-human IgG which was from Jackson Immuno Research (West Grove, PA).

2.6 Intracellular Cytokine Staining

Following surface staining, in cases were staining of intracellular contents were desired, samples in a 96-well plate were washed in FACS buffer and subsequently resupended in 100uL of Cytofix/Cytoperm (BD Pharminogen; Missaussauga, ON) and incubated at 4˚C for 20 minutes. Samples were then washed by placing 100uL of 1X diluted permwash (BD Pharminogen; Missaussauga, ON, additional to the Cytofix/Cytoperm) in each well followed by a second wash with 200uL of permwash. Cells were then stained with antibody diluted in permwash (25uL/well) and left for 20 minutes at 4˚C. Antibodies used were for IFNγ (Pacific Blue, 1:100 or APC, 1:100). Following ICS, samples were washed and resuspended in FACS buffer and run for flow cytometry.

2.7 Viruses Used

Viruses used in these studies were generated by Dr. Brian Lichty's lab. The VSV-ERK is a wild type vesicular stomatitis virus engineered as described previously[159] to encode the mERK target of DUC18 T-cells. The Maraba virus harbours point mutations in the M and G proteins in order to render it more attenuated by impeding its ability to antagonize the IFN response[160] and

was engineered as described previously[129]. The adenovirus expressing mERK used in the supplementary section harbours an E1/E3 deletion as described previously[129].

2.8 Mouse Models and Monitoring

P14, DUC18 and NSG mice were bread and raised in house in the Central Animal Facility at McMaster University. Wild-type BALB/c and C57Bl/6 mice were purchased from Charles River and stored in the Central Animal Facility. All mice used in experiments were 6-8 weeks old unless otherwise specified. For procedures requiring anesthetic, gaseous isofluorane (Fresnius Kabi, Richmond Hill, Ontario) was used. All mouse experiments were carried out with approval from the Animal Research Ethics Board (AREB) at McMaster.

Injections were performed using 29G ½'' monoject insulin syringes (Covidien; Gananoque, ON). For all injections, cultured cells were washed twice with PBS prior to injecting. For the establishment of subcutaneous tumors, cells were given 6 or 7 days prior to treatment. CMS-5 cells were given at doses of 5E5 (NSG) or 1E6 (wild type) cells in 30uL. For chimeric tumors, 100 RenCa cells were mixed in with 1E6 CMS-5 cells and given in 30uL. For lung metastasis study, 1E5 RenCa cells were given in 200uL of PBS via tail vein injection 5 days prior to initial treatment.

Unless otherwise specified mice were treated with 1E6 T-cells in 200uL of PBS by tail-vein injection (intravenous, I.V.). Boosting was performed the following day with 2E8 PFU of Maraba-ERK for NSG mice or 5E8 PFU of VSV-ERK for wild-type mice. Viruses were also administered I.V. in 200uL of PBS. Ad-mERK was administered intramuscularly in 50uL to each hind leg to achieve a total of 5E8 PFU/animal.

For tumor monitoring, subcutaneous tumors were measured using electronic calipers to approximate length, width and height in order to obtain volume. For lung metastasis, mice were euthanized by isoflurane (Fresnius Kabi, Richmond Hill, Ontario) overdose and chest cavities were opened by cutting upwards from the peritoneal cavity. 15% India Ink (Speedball Art, Toronto, ON) (diluted in PBS) was administered via cardiac perfusion until lungs stained black. Lungs were subsequently excised and placed in Fekete's solution (70% EtOH, 3.75% PFA, 0.75M acetic acid) in which metastasis destained to a white colour amenable to counting. For luciferase imaging, mice were anesthetized and injected with 200uL of D-Luciferin (15mg/mL, Perkin-Elmer, Waltham, Mass) intraperitoneally (IP). 14 minutes following injection, mice were imaged using the In Vivo Imaging System (IVIS) from Perkin-Elmer.

MS-275 was purchased from Selleckchem (Burlington, ON) and resuspended in 15:2.5 EtOH:DMSO at 0.1mg/17.5uL. Doses of 17.5uL were administered to mice diluted with an additional 32.5uL of PBS. Treatments were administered I.P. for five days beginning on the day of virus injection.

2.9 Blood Collection

For analysis of immune response in peripheral blood, 5 drops of blood were collected into 1.5mL ependorf tubes with 50uL of 0.6ng/mL heparin (in Hank's Buffer) via retro-orbital bleed using heperanized capillary tubes (Fischer, Nepean, ON). Following bleeding, mice were treated with Tear Gel (Alcon, Fort Worth, TX). Volume of blood in each sample was then measured during transfer to a FACS tube. Blood in FACS tubes was subjected to two rounds of ACK lysis (5min) followed by dilution in HANK's buffer and pelleting.

To quantify activated T-cells, 100uL of T-cell media containing 100ng/mL of peptide of interest was placed in a round-bottom 96-well plate. Blood samples were resuspended in 50uL Hank's buffer and added to each well for stimulation (an unstimulated, peptide-free well with pooled residual samples was also included). Stimulation was carried out for 1 hour in incubators at 37˚C followed by addition of 50uL T-cell media with 0.2uL Golgi Plug (BD Pharmingen) and stimulation for an additional 3 hours. Subsequently cells were subjected to surface and intracellular cytokine staining as described above.

2.10 Tumor Isolation and Staining

For assessment of *in vivo* cell surface expression on tumors, masses were excised from euthanized mice and weighed. Tumors were homogenized as far as possible in Hank's buffer using scissors. Tumor homogenate was placed in digestion buffer containing 0.5mg/mL Collagenase Type 1 (Gibco, Grand Island, NY), 0.2mg/mL DNAse (Roche, Mississauga, ON) and 0.02mg/mL Ovine Hyalurinidase (MP Biomedicals, Santa Ana, CA) in Hank's Buffer. 10mL of digestion buffer was used for every 0.25mg of tumor. Subsequently, digest was filtered through a 70um Falcon cell strainer (Fisher, Nepean, ON) and centrifuged. Suspension was resuspended in FACS buffer for staining and further analysis.

For tumor staining, αCD45 (Pacific Blue) was used to distinguish immune and non-immune cells. For NKG2D ligand staining, primary antibody for NKG2DL was used (1:10, R&D Systems, Minneapolis, MN) for 20min. Samples were then washed twice with PBS and αhuman IgG (PE, 1:100) was used.

Chapter 3

Results

3. Results

3.1 *In vitro* development and characterization of dual-specific T-cells for use in the ACT + OVV regimen

3.1.1 Transduction Efficiency is Influenced by T-cell Culture Conditions and Activation Strategy

Immunotherapies have recently demonstrated tremendous promise as treatments for neoplastic disease however the emergence of antigen escape variants and the inherent heterogeneity of cancer present significant challenges in establishing durable cures[133–136]. Previous work performed in our lab has demonstrated the efficacy of utilizing a combined adoptive cell transfer with oncolytic viral vaccine boosting strategy for treating established solid tumors but antigen negative relapse remains an issue in a variety of disease models (see **Figure 1**). Thus while oncolysis and attack of the tumor by adoptively transferred and boosted T-cells can result in antigen spreading in some models, the therapy would benefit from additional strategies to directly target tumor heterogeneity. To this end we sought to engineer T-cells capable of responding to two distinct tumor antigens but still having the capacity to respond to OVV-boosting for use in our therapy.

To achieve this goal, transduction of an NKG2DL-targeting CAR previously described[153] was carried out onto DUC18 murine T-cells which exhibit native specificity for the mERK immunodominant peptide expressed by the BALB/c CMS-5 fibrosarcoma cell line[156] (see **Figure 2A**). The CMS-5/DUC18 system has been well characterized in our lab and we have available boosting OVVs which also express mERK making this a logical model to work in. Since our has pilot data indicating that a memory-like phenotype elicited by our specific cytokine cocktail and peptide activation is critical to the boostability of our adoptively transferred cells, transduction

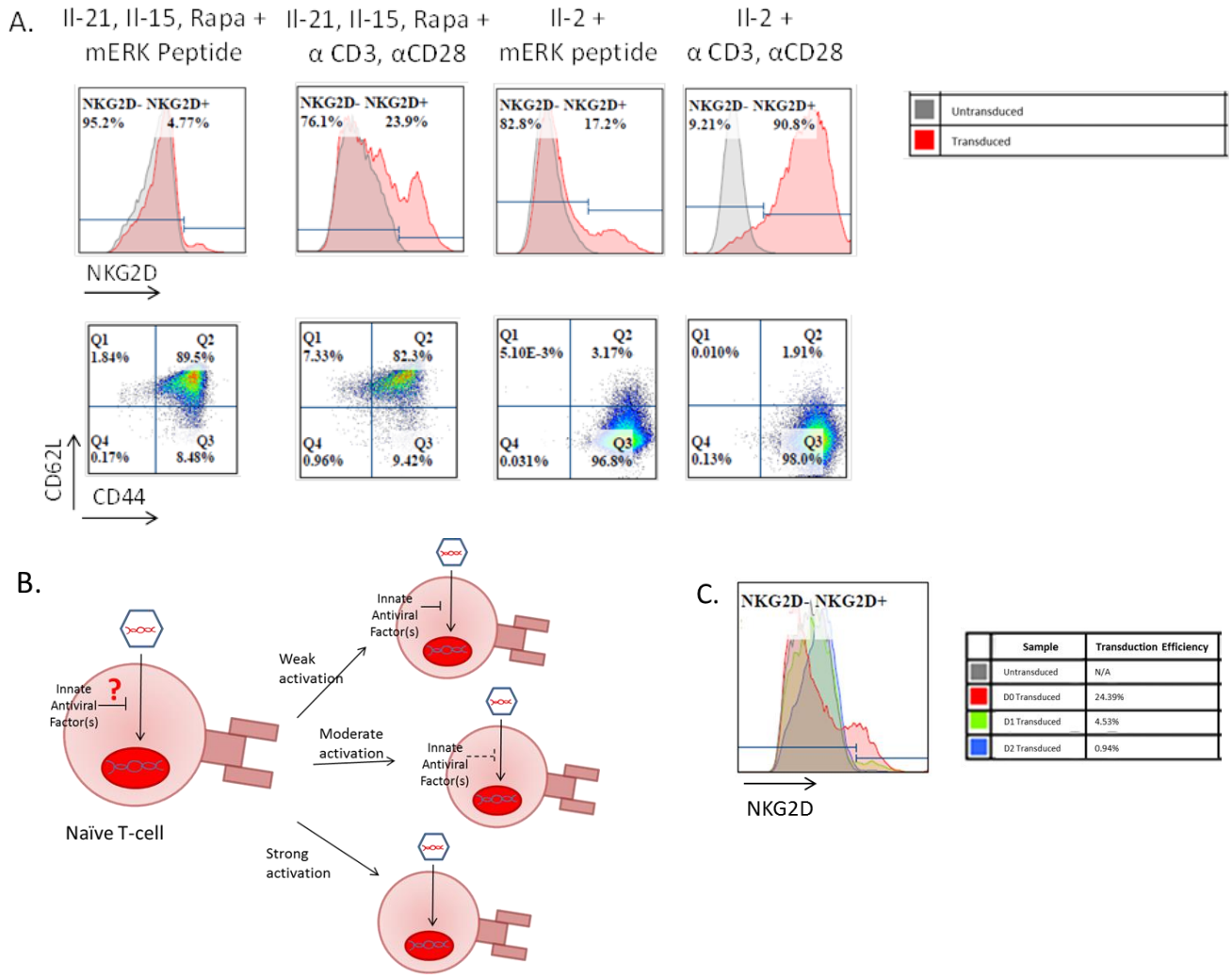

Figure 2. Retroviral-mediated transduction of murine T-cells is impacted by culture conditions and activation method. Additional strategies are required to overcome these limitations. A. DUC18 T-cells were cultured from splenocytes in a variety of conditions and transduced with NKz10 retrovirus 2 days following beginning of culture. Subsequently cells were cultured for 5 additional days and then stained for NKG2D receptor as well as CD44 and CD62L expression levels relative to untransduced controls cultured in the same way. Transduction was markedly increased in T-cells cultured with Il-2 and antibody based activation relative to either Il-21, Il-15 and rapamycin or peptide-based activation strategies. Phenotypically, cells acquire a memory-like phenotype using Il-21, Il-15 and rapamycin and an effector phenotype using Il-2. **B.** Potential model to explain the effects of culture conditions and activation strategy on transduction efficiency. Innate antiviral pathways are activated in T-cells cultured in conditions with weaker activation and eliciting a memory-like phenotype. These pathways are either not activated or overruled by strong activation signals and conditions eliciting an effector-like phenotype. The activity of these pathways in naïve T-cells was unclear. **C.** DUC18 T-cells transduced prior to acquisition of a memory phenotype display superior transduction relative to those cultured in memory conditions. Splenocytes from a DUC18 mouse were isolated and transduced in parallel or 1 or 2 days following addition of peptide and memory cytokines to culture media. After 1 week in culture, transduction efficiency was superior when performed prior to addition of memory cytokines and peptide and decreased dramatically with each day of culture before transduction.

efficiency of DUC18 cells activated and cultured for an effector phenotype as previously described[153] to cells cultured utilizing various permutations of cytokines and activation strategy (peptide or antibody) that more closely resembled our culture conditions (**Figure 2A**) were compared. Strikingly it was observed that while cells cultured as previously described with Il-2 and antibody based activation[153] were transduced with efficiencies on the order of 90%, substituting activation signal and cytokine environment significantly decreased the transduction efficiency to roughly 5%. Phenotypic analyses confirmed that cells cultured with Il-2 developed an effector-like phenotype as revealed by CD44 and CD62L expression while cells cultured with our memory conditions exhibited the appearance of central memory T-cells (**Figure 2A**).

