COOPERATION BETWEEN ENDOGENOUS LYMPHOCYTES AND ACT

ENDOGENOUS LYMPHOCYTES PLAY A CRITICAL ROLE IN THE ELIMINATION OF SOLID TUMORS IN THE CONTEXT OF ADOPTIVE CELL THERAPY COMBINED WITH ONCOLYTIC VACCINATION

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Lay Abstract

Current approaches to the T cell therapy of cancer are hindered by poor cell quality. It is simple to grow higher quality T cells, but it is difficult to grow very large numbers of them. Furthermore, higher quality T cells need a signal in order to “switch on” before they can start killing cancer cells. Here, we use a cancer-targeting virus as a signal for these cells to activate, grow to very large numbers in the patient, and destroy their tumor. Our vaccine also switches on other immune cells in the patient, which help guarantee the destruction of the tumor. The significance of this work is that it will improve T cell therapy for cancer by opening the possibility of using higher-quality T cells which are much better at killing cancer than the currently used type of T cells.

Abstract

A major obstacle in the implementation of adoptive cell therapy (ACT) for solid tumors is $CD8^+$ T cell quantity and functional quality. In order to address this issue, the ACT field has directed considerable effort toward the generation of less-differentiated memory T cells (Tm), which demonstrate superior effector function and engraftment over effector T cells. An obstacle in using Tm for ACT is their requirement for *in vivo* activation before full effector function can be acquired. We sought to determine if a rhabdovirus expressing a defined tumor antigen (i.e. a rhabdoviral oncolytic vaccine) could activate adoptively-transferred Tm *in vivo* and eliminate established tumors. We used *ex vivo* cultured DUC18 TCR-transgenic Tm combined with a rhabdoviral oncolytic vaccine to target established CMS5 fibrosarcomas in both balb/c and NRG mice, and we compared the efficacy of the combination treatment versus monotherapies. Our data demonstrate that the rhabdoviral oncolytic vaccine was capable of expanding adoptively-transferred Tm in order to eliminate established tumors. Furthermore, synergy between ACT and oncolytic vaccination was required for optimal therapeutic outcome. Interestingly, we observed a population of endogenous, tumor-primed lymphocytes which appeared to be required for complete tumor elimination and subsequent memory formation. This was in contrast to the current consensus in the ACT field which is that endogenous lymphocytes are detrimental to therapeutic outcome, thus necessitating lymphodepletion prior to the commencement of therapy. Our data suggest that endogenous lymphocytes may be a beneficial cell population which is overlooked by current approaches to ACT.

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List of Abbreviations

ACT – Adoptive Cell Therapy

APC – Antigen-presenting Cells

CAR – Chimeric Antigen Receptor

CD – Cluster of Differentiation

CTLA-4 – Cytotoxic T-lymphocyte Associated Protein 4

DAMPs – Danger-associated Molecular Patterns

DC – Dendritic Cell

dpi – Days Post-infection

ERK2 – Mitogen-activated protein kinase 1 (ERK2 is an alternate name of MAPK1)

FOXP3 – Forkhead Box P3

GM-CSF – Granulocyte-Macrophage Colony Stimulating Factor

GFP – Green Fluorescent Protein

IDO – indoleamine 2, 3-dioxygenase

IFN – Interferon

IL - Interleukin

LDLR – Low Density Lipoprotein Receptor

MAGE-A – Melanoma-associated Antigen

MDSC – Myeloid Derived Suppressor Cell

MHC – Major Histocompatability Complex

Mrb – Maraba Virus

mTORC1 – mechanistic target of rapamycin complex 1

NK Cell – Natural Killer Cell

NKG2D – Killer Cell Lectin-like Receptor Subfamily K, Member 1

NY-ESO-1 – New York - Esophageal Cancer -1

PBMC – Peripheral Blood Mononuclear Cells

PBS – Phosphate-buffered Saline

PD-1 – Programmed Death 1

PD-L1 – Programmed Death Ligand 1

pfu – plaque-forming units

TCR – T Cell Receptor

Tm – Memory T Cell

TME – Tumor Microenvironment

TNF – Tumor Necrosis Factor

TGF – Transforming Growth Factor

Treg – Regulatory $CD4^+$ T Cell

VEGF – Vascular Endothelial Growth Factor

VSV – Vesicular Stomatitis Virus

Declaration of Academic Achievement

I hereby declare that the research presented within this document was completed by myself, Boris Simovic, with the assistance of Dr. Lan Chen (assisted with NRG mouse experiments and intravenous tail vein injections when necessary), and Talveer S. Mandur (intravenous tail vein injections). I contributed toward the design of this study under the guidance of my supervisor, Dr. Yonghong Wan, and performed data collection and analysis.

Chapter 1: Background

1. Introduction and Rationale

Rhabdoviruses such as vesicular stomatitis virus and maraba virus are potent oncolytic agents due to their ability to selectively replicate in a variety of tumor histologies [1]. By targeting the ubiquitously-expressed low density lipoprotein receptor family, rhabdoviruses are capable of infecting a broad array of mammalian tumour histologies [2]. Such a broad host range and selectivity for malignantly transformed cells makes rhabdoviruses ideal candidates for cancer therapy vectors. Recombinant rhabdoviruses have been administered intravenously as well as intratumorally in pre-clinical models with impressive efficacy, and an even more impressive safety margin [3, 4, 5, 6, 7]. This safety profile is a result of the ability of recombinant rhabdoviruses to induce a potent type I interferon (IFN) response when they infect cells with intact interferon signalling pathways. In healthy tissues, a rapid type I IFN response protects the cells from viral infection which protects from collateral damage of non-malignant tissue. Conversely, a significant number of tumors across a broad range of histologies display defects in type I IFN signalling which renders them vulnerable to rhabdovirus-mediated infection and destruction [8].

Despite the power of oncolytic virotherapy it is highly unlikely that every cell within a tumor will be infected – even when very high doses of virus are used. Given enough time, the presence and unchecked growth of uninfected cells within the tumor will lead to eventual relapse. It has now become abundantly clear that oncolytic virotherapy must engage, and cooperate with, the patient's immune system if optimal therapeutic efficacy is to be achieved. In fact, the ability of rhabdoviruses to induce potent type I IFN responses demonstrates that they are very powerful immunomodulators. This property of rhabdoviruses offers the promise of

harnessing the patient's immune system and directing it to attack and eliminate established tumors [9, 10, 11] . In the context of heterologous prime-boost vaccination cancer immunotherapy we have previously demonstrated that by using a rhabdovirus engineered to express a defined tumor antigen (i.e. a "rhabdoviral oncolytic vaccine") to engage an existing tumor antigen-specific memory $CD8^+$ T cell population we were able to eliminate established tumors [12, 13, 14]. However, the efficacy of heterologous prime-boost vaccination therapy is highly dependent on the successful generation of a sufficient number of anti-tumor $CD8^+$ T cells by the priming vaccine, and this may not be equally effective in all patients.

Adoptive cell therapy (ACT) has recently emerged as a powerful immunotherapeutic paradigm for the treatment of a broad array of cancers [15]. ACT involves the isolation of $CD8^+$ T cells from a cancer patient's peripheral blood or surgically-resected tumor followed by *ex vivo* culture in order to expand their number and to confer effector function. Once a sufficient quality and quantity of anti-tumor $CD8^+$ T cells has been achieved, the resultant cellular product is infused into the patient. However, cultured effector $CD8^+$ T cells do not persist long enough to mediate tumor regression without pre-infusion lymphodepletion. Lymphodepletion is used to remove regulatory $CD4^+$ T cells and other endogenous lymphocytes which could outcompete the transferred cells for cytokines and growth factors [16]. Advances in *ex vivo* culture techniques and genetic engineering have made the generation of T cell receptor (TCR) transgenic $CD8^+$ T cells specific for a given tumor antigen readily available [17, 18]. The culture of $CD8^+$ T cells in the presence of defined combinations of cytokines and small molecule inhibitors has given researchers the power to alter T cell differentiation toward longer-lived, higher-quality memory T cell subsets [19, 20, 21, 22, 23, 24].

Despite these advances the requirement for lymphodepletion remains which, due to the physical toll taken by lymphodepleting agents such as chemotherapy and irradiation, limits the amount of patients capable of receiving this potentially curative therapy. Moreover, a very large dose of $CD8^+$ T cells is required by conventional ACT protocols (i.e. tens of billions of cells) in order to achieve desirable clinical outcomes. This serves as a further barrier to the success of ACT as *ex vivo* culture protocols for the generation of so many anti-tumor $CD8^+$ T cells require cells to be grown for as long as two months, which may inevitably result in terminal differentiation and replicative senescence of the T cells. Furthermore, during this time some patients may deteriorate to the point that they are no longer able to receive the therapy [15]. Finally, tumor infiltration and the immunosuppressive tumor microenvironment remain a challenge facing the successful implementation of ACT.

We have previously demonstrated that rhabdoviral oncolytic vaccination could engage and rapidly expand pre-existing central memory $CD8^+$ T cells, which results in increased tumor infiltration by activated T cells [14, 25]. Presently we sought to determine if rhabdoviral oncolytic vaccines were able to enhance the efficacy of ACT using *ex vivo* generated anti-tumor central memory $CD8^+$ T cells. Specifically we believed that a modest dose of central memory $CD8^+$ T cells could be sufficient for *in vivo* expansion by rhabdoviral oncolytic vaccination to achieve therapeutic benefit in animals bearing established tumors. This would overcome the requirement for lengthy culture protocols to generate the large doses of T cells used by current ACT protocols. This approach may also allow for lymphodepletion to be bypassed due to the inflammatory, vaccination, and tumor targeting effects of oncolytic vaccines that provide support for T cell survival, tumor infiltration, and anti-tumor activity.

In summary, we predict that the marriage of these two promising immunotherapeutic platforms will offer an effective method of eliminating established tumors by providing large numbers of functional $CD8^+$ T cells, and the mechanisms to overcome tumor-mediated immunosuppression.

2. Cancer and the Immune System

2.1 Immunosurveillance and Cancer Immunoediting

Pre-cancerous cells arise frequently in the human body as a result of mutations arising from genetic and environmental factors, and in the vast majority of cases these mutations trigger apoptotic pathways which lead to the death of the aberrant cell before it can establish a tumor. A small number of aberrant cells will be unable to engage these intrinsic pathways of tumor control and continue to grow, but they are quickly identified and killed by the immune system in a process termed immunosurveillance [26]. However, sustained immune attack on tumors creates a selective pressure for cells capable of evading or shutting down the immune response in a process termed cancer immunoediting. Cancer immunoediting is characterized by three distinct phases: elimination, equilibrium, and escape.

The expression of inflammatory factors induced by the disruption of surrounding tissues, and the release of danger-associated molecular patterns (DAMPs) from the turnover of dead/dying cells within the burgeoning tumor recruit innate effector cells and professional antigen-presenting cells (e.g. dendritic cells) to the tumor site [27]. Markers of cellular stress such as NKG2D ligands expressed by tumor cells allow them to be targeted and killed by natural killer (NK) cells and γδ T cells, while indirect killing can be mediated by macrophages and dendritic cells (DC) via the production of IFNγ. Tumor-infiltrating dendritic cells will take up released

tumor antigens, while the DAMPs released by dying tumor cells will induce their maturation as they travel to the lymphoid organs [28]. Within the lymphoid organs mature DC present the released tumor antigens on class I major histocompatability complexes (MHC) to $CD8^+$ αβ T cells, and on class II MHC to $CD4^+$ T cells leading to the activation of adaptive anti-tumor immunity. Activated $CD4^+$ T cells play several key roles within the tumor and in the periphery; within the lymphoid organs they provide Th1 cytokines such as IFNγ, along with IL-2, in order to facilitate the activation of $CD8^+$ T cells and their subsequent expansion [29]. Within the tumor helper $CD4^+$ T cells produce cytokines such as IFNγ and TNFα which have pro-apoptotic and anti-angiogenic effects on the growing tumor. IFNγ and IL-2 produced by $CD4^+$ T cells within the tumor microenvironment maintain the activation status and effector function of tumor-infiltrating $CD8^+$ T cells as well as recruiting NK cells and type I macrophages, which further potentiates their contribution to tumor regression [29]. Activated $CD8^+$ T cells rapidly expand and migrate into the tumor site and directly kill any remaining tumor cells [30]. Such events comprise the elimination phase of cancer immunoediting, and most developing tumors do not make it past this stage.

Because tumors are composed of a heterogeneous population of cells, some cells within a developing tumor may be capable of surviving the elimination phase and enter the equilibrium phase. The equilibrium phase is characterized by a relative balance of immune-mediated attack on the tumor and the suppression of the immune response by the surviving tumor cells and can last for a patient's lifetime [30]. While the exact mechanisms controlling this process are still under investigation, it is currently known that the balance of immune effector cells versus tumor-associated suppressive cells, the relative ratio of anti-tumor cytokines (e.g. IL-12) to tumor-promoting cytokines (e.g. IL-23), the ability of tumor cells to resist immune-mediated

cytotoxicity, and the ability of the tumor cells to present sufficient levels of antigen for recognition by $CD8^+$ T cells via class I MHC all play a role in the process [31]. Regardless of the exact mechanisms directing this process, over time the equilibrium phase allows for the selection of less immunogenic, more immunosuppressive tumor cell subsets which can lead to the complete evasion of the immune system which characterizes the escape phase.

The escape phase is typically when disease becomes clinically apparent due to the unchecked growth of the malignant cells causing disruptions in the function of surrounding tissues. During the escape phase tumors become almost completely resistant to immune attack, which leads to the uncontrolled outgrowth of the tumor. This is largely due to the formation of an immunosuppressive microenvironment within the tumor which is characterized by a variety of suppressive cell types, soluble factors, and cell-surface inhibitory ligands expressed by both tumor cells and infiltrating suppressive cells.

2.2 The Immunosuppressive Tumor Microenvironment

The immunosuppressive nature of the tumor microenvironment (TME) is the culmination of a number of different factors which allows for tumors to escape immune attack. Factors secreted by tumor cells such as TGF-β and IL-10 can suppress anti-tumor T cell responses by inhibiting the maturation of tumor-infiltrating DC, and instead directing their differentiation into a tolerogenic phenotype [32, 33]. Additionally, tumor cells express many intracellular enzymes (e.g. indoleamine 2, 3-dioxygenase, IDO) and cell-surface factors which can directly inhibit the efficacy of infiltrating $CD8^+$ T cells. Surface-bound FasL can lead to apoptosis of tumor-infiltrating $CD8^+$ T cells by engaging the Fas-FasL axis, while surface-bound PD-L1 can interact with PD-1 on the surface of infiltrating $CD8^+$ T cells to induce a phenomenon known as T cell

exhaustion which is characterized by defects in cell survival, proliferation, and effector function which culminates in the physical deletion of exhausted T cells [34, 35]. Tumor cells are also able to suppress the expansion of infiltrating $CD8^+$ T cells by depleting tryptophan via expression of IDO – an ability they share with certain suppressive myeloid cell types found within the tumor microenvironment [36, 37, 38].

Suppressive myeloid cells, which include myeloid-derived suppressor cells, tumor-infiltrating DC, and tumor-associated macrophages, are also capable of suppressing T cell responses within the tumor microenvironment through the secretion of IL-10, TGF-β, and VEGF [39, 40]. Subsets of these cells are also capable of producing IDO, thus contributing to the suppression of T cell proliferation within the tumor.

Regulatory $CD4^+$, $FOXP3^+$ T cells (Treg) are also recruited to growing tumors where they also skew the T cell response toward tolerance rather than immune activation through the expression of IL-10, TGF-β, and CTLA-4. CTLA-4 is an immune checkpoint molecule expressed on the surface of Treg which acts to competitively bind CD80 and CD86 on the surface of mature DC [41, 42]. By inhibiting the interaction between CD28 on the surface of a $CD8^+$ T cell and CD80/CD86 on mature DC, Treg-expressed CTLA-4 is able to induce anergy and/or apoptosis in tumor-infiltrating $CD8^+$ T cells.

Collectively these factors within the TME serve to prevent immune-mediated attack, and to promote the continued outgrowth of the tumor.

2.3 Adaptive Anti-tumor Immunity and the Birth of Immunotherapy

While the microenvironment of growing tumors is profoundly immunosuppressive, the presence of tumor-reactive $CD8^+$ T cells has been observed in the peripheral blood and within

the tumors of cancer patients. Tumor antigen-specific $CD8^+$ T cells have been described in a number of tumor histologies including melanoma, fibrosarcoma, breast cancer, lung cancer, and glioblastoma; the magnitude of their presence in a patient's circulation and/or tumor is correlated with positive prognostic outcome which highlights the integral role of $CD8^+$ T cells in anti-tumor immunity [43, 44, 45, 46, 47].

One of the earliest observations that sparked interest in this area of research was from patients with advanced melanoma who were receiving intravenous IL-2. It was observed that patients who experienced auto-immune vitiligo as a side-effect of therapy often had a better prognosis than those who did not [48, 49]. This phenomenon suggested a T cell-mediated mechanism of therapeutic efficacy, and prompted numerous investigators to seek out more reliable and effective methods of restoring anti-tumor immunity giving birth to the field of cancer immunotherapy [50].

3. $CD8^+$ T Cells as a Tool for the Treatment of Cancer

3.1 Adoptive Cell Therapy

Among the most promising platforms for cancer immunotherapy which has emerged in recent years is adoptive cell therapy. Originally pioneered by Steven Rosenberg's group, the principle behind adoptive cell therapy is simple; tumor-reactive $CD8^+$ T cells are removed from the influence of the TME and cultured *ex vivo* before reinfusion into the patient. Tumor antigen-specific $CD8^+$ T cells are typically derived from either surgically resected tumors, or from the peripheral circulation of the patient in question [51]. Because the frequency of such cells can be quite low (e.g. $CD8^+$ T cells specific for the melanoma/sarcoma-associated cancer-testis antigen NY-ESO-1 are typically found at a frequency of <1/10 000 $CD8^+$ T cells in the circulation, and

tumor-infiltrating $CD8^+$ T cells are not numerous enough to mediate tumor regression) one of the purposes of *ex vivo* culture is to expand their number to a therapeutically relevant amount [18]. In the context of tumor-infiltrating $CD8^+$ cells, *ex vivo* culture also removes the suppressive influence of the TME and serves to restore the effector function of these enriched anti-tumor $CD8^+$ T cells such that they can effectively mount an attack on, and eliminate a patient's tumor [24].

While simple in concept, applying ACT in practice has been beset by numerous challenges. While *ex vivo* culture of tumor-reactive $CD8^+$ T cells in the presence of IL-2 allowed for a massive expansion of their numbers and does restore effector function, these cells were found to engraft very poorly in patients. The solution to this problem was to infuse patients with high doses of IL-2 in order to maintain the activation, and promote the survival of the infused $CD8^+$ T cells [52, 53]. While this proved effective it was associated with sometimes life-threatening side-effects such as vascular leak syndrome, which resulted from a massive IL-2-induced release of cytokines by both the infused T cells and other immune cells present in the patient's circulation [54]. As the ability of clinicians to manage these side-effects via anti-inflammatory treatments (e.g. IL-6 administration) has improved, IL-2 infusion has become a routine step in current ACT protocols [55, 56].

To further enhance the engraftment and function of transferred T cells, it has become standard practice to lymphodeplete patients prior to the infusion of the cellular product. Lymphodepletion is accomplished by using either standard chemotherapeutics (e.g. cyclophosphamide and fludarabine) or total-body irradiation [53]. The rationale behind this is to deplete regulatory lymphocyte populations (i.e. Treg) which have been demonstrated to have a negative impact on therapeutic outcome by dampening the anti-tumor $CD8^+$ T cell response [57, 58].

Additionally, lymphodepletion enhances the survival of the transferred cells by carving out a niche for them by removing endogenous, non-tumor-reactive lymphocytes which are thought to compete with the transferred cells for homeostatic cytokines such as IL-7 and IL-15 [58].

However, pre-treatment lymphodepletion and post-infusion IL-2 administration are extremely physically demanding on patients. This necessitates the keeping of ACT patients in intensive-care unit-like conditions due to the possibility of the rapid manifestation of life-threatening side-effects. Additionally the demanding nature of these regimens limits the number of patients able to safely undergo ACT, which continues to be a significant hurdle in the development of this therapeutic platform and makes it out of reach for those who need it most.

3.2 Advances in the *ex vivo* Generation of $CD8^+$ T Cells for ACT

Advances in genetic engineering such as lentiviral transduction, targeted mutagenesis, and directed evolution of T cell receptor (TCR) genes has largely removed the requirement for isolating tumor antigen-specific $CD8^+$ T cells from patients [17]. This has given researchers and clinicians the power and flexibility to target any tumor antigen for which a TCR sequence is known. While a select few clinical trials demonstrate that such efforts must be made with extreme care to avoid off-tumor toxicity, these technologies promise to extend ACT to virtually any tumor histology [59, 60, 61].

Despite the improvements in therapeutic efficacy due to high-dose IL-2 infusion and preparative lymphodepletion, a large fraction of patients continue to be unresponsive to therapy which suggests sub-optimal effector function and survival of the adoptively-transferred cellular product [51]. As a result, there has been a concerted effort in the ACT field to direct T cell differentiation states toward longer-lived memory T cell subsets with superior effector function

(compared to those cultured in IL-2) using defined combinations of cytokines and small molecule inhibitors [20, 24, 62, 63].

3.3 CD8$^+$ T Cell Differentiation States and ACT

CD8$^+$ T cell differentiation exists on a spectrum ranging from long-lived, self-renewing naive CD8$^+$ T cells to short-lived terminally differentiated effector CD8$^+$ T cells; the ability to survive, proliferate, and exert effector function are dependent on where a particular T cell lies on this spectrum. Following thymic selection naive CD8$^+$ T cells primarily migrate into the "T cell zones" of the secondary lymphoid organs (but they are also capable of re-circulating in the periphery) where they are put into an optimal position for antigen presentation from class I MHC on the surface of APC such as DC and B cells. Upon encounter with their cognate antigen activated naive CD8$^+$ T cells exhibit the greatest potential for proliferation and the resultant effector CD8$^+$ T cell progeny are the most effective at killing infected and/or transformed cells [64, 65]. Once the insult is resolved the majority of the effector CD8$^+$ T cells die because terminal differentiation results in an inability to undergo further proliferation. But a small minority of antigen-experienced memory CD8$^+$ T cells continue to persist long-term in various compartments to provide ongoing surveillance and a rapid secondary response against another encounter with that antigen [64].