Since our lab has generated pilot data demonstrating the requirement of our culture conditions for cells used in the ACT + OVV regimen, we sought a strategy to improve the transduction of these cells specifically. It has previously been demonstrated that resting T-cells possess an early "block" in infection with some viruses but that this is lost following activation[161]. It therefore conceivable that a similar block would be present in T-cells cultured using our protocol and that this same block would be active to the same extent in naïve T-cells (**Figure 2B**). In the conventional transduction protocol, T-cells are activated and cultured for two days prior to transduction[153]. The transduction of DUC18 T-cells prior to and one or two days following activation and culture in our cytokine cocktail was thus investigated. Interestingly, T-cells transduced with frozen aliquots of the same virus prior to culture exhibited markedly greater transduction than those transduced following 1 or 2 days of culture (**Figure 2C**) (roughly 25% on day 0 compared to 5% and 1% for days 1 and 2 respectively). However, cells with greater transduction consistently grew to lower yields than their untransduced counterparts. **While a variety of other strategies/conditions were explored, the optimal**

balance between transduction efficiency, expansion and maintenance of memory phenotype was obtained by the transduction prior to activation protocol. Therefore we proceeded utilizing this strategy and accepting a transduction efficiency of roughly 25% for further characterization of the dual-specific T-cells' targeting capacity and ability to address tumor heterogeneity *in vitro* and *in vivo*.

3.1.2 CAR-Transduced Cells Are Functionally Dual-Specific *in Vitro*

After solidifying a protocol to transduce T-cells while maintaining the memory-like phenotype required for the ACT + OVV regimen, efforts were taken to characterize the performance of the NKz10 CAR. To establish the functionality of the CAR *in vitro*, the expression of NKG2D ligands on cultured tumor cell lines was explored. The vast majority of cell lines screened were high expressers of NKG2DL *in vitro* with B16F10 melanoma cells being the only cell line on which expression was completely absent (**Figure 3A**). Because the BALB/c cell lines CMS-5, RenCa and 4T1.2 all strongly expressed NKG2DL *in vitro* (**Figure 3A**) these cell lines were employed in killing assays. Furthermore, members of our group had recently isolated a CMS-5 cell line (CMS-5r) which was confirmed by sequencing to lack expression of the mERK epitope (**Figure 1E**) and this cell line was also utilized. As expected, both transduced and untransduced DUC18 cells exhibited dose-dependent killing of cultured CMS-5 cells (**Figure 3B**). Only DUC18 cells transduced with the NKG2D-CAR however were able to kill cultured 4T1.2, RenCa or CMS-5r cells (**Figure 3B**).

To further establish the functionality of the NKG2D-CAR on transduced cells, co-incubation experiments with NKG2DL-expressing cell lines and transduced cells were performed. Cells were incubated overnight and staining for IFNγ production was carried out the following day to assay T-cell activation. Following co-incubation live CD8+ T-cells were separated based on

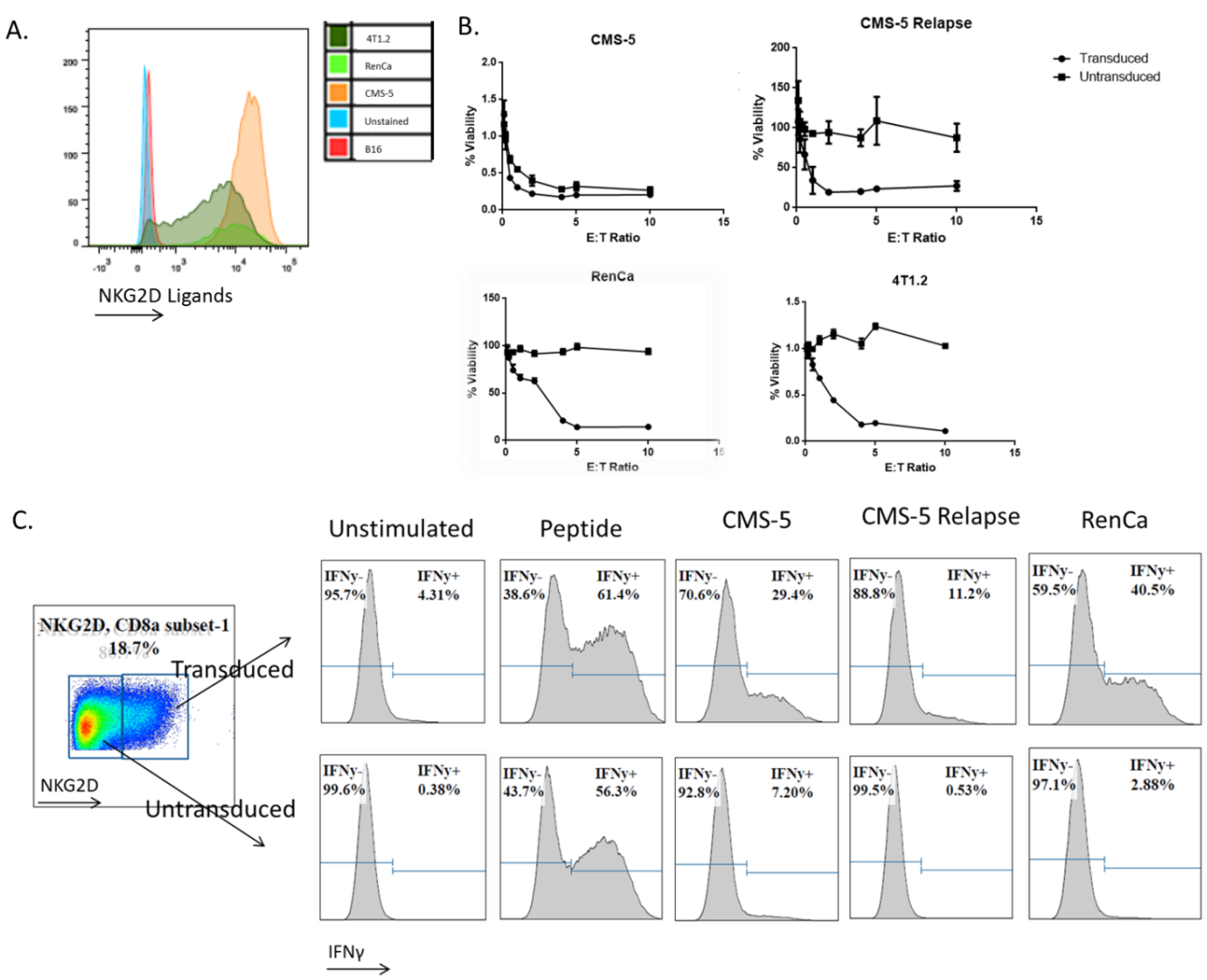

Figure 3. NKz10-transduced cells mediate cytotoxicity and demonstrate responsiveness to NKG2D ligand-expressing cells *in vitro*. A. Expression of NKG2D ligands on cultured cell lines. Tumor cells were grown in culture and stained for NKG2D ligands relative to an unstained control. Strongest expression was observed on CMS-5 cells while RenCa and 4T1.2 cells also displayed upregulation of NKG2DL. Ligands were absent on cultured B16F10 cells. **B.** Transduced cells mediate toxicity against NKG2DL-expressing cell lines. Killing assays were performed by co-incubation of transduced or untransduced DUC18 T-cells with tumor targets for 18-24 hours followed by washing of T-cells and assessment of target viability by resazurin salt. Both transduced and untransduced cells displayed dose-dependent cytotoxicity against the mERK-expressing CMS-5 cell line whereas only transduced cells were able to kill the mERK-negative CMS-5 relapse, RenCa and 4T1.2 cell lines. **C**. Interferon gamma production by transduced and untransduced cells in co-culture with NKG2DL-expressing targets. Cells within a transduced culture were co-cultured overnight with various targets. Subsequently cells were stained for viable CD8a-expressing T-cells and divided into transduced and untransduced groups based on NKG2D-expression. IFNγ production was assessed in each of these groups. Transduced cells consistently produced higher levels of IFNγ and were the only cells to display robust production of the cytokine in response to mERK negative targets.

NKG2D expression (as an indicator of transduction) and IFNγ production was measured. While both transduced and untransduced cells produced IFNγ in response to peptide and CMS-5 cell stimulation, only NKG2D-CAR transduced cells demonstrated reliable IFNγ production in response to mERK negative, NKG2DL positive cell lines. Interestingly the basal level of IFNγ production was greater in transduced relative to untransduced cells (**Figure 3C**). **As such NKz10-transduced DUC18 cells are able to kill and produce cytokines in response to cell lines which express NKG2DL *in vitro.***

3.2 *In vivo* assays for NKz10 CAR function on dual-specific T-cells in the ACT + OVV regimen and efficacy of this strategy in targeting tumor heterogeneity.

3.2.1 Preliminary characterization of NKz10 CAR function subsequent to boosting in the ACT + OVV regimen.

In order to investigate the capacity of the NKz10 CAR to target antigenically heterogeneous tumors in the context of dual-specific DUC18 cells in the ACT + OVV regimen, we first sought to establish the *in vivo* functionality of the NKz10 CAR alone. To this end, we sought an NKG2DL-expressing tumor model on a BALB/c background. One of the cell lines screening positive for *in vitro* expression of NKG2DL in initial assays was the RenCa renal carcinoma line (**Figure 3A**). Because NKG2D dependent control of RenCa lung metastases by NK cells has previously been reported *in vivo*[162], it was reasoned that this might represent a useful model in which to investigate the functionality of the NKz10 CAR.

To assay the ability of boosted transduced memory DUC18 T-cells to control RenCa lung metastases, mice were injected I.V. with 1E5 RenCa cells. Five days following tumor injection

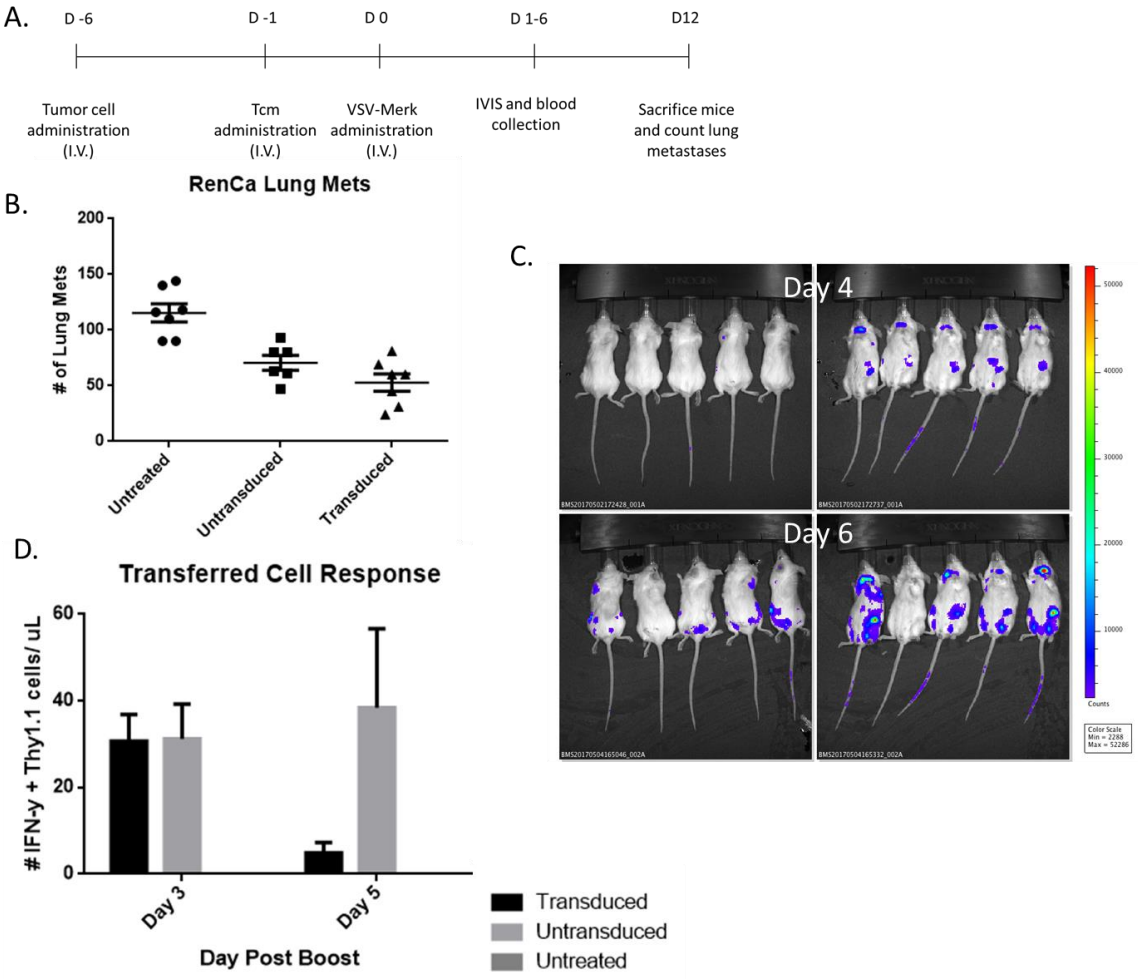

Figure 4. Dual-specific cells mediate marginal tumor control and exhibit limited persistence in the RenCa lung metastasis tumor model. A. Experimental outline: Mice were injected with 1E5 RenCa cells I.V. and given T-cells and boosting virus after 5 and 6 days respectively. 19 days following tumor inoculation mice were euthanized and lung metastases were counted by staining of lungs with India ink and immersion in Fekete's solution. **B.** VSV-mERK boosted NKz10 transduced DUC18 cells marginally reduce RenCa lung metastasis relative to untransduced DUC18 cells boosted in the same way. Both groups demonstrate increased control relative to that observed in untreated mice. **B.** Transduced cells display marginal localization to the lung but limited persistence by IVIS imaging. Transduced cells were visualized by luciferase expression by IVIS on days 4 and 6 following boost. Dorsal and ventral images are shown. **C.** Transduced cells display poor persistence of response in the blood. Mice treated with VSV-mERK boosted transduced and untransduced DUC18 T-cells or not treated were bled and T-cells were stimulated with mERK peptide. # of IFNγ-secreting Thy1.1+ cells were quantified by ICS.