"Memory CD8$^+$ T cell" is a blanket term that actually encompasses three distinct subsets of long-lived antigen-experienced CD8$^+$ T cells, each of which represents a step along the spectrum of CD8$^+$ T cell differentiation: stem cell memory CD8$^+$ T cells, central memory CD8$^+$ T cells, and effector memory CD8$^+$ T cells [64]. Minimally-differentiated stem cell memory CD8$^+$ T cells remain capable of homing to the secondary lymphoid organs, an ability they share with

central memory $CD8^+$ T cells, due to expression of lymphoid homing surface markers (e.g. CD62L); they also exhibit a proliferative capacity second only to naive $CD8^+$ T cells and a robust capacity for self-renewal (hence "stem cell memory") [66, 67]. Additionally effector T cells derived from stem cell memory $CD8^+$ T cells display potent effector function, which would translate well into ACT [68]. In contrast, effector memory $CD8^+$ T cells are incapable of homing to the secondary lymphoid organs due to loss of CD62L expression; they also display a very limited capacity for proliferation and appear incapable of self-renewal. Furthermore their progeny tend to display poor survival compared to less differentiated subsets as most of the progeny $CD8^+$ T cells will be terminally differentiated, and they have inferior effector function compared to effector $CD8^+$ T cells derived from less-differentiated T cell subsets [68]. Central memory $CD8^+$ T cells lie somewhere between stem cell memory $CD8^+$ T cells and effector memory $CD8^+$ T cells on the spectrum of T cell differentiation; they possess robust proliferative capacity, and their effector progeny are very capable killers. However central memory $CD8^+$ T cells do not share the self-renewal capacity of stem cell memory $CD8^+$ T cells, and they display less potent effector function than stem cell memory $CD8^+$ T cells [21, 68].

In theory the ideal $CD8^+$ T cell subsets for use in ACT are naive $CD8^+$ T cells or stem cell memory $CD8^+$ T cells, but this is currently not practical due to the requirement for *ex vivo* expansion of $CD8^+$ T cells in order to achieve a clinically meaningful number of these cells. The differentiation state of $CD8^+$ T cells is inextricably linked with proliferation – the more a $CD8^+$ T cell proliferates, the further along the differentiation spectrum it progresses. Since activation and proliferation invariably lead to $CD8^+$ T cell differentiation it is not currently possible to use naive $CD8^+$ T cells for ACT due to their very low frequency. While it is readily possible to culture stem cell memory $CD8^+$ T cells *ex vivo* through a variety of methods (e.g. stimulation of Wnt

signalling using small-molecule inhibitors of GSK3β) it is currently not possible to avoid the concurrent prohibitive decrease in final cell yield [69]. In practice a balance must be struck between ideal differentiation state and cell yield when culturing $CD8^+$ T cells for ACT.

As a result, the greatest focus in the ACT field has been on generating $CD62L^+$ $CD44^+$ central memory $CD8^+$ T cells owing to their good *ex vivo* proliferative capacity, their capacity for successful engraftment and long-term persistence in hosts, and their robust effector function upon activation [21, 70, 71]. As a consequence of their capacity for long-term persistence, the use of central memory T cells offers the promise of life-long protection against tumor relapse. Additionally because central memory T cells can expand substantially *in vivo*, unlike terminally differentiated effector T cells generated by culture in the presence of IL-2, their use offers the possibility of overcoming the need for lengthy *ex vivo* culture protocols because a small number of adoptively-transferred central memory T cells could expand several-fold once infused into the patient. Despite this, an obstacle in the use of memory T cells for ACT exists in the form of their requirement for *in vivo* activation in order to fully acquire effector function.

4. Oncolytic Virotherapy

4.1 Principles and Shifting Paradigms

The term "oncolytic virus" is a catch-all for a variety of viruses from several diverse families (including, but not limited to, *Rhabdoviridae*, *Herpesviridae*, and *Poxviridae*) which share the common property of being able to selectively infect, replicate in, and kill malignantly transformed cells while sparing healthy tissues. Unlike cytotoxic chemotherapies which indiscriminately kill dividing cells, oncolytic viruses offer the potential for targeted therapies with fewer side effects than the current standard of care for many cancers [72]. The oncolytic

properties of viruses have been studied since the mid-twentieth century. Initial interest arose from observations in lymphoma patients where those who developed influenza infections experienced a transient regression in their disease [73]. Early attempts at oncolytic virotherapy were crude and unrefined – sometimes involving direct administration of infected bodily fluids, but with modern advances in genetic engineering and virus preparation the field has become a very promising avenue for the discovery of novel cancer therapeutics [72].

Several aspects of a growing tumor create an ideal environment for viral replication such as unchecked cell growth providing a large reservoir of host cells, defects in antiviral immune pathways offering a high degree of permissiveness in comparison to surrounding healthy tissues, and promiscuous translation of proteins which allows for the rapid production of viral progeny [72]. Indeed, the immunosuppressive microenvironment which makes tumors so adept at evading the immune response also makes them very attractive targets for viral infection.

While there have been numerous studies in which oncolytic virotherapy acting alone produced encouraging results in pre-clinical models, the recapitulation of these events in the clinic has proved to be a significant challenge [73, 74, 75]. Perhaps the largest hurdle for oncolytic virotherapy to overcome is the stark reality that it is highly unlikely (if not impossible) that every cell within a tumor will be infected and killed as a result of viral infection. This reality has caused a shift in the rational design of therapies using oncolytic viruses.

In its infancy, the field of oncolytic virotherapy viewed the immune system as a barrier to successful therapy. The development of neutralizing antibody responses and cellular immunity against the viruses used hindered the spread of viruses within the patient, which often-times would severely limit the efficacy of oncolytic virotherapy. However, in recent decades there has

been a change in how the immune system is viewed in the field. Because many of the viruses used in oncolytic virotherapy are very immunogenic, researchers have begun to see this property as a valuable tool in combating cancer. Instead of seeking to suppress the immune response against the viral vector, current efforts in the development of oncolytic virotherapy seek to harness the patient's immune system for the purpose of attacking their tumor [76]. Methods in which this is done include the insertion of transgenes encoding cytokines which lead to the activation and maturation of professional antigen-presenting cells (APC) such as GM-CSF, pro-inflammatory cytokines known to enhance cellular immunity against tumors (e.g. IL-12), and the insertion of defined tumor antigens in order to redirect the anti-viral immune response to target tumors expressing the same antigen [77].

4.2 Vesicular Stomatitis Virus and Maraba Virus

Vesicular stomatitis virus (VSV) and Maraba virus (Mrb) are members of the family *Rhabdoviridae*, and are both members of the genus *Vesiculovirus*. As such they have a ~11 kb single-stranded negative-sense RNA genome encoding 5 genes (N, P, M, G, L) which are produced in decreasing abundance based on the proximity of their coding region to the 3' end of the genome (i.e. N, the nucleoprotein is produced in the highest abundance and L, the "large protein" which is a component of the viral RNA-dependent RNA polymerase, is produced in the lowest abundance) [78, 79].

The short replication time of these two viruses and the large amount of progeny virions generated makes generating high titres relatively easy compared to other viruses used for oncolytic virotherapy. The viral glycoprotein (the "G" protein), which mediates attachment and entry into host cells via interaction with members of the low density lipoprotein receptor (LDLR)

family, which are ubiquitously-expressed by mammalian cells, allows these viruses to infect virtually any tumor histology [78]. Additionally, due to a general lack of contact with these viruses by the general public, the vast majority of patients likely to be treated with them will not have pre-existing neutralizing antibodies. Another highly desirable feature of these two vesiculoviruses is their amenability to genetic engineering which allows for the insertion of immunostimulatory transgenes with relative ease [8]. Various groups have experimented with the use of GM-CSF and type I IFN expressing rhabdoviruses in an effort to facilitate the activation and maturation of APCs in order to engage adaptive anti-tumor immunity; others have inserted genes for cytokines (e.g. IL-12) which augment the cytotoxic effector functions of NK cells and $CD8^+$ T cells in the TME [9, 11, 80, 81]. This amenability to genetic engineering has also resulted in an impressively large safety window for the therapeutic use of these viruses by creating attenuated strains with defects in their matrix protein (the "M" protein).

Normally, the M protein allows vesiculoviruses to shut down type I IFN responses in infected cells through blocking host mRNA transcription, export and translation, but attenuated strains of vesiculoviruses lack this ability which enhances their safety profile [82, 83, 84, 85]. Normal cells undergoing infection with M protein mutant vesiculoviruses will have intact type I IFN signalling which renders any such infection non-productive. In contrast, cancer cells, a significant proportion of which have defects in the type I IFN pathway, will undergo productive infection leading to the death of the infected cell.

The problem with oncolytic viral monotherapy is that it is highly unlikely to result in the infection of every cell within a tumor which will lead to inevitable relapse unless anti-tumor immunity is engaged. It is within this realm that rhabdoviruses have demonstrated their greatest promise as therapeutic agents.

4.3 Rhabdoviral Oncolytic Vaccination: Heterologous Prime-boost and Beyond

Perhaps the most promising use of rhabdoviruses for the treatment of cancer is as therapeutic vaccines. By inserting a gene encoding a defined tumor antigen, systemically administered rhabdoviruses can potently and specifically engage anti-tumor $CD8^+$ T cell populations within patients. We have previously demonstrated this paradigm in the context of a heterologous prime-boost vaccination strategy for the immunotherapy of cancer. The principle behind heterologous prime-boost vaccination revolves around first generating a stable antigen-specific memory T cell (Tm) population using a priming vaccine. In this context the priming vaccine can be an unrelated virus such as an adenovirus engineered to express a target tumor antigen; when administered to a tumor-bearing host this vaccine will lead to the priming of $CD8^+$ T cells specific for the target antigen. As this primary response contracts a population of central memory T cells forms and is sequestered within the T cell zones of the lymphoid organs due to their expression of the lymphoid homing surface molecule CD62L [14, 86]. This localization within the T cell zones of the lymphoid organs puts central memory T cells within close proximity to DCs providing an optimal scenario for antigen presentation and reactivation.

In order to take advantage of the proliferative capacity and robust effector function of these tumor antigen-specific central memory T cells, a rhabdoviral vaccine expressing the same tumor antigen (our so-called "rhabdoviral oncolytic vaccine") is administered systemically in order to activate them. There are several reasons why rhabdoviruses are ideal for this role; they still retain their original oncolytic function which can serve to directly debulk the tumor and the expression of the tumor antigen during the infection cycle can upregulate the expression of the target within the tumor microenvironment creating an *in situ* vaccination effect [78]. Additionally, systemic dissemination of the rhabdoviral oncolytic vaccine results in the virus entering the

spleen where it is able to infect follicular B cells. What is particularly interesting about this property is that the follicular B cells act as a sort of "antigen reservoir" which results in the cross-presentation of the antigen by splenic DCs to the central memory T cell population generated by the priming vaccine [87]. Together, these features of rhabdoviral oncolytic vaccines lead to a massive anti-tumor immune response that is capable of eliminating established tumors. This combination is currently undergoing a phase I/II clinical trial in human patients with advanced MAGE-A3$^+$ solid tumors (clinicaltrials.gov: NCT02285816).