mice were provided with either no treatment or VSV-mERK boosted transduced or untransduced DUC18 cells (**Figure 4A**). Lungs were taken from euthanized mice and metastases quantified on day 19 following tumor inoculation (based on pilot experiments, data not shown). While improved tumor control was observed in both the untransduced and transduced groups relative to the untreated control, the difference between transduced and untransduced groups was much less impressive and only bordered on significance (**Figure 4B**). Further analysis performed by IVIS imaging indicated that transduced T-cells only displayed minimal trafficking to the lung, and their persistence was severely compromised (**Figure 4C**). Interestingly, quantification of activated transferred cells in the blood indicated that these cells maintained a much longer response in the untransduced relative to transduced groups (**Figure 4D**). **Thus while capable of expanding in response to the OVV boosting *in vivo*, both the persistence and tumor-trafficking of dual specific cells were severely compromised in this model and significant NKG2DL-based killing was not observed.**

3.2.2 Dual Specific Cells can Prolong Remissions in a Relapse Model of Cancer

Although NKz10-transduced cells did not mediate significant impact against RenCa lung tumors it was reasoned that part of the lack of therapeutic effect in this model could be due to poor trafficking of T-cells to the tumors. A variety of factors could come in to play, for example the extent and characteristics of tumor vasculature have been identified as contributors to T-cell infiltration into solid tumors[163]. Therefore we sought to employ a model in which tumor infiltration by transferred cells would be expected to occur in order to address our primary hypothesis regarding the targeting of tumor heterogeneity.

The CMS-5 fibrosarcoma model has been characterized extensively in our lab. Treatment of intradermal CMS-5 tumors using memory-cultured DUC18 T-cells boosted with oncolytic rhabdoviral vaccines has been shown to result in elimination and durable cures of established tumors in wild-type BALB/c mice. This would imply and we have shown that transferred T-cells do indeed traffic to the tumor in this model. Interestingly these cures are only durable in immunocompetent animals. NRG mice, which lack an adaptive immune system, display relapse following transient tumor regression (**Figure 1C**). Sequencing has demonstrated that these relapsing tumors lose mERK expression on a genomic level (**Figure 1D**) and represent antigen negative variants which likely arose over the course of therapy. Further characterization has revealed that these variants are eliminated by an endogenous immune response mounted over the course of therapy in immunocompetent animals, explaining the lack of relapse in this context. In fact BALB/c mice cured of CMS-5 tumors by rhabdovirus-boosted DUC18 T-cells are resistant to even CMS-5r tumors (which lack mERK) whereas naïve animals are not. This can be taken to imply the establishment of immunological memory against the CMS-5 cell line based on epitopes other than the mERK target of DUC18 cells. While the complete cures obtained in wild-type animals make this a difficult model in which to observe additional benefit to dual-antigen targeting, the antigen-negative relapse seen in NRG mice provides an ideal arena in which to assess the ability of dual-specific T-cells to target tumor heterogeneity.

Not only can intratumoral trafficking of transferred cells be expected in this model but it may also provide a means of increasing T-cell persistence. Lymphodepletion is a strategy commonly employed in clinical ACT regimens in order to reduce competition for homeostatic cytokines and growth factors between endogenous lymphocytes and transferred cells, improving transferred cell survival[164]. Thus the natural lymphodepletion existing in NRG mice also provides a potential

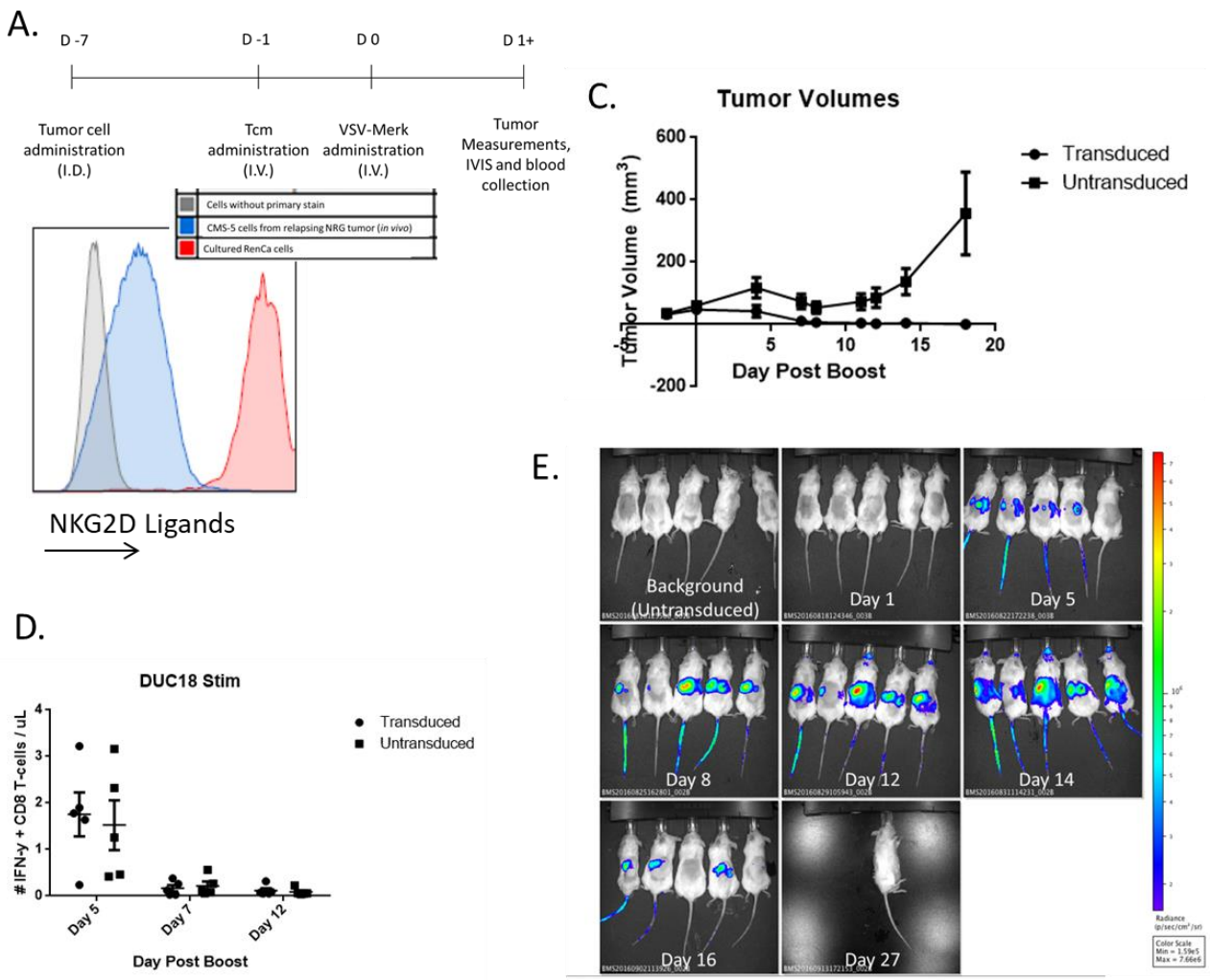

Figure 5. Dual-specific cells mitigate prolong remission in a relapse fibrosarcoma model. A. Experimental layout for intradermal tumor experiments: Tumors were established for 6 days prior to administration of T-cells followed by MRB-mERK one day later. **B.** Relapsing CMS-5 tumors from mice treated with DUC18 cells boosted after 1 day with MG1-mERK in NRG mice express NKG2DL *in vivo*. Tumors were excised and homogenized following treatment. Subsequently cells were stained and levels of NKG2DL on the live CD45 negative population were compared to cells stained without the NKG2DL primary antibody and *in vitro* cultured RenCa cells for negative and positive controls respectively. **C.** CMS-5 tumors treated with MRB-mERK boosted untransduced DUC18 cells relapse whereas remission is prolonged when cells are transduced with the NKz10 CAR. Tumor volumes are displayed (n=5 mice/group). **D.** Immune responses in transduced and untransduced groups are comparable in magnitude. Mice were bled at indicated time points following boost and T-cells were stimulated with mERK peptide. INFγ-producing Thy1.1+ cells were quantified by flow cytometry. **E.** IVIS monitoring reveals localization of transduced cells between spleen and tumor in treated mice. Mice were given D-luciferin I.P. and imaged using IVIS after 14 minute incubation (the NKz10 retrovirus contains a luciferase reporter gene).

means of increasing persistence of transferred cells, another limitation identified in the initial RenCa lung metastasis experiments.

To this end we explored the ability of OVV-boosted dual-specific DUC18 cells to treat and prevent relapse of CMS- 5 tumors in NRG mice (**Figure 5A**). Firstly, the expression of NKG2DL on cells staining low for CD45 isolated from a relapsing NRG tumor was investigated. While these cells displayed lower levels of NKG2DL expression than CMS-5 cells from culture, these ligands were nevertheless expressed on the tumor cells *in vivo* (**Figure 5B**). Treatment of CMS-5 tumors in NRG mice with NKG2D-CAR transduced DUC18 cells led to prolonged remission relative to those treated with untransduced DUC18 cells (**Figure 5C**). Furthermore, activated cells were observed in the blood at comparable levels for both groups (**Figure 5D**). Since the NKz10 construct features a luciferase reporter gene, it was also possible to track the localization of transduced cells *in vivo*. This revealed initial localization and expansion of the T-cells in the spleen and tumor followed by the eventual contraction of the T-cell response. Interestingly, T-cells could be viewed in the mice at time points over 14 days following boosting (**Figure 5E**). While the results of this experiment were promising, the NRG model has several important limitations. Significantly because these mice are severely immunocompromised they cannot mount an effective adaptive response against the boosting virus and eventually succumb to this infection after 2-3 weeks. This therefore precludes the long-term follow up of relapse. **Therefore targeting NKG2DL as a second antigen in the context of OVV-boosted DUC18 cells treating CMS-5 tumors in NRG mice was successful in demonstrating a benefit to dual-antigen targeting. Despite this success, limitations to the NRG system make the development of a wild-type model desireable.**

3.2.3 NKz10-transduced DUC18 T-cells can traffic to mERK-negative skin tumors in wild-type BALB/c mice.

Encouraged by the success of OVV-boosted dual-specific cells in mitigating relapse in the CMS-5 model in NRG mice we sought to expand this success to a wild-type model. To this end we wanted to investigate the NKz10 activity of the dual specific T-cells both independently of and in combination with their native mERK targeting capacity. The expression of NKG2DL on relapsing CMS-5 cells in NRG mice provided encouragement for this model in the context of wild-type mice. Thus, a bilateral tumor experiment was undertaken. In this investigation, CMS-5 tumors made from parental CMS-5 cells or mERK-negative CMS-5r cells isolated from relapsing NRG tumors (**Figure 1E**) were given to mice on either side of the posterior. Mice were subsequently treated with transduced or untransduced DUC18 T-cells boosted with VSV-mERK.

IVIS imaging revealed that transduced cells did in fact traffic to both parental and relapsed tumors, albeit at a lower frequency in relapse tumors (**Figure 6A**). Despite T-cell infiltration in both tumors, only parental tumors were controlled by the therapy with relapse tumors growing out in both transduced and untransduced groups with little apparent difference (**Figure 6B**). To gain insight into this phenomenon CMS-5 tumors were excised from wt BALB/c mice and assayed these for NKG2D ligand expression, using tumor from NRG mice as a positive control. Surprisingly, while NKG2D ligands were expressed on CD45-negative live cells from NRG tumors they were absent on tumors isolated from the wild type counterparts (**Figure 6C**). **Therefore there may be differential expression of NKG2DL on skin tumors established in NRG and wild type mice. While the dual-specific cells were capable of trafficking to mERK-negative tumors they were not able to mediate significant therapeutic effect against**

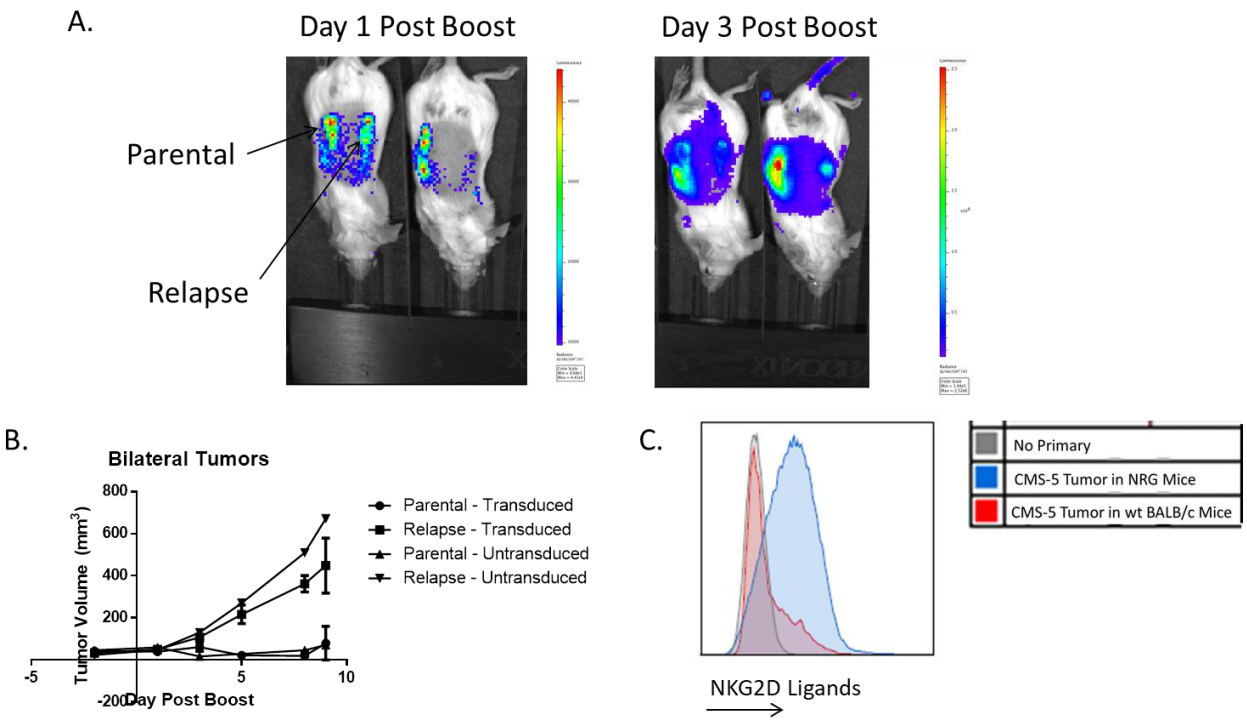

Figure 6. VSV-mERK boosted transduced DUC18 cells traffic to mERK negative CMS-5r tumors *in vivo* but mediate negligible clinical benefit in wild-type BALB/c mice. Wild-type BALB/c mice were given parental and mERK-negative relapse CMS-5 tumors and treated with transduced or untransduced memory cultured DUC18 T-cells one week after tumor inoculation. T-cells were boosted with VSV-mERK one day following their administration. **A.** Transduced cells traffic to both parental CMS-5 and relapse tumors (labelled) *in vivo*. 1 and 3 days following boosting, transduced T-cells were visualized based on luciferase expression by administration of D-luciferin I.P. and IVIS imaging. **B.** Both transduced and untransduced cell treatments mediate control of parental CMS-5 tumors but display limited control of relapse tumors. Tumor volumes following boost are displayed. **C.** While CMS-5 tumors isolated from NRG mice express NKG2D ligands, the expression is significantly reduced on CMS-5 tumors derived from wt BALB/c mice. Cells were isolated from relapsing and newly established tumors in NRG and wt BALB/c mice respecitively and processed. NKG2DL staining on live cells staining negative for CD45 is displayed.

these neoplasms, possibly due to a lack of NKG2DL-expression.

3.2.4 Utilization of Pharmacological Strategies to Enhance the Efficiency of NKG2D Ligand Targeting and Dual-Specific T-cells *in vivo*

Taken as a whole the data presented thus far suggest that in the wild-type models employed until this point the use of dual-specific T-cells targeting NKG2DL is hindered by poor T-cell persistence and target ligand expression on tumors. One of the compounds under investigation in our lab for its ability to prolong T-cell persistence in challenging tumor models is the histone deacetylase inhibitor (HDACi) MS-275 (Entinostat). Interestingly HDACis including MS-275 specifically have been shown to upregulate NKG2D ligands on certain cancers[165]. To this end chimeric tumor experiments were carried out as a means of investigating the potential of MS-275 to ameliorate the previously observed issues to using dual-specific T-cells in wild-type animals. BALB/c mice with were inoculated with 5E5 parental CMS-5 cells mixed with 100 RenCa cells. One week after tumor injection, mice were treated with VSV-mERK boosted transduced or untransduced memory DUC18 cells in the presence of MS-275 (**Figure 7A**). Stimulation of transferred T-cells in the blood demonstrated that MS-275 led to prolonged persistence of both transduced and untransduced cells (**Figure 7B**). The collapse of transferred cells, regardless of transduction status in the CMS-5 model, is a phenomenon previously observed in our lab.

Unfortunately a proportion of the tumors treated with boosted DUC18 cells relapsed in both groups regardless of transduction of the T-cells (**Figure 7C**). Staining of the CD45 negative population from the tumor indicated that relapsing tumors in groups treated with transduced or

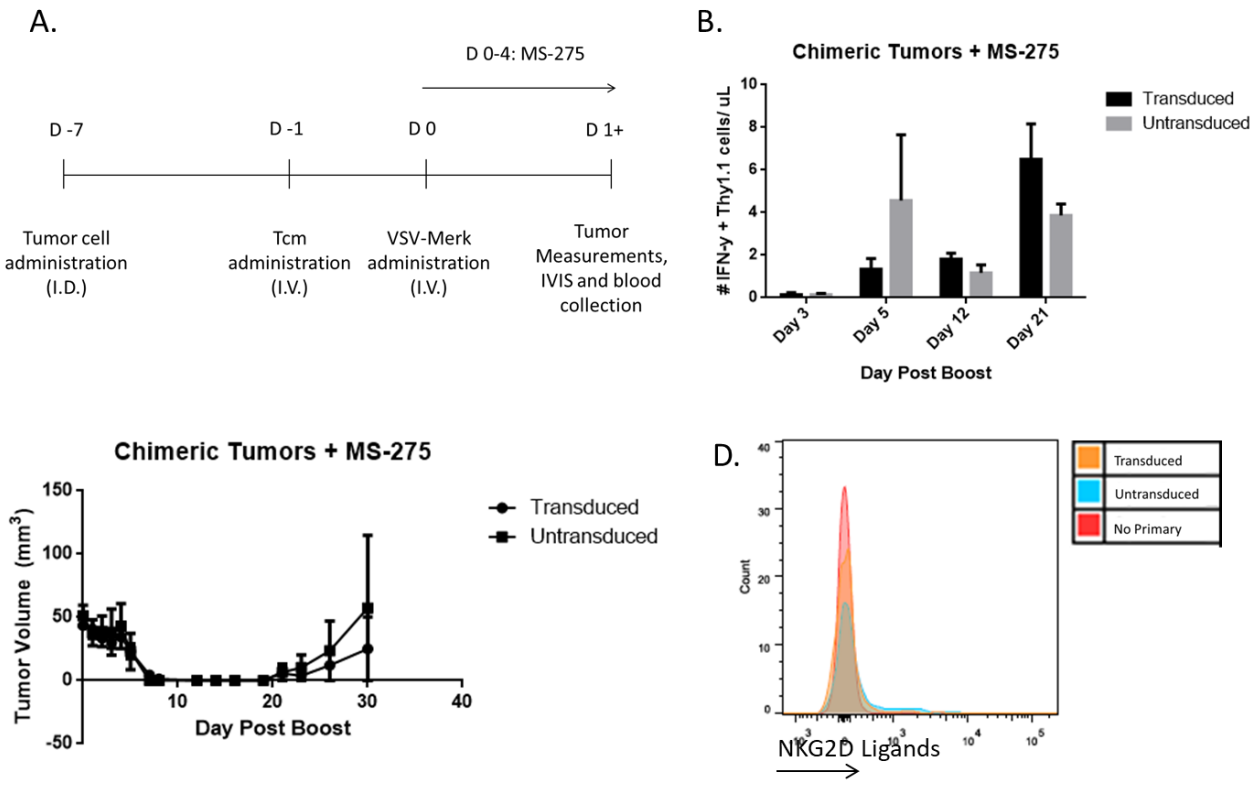

Figure 7. The addition of MS-275 prolongs the life of transferred T-cells but this does not prevent relapse in a chimeric tumor model. A. BALB/c mice were given chimeric tumors consisting of 100RenCa cells in 5E5 CMS-5 cells and treated with VSV-mERK boosted transduced or untransduced DUC18 T-cells (n=3 per group) with administration of MS-275 for 5 days starting on the day of the boost. **B.** Blood was collected at indicated time points and stimulated with mERK peptide to quantify IFNγ-producing Thy1.1 cells by intracellular cytokine staining. **C.** Tumor volumes were measured as a product of length, width and height using electronic calipers and are displayed for both groups as a function of time. **D.** Relapsing tumors from mice treated with transduced or untransduced DUC18 cells were isolated and homogenized. NKG2DL expression on the viable, CD45 low fractions is shown.

untransduced DUC18 cells did not appreciably express NKG2DL relative to negative control despite initial treatment of the mice with MS-275 (**Figure 7D**). **Thus while MS-275 was successful in prolonging the lifespan of transferred CAR-bearing cells, absence or downregulation of NKG2DL expression on the tumor appears to have limited the ability of dual-specific T-cells to eliminate antigen negative variants.**

Chapter 4

Discussion

4. Discussion

While adoptive cell transfer has demonstrated considerable promise in the treatment of hematological malignancies, barriers remain to its achieving broader curative potential. Persistence of transferred cells, culturing sufficient cells, strategies to mitigate toxicities and addressing intratumoral heterogeneity have all been identified as critical considerations in the improvement of T-cell therapies[97,103–106,135]. To overcome some of these issues our previously developed an approach in which tumor-specific T-cells are cultured for a memory-like phenotype and boosted *in vivo* with an oncolytic viral vaccine to drive antitumor effect (**Figure 1A**). While we have data in a variety of models establishing this regimen as a powerful approach in the treatment of cancer, we hypothesized that it could be improved by the inclusion of a mechanism built in to transferred cells to address tumor heterogeneity. Indeed antigen negative escape has been observed in the clinical setting in therapies with a single target and can severely impede the establishment of durable cures[69,134,135,166,167].

Thus the goal pursued in this work involved the generation and characterization of dual-specific T-cells which would recognize two distinct tumor antigens while maintaining responsiveness to the OVV boosting employed in the protocol developed in our lab. In this context the antigen-specificity used for boosting is a TSA endogenous to the T-cells, while the second target could be rationally selected. A CAR specific for ligands of the NKG2D receptor as the second targeting domain was chosen for a variety of reasons. The use of a CAR allows for circumventing escape mechanisms such as MHC downregulation and therefore the dual specific T-cells would be able to reap the benefit of targeting tumor cells through both MHC-dependant and independent mechanisms. NKG2DL specifically represented an attractive target as it has been shown to be upregulated both on tumor cells themselves as well as components of the TME

including the vasculature and regulatory immune cells[150–152]. Thus even if the immune pressure of targeting NKG2D ligands on the tumor resulted in downregulation of these ligands by cancer cells, this strategy may nevertheless be able to indirectly achieve tumor killing via attack of the TME.

The first goal in the development of this protocol was the generation of T-cells which carried an NKG2DL-targeting CAR in addition to their endogenous tumor-specific TCR. One of the primary models used by our lab involves the use of DUC18 transgenic mice which produce T-cells specific for the mERK peptide expressed by the murine CMS-5 fibrosarcoma. We also have VSV, Maraba virus and Adenoviral vectors which express the mERK antigen. In terms of targeting NKG2DL, the NKz10 CAR has previously been characterized[153] and consists of the NKG2D receptor linked to a CD3ζ domain and the DAP10 adaptor protein through which NKG2D signalling is carried out. This construct was demonstrated to mediate significant toxicity in mouse models when employed on high numbers of effector cells[153] but the effect of memory T-cells bearing this construct was not evaluated. Indeed it is possible that through the administration of less activated T-cells followed by their controlled activation and recruitment to the tumor it would be possible to bypass toxicities associated with bulk infusion of highly active T-cells. For these reasons, engineering DUC18 cells with the NKz10 CAR was undertaken.

Surprisingly, initial efforts to engineer these transgenic T-cells were met with substantial difficulty. While traditional transduction protocols resulted in transduction efficiencies on the order of 90%, transduction efficiency was severely compromised in the culture conditions used by our lab to elicit a memory phenotype (efficiencies of roughly 5%) (**Figure 2A**). It is unclear what about the memory cocktail causes the reduction in transduction efficiency although previous work has documented that effector but not memory T-cells display an innate antiviral

block to infection with myxoma virus[161]. While the exact nature of this blockade is not known it was hypothesized that it may also be limiting the retroviral-mediated transduction of the memory T-cells presently employed. It was however unknown as to whether such a blockade would be active in naïve T-cells (**Figure 2B**). Interestingly it was found that the transduction efficiency was significantly greater in naïve T-cells as opposed to those cultured in the memory cocktail for one or two days prior to transduction (**Figure 2C**). Taken together these data indicate that the cytokine cocktail used to elicit a memory phenotype in our studies limits the transducibility of our cells but the exact reasons for this are not clear.

That naïve DUC18 T-cell transduction was greater than that of cultured memory cells implies that this phenomenon is not simply the result of the inability of retrovirus to transduce non-dividing cells since one would assume basal proliferation of memory-cultured T-cells to be greater than that of naïve T-cells. This could be taken to imply an innate block to transduction though characterization of this phenomenon was not pursued in great depth. Interestingly pilot work with P14 T-cells demonstrated markedly enhanced transduction of cells harbouring a knockout of the IFNAR receptor (**Figure S1**) further supporting the notion of the involvement of innate antiviral pathways.