However, there are some limitations to the prime-boost approach which may prove to be problematic in the clinic. Because the priming vaccine generates an effector CD8$^+$ T cell response, it necessitates a waiting period for this response to subside. If this is not taken into account and the rhabdoviral oncolytic boosting vaccine is administered prematurely, the primary effector response will lead to the rapid killing and clearance of infected APCs. Ultimately, this would result in a substantial attenuation of central memory T cell engagement and a decrease in therapeutic efficacy [86]. Moreover, the reliance on the priming vaccine successfully generating a meaningfully large population of anti-tumor CD8$^+$ T cells is a more troubling issue. The priming vaccine may have differential degrees of efficacy from patient to patient, due to their disease staging and previous treatments, and this may lead to suboptimal therapeutic efficacy in those patients which do not generate a large enough central memory population [88, 89]. This limitation may be addressed by ACT because, conceptually, a large number of central memory T cells specific for virtually any tumor antigen could be generated *ex vivo*. However, in practice this process has several technical challenges: the long culture times required, the reduced cell yield that comes with limiting T cell differentiation, and central memory CD8$^+$ T cells require *in vivo* activation in order to acquire full effector function. We believe that combining ACT with

rhabdoviral oncolytic vaccination could help address some of these barriers to the success and broader implementation of ACT.

5. Hypothesis

We hypothesize that rhabdoviral oncolytic vaccination will safely enhance ACT for the elimination of established, solid tumors by facilitating the *in vivo* activation and proliferation of transferred central memory $CD8^+$ T cells, enhancing their effector function, and directing their migration to disease sites.

6. Specific Aims

1. Determine if rhabdoviral oncolytic vaccination can engage adoptively-transferred, *ex vivo* cultured tumor antigen-specific central memory $CD8^+$ T cells in a tumor-bearing and tumor-free context.
2. Determine if ACT and rhabdoviral oncolytic vaccination combination therapy can eliminate established tumors in an immunocompetent mouse model compared to monotherapies.
3. Analyze the *in vivo* engraftment, effector function, and persistence of adoptively-transferred anti-tumor central memory $CD8^+$ T cells in the context of combination therapy with rhabdoviral oncolytic vaccination.

Chapter 2: Materials and Methods

1. Animals

Female Balb/c mice (6-8 weeks of age upon receipt) were purchased from Charles River Laboratories (Wilmington, MA) and housed in a specific pathogen-free room in the McMaster University Central Animal Facility (Hamilton, ON). Balb/c mice were housed in conventional mouse micro isolators fitted with HEPA air filters. NRG (NOD, RAG1$^{-/-}$, IL2R$\gamma^{-/-}$) breeders were kindly provided by Dr. Ali Ashkar and mice were bred under ultra-clean conditions. DUC18-Thy1.1 TCR transgenic mice, which recognize an H-2K^{d}-restricted CD8^{+} T cell peptide epitope corresponding to amino acids 136-144 of a mutated ERK2 protein found in the murine fibrosarcoma cell line CMS5, were kindly provided by Dr. Brian Lichty [90]. All animals were housed under a controlled 24-hour light cycle (12 hours of light and 12 hours of darkness) and a constant temperature of 22ºC. Experimental animals were fed an irradiated diet (18.6% protein, 6.2% fat, and 3.5% fibre) (2918, Teklad Global Diets, Indianapolis, IN) and sterilized water. All experiments were compliant with Canadian Council on Animal Care guidelines, and received internal approval through the McMaster University Animal Research Ethics Board.

2. Rhabdoviruses

Wild-type vesicular stomatitis virus (Indiana serotype) was engineered to express an H-2K^{d}-restricted CD8^{+} T cell peptide epitope corresponding to amino acids 136-144 of a mutated ERK2 protein found in the murine fibrosarcoma cell line CMS5, as described previously [14, 91]. The resultant rhabdoviral oncolytic vaccine was dubbed "VSVErkM". Maraba virus MG1, which contains attenuating mutations in both its M protein and its G protein, was engineered to express the same epitope as VSVErkM as described previously [14, 25]. The resultant rhabdoviral oncolytic

vaccine was termed "MrbMG1ErkM". Finally, a wild-type vesicular stomatitis virus expressing GFP was provided by Dr. Bryan Lichty for use as a negative control in some experiments.

3. Peptide

The H-2K^d-restricted peptide arising from a mutated ERK2 in CMS5, "ErkM" (ErkM$_{136-144}$ QYIHSANVL) was purchased from Biomer Technologies (Pleasanton, CA). The peptide was dissolved in PBS supplemented with 0.5% BSA and stored at -20ºC.

4. Cell Line and Tumor Challenge

CMS5 fibrosarcoma cells, expressing a H-2K^d-restricted neoantigen arising from a single K > Q substitution in ERK2$_{136-144}$ (ErkM$_{136-144}$ QYIHSANVL), were cultured at 37ºC and 5% CO_2 in DMEM supplemented with 10% FBS, Penicillin/Streptomycin (100 U/mL, and 100ng/mL, respectively), and L-glutamine (2mM) until the cells were confluent. CMS5 cells were washed twice with PBS and resuspended at a concentration of 10^6 cells/30 µL in PBS. Balb/c and NRG mice (6-8 weeks old) were challenged via intradermal injection of 30 µL of the CMS5 preparation. Tumors were allowed to grow to a mean volume of approximately 150 mm^3 prior to the commencement of treatment. Rechallenge experiments followed the same preparation protocol, but only 10^5 cells/30 µL were injected into previously cured Balb/c mice.

5. DUC18 Central Memory CD8$^+$ T Cell Culture

DUC 18 CD8$^+$ T cells are engineered to express a T cell receptor specific for the ErkM epitope. Bulk splenocytes from Thy 1.1 congenic DUC18 TCR-transgeneic mice were isolated via physical disruption of spleens, and cultured for 7 days (37ºC, 5% CO_2) in RPMI 1640 supplemented with 10% FBS, Penicillin/Streptomycin (100 U/mL, and 100ng/mL, respectively),

L-glutamine (2mM), and 2-mercaptoethanol (55 µM). Bulk splenocytes were stimulated with 100 ng/mL of ErkM peptide, and proliferation/memory differentiation were induced via the addition of 10 ng/mL IL-15, 10 ng/mL IL-21, and 20 ng/mL rapamycin to the culture medium.

6. Adoptive Transfer and Oncolytic Vaccine Administration

When tumors reached an approximate mean volume of 150 mm^3, DUC18 memory $CD8^+$ T cells were harvested, washed twice in PBS, and injected intravenously into tumor-bearing Balb/c and NRG mice at a dose of 10^6 cells/200 µL of PBS. After 24 hours, 2-5 x 10^8 pfu of a rhabdoviral oncolytic vaccine expressing the ErkM epitope, either VSV (VSV-ErkM) or MrbMG1-ErkM, were injected intravenously into the animals in 200 µL of PBS.

7. Blood Collection and Peptide Stimulation

Blood was collected from treated mice via retro-orbital bleeding, and red blood cells were lysed using ACK lysis buffer (0.15 mol/L NH_4Cl, 10.0 m mol/L $KHCO_3$, 0.1 m mol/L Na_2EDTA, PH 7.2-7.4) prior to peptide stimulation. Enriched peripheral blood mononuclear cells (PBMC) were stimulated with 1 µg/mL ErkM peptide, or wild-type ERK2 peptide, for 4 hours in the presence of Brefeldin A to block cytokine secretion.

8. Surface/Intracellular Staining and Flow Cytometry

Cultured DUC18 memory $CD8^+$ T cells were stained with the following fluorochrome conjugated antibodies: anti-mouse CD8a – BV711 (Biolegend), anti-mouse CD44 – FITC (Biolegend), and anti-mouse CD62L – Alexafluor 700 (Biolegend). Stimulated PBMC from blood samples were stained with the following fluorochrome conjugated antibodies: anti-mouse CD8a – BV711 (Biolegend), anti-mouse Thy1.1 – PE (Biolegend), anti-mouse IFNg – APC

(Biolegend), and anti-mouse TNFa – FITC (Biolegend). For intracellular staining, cells were permeabilized using BD Cytofix/Cytoperm buffer (BD Biosciences). Fluorescence was detected using a BD LSRFortessa flow cytometer (BD Biosciences), and the data was analyzed using FlowJo (Version 10) flow cytometry analysis software (Treestar).

9. Survivor Leukocyte Extraction and Tetramer Staining

Cured mice (defined as being >60 days tumor-free following treatment) were euthanized, and blood and spleen samples were collected as described elsewhere in this report. Bone marrow samples were collected from the same animals by removing both femurs, and subsequent flushing of the bone with PBS using a 25 gauge needle. To determine the frequency and origin of anti-ErkM $CD8^+$ T cells in each compartment, bulk leukocytes were stained with anti-mouse CD8a – BV711 (Biolegend), anti-mouse Thy1.1 – PE (Biolegend) antibodies, and a PerCP – conjugated H-$2K^d$ tetramer loaded with the ErkM epitope (kindly provided by Dr. Kyle Stephenson). Fluorescence was detected using a BD LSRFortessa flow cytometer (BD Biosciences), and the data was analyzed using FlowJo (Version 10) flow cytometry analysis software (Treestar).

10. $CD8^+$ T Cell Depletion Experiments

Depletion of $CD8^+$ T cells was achieved using 2 doses of anti-mouse CD8 antibody administered intraperitoneally (i.p.) at a dose of 250 µg/200 µL spaced 48 hours apart. Depletion was maintained using a bi-weekly i.p. injection of anti-mouse CD8 antibody at a dose of 250 µg /200 µL. Flow cytometry was used to verify depletion using an anti-mouse CD8a – BV711 antibody (Biolegend).

Chapter 3: Results

1. *Ex vivo* Cultured Central Memory CD8$^+$ T Cells Expand and Persist in Response to Oncolytic Viral Vaccination in a Tumor-free Host

We have previously demonstrated the ability of vaccine-induced central memory CD8$^+$ Tm to robustly expand and migrate into tumors in response to rhabdoviral oncolytic vaccination; presently, this study sought to determine if the same paradigm applies to *ex vivo* cultured central memory CD8$^+$ Tm as a novel approach to ACT [14, 78]. The consensus in the ACT field is that it is desirable to use less-differentiated CD8$^+$ T cell subsets (e.g. central memory CD8$^+$ T cells) for adoptive transfer due to their superior engraftment, proliferative capacity, and effector function [15], [17]. To remain consistent with the state of the field, we elected to use bulk splenocytes collected from DUC18-Thy1.1 TCR-transgenic mice as a source of tumor antigen-specific CD8$^+$ T cells which we subsequently cultured into central memory CD8$^+$ T cells. DUC18-Thy1.1 mice, which are based on the Balb/c strain, are characterized by 30% - 50% of their CD8$^+$ T cells containing a TCR specific for the ErkM neoepitope (ErkM$_{136\text{-}144}$ QYIHSANVL) arising from a mutation in the ERK2 gene in CMS5 – a methylcholanthrene-induced fibrosarcoma of Balb/c mice [90, 91].