Knowledge of the effects of cytokine cocktail on transducibility of T-cells will likely have important implications as the field of ACT explores the use of less differentiated cells in adoptive transfer regimens. Indeed evidence is emerging that similar challenges may exist in the transduction of other immune cell types. NK cells cultured in Il-21 appear more refractory to transduction (personal communication with Dr. Dean Lee, Nationwide Children's Hospital, Ohio). Interestingly Il-21 is also a key component of the cytokine cocktail employed in our lab and therefore presents a possible candidate driver of the transduction-resistant phenotype

observed in our cells. Further exploration into whether Il-21 is a key mediator of resistance to transduction as well as the mechanisms governing this phenomenon on a transcriptional level could prove informative both for improving T-cell based therapies and from the standpoint of knowledge regarding retroviral infection of T-cells.

We have preliminary data showing that the memory-like phenotype elicited by our cytokine cocktail and peptide activation is crucial to having cells which adequately respond to OVV-boosting *in vivo*. Since the early transduction of DUC18 cells allowed for the use of peptide-based activation and memory-driving culture conditions, this model was selected for further investigation. Firstly, killing and IFN-γ production were shown in response to mERK positive and negative cell lines with confirmed NKG2DL expression *in vitro* (**Figure 3**). Interestingly the basal cytokine production by transduced cells was greater than that of untransduced cells with roughly 4% of cells showing IFNγ production even in unstimulated samples. Indeed even in the case of the mERK-positive CMS-5 cell line the response elicited by transduced cells far eclipsed that of the untransduced cells (**Figure 3C**). These observations could be attributed to tonic CAR signalling as well as recognition of both the mERK and NKG2DL targets on CMS-5 cells. It is also possible that transduced cells have a more effector-like phenotype either because such cells are more susceptible to transduction or because of signalling carried out through the CAR following transduction. Although these cells show expression of CD62L and CD44 (**Figure 2A**) which would imply a memory phenotype, it is possible that they have more of an effector-memory phenotype as opposed to the memory-like phenotype achieved by untransduced cells.

Another difference observed between transduced and untransduced cells *in vitro* was a reduced yield of transduced cells relative to untransduced cells cultured in the same way. It is unclear whether this represented impaired proliferation following transduction or active killing

of NKG2DL-expressing T-cells in culture by the NKz10-transduced cells. Indeed NKG2D-CAR mediated killing of T-cells in cultures following transduction has previously been documented[168]. While these studies demonstrated that this effect resulted in an enrichment of transduced cells relative to those not becoming transduced in their culture it is useful to bear in mind that NKG2D-CAR transduced T-cells can likely mediate toxicity against other T-cells. This could prove relevant *in vivo* in preclinical and clinical trials employing NKG2DL as a target.

Following *in vitro* characterization of the NKz10-transduced DUC18 cells, we sought to investigate the potential of these cells to mitigate antigen-negative relapse in an *in vivo* model of cancer. Moving into wild-type BALB/c mice, the RenCa cell line has previously been observed to exhibit NKG2D-dependent killing by NK cells *in vivo*. Specifically a greater tumor burden was observed in animals which were given blocking antibody to NKG2D in a lung metastasis model[162]. Thus we reasoned that this model may represent an NKG2DL-positive target to confirm NKG2D-CAR activity independently of mERK recognition *in vivo*. VSVmERK boosted DUC18 cells transduced with the NKz10 CAR did not however lead to a significant reduction in lung metastasis relative to untransduced controls in an adapted version of the RenCa metastasis model (**Figure 4B**). Both therapies did reduce tumor burden relative to untreated controls. Therefore it is conceivable that oncolysis or *in situ* vaccination by the boosting virus played a role in some level of tumor reduction but that the effects of the NKz10 were absent or marginal. Analysis of transduced T-cells in the blood by ICS and by IVIS imaging (**Figures 4C & D**) reveal that transduced cells persist poorly relative to untransduced controls and do not traffic appreciably to the lung.

The poor persistence of transferred cells could be due to a measure of activation induced cell death or exhaustion via tonic signalling through the CAR. Indeed it has previously been shown

that T-cells transduced with highly activated CARs demonstrate increased susceptibility to AICD based on upregulated FasL as a result of CAR signalling[169]. Since NKG2DL may be expressed in areas other than the tumor it is possible the T-cells are encountering this antigen in anatomical locations other than the tumor, accounting for the lack of antitumor effect. Alternatively NKG2DL-encounter may not be necessary and CAR signalling independent of antigen could be the cause of this drop off. Such a notion is supported by the higher baseline IFNγ production observed in unstimulated but transduced cells (**Figure 3C**). Another potential source of the poor persistence of transduced cells *in vivo* would be killing of transferred cells by NKG2D-CAR cells via recognition of NKG2DL.

While the impaired persistence of transduced cells could serve as a possible explanation for the poor antitumor efficacy observed in this model (**Figure 4B**) other factors may also be at play. It is likely that the poor lung-infiltration by transduced cells (**Figure 4D**) was a contributing factor. This could be reflective of low or absent NKG2DL expression on RenCa cells despite high *in vitro* expression which would be consistent with observations in CMS-5r tumors established in wild-type mice (**Figure 6C**, discussed subsequently). Another possibility would be that RenCa lung metastasis do in fact express NKG2DL but that the NKz10-CAR T-cells are not efficiently held in the lung by interactions between CAR and antigen or that transferred cells did not reach the lung following expansion. While results presented in **Figure 6A** seem to argue that transduced cells can be retained in mERK-negative tumors (even when the presence of NKG2DL is low at best, **Figure 6C**) the different anatomical locations and different tumor models could affect this CAR T-cell localization. T-cell trafficking to the tumor has been identified as a difficulty in some solid tumors with a variety of possible causes[170,171]. For example differences in high endothelial venule (HEV) density can impact T-cell infiltration and patient outcomes in

the clinic[172]. It would however be surprising that following activation these T-cells never entered the lung. That being said differences in chemokine milieus within tumors could be invoked to partially explain differences in T-cell tumor infiltration[171,173,174].

Despite the initial discouraging results obtained in the RenCa lung metastasis model, experiments conducted with CMS-5 tumors in immunodeficient mice provided encouraging evidence for the use of dual-specific T-cells in targeting tumor heterogeneity. While relapsing tumors in NRG mice do express NKG2D ligands, their expression level is significantly lower than the same cells cultured *in vitro* (**Figure 5B**). It has previously been observed that cytokines produced *in vivo*, namely types I and II IFNs, downmodulate NKG2D ligands on 3'-methylcholanthrene induced tumors[175]. Furthermore the use of a rhabdovirus as a boosting agent may have further compromised the *in vivo* expression of NKG2D ligands. While it has been shown that VSV downregulates NKG2DL on infected cells[176] we originally reasoned that the actual number of tumor cells remaining infected long term and also surviving infection would be negligible. However others in the Wan group have characterized rhabdoviruses as extremely potent inducers of IFN. Thus rhabdovirus infection may downregulate NKG2DL even on non-infected cells through signals initiated in infected cells. This reveals a potential drawback to the use of VSV as a boosting agent and in cancer therapies in general if the exorbitant amount of IFN generated by infection negatively acts on antitumor immunity. This area is under active investigation by others in our lab. While experiments were undertaken to use different boosting vectors none were successful. The use of an adenoviral boost only infrequently achieved tumor regression (**Figure S3**) while the use of vaccinia expressing mERK led to the rapid collapse of transferred cells in the DUC18/CMS-5 model (work performed by Dr. Scott Walsh, data not shown). Because of these factors, work was continued with rhabdoviral boosting.

Regardless of *in vivo* reduction in NKG2DL expression, the use of dual-specific T-cells led to an extension of remissions in the CMS-5-bearing NRG model (**Figure 5C**). This was observed despite comparable numbers of mERK-responsive T-cells in the blood (**Figure 5D**). Indeed across multiple repetitions of the experiment illustrated in **Figures 5B & C**, transduced cells continually produced more frequent and longer remissions with comparable or lower numbers of activated cells than observed in untransduced controls (data not shown). Taken together these data suggest a benefit to dual-antigen targeting which could potentially be curative in some cases. Therefore, in a context where the second antigen is expressed on the tumor, dual-antigen targeting appears to be effective at increasing therapeutic potency and extending remission as hypothesized. This serves as rationale for inclusion of built-in mechanisms to address tumor heterogeneity in immunotherapies as they transition to the clinic.

The inclusion of a luciferase reporter gene in the transduced cells allowed for the tracking of the cells *in vivo*. Primary localization of the cells was observed to shift between secondary lymphoid organs and tumor (**Figure 5E**). This suggests a partial explanation for the lack of on-target-off-tumor toxicities observed in this study as it seems likely that activated T-cells were mostly restricted to the tumor or areas with limited NKG2DL expression. This could prove extremely relevant as treatment associated autoimmune toxicities represent a serious concern for T-cell therapies[107,108]. The use of an OV capable of recruiting immunotherapies to the tumor could therefore prove valuable whether or not it is being used as a boosting agent.

Despite the success of the NRG model in demonstrating the efficacy of dual-specific T-cells in prolonging remission, this model has important limitations. The lack of an endogenous adaptive immune system impairs the ability of these animals to clear the boosting virus. Ultimately this results in neurotoxicity which would not be observed in immunocompetent hosts.

Thus the long term observation of remission in these mice is not possible making it difficult to suggest the treatment is curative and thus to fully assess the hypothesis. For this reason we sought to develop a wild-type model in which to apply dual-specific T-cells. While CMS-5 tumors reliably relapse in NRG mice this phenomenon is not observed in wild-type BALB/c mice. Work in our lab has demonstrated that this is due to the development of an endogenous immune response over the course of therapy (manuscript in preparation). Although this is true in the CMS-5 model, antigen-negative relapse is observed in preclinical work[136] and clinical trials[134,135] making the development of antigen-negative relapse models desirable. Furthermore clinical tumors are inherently heterogeneous[177] and it is unlikely that all cells within a neoplasm will express the same target antigen.

Two distinct but complimentary approaches were thus undertaken to assess the ability of dual-specific T-cells to address heterogeneous antigen expression by a tumor in the context of ACT + OVV. In the first experiment, bilateral CMS-5 tumors were used in wild-type BALB/c mice wherein one tumor was composed of parental cells while the other was composed of the mERK negative CMS-5r cell line. While IVIS was able to show the presence of transduced T-cells in both tumors, albeit at lower levels in the mERK-negative tumors (**Figure 6A**), this did not translate into therapeutic benefit against the CMS-5r tumors (**Figure 6B**). Interestingly CMS-5r cells isolated from these WT BALB/c mice appeared not to appreciably express NKG2DL *in vivo* despite the same tumors expressing these ligands more strongly in NRG mice (**Figure 6C**). This would imply that T-cell persistence in the tumor is not entirely antigen dependent or that low level NKG2DL expression is enough to hold cells in the tumor.

There are a variety of explanations for the discrepancy in NKG2DL expression. For example further downregulation of ligands based on cytokine environment that is distinct between NRG

and WT mice is possible. Furthermore NK-cell mediated immunoediting present in WT but not NRG mice could have put a selective pressure on the growing tumor, dictating subsequent expression of NKG2DL. Indeed spontaneous tumors established in wild type mice as opposed to NKG2D (receptor) deficient mice have been shown to exhibit lower levels of NKG2D ligands although they occur less frequently[178]. This would heavily imply a role of NKG2D in immune editing. Given that tumors do still occur in NKG2D competent animals as well as the documented tumor mechanisms for downregulating NKG2DL[179–181] it is possible that in the immune competent models used here, tumors were edited to limit NKG2DL expression which would explain the lower expression of these ligands and failure of therapy targeting NKG2DL to show appreciable benefit in wild type animals. The lack of benefit would also imply that NKG2DL-mediated targeting of the TME (rather than tumor cells directly) is not sufficient to prevent relapse in the immune competent systems employed here (preliminary data does show expression of NKG2DL on infiltrating immune cells, see **Figure S2C**). Nevertheless successful NKG2DL-targeting has been used in other models of CAR therapy implying that some established tumors do retain expression of NKG2DL despite an immunocompetent host[144,147–149].

Collectively the RenCa lung metastasis and bilateral tumor experiments identified some challenges to employing NKz10-CAR transduced DUC18 cells in a WT BALB/c background. Firstly, persistence of CAR-transduced cells appears to be compromised relative to untransduced cells. Whether this is due to antigen engagement or inherent susceptibility of CARs to AICD is unknown. Furthermore, expression of NKG2DL *in vivo* appears lower that *in vitro* at least in some tumor models. This effect may be exacerbated in WT relative to immunodeficient mice. Thus a strategy to enhance the functionality of these dual-specific cells in the wild-type context

would have to address both the persistence of transduced cells as well as the expression of their target *in vivo*.

Blocking of histone deacetylases (HDACs) has recently demonstrated promise both for its direct anticancer activity and enhancement of other therapeutic strategies including immunotherapy[182,183]. Acetylation impedes the binding of histone proteins to DNA and results in in increased transcription while HDACs restrict this process. By limiting access of the transcriptional machinery to genes involved in cell cycle regulation, apoptosis and a variety of tumor suppressive pathways HDACs can foster neoplastic development[182]. Indeed it has been demonstrated that the administration of HDAC inhibitors (HDACis) can promote the upregulation of NKG2DL on a variety of tumor samples[165,184]. Furthermore HDACis have been employed for their direct activity on immune cells in the context of immunotherapy[183]. Notably one study demonstrated that a variety of HDACis resulted in lower levels of FasL on tumor infiltrating CD4+ T-cells which correlated with better cell survival and reduced tumor burden[185]. Thus HDACis have the potential to address both the issues which were hypothesized to be limiting the efficacy of the NKz10-transduced cells *in vivo*.