DUC18 central memory Tm were generated via *ex vivo* culture of bulk ErkM peptide-stimulated DUC18-Thy1.1 splenocytes in the presence of IL-15, IL-21, and the mTORC1-inhibitor rapamycin (all of which are previously identified factors capable of influencing CD8$^+$ T cell differentiation in *ex vivo* culture) over the course of 7 days [63, 92, 93]. Following 7 days of culture in the presence of this cytokine and small molecule inhibitor cocktail, flow cytometric

analysis revealed that the composition of the final product was >95% $CD8^+$ T cells – the vast majority of which demonstrated a $CD62L^+$ $CD44^+$ central memory-like phenotype (Figure 1a).

Despite the potential for robust anti-tumor effector function, adoptively transferred Tm require activation before they are therapeutically useful. Currently activation is achieved *ex vivo* prior to the cellular infusion, but these cells still require support through the administration of high-dose IL-2 infusion which can result in life-threatening side-effects. However, a major advantage of using rhabdoviral oncolytic vaccines in the context of heterologous prime-boost vaccination is their ability to stimulate potent $CD8^+$ T cell-mediated anti-tumor immune responses *in vivo* [14, 94]. Thus, we sought to determine if our *ex vivo* generated anti-tumor Tm were capable of expanding *in vivo* in the context of ACT combined with rhabdoviral oncolytic vaccination administered to tumor-free and tumor-bearing hosts.

Using IFNγ production by peptide-stimulated peripheral blood mononuclear cells (PBMC) as a metric of the frequency of anti-ErkM $CD8^+$ T cells in circulation, we observed that *ex vivo* cultured Tm could undergo massive *in vivo* expansion in response to rhabdoviral oncolytic vaccination in the absence or presence of a tumor. While the magnitude of the peak of the $CD8^+$ T cell response at day 5 post-infection (dpi) was virtually identical in tumor-free and tumor-bearing animals, the magnitude of the response 12 dpi was reduced in tumor-bearing hosts compared to tumor-free hosts. However, such differences in response magnitude appeared to resolve by 19 dpi (Figure 1b).

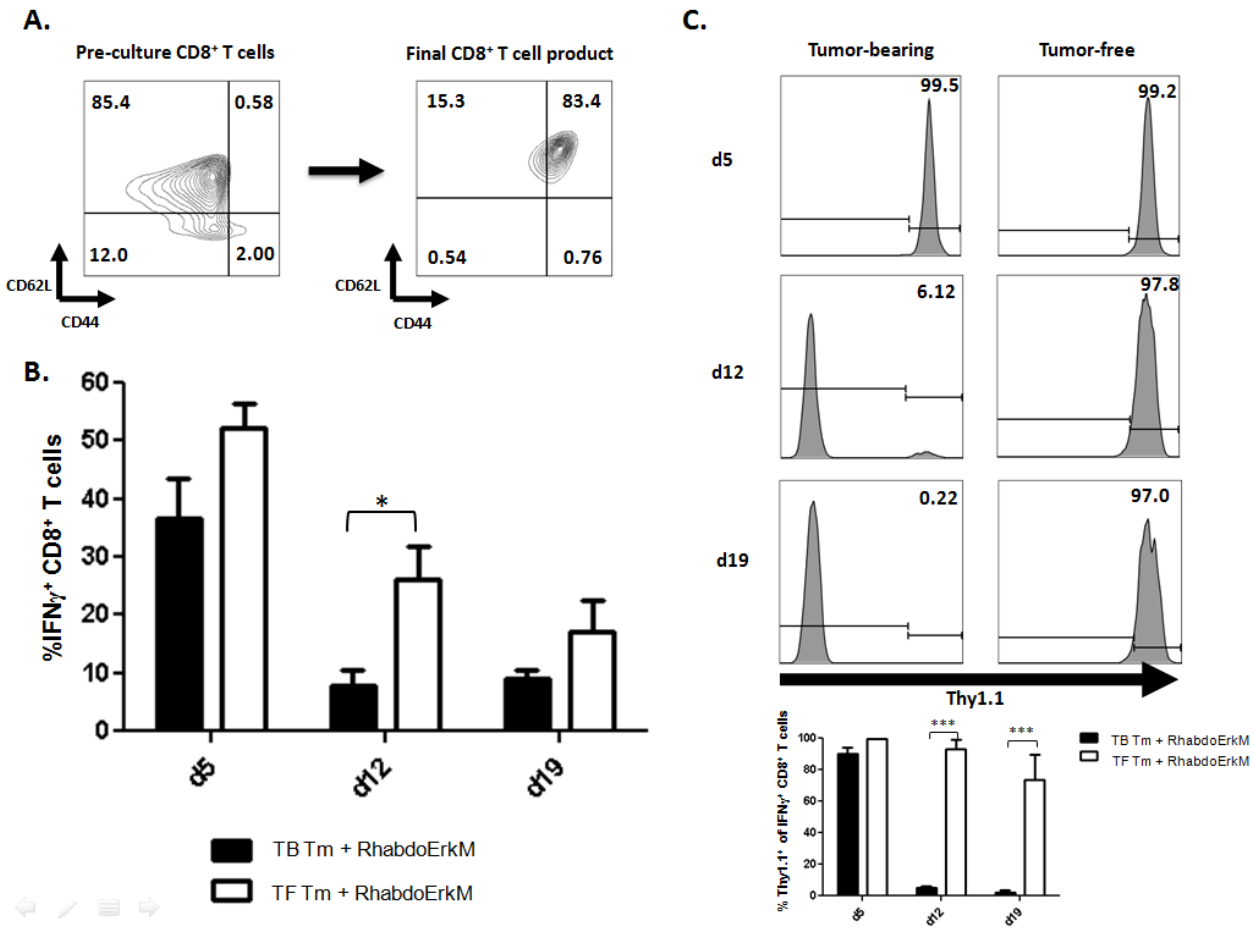


Figure 1. Expansion and persistence kinetics of *ex vivo* cultured DUC18 Tm in tumor-bearing and tumor-free hosts

(A) Staining for CD44 and CD62L expression in naive and cultured DUC18 Tm illustrating the phenotypic reprogramming imparted by IL-15, IL-21, and rapamycin.

(B) IFNγ staining of ErkM peptide-stimulated $CD8^+$ T cells collected from the peripheral blood of tumor-bearing and tumor-free animals 5, 12, and 19 dpi. (* = $P < 0.05$, two-way ANOVA).

(C) Frequency of $Thy1.1^+$ DUC18 $CD8^+$ T cells among the total pool of ErkM peptide-stimulated $CD8^+$ T cells enriched from the peripheral blood of tumor-bearing and tumor-free animals; samples were taken 5, 12, and 19 dpi. Flow cytometric analysis revealed a rapid decrease in the frequency of $Thy1.1^+$ DUC18 $CD8^+$ T cells in tumor-bearing, but not tumor-free animals. The bar graph below provides a summary of the kinetics of the $Thy1.1^+$ $CD8^+$ T cell response against the ERKM model tumor antigen (*** = $P < 0.001$, two-way ANOVA).

Abbreviations: TB = tumor-bearing, TF = tumor-free, dpi = days post-infection (with the rhabdoviral oncolytic vaccine)

Because DUC18 $CD8^+$ T cells express a congenic marker ($Thy1.1^+$) different from host Balb/c mice ($Thy1.2^+$), we were able to track their fate among the endogenous $CD8^+$ T cell pool within tumor-free and tumor-bearing animals. In tumor-free animals, the anti-ErkM response was dominated by $Thy1.1^+$ DUC18 $CD8^+$ T cells at all time points examined. This observation demonstrated to us that the culture conditions used did not negatively impact the ability of these *ex vivo* differentiated Tm to respond to rhabdoviral oncolytic vaccination, nor their ability to persist long-term following clearance of the virus. Importantly, this also established that the rhabdoviral oncolytic vaccine itself is not detrimental to the survival of the adoptively-transferred *ex vivo* cultured Tm. In contrast to tumor-free animals, tumor-bearing animals displayed a progressive loss of the Thy1.1 signal over the course of 19 dpi. Initially the anti-ErkM response was dominated by the adoptively-transferred DUC18 $Thy1.1^+$ $CD8^+$ T cells, but over the course of the next two weeks the DUC18 $CD8^+$ T cells became all but undetectable in the blood and were instead replaced by ErkM-specific $Thy1.1^-$ $CD8^+$ T cells of endogenous origin (Figure 1c). This suggested to us that some tumor-associated factor may have been causing the death of a large number of our transferred cells. Despite this, we reasoned that the magnitude of the response induced within the first week post-infection with our rhabdoviral oncolytic vaccine would be more than sufficient to eliminate established tumors.

2. Synergy Between ACT and Rhabdoviral Oncolytic Vaccination is Required for Complete Tumor Elimination

Our observation of an endogenous anti-ErkM $CD8^+$ T cell response to oncolytic vaccination is in line with previous reports in the literature of spontaneous priming of endogenous anti-tumor $CD8^+$ T cells in pre-clinical models, clinical reports, and in the CMS5 model [27, 45, 95]. More encouraging are observations from recent Phase III clinical trials of T-VEC

(an oncolytic viral vaccine made from a modified herpes simplex virus 1 expressing GM-CSF) in which intratumoral injection of the virus induced regression of distal uninjected lesions [77]. The authors of the study attributed this effect to the maturation-stimulating effects of GM-CSF on DC which lead to the priming of an anti-tumor $CD8^+$ T cells response in patients with advanced melanoma; this highlights that endogenous lymphocytes can play a very powerful, but currently under-exploited, role in tumor regression [77, 96]. As a result we were prompted to not only ask the question of whether the combination of ACT and rhabdoviral oncolytic vaccination could eliminate established tumors, but also how much each component of the treatment contributed to the ultimate therapeutic outcome.

To this end, we compared the therapeutic efficacy of ACT alone, rhabdoviral oncolytic vaccination alone, a combination of ACT and a rhabdovirus expressing an irrelevant protein (GFP), and ACT combined with rhabdoviral oncolytic vaccination to untreated controls in treating established CMS5 tumors in Balb/c mice. Our rationale for this was to determine if rhabdoviral oncolytic vaccination on its own could engage a sufficient amount of endogenous anti-ErkM $CD8^+$ T cells in order to control tumor growth. If applicable, ACT was performed 24 hours prior to the rhabdoviral oncolytic vaccination; Figure 2a outlines a schematic of the timing of treatment which was followed in all groups tested.

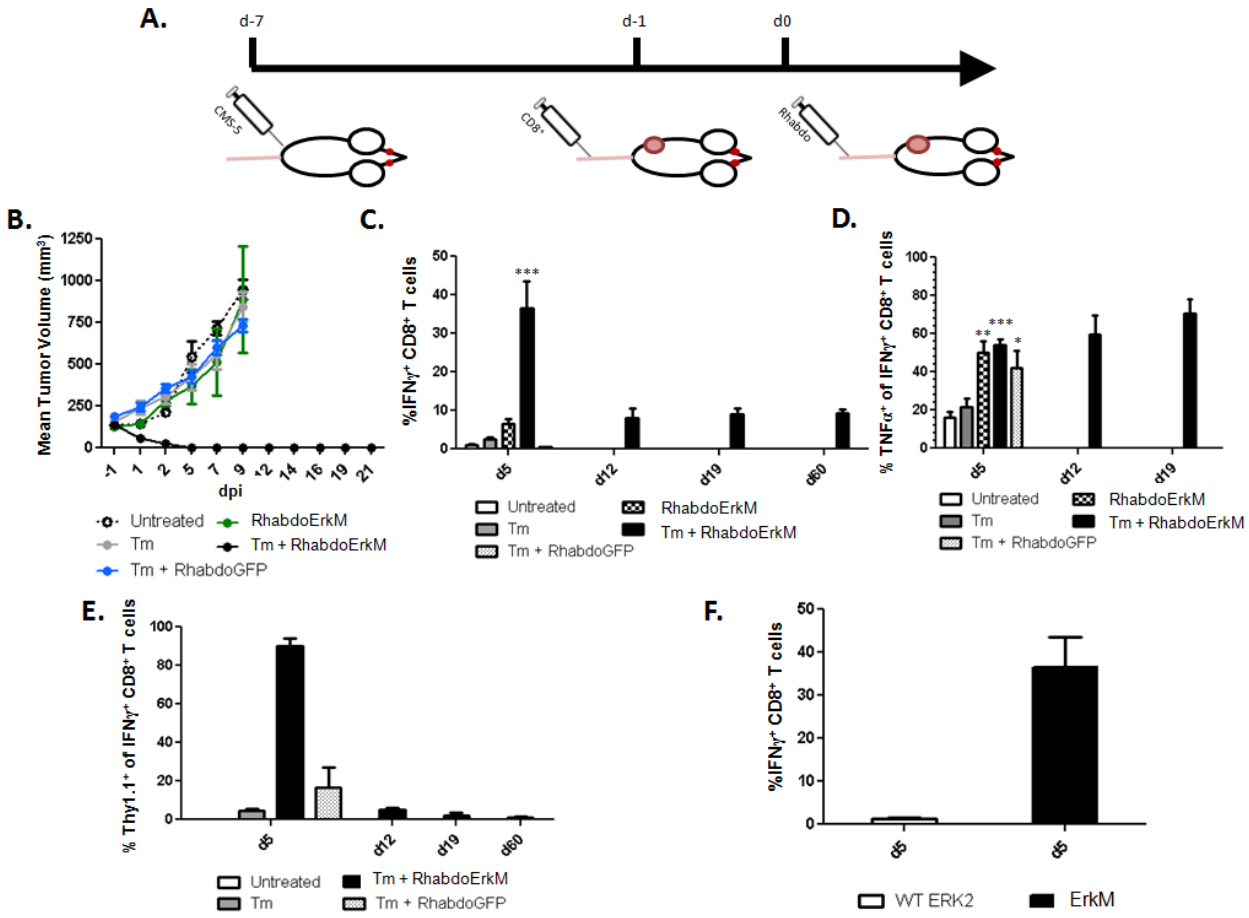


Figure 2. Comparison of adoptive transfer and rhabdoviral oncolytic vaccine monotherapies versus combination therapy

(A) Schematic representation of experimental design; Balb/c mice were implanted with intradermal CMS5 tumors 7 days prior to infection with an intravenously delivered rhabdoviral oncolytic vaccine. *Ex vivo* cultured DUC18 central memory $CD8^+$ T cells were adoptively-transferred intravenously 24 hours prior to rhabdoviral oncolytic vaccine administration. Monotherapies followed the same basic timeline with relevant treatments removed.

(B) No monotherapies resulted in meaningful delay in tumor growth compared to untreated controls. Only the combination of adoptively-transferred DUC18 central memory $CD8^+$ T cells with rhabdoviral oncolytic vaccination was capable of inducing complete elimination of established CMS5 tumors. Days following rhabdoviral oncolytic vaccination are measured on the X axis.

(C) IFNγ production was detected in ErkM peptide-stimulated $CD8^+$ T cells collected from the peripheral blood to measure the magnitude of the anti-ErkM immune response induced by the chosen treatment. Blood was collected 5, 12, and 19 days following

rhabdoviral oncolytic vaccination. All monotherapies failed to produce an immune response significantly different from untreated controls. Adoptive-transfer of DUC18 central memory $CD8^+$ T cells combined with rhabdoviral oncolytic vaccination induced a robust anti-ErkM $CD8^+$ T cell response which was detectable at least as far as 60 days post-vaccination (*** = $P < 0.001$, one-way ANOVA with Dunnett's multiple comparison test against untreated control).

(D) TNFα staining in the population of ErkM peptide-stimulated, IFNγ producing $CD8^+$ T cells (i.e. the responsive ones) isolated from peripheral blood as a measure of the quality of $CD8^+$ T cell effector function in a given group. All treatment groups receiving some form of rhabdovirus displayed a greater frequency of $IFN\gamma^+$, $TNF\alpha^+$ $CD8^+$ T cells compared to untreated controls (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, one-way ANOVA with Dunnett's multiple comparison test against untreated control).

(E) Thy1.1 congenic marker staining to determine the proportion of the anti-ErkM $CD8^+$ T cell response mediated by adoptively-transferred DUC18 $CD8^+$ T cells.

(F) Assessment of cross-reactivity of $CD8^+$ T cells from adoptive transfer plus rhabdoviral oncolytic vaccine treated animals. The concentrations of wild-type Erk2 peptide, and ErkM peptide were 1 µg/mL in their respective wells; stimulation was carried out for 4 hours in parallel. A 10-fold difference in the magnitude of the T cell response, as measured by IFNγ production, illustrates the safety of targeting the ErkM epitope in this model.

Abbreviations: RhabdoERKM = rhabdoviral oncolytic vaccine expressing ErkM epitope, RhabdoGFP = GFP-expressing rhabdovirus, Tm = DUC18 memory $CD8^+$ T cells

We observed that only the combination of ACT and rhabdoviral oncolytic vaccination was capable of completely eliminating established CMS5 tumors (Figure 2b). This observation was somewhat surprising given that we observed such a robust endogenous anti-tumor immune response in combination therapy treated animals, so we speculated that such a response could occur in the absence of ACT and at least recapitulate some therapeutic efficacy. However, when we analyzed the circulating PBMC of the group receiving rhabdoviral oncolytic vaccination on its own we found that, without the presence of adoptively-transferred DUC18 Tm, the resultant T cell response was insufficient to control tumor growth in any meaningful way. This also indirectly revealed to us that viral oncolysis plays a minimal role in actual tumor debulking; instead, it suggested that the primary role of the rhabdoviral oncolytic vaccine was as an immunomodulator. We also observed that ACT monotherapy was no better than untreated controls, and this was reflected once more via IFNγ staining which showed no significant expansion of the transferred cells (Figure 2c). This is consistent with previous observations made in the ACT field which indicated a necessity for the activation of less-differentiated $CD8^+$ T cell subsets (i.e. naive or memory) [21, 65]. Similarly, we observed no significant anti-ErkM $CD8^+$ T cell expansion in the ACT + GFP-expressing rhabdovirus treated animals, which also corresponded with no therapeutic benefit compared to untreated controls. This observation confirmed our speculation that an antigen expressing rhabdovirus (i.e. a rhabdoviral oncolytic vaccine) was required in order to selectively drive the expansion of anti-ErkM $CD8^+$ T cells in tumor-bearing animals (Figure 2c). Thus we concluded that synergy between ACT and rhabdoviral oncolytic vaccination was required for optimal therapeutic outcome.

The success of $CD8^+$ T cell-mediated immunotherapy depends not only on the magnitude of the anti-tumor immune response, but also the quality of the $CD8^+$ T cells that are brought to

bear. This is most apparent in the phenomenon of T cell exhaustion which is observed in virtually all cancer patients. Exhausted T cells have greatly diminished, if not completely abolished, effector function [97]. Because progressive loss of effector cytokine production (e.g. IFNγ, TNFα, and IL-2) is among the defining features of $CD8^+$ T cell exhaustion, we decided to use the co-production of IFNγ and TNFα as a metric of T cell functional quality.

Consistent with the requirement for some form of *in vivo* activating signal, ACT of central memory DUC18 Tm alone showed no improvement in IFNγ and TNFα co-production compared to untreated animals. Interestingly, all animals receiving some form of rhabdovirus (whether or not it contained the ErkM antigen) displayed an improvement in anti-ErkM $CD8^+$ T cell functional quality as measured by IFN and TNF co-production (Figure 2d). This observation was not entirely unexpected as it is well-established that the inflammatory factors produced by an acute viral infection (i.e. type I IFN) can act as a co-stimulatory signal during $CD8^+$ T cell activation; indeed, without co-stimulation of some kind $CD8^+$ T cells become anergic [98]. This demonstrated to us that rhabdoviral oncolytic vaccination not only facilitated the *in vivo* activation and expansion of adoptively-transferred central memory T cells, but that it also plays a key role in enhancing their effector function.

Using the Thy1.1 congenic marker expressed by the adoptively-transferred DUC18 Tm, we were able to monitor the kinetics of the anti-ErkM $CD8^+$ T cell response and the relative contribution of endogenous versus exogenous T cells to the ultimate outcome of therapy. We found that only animals that received ACT combined with rhabdoviral oncolytic vaccination demonstrated expansion of adoptively-transferred $Thy1.1^+$ DUC18 $CD8^+$ T cells. This observation further supported our rationale for using a rhabdoviral oncolytic vaccine to drive the expansion of adoptively-transferred anti-tumor $CD8^+$ T cells. Once again, we observed a

disappearance of Thy1.1$^+$ CD8$^+$ T cells from the circulation by 12 dpi, but the overall magnitude of the anti-ErkM response remained quite substantial (Figure 2e and Figure 2c, respectively).

While tumor-associated antigens, especially neoepitopes like ErkM, are very attractive targets for cancer immunotherapy, a great deal of caution must be exercised when designing such treatments in order to avoid off-tumor toxicity. This caveat is perhaps best exemplified in a small number of ACT clinical trials involving TCR transgenic CD8$^+$ T cells to target melanoma and synovial cell sarcoma. A small number of patients in two separate clinical trials experienced fatal off-tumor toxicity as a result of on-target and off-target (i.e. TCR cross-reactivity with an epitope in healthy tissue) T cell activation. Both of these trials targeted the cancer-testis antigen MAGE-A3, which is commonly expressed by melanomas and a great number of other cancers, [99, 100, 101, 102] . In a trial conducted by Morgan et al., three of nine enrolled patients suffered treatment-related neurotoxicity which resulted in two fatalities. Upon analysis of neural tissue from the deceased patients, the cause was found to be extensive infiltration of the white matter by CD8$^+$ T cells. Digging deeper, it was found that a related protein, MAGE-A12 was expressed in neural tissue and contained an identical epitope to the one targeted on MAGE-A3 [61]. In another trial, an engineered TCR targeting MAGE-A3 displayed unexpected cross-reactivity with an epitope derived from titin in cardiac muscle. This off-target reactivity resulted in patients experiencing fatal immune-mediated destruction of cardiomyocytes leading to heart failure [59].