For these reasons, moving into the chimeric tumor model, mice were co-treated with an HDACi, MS-275 (Etinostat). Tumors were made of parental CMS-5 cells with a small number of mERK-negative RenCa cells were treated with transduced or untransduced DUC18 T-cells in the ACT + OVV regimen co-administered with MS-275 (**Figure 7A**). MS-275 did indeed improve the persistence of both transduced and untransduced cells and activated Thy1.1 T-cells were observed in the blood as many as 21 days following boosting in both groups (**Figure 7B**). In the conventional ACT + OVV protocol we observe a peak response at Day 5 followed by rapid contraction by day 12 (manuscript in preparation). Despite the improved persistence of the

transferred cells, relapse was ultimately observed in both groups (**Figure 7C**). Analysis of the CD45 negative cell compartment within the tumor demonstrated that tumor cells did not appreciably express NKG2DL in either treatment group (**Figure 7D**). Thus this data suggests that co-targeting of NKG2DL in addition to the primary therapeutic target was insufficient to mitigate relapse in this particular model and a lack of NKG2DL expression could be involved to some extent. While the use of NKG2DL-targeting CARs has been shown to control NKG2DL expressing tumors *in vivo*, most of these results were observed in cell lines on a C57BL/6 background[144]. Furthermore variability in NKG2DL expression on established tumors has also been observed *in vivo*[145] and it is thus possible that NKG2D plays a protective but sculpting role in many models of cancer[186].

Although absence of NKG2DL expression on the tumor represents a likely explanation for the failure of these dual-specific to mediate long term protection in chimeric tumor models, other possibilities cannot be excluded. The capacity of NKz10-transduced cells to mediate NKG2DL-dependent killing *in vivo* following boosting could also be called into question. While the results obtained in the NRG experiments (see **Figure 5**) imply that the CAR is indeed functional following boosting, this has yet to be shown *in vivo* in a context where CAR killing alone is assayed. While such experiments were undertaken they were compromised by the poor tumor control associated with Ad-mERK boosting in BALB/c mice (see **Figure S3**). Such experiments would also require tumor cells which unambiguously and reliably express NKG2DL *in vivo.* One option would be to carry such experiments out in the context of well-established NKG2D CAR-models in C57Bl/6 mice however differences in the transducibility of transgenic mice on this background compromised initial efforts in this area (see **Figure S1**). Thus screening of various tumor lines for *in vivo* expression of NKG2DL and their responses to both MS-275 and infection

with VSV represents an important next step. Stimulation of T-cells from peripheral blood of mice treated in the transduced groups with NKG2DL-expressing tumor cell lines was also undertaken but was not successful. This however could be caused by a need for optimizing cell-based stimulation of *in vivo* isolated T-cells and should not in itself be taken to imply that no NKG2DL-responsive cells were expanded *in vivo*.

While the lack of durable cure in this chimeric tumor model was disappointing the effects of MS-275 in prolonging survival of transferred cells could represent a finding of great interest to the field. Indeed disappearance of transferred cells prior to tumor eradication has been attributed to incomplete regressions in the clinic[187,188] and strategies to extend T-cell lifespan should be of great interest. Because MS-275 also produces lymphodepletion it would be conceivable that part of the impact of the drug on transferred T-cells comes as the result of reduction in competition for homeostatic cytokines[164,189]. To gain insight into this phenomenon out adoptive transfer experiments were carried out in RAG KO mice which lack endogenous lymphocytes. Even in this context, MS-275 resulted in increased activity of transferred cells (**Figure S4**) implying effects on T-cell biology beyond elimination of resource completion. This therefore represents an intriguing area for future exploration.

When considered as a whole the results presented here provide preliminary encouragement for the use of dual-specific T-cells in the ACT + OVV regimen although they call into question NKG2DL as a broad target for *in vivo* work in tumor immunotherapy. The transduction protocol developed here provides a useful strategy with which to achieve more reliable transduction of T-cells with a memory phenotype and demonstrate their *in vitro* dual-specific responsiveness. The results obtained in NRG mice are encouraging for the ability of dual-specific T-cells to prolong remission and eliminate antigen-negative variants *in vivo* but the limitations of this model

precludes the long-term analysis of cure. That the results in wild type mice failed to replicate the successes in the NRG mice could be reflective of poor NKG2DL expression in these models. NKG2DL expression *in vivo* on non-immune cells within the tumor was not detected which could suggest that these ligands are not as ubiquitously expressed as thought at least in the models explored here. The use of pharmacological strategies to improve the survival of transduced T-cells *in vivo* is however encouraging and may generate more widespread interest in the field. The combination of these strategies with other tactics to increase NKG2DL expression *in vivo* or if they were used in the appropriate model may indeed allow for proof of principle for the use of dual-specific T-cells in the ACT + OVV regimen. Data exists suggesting that NKG2DL may still represent a good target in many human tumors[144] and with clinical trials currently targeting these ligands (NCT02203825) it could be that the poor-expression of NKG2DL on BALB/c tumors observed here will not be reflective of what is seen in the clinic. Alternatively targeting of a different second antigen whether it is part of the tumor directly or a robustly expressed component of the microenvironment may prove more successful. As such the results and methodologies presented in this dissertation may serve as a framework for a variety of future works and specifically for those investigating the use of dual-specific T-cells in protocols such as the ACT + OVV regimen.

Chapter 5

Supplementary Figures

5. Supplementary Figures

The following section consists of standalone experiments which either were not successful to the extent that they fit in the primary story told here or represented a different direction from the focus of the thesis. Since they may nevertheless prove useful and informative they have been included in the supplementary section that follows.

5.1 Transduction with the NKz10 CAR is further compromised in P14 mice but rescued in P14IFNKO

Since the bulk of the literature using *in vivo* mouse models to eliminated NKG2DL positive tumors was performed on a C57Bl/6 background, it would be ideal to employ these models to gain insight into the functionality of the NKz10 CAR following boosting. To this end, transduction of P14 splenocytes to generate memory T-cells with a TCR recognizing the LCMV gp33 peptide was attempted. Since we have available B16F10 melanoma cells expressing gp33 as well as a variety of boosting viruses (VSV, VacV and adenovirus) which also express this antigen, successful transduction of P14 cells would pave the way for a variety of experiments interrogating the use of dual-specific T-cells targeting NKG2DL and a TSA.

In P14 T-cells, a similar trend to that which was observed in DUC18 cells was seen. Culture with memory cytokines resulted in lower transduction than effector cytokines however the overall transduction efficiency was drastically reduced by comparison to the DUC18 cells (**Figure S1A**). The difference in transducibility of DUC18 and P14 cells could in part be attributable to strain specific differences that were previously documented. Indeed VanSeggelen et al observed reduced transduction efficiency of C57Bl/6 derived T-cells relative to BALB/c cells however the difference was not as striking as that which was observed in the present

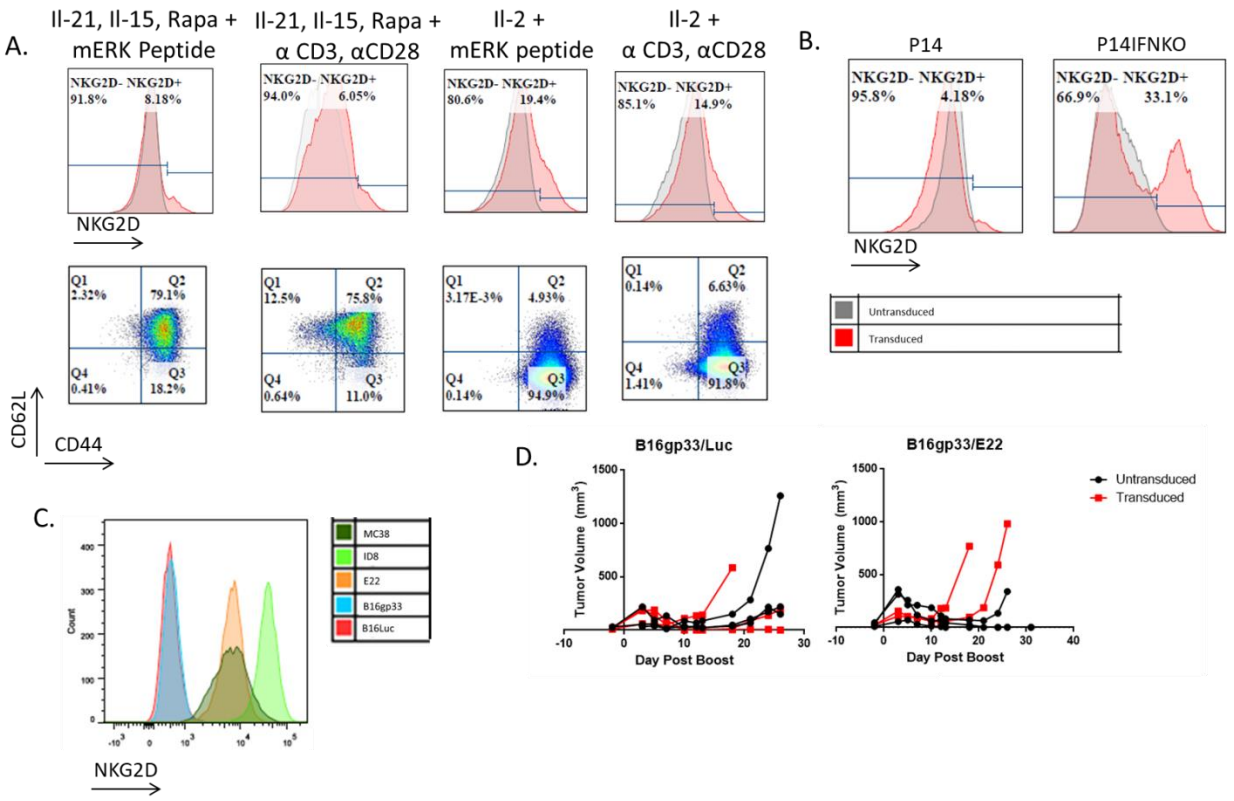

Figure S1. Characterization of NKz10 Transduction and preliminary *in vivo* work on a C57Bl/6 background. A. Transduction efficiency of P14 cells is decreased relative to results obtained for DUC18 but follows similar trends with regards to cytokine environment. P14 splenocytes were isolated from a euthanized mouse and cultured with indicated cytokines and peptide or antibody based activation. Transduction with NKz10 retrovirus was performed two days after activation and assessed following 5 more days in culture using staining for the NKG2D receptor. Staining for CD62L and CD44 was also performed as an indicator of phenotype. **B.** The transduction experiment was repeated using Il-21, Il-15 and rapamycin with peptide-based activation using P14 or P14IFNKO mice. **C.** Expression of NKG2DL on *in vitro* cultured tumor cells of a C57Bl/6 background was assayed by flow cytometric staining for NKG2DL and viability. **D.** Transduced P14 cells boosted with VacVgp33 were used to treat chimeric tumors consisting of B16gp33 cells with small amounts of B16 Luciferase or E22 cells. Tumor volumes are displayed on a per mouse basis.

work[153]. The greater difference could be attributable to the transgenic nature of the T-cells employed in this study or other as yet unknown factors. This difference however was consistent over multiple experiments.

Interestingly P14 T-cells, unlike those from DUC18 mice, were resistant to transduction even in their naïve state (data not shown) further supporting the idea of strain-specific differences. Fortunately our group has available P14IFNKO cells and we wondered whether knockout of the IFNAR receptor would alleviate some of the potential innate antiviral factors limiting transduction of P14 cells. Indeed P14IFNKO cells displayed almost 8 times greater transduction on day 2 following culture than their wild-type counterparts (**Figure S1B**). Culture with an IFN blocking antibody was not able to recapitulate this effect in wild-type DUC18 or P14 cells however. This could be indicative of incomplete blocking when employing the antibody or unanticipated effects of the IFNKO genotype.

To avoid the confounding factor of T-cell non-responsiveness to type I IFN, preliminary experiments in C57Bl/6 mice were conducted using low-level transduced P14 cells. Bl/6 tumor cell lines were first screened for NKG2DL expression. In accordance with previous reports, the B16F10 line did not express NKG2DL[190] however this target was expressed by MC38 colon adenocarcinoma, E22 (a derivative of EL-4) lymphoma and ID8 ovarian carcinoma (**Figure S1C**). Chimeric tumors were then generated using 1E5 B16gp33 expressing cells combined with either 100 B16F10 cells expressing luciferase or 100 E22 cells and grown for one week prior to treatment with VacVgp33 boosted transduced or untransduced memory P14 cells. The goal of this experiment was to determine whether a small population of antigen negative cells could be eliminated using the TME-targeting capacity of the NKG2D-CAR when the target cell line did not actually express this ligands (B16F10-Luciferase) or if expression of NKG2DL on the tumor

cell target itself was needed (E22). Unfortunately relapse was observed in both instances and there was no clear benefit to using dual-specific cells in either context (**Figure S1D**). Furthermore this regimen as well as NKz10-transduced P14 cells injected intraperitoneally was not able to reduce the number of peritoneal tumors on mice injected I.P. with 2E7 ID8 cells one week prior to treatment (data not shown). Although these represent pilot experiments with minimal mice per group, the lack of obvious benefit in any of these trials suggests that these cells are not effective with the current level of transduction.

5.2 ACT + OVV using antibody-activated, NKz10-transduced DUC18 cells boosted with MRB-mERK does not prevent antigen-negative relapse in CMS-5/CT26 chimeric tumors

Because antibody mediated activation resulted in greater transduction efficiency for memory cultured DUC18 T-cells they were initially used to explore the treatment of chimeric tumors comprised of 1E6CMS-5 cells mixed with 1000 CT26 cells established for one week. While both NKz10-transduced and untransduced cells boosted with MRB-mERK resulted in initial tumor regression and performed better than a virus alone control, there was no distinguishable difference between the transduced and untransduced groups (**Figure S2A**). Interestingly wild-type BALB/c cells transduced with the NKz10 construct did produce some delay in tumor growth compared to the virus alone control which could be taken to imply some benefit to targeting NKG2DL in this model (**Figure S2A**). While the transduced DUC18 cells displayed expansion following boost as evidenced by an increase in luciferase signal on day 4 from day 1, this was not observed at significant levels in the cells of wild-type origin (**Figure S2B**). This stands to reason as only cells with mERK responsiveness should be expanded by the virus. A dramatic drop-off in transduced cell persistence between days 4 and 6 was also observed

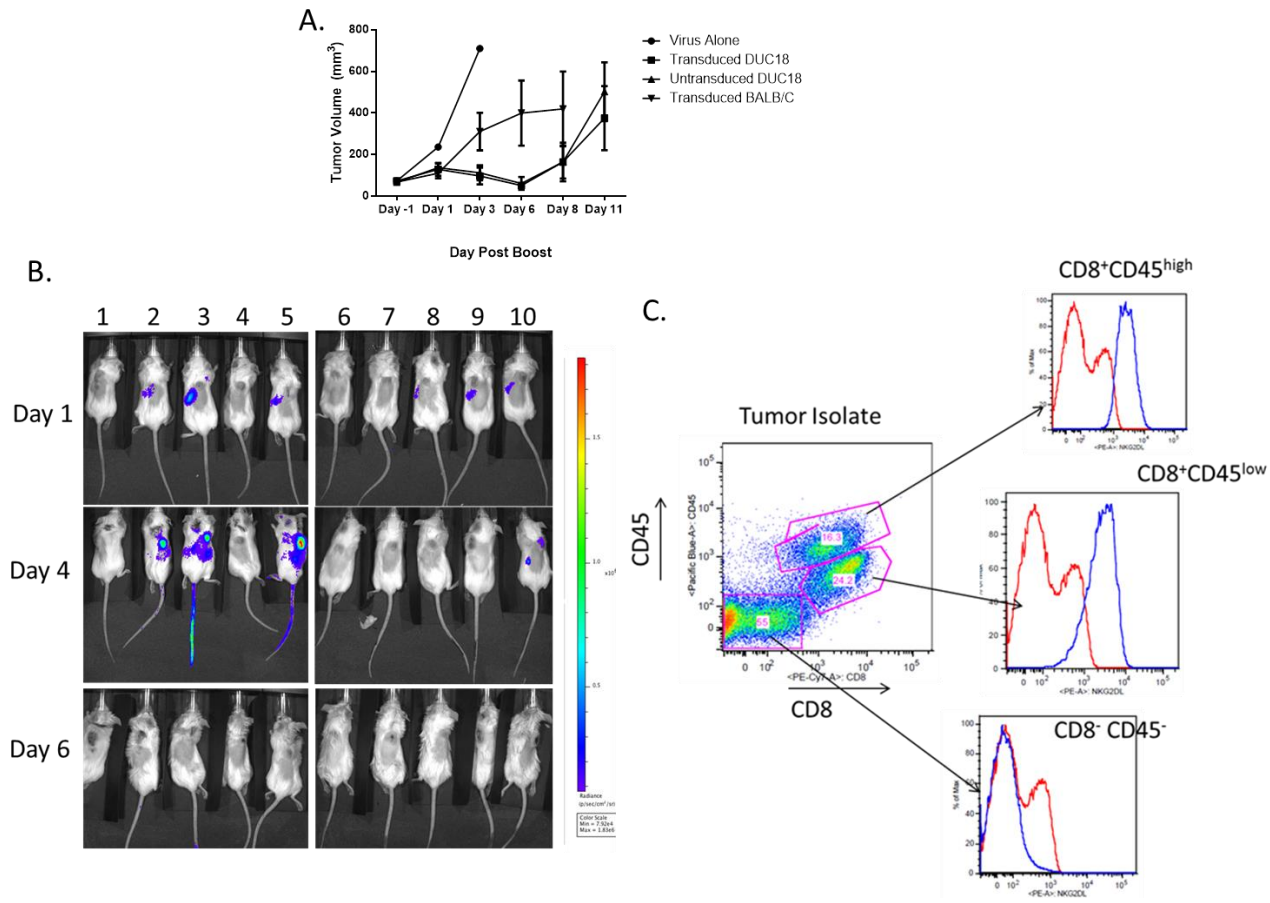

Figure S2. Treatment of CMS-5/CT26 chimeric tumors with antibody-activated, memory-cultured DUC18 T-cells boosted with MRB-mERK. A. BALB/c mice bearing 1-week established tumors from 1E3 CT26 cells in 1E6 CMS-5 cells were treated with MRB-mERK or MRB-mERK in combination with antibody-activated, memory cultured DUC18 cells (+ or – transduction) or transduced WT BALB/c T-cells. Tumor volumes were recorded as a function of time. (Tumor D -7, T-cells D -1, virus D0) **B.** IVIS imaging of mice on days 1, 4 and 6 were used to trace transduced cells. 1 – virus alone control; 2,3,5 – transduced DUC18 cells, 4,6,7 – untransduced DUC18 cells; 8,9,10 – Transduced BALB/C cells. Exposure was done for 10sec on days 1 and 4 and 2min on day 6. **C.** Relapsing tumor was excised, processed, stained for NKG2DL as well as CD45 and CD8a and run on flow cytometry to determine NKG2DL-expression on different components of the tumor.

regardless of wild-type BALB/c or DUC18 origin (**Figure S2B**). Although this drop-off is seen in our standard protocol it was much more rapid in this model. This could be attributable to the boosting vector used (the VSV typically used in BALB/c mice is wild-type whereas the Maraba virus used herein was the MG1 mutant) as well as the antibody-mediated activation strategy. The Maraba virus vector was used in hopes of establishing it as another vector useable in our therapy however follow-up work in our group has demonstrated that while it may be functional in boosting memory T-cells *in vivo* the response produced by it is distinct from what we typically see with wild-type VSV (data not shown). Furthermore it is possible that the antibody-mediated activation provided a signal of greater strength than the typical peptide-based activation. This could result in a more effector memory (as opposed to central memory)-like phenotype and impaired persistence of the transferred T-cells. For these reasons experiments performed in wild-type BALB/c mice after this trial involved the use of VSV-mERK and the subsequently developed transduction before activation protocol described in **Figure 2C**.

In an effort to further dissect the failure of this therapy to eliminate antigen-negative variants, relapsing tumors were isolated from euthanized mice and processed for flow cytometry. Staining of CD45, CD8 negative populations demonstrated that these cells did not express NKG2DL strongly relative to unstained control. Interestingly CD45 fractions which divided into CD8 high and low subpopulations both expressed NKG2DL implying that at least some infiltrating immune cells do express NKG2DL in this model (**Figure S2C**).

5.3 Boosting with adenovirus results in T-cell expansion but does not produce consistent and significant delay in tumor growth in the contexts used here.

Because of the potential for VSV to directly downregulate NKG2DL directly[176] or as a result

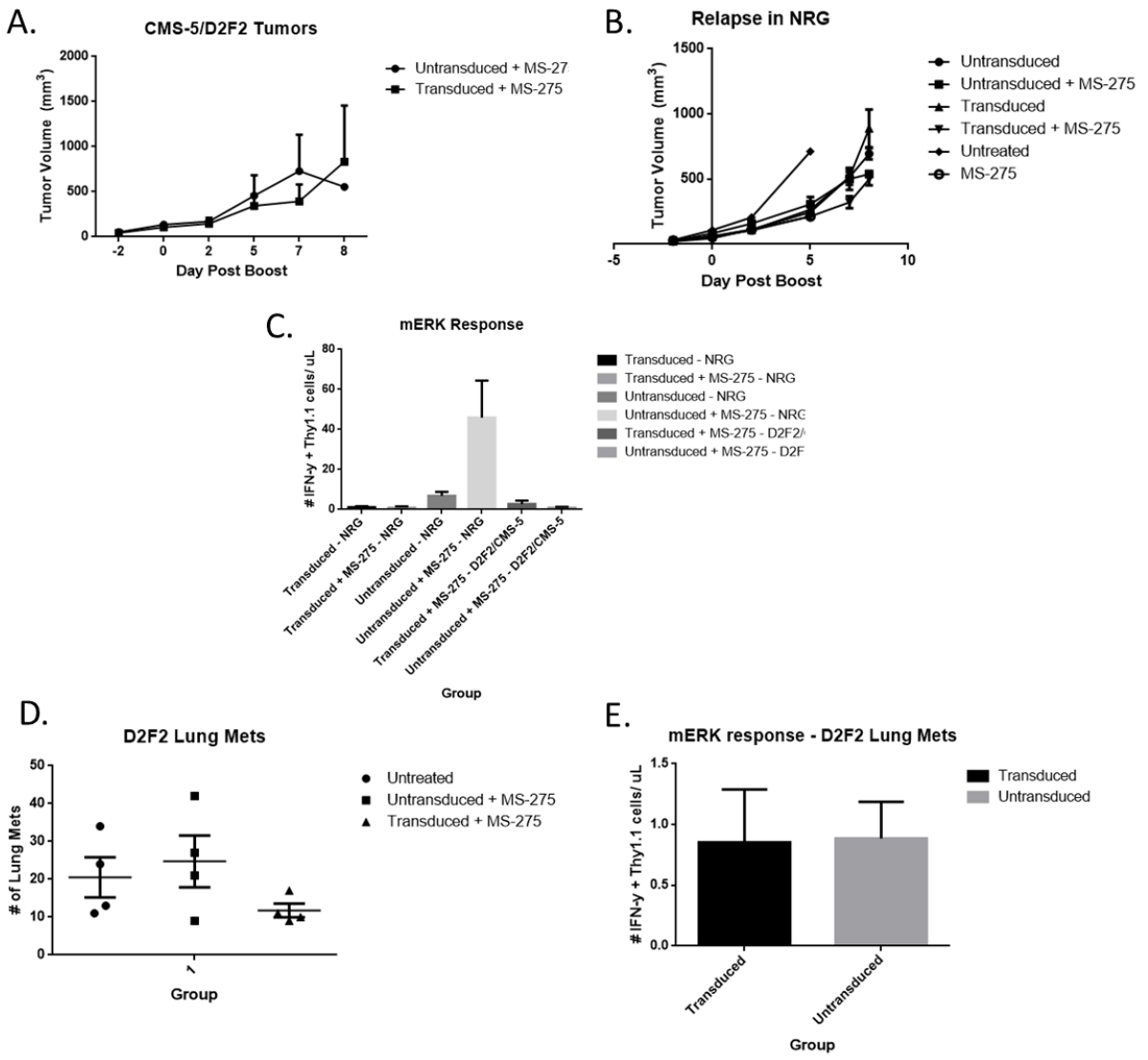

Figure S3. Adenoviral boosting leads to marginal tumor control regardless of additional NKG2DL targeting in various models of cancer. A. Chimeric tumors generated from 1E6 CMS-5 cells with 100 D2F2 cells were established for one week in BALB/c mice and then treated with Ad-mERK boosted NKz10-transduced or untransduced DUC18 T-cells with co-administration of MS-275. Tumor volumes as a function of days post boosting are shown. . (Tumor D -7, T-cells D -1, virus D0) **B**. mERK-negative CMS-5R cells were used to establish tumors in NRG mice and treated with Ad-mERK boosted NKz10-transduced or untransduced DUC18 cells with or without co-administration of MS-275. Untreated or MS-275 alone mice were included. Tumor volumes are displayed as a function of time. **C.** mERK-responsive cells in peripheral blood of mice receiving adoptive transfer in (A) and (B) were quantified using mERK-stimulation followed by ICS for IFNγ. **D.** D2F2 lung tumors were established in BABL/c mice by I.V. injection of 1E5 D2F2 cells 5 days prior to treatment. Subsequently mice were either not treated or treated with Ad-mERK boosted NKz10-transduced or untransduced DUC18 T-cells in combination with MS-275. Metastasis were counted on day 20 following the establishment of tumors. **E.** mERK-responsive Thy1.1 T-cells were quantified in the blood of mice treated in (D) on day 5 using ICS for IFNγ.

of high amounts of IFN produced upon infection[175] a set of *in vivo* experiments was conducted using Adenovirus as a boosting vector. In chimeric tumors composed of CMS-5 and D2F2 cells (D2F2 cells were used based on results presented in a previous study[191] and because other cell lines used to this point did not seem to express NKG2DL in BALB/c mice *in vivo*), Ad-Merk boosted DUC18 cells did not reliably result in tumor regression regardless of transduction (**Figure S3A**). Although one tumor did completely regress in the transduced group and is being monitored for relapse another tumor in the same group did not obviously respond while the other demonstrated only a slight delay in growth. In an experiment conducted in parallel, NKz10-transduced DUC18 cells were used following Ad-mERK boosting as a treatment for mERK-negative CMS-5R tumors in NRG mice. There was at best a minor delay in growth using transduced cells (**Figure S3B**) and tumors rapidly grew to endpoint size in all instances. Because of the poor response in the CMS-5/D2F2 tumors however it is difficult to conclude anything from this negative result since it could simply be the result of poor antitumor efficacy of Ad-mERK boosting at least at the doses used here. Across both experiments the number of activated T-cells was in general small with the exception of the untransduced group co-administered with MS-275 in the NRG mice (**Figure S3C).** Interestingly the administration of MS-275 appeared to negligibly improve the lifespan of transduced T-cells following Ad-mERK boosting in any of these experiments (**Figure S3C**).