Given that the ErkM epitope differs in only one amino acid from the same epitope in wild-type ERK2, we sought to determine if we would observe any toxicity associated with our treatment. Cross-reactivity with the wild-type epitope was assessed by stimulating PBMC collected from combination treated animals 5dpi (i.e. the peak of the anti-ErkM CD8$^+$ T cell response) with either the ErkM peptide, or the corresponding wild-type ERK2 peptide. After four

hours of stimulation, we observed a 10-fold difference in the frequency of IFNγ – producing $CD8^+$ T cells (Figure 2e). This suggested to us that the DUC18 TCR had a far greater affinity for the ErkM epitope compared to the wild-type epitope. This was also reflected in the lack of any apparent, observable toxicity in the treated animals, and collectively these data demonstrate that this treatment approach is free of off-target toxicity.

3. Endogenous, Tumor-primed $CD8^+$ T Cells Establish Memory in Multiple Compartments After Tumor Elimination

A major goal of ACT is to establish long-term immunosurveillance against tumor relapse. Circulating tumor antigen-specific $CD8^+$ T cells would protect a patient from relapses arising anywhere in the body – as long as the relapsed tumor expresses the same antigen as the initial tumor. While we did not observe persistence of adoptively-transferred central memory DUC18 Tm following tumor elimination, we did observe an anti-ErkM $CD8^+$ T cell population within the circulation of cured mice which persisted for at least 60 dpi. Such long-term persistence is a property of memory T cells, and their presence in the blood suggested that they were possible effector-memory $CD8^+$ T cells. Because effector-memory $CD8^+$ T cells and central memory $CD8^+$ T cells differ in their localization, with effector-memory $CD8^+$ T cells being confined to the periphery and central memory $CD8^+$ T cells being able to home to the secondary lymphoid organs and bone marrow, we resolved to characterize the distribution of this putative anti-ErkM memory T cell population [103, 104]. Using an H-$2K^d$ tetramer loaded with the ErkM epitope we examined spleen, blood, and bone marrow samples which were collected from Balb/c mice which had been previously cured (i.e. 60+ days tumor-free) for the presence of endogenous or exogenous anti-ErkM $CD8^+$ T cells.

Tetramer staining revealed that ErkM-specific $CD8^+$ T cells were present in all three compartments examined. We found that the highest frequency of anti-ErkM $CD8^+$ T cells was in the blood, and it was lowest in the bone marrow. Thy1.1 staining showed little-to-no presence of adoptively-transferred DUC18 $CD8^+$ T cells in all compartments examined, which demonstrates that the overwhelming majority of the persisting anti-ErkM $CD8^+$ T cells are of endogenous origin (Figure 3a). However, simple persistence and binding to a tetramer are not sufficient evidence to establish whether the persisting anti-ErkM $CD8^+$ T cells were indeed true memory cells capable of long-term protection.

In order to determine if these cells were indeed a bona-fide memory population, we challenged previously cured Balb/c mice, and naive untreated mice, with a second intradermal injection of CMS5 cells. If the persisting anti-ErkM $CD8^+$ T cells were indeed functional memory T cells, we expected to observe an increase in the frequency of ErkM-specific T cells in the peripheral circulation. Using IFNγ staining, we observed an approximately 2.5-fold increase in the frequency of anti-ErkM $CD8^+$ T cells five days after tumor challenge (~10% before challenge, and ~25% after challenge). In contrast, untreated naive Balb/c mice failed to mount a detectable anti-ErkM $CD8^+$ T cell response in the same timeframe (Figure 3b). Interestingly, despite observing a small population of $Thy1.1^+$ anti-ErkM $CD8^+$ T cells in the circulation, we were unable to detect them 5 days after tumor challenge. This suggests that they either failed to expand in response to rechallenge, or that they were outcompeted by the endogenous anti-ErkM T cell response. Thus, it appeared to us that the endogenous anti-ErkM $CD8^+$ T cells were sufficient to mount a robust recall response upon secondary exposure to their cognate antigen.

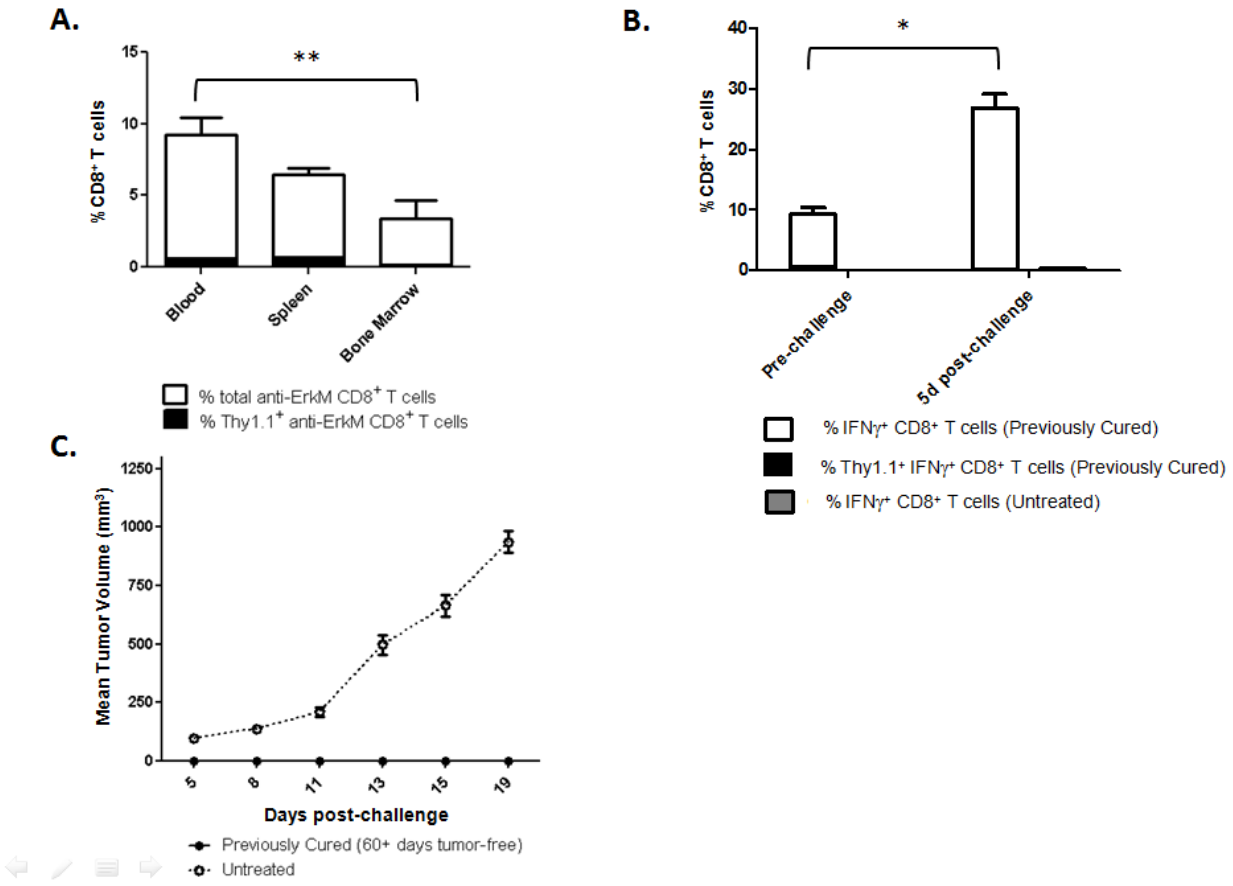


Figure 3. Protective memory is formed by endogenous, tumor-primed CD8$^+$ T cells

(A) ErkM-tetramer staining on blood, spleen, and bone marrow from tumor-bearing Balb/c mice treated with the combination therapy which had remained tumor-free 60 days following treatment administration. White bars represent total frequency of ERKM-specific CD8$^+$ T cells among all CD8$^+$ T cells from that compartment. Superimposed black bars represent the total frequency of Thy1.1$^+$, ErkM-specific CD8$^+$ T cells among all CD8$^+$ T cells (** = $P < 0.01$, one-way ANOVA with Tukey's multiple comparison test).

(B) Peripheral anti-ErkM CD8$^+$ T cell responses prior to, and 5 days after, CMS5 rechallenge in previously cured animals. White bars represent total frequency of ErkM-specific CD8$^+$ T cells among all CD8$^+$ T cells from that compartment. Superimposed black bars represent the total frequency of Thy1.1$^+$, ErkM-specific CD8$^+$ T cells among all CD8$^+$ T cells. Untreated controls are included as (very small) grey bars (* = $P < 0.05$, Student's t-test).

(C) Tumor growth kinetics following CMS5 challenge of previously cured and naive (untreated) animals. Untreated animals uniformly developed tumors, while no previously cured animal developed a tumor.

Ultimately, the utility of a population of memory cells is measured in their ability to protect against rechallenge with a given antigen. To this end, we observed the challenged mice (cured mice, and untreated naive mice) for development of tumors. Unsurprisingly, the naive untreated animals uniformly developed CMS5 tumors which grew to end-point size in 19 days. In comparison, none of the previously cured animals developed a tumor which, when taken together with our T cell response data from the circulation, demonstrate that the persisting endogenous anti-ErkM $CD8^+$ T cells were able to form a stable and protective memory population (Figure 3c). These data bring up the interesting notion that in the context of an immunogenic tumor (e.g. CMS5) adoptively-transferred anti-tumor $CD8^+$ T cells may be dispensable for the formation of anti-tumor memory provided that the endogenous $CD8^+$ T cell compartment remains intact throughout the duration of treatment. While tantalizing, this idea would require validation in other tumor models (both immunogenic and poorly immunogenic) before a concrete relationship between tumor immunogenicity, endogenous anti-tumor effector function/memory, and ACT could be established. If this observation is consistent in other models, then it may prompt a re-evaluation of the benefits and costs of lymphodepletion.

4. A Lack of Endogenous Lymphocytes in the Early Phases of Tumor Regression Results in Incomplete Elimination of Established Tumors and Leads to Eventual Relapse

Our unexpected observation that an endogenous anti-ErkM $CD8^+$ T cell response could not only form, but also establish protective memory, during treatment of established CMS5 tumors using the combination of ACT and rhabdoviral oncolytic vaccination prompted us to ask whether these cells are required for successful tumor elimination. This is an especially pertinent question because of the current consensus in the ACT field which regards pre-infusion lymphodepletion as an absolutely essential component of successful therapy. Because

lymphodepletion enhances the survival of transferred $CD8^+$ T cells in conventional ACT by eliminating competing endogenous lymphocytes, we sought to determine if a lack of endogenous lymphocytes would allow adoptively-transferred $Thy1.1^+$ DUC18 Tm to exhibit the same persistence we initially observed in tumor-free animals (where an endogenous anti-ErkM does not develop) [105].