In a parallel experiment, Ad-mERK boosted NKz10 transduced and untransduced DUC18 cells were used to treat D2F2 lung metastases along with co-administration of MS-275. A very marginal benefit to using transduced cells was observed and this could be subject to inherent variation in the lung metastases experiments (**Figure S3D**). The numbers of activated T-cells at day 5 was relatively consistent between groups but was low compared to previous

experience in terms of absolute number (**Figure S3E**). T-cells expressing Thy 1.1 were difficult to detect by day 13 (data not shown) despite the administration of MS-275.

Collectively these data argue against the use of Ad-mERK, at least at this dose, as a boosting vector in this model.

5.4 The effects of MS-275 extend beyond lymphodepletion.

In order to delve further in to the effects of MS-275 on transferred T-cells, an ACT + OVV experiment was carried out in RAG knockout mice which lack an endogenous T and B-cell compartment. Lymphodepletion is commonly used in ACT protocols to limit endogenous lymphocytes competing with transferred cells for cytokines and fuel[164,189]. Because lymphopenia is induced by MS-275, we reasoned that this effect of the drug could be partially or entirely responsible for the improved T-cell activation and persistence observed in our mouse models. To investigate this possibility RAG knockout (RAG KO) mice were used to carry out adoptive transfer of DCT (dopacrhome tautomerase)-specific T-cells with a memory like phenotype and boosting with VSVdM51-hDCT with and without co-administration of MS-275 to treat B16F10 tumors (which express DCT). Since the RAG KO mice lack an endogenous T-cell and B-cell compartment any effects observed by MS-275 in this context could be attributed to factors other than lymphodepletion. Indeed significantly higher numbers of activated CD8+ T-cells responding to DCT were observed five days post boosting in the MS-275 co-treated group, implying factors beyond lymphodepletion were at play. This response normalized by day 12 (at which point tumors were absent on cured mice) (**Figure S4A**). Tumors regressed in both groups although regression was more widespread in MS-275 co-treated mice with all mice in this group maintaining regression while one of four in the group not treated with MS-275 displayed only

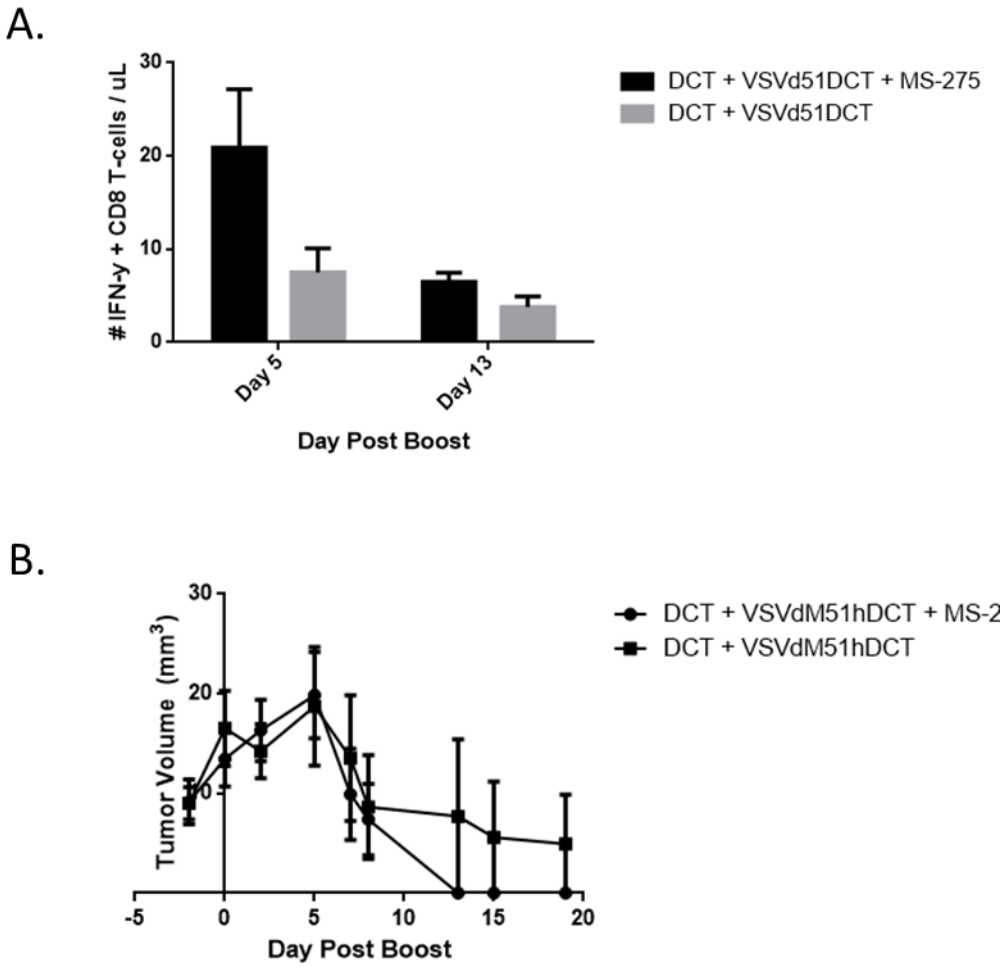

Figure S4. The beneficial effects of MS-275 on transferred T-cells extend beyond lymphodepletion. DCT-specific T-cells were administered to RAG KO mice lacking an endogenous adaptive immune system and bearing B16F10 tumors. Boosting was performed using a VSVdM51 vector expressing hDCT followed by 5 days with or without injection of MS-275. Tumor was given D -4, followed by Tcm D-1 and virus D0 **A.** Live CD8+ T-cells producing IFNγ were quantified by flow cytometry on days 5 and 13 post administration of virus. **B.** Tumor volumes were recorded as a product of length, width and height and were plotted as a function of days following boosting.

partial

regression (**Figure S4B**).

5.5 Applications of dual-specific T-cells as a universal boosting TCR

In addition to work on the NKG2D project the use of dual-specific T-cells in the context of a universal boosting TCR was also explored. While the ACT + OVV regimen represents an attractive therapeutic strategy it could become costly to manufacture T-cells and viruses on a patient by patient basis and therapies that are "off the shelf" are attractive in this regard. That being said ACT strategies have been lauded for their ability to act as personalized medicines[87]. The ideal therapy would thus be off the shelf in terms of manufacture but personalized to an individual's cancer. As a means of achieving these diverging goals we proposed the use of a universal boosting TCR. In this strategy tumor specific T-cells would be isolated from a patient. The endogenous antigen specificity of these T-cells need not be known so long as they responded to a tumor antigen. These cells would then be transduced with a TCR that, while matched to a patient's haplotype, could be used for any patient with the proper haplotype and used with a universal boosting OVV expressing the cognate antigen of the transduced TCR. This would enable the use of virtually any population (or populations) of tumor specific T-cells such as TILs to be used in the ACT + OVV regimen (**Figure S5A**).

As proof of principle for this strategy, we transduced P14 cells cultured for a memory phenotype with an OVA-reactive TCR. While the transduction efficiency was only marginal this enabled us to adoptively transfer these cells and boost them *in vivo* with a MG1-OVA vector to treat B16F10 tumors expressing gp33. This resulted in temporary tumor control relative to mice treated with untransduced P14 cells and MG1-OVA (**Figure S5B**). While tumor regression was not complete this could be due to the vector used or

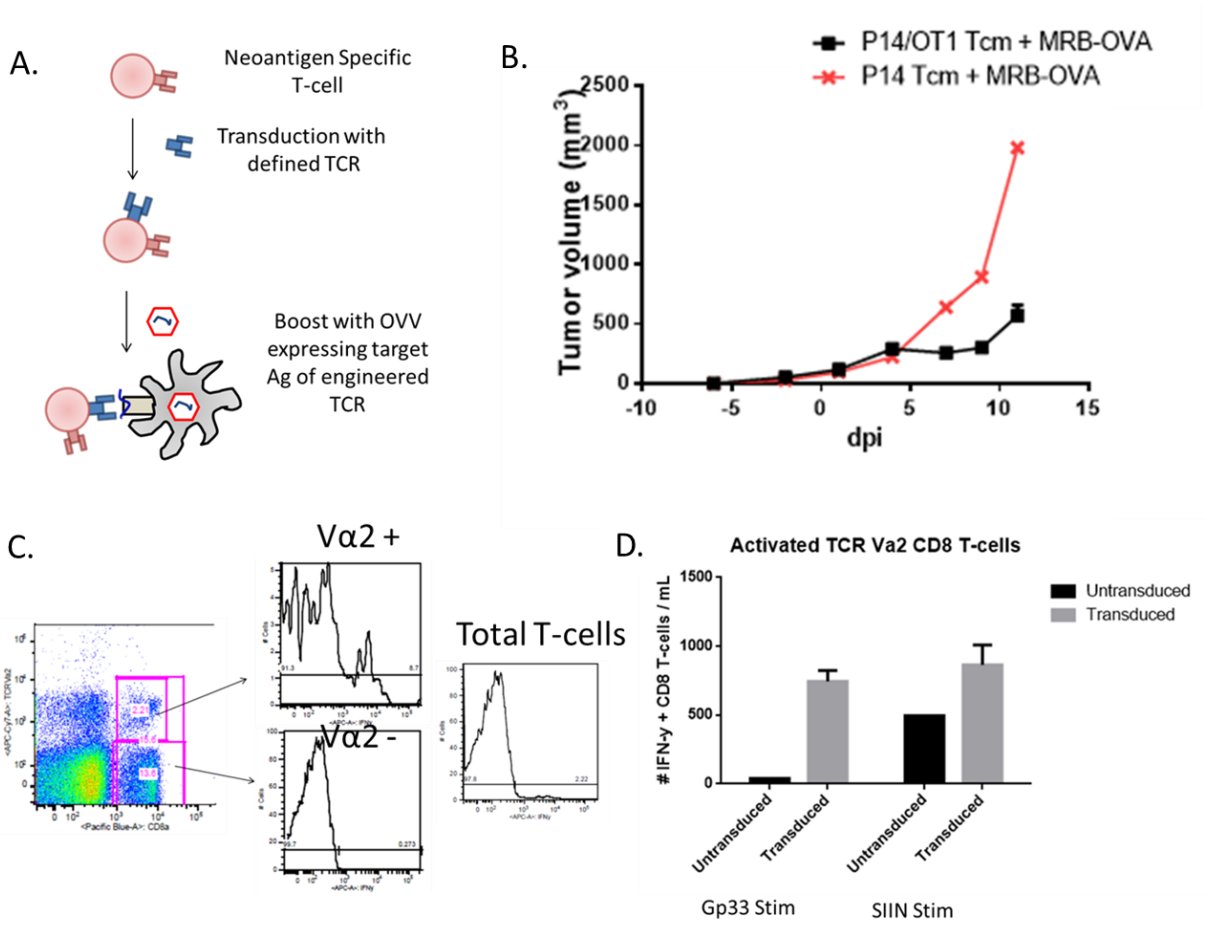

Figure S5. Concept and proof of principle for the use of dual-specific T-cells in a universal boosting TCR regimen. A. Concept of the universal boosting TCR. In this methodology tumor-specific T-cells targeting an antigen with a known or unknown identity are transduced with a defined TCR. Boosting is carried out *in vivo* using an OVV-expressing the target of the transduced TCR in order to achieve responsiveness through the endogenous TCR. **B.** Preliminary results using a model of a universal boosting TCR. P14 T-cells specific for the LCMV gp33 epitope were transduced with a TCR targeting the OVA antigen. These cells were used in combination with a MRB-OVA boosting vector to treat B16F10 cells engineered to express gp33. While no tumor control was observed using untransduced cells, the group receiving P14 cells transduced with the OVA TCR demonstrated transient tumor control. (Tumor D -6, Tcm D-1, virus D0) **C.** Intracellular cytokine staining to identify gp33 responsive cells in a mouse receiving OVA TCR-transduced P14 cells boosted with MRB-OVA. Peripheral blood was isolated via retro-orbital bleed and T-cells were stimulated with gp33 peptide. Staining for the Vα2 TCR was used as a marker for P14 cells. **D.** # of IFNγ-producing T-cells in peripheral blood were quantified via ICS in response to stimulation with either gp33 or SIIN in mice receiving OVA TCR-transduced or untransduced T-cells with MRB-OVA boosting.

the low level of transduction on the cells employed here. We further showed that in mice treated with the MRB-OVA boosted transduced P14 cells, gp33 responsiveness was indeed from cells bearing a Vα2 TCR (the gp33-reactive TCR on P14 cells is Vα2) (**Figure S5C**). Interestingly T-cells reactive to the SIIN peptide target of the OVA TCR were observed in both mice receiving transduced or untransduced P14 cells while only mice receiving the transduced cells displayed responsiveness to gp33 (**Figure S5D**). This could be reflective of endogenous cells primed by the MRB-OVA vaccination, especially since OVA is a relatively immunogenic target.

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