In order to address these questions we repeated our tumor challenge and combination treatment experiment (Figure 2a) in NRG mice. NRG mice, which lack all lymphocytes, offer an ideal scenario to recapitulate the scenario of pre-treatment lymphodepletion without introducing the confounding effects of chemotherapy on tumor growth. NRG animals exhibited robust tumor regression following treatment, and the tumors were no longer detectable by 10dpi. However, all treated animals relapsed in the following 2-3 days and the relapsed tumors grew uncontrollably until endpoint tumor size was reached (Figure 4a). To verify that there were no issues with rhabdoviral oncolytic vaccination-induced expansion of the adoptively-transferred DUC18 $CD8^+$ T cells, we stained ErkM peptide-stimulated PBMC for IFNγ production at 5, 12, and 19 dpi. IFNγ staining revealed robust DUC18 $CD8^+$ T cell expansion whereas untreated animals predictably showed no $Thy1.1^+$ $CD8^+$ T cell presence (Figure 4b). Contrary to our expectations, despite the complete lymphopenia, DUC18 $CD8^+$ T cells persisted no better than in lymphoreplete Balb/c animals. This was surprising given that the consensus of the ACT field is that lymphodepletion augments the capability for survival in adoptively-transferred $CD8^+$ T cells [105]. Instead, this supports our observations in tumor-bearing versus tumor-free Balb/c mice: some tumor-intrinsic factor was causing the death of the adoptively-transferred DUC18 $CD8^+$ T cells. We speculate that this factor could likely be PD-L1 which is a major player in T cell exhaustion – which leads to their physical deletion [106]. Collectively, these data suggested that there were no

issues with the initial expansion of the adoptively-transferred DUC18 $CD8^+$ T cells and that whatever was causing relapse was occurring after the peak of the vaccination-induced $CD8^+$ T cell response.

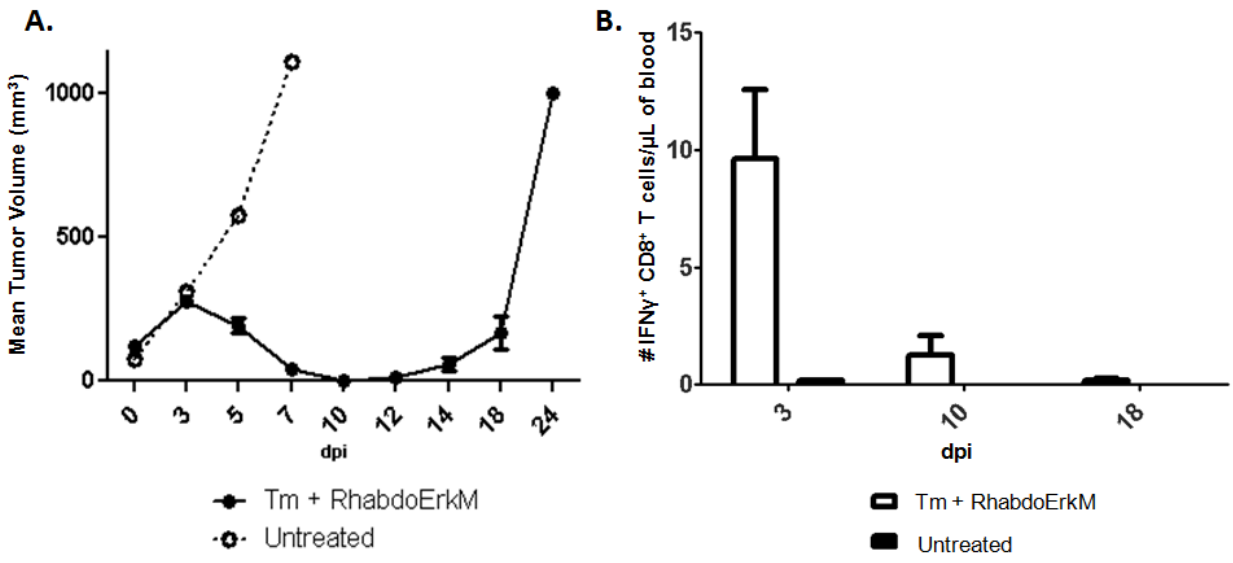


Figure 4. Endogenous anti-tumor $CD8^+$ T cells are required for the elimination of established CMS5 fibrosarcomas

(A) CMS5 tumor challenge of NRG mice was initially controlled by combination treatment with adoptively-transferred DUC18 central memory $CD8^+$ T cells and rhabdoviral oncolytic vaccination resulting in an ostensibly complete response. However, tumors eventually relapsed and grew uncontrollably, which suggested a role for endogenous lymphocytes in ensuring complete tumor elimination.

(B) IFNγ production was detected in ErkM peptide-stimulated $CD8^+$ T cells collected from the peripheral blood of treated, and untreated, tumor-bearing NRG mice. This was done in order to measure the magnitude of the anti-ErkM immune response induced by combination treatment with ACT and rhabdoviral oncolytic vaccination in a lymphodepleted context. Blood was collected 3, 10, and 18 days following rhabdoviral oncolytic vaccination.

Because NRG mice lacked endogenous lymphocytes, there was no opportunity for an endogenous anti-ErkM $CD8^+$ T cell response to be generated. We speculated that the endogenous $CD8^+$ T cells we observed in treated, tumor-bearing Balb/c animals were indeed required to prevent tumor relapse. We believed two possibilities existed; either the endogenous $CD8^+$ T cells participated in the complete elimination of established CMS5 tumors, or the endogenous $CD8^+$ T cells suppressed the outgrowth of a small number of surviving CMS5 cells. To test these possibilities we treated tumor-bearing, Balb/c mice with the combination of ACT and rhabdoviral oncolytic vaccination, and then we used an anti-CD8 antibody to deplete the relevant T cells at two time points and compared them to animals given an isotype control antibody.

Flow cytometric staining for CD8 in PBMC demonstrated complete depletion of $CD8^+$ T cells at both 7 dpi and 60 dpi (Figure 5a). We reasoned that if endogenous $CD8^+$ T cells were playing a role in preventing the outgrowth of residual CMS5 tumors, then depletion 7 dpi (i.e. when tumors generally become undetectable) would recapitulate the regression-relapse phenomenon we observed in NRG animals. If this was the case then we also expected that depletion 60 dpi would recapitulate the therapeutic outcome we observed in NRG animals. To our surprise, neither of these two depletions yielded the expected phenotype and all CD8-depleted animals remained free of relapse (Figure 5b). This told us that whatever the role of endogenous anti-ErkM $CD8^+$ T cells in eliminating established tumors was, it occurred during the earliest phases of tumor regression while the DUC18 $CD8^+$ T cells were still present in the system. Interestingly, these data appear to suggest that lymphodepletion may be detrimental to the ultimate therapeutic outcome of ACT. It is possible that, in the effort to create as ideal of an environment as possible for adoptively-transferred $CD8^+$ T cells, lymphodepletion may be

removing therapeutically beneficial lymphocyte populations and inadvertently limiting therapeutic efficacy.

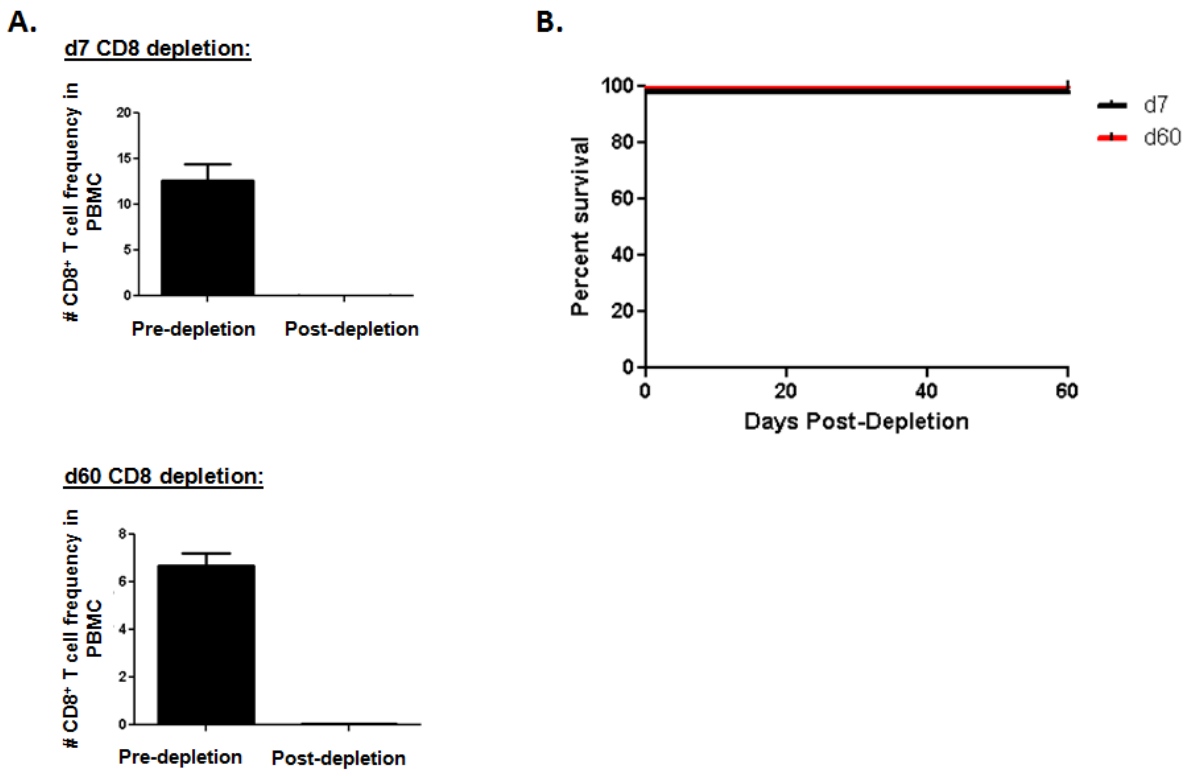


Figure 5. Post-treatment depletion of CD8$^+$ T cells fails to induce relapse of CMS5 tumors

(A) Frequency of CD8$^+$ T cells in the peripheral blood of CMS5-challenged Balb/c mice prior to, and 1 day after, antibody-mediated CD8$^+$ T cell depletion. Animals were depleted at 7, or 60, days following combination therapy with ACT and rhabdoviral oncolytic vaccination.

(B) Survival curves of CD8$^+$ T cell depleted animals. No tumor relapse was observed in the day 7 and day 60 depletion groups; animals were monitored for 60 days following CD8$^+$ T cell depletion.

Chapter 4: Discussion

Despite impressive advances made in ACT there are still several daunting challenges which must be overcome for ACT to become a staple of the oncologist's toolkit. Among these challenges is making the jump from haematological malignancies to solid tumors which establish an immunosuppressive microenvironment that impedes both endogenous and adoptively-transferred adaptive anti-tumor immunity by suppressing $CD8^+$ T cell proliferation and effector function within the TME. The need to overcome the influence of the TME is reflected in the suboptimal response rates observed in clinical trials of ACT for solid tumors in which only a fraction of patients experience a clinical response, and an even smaller fraction of these responses is complete and durable [15]. Since oncolytic recombinant rhabdoviruses are potent immunomodulators which can induce a great deal of inflammation they offer the possibility of subverting the tumor's ability to protect itself from T cell mediated attack by inducing the production of type I IFNs within the TME, thus augmenting $CD8^+$ T cell effector function. This was reflected in our study by the uniformly complete and durable responses in animals treated with adoptive transfer combined with rhabdoviral oncolytic vaccination.

One of the most significant obstacles in ACT is the requirement for very large doses (more than 10^{10} cells/infusion) of $CD8^+$ T cells; the culture protocols required to generate such staggering numbers are very labour intensive and require as long as 2 months to complete [18]. Genetic engineering allowing for the insertion of TCR or chimeric antigen receptors (CAR) into a patient's autologous $CD8^+$ T cells have made it possible to generate large numbers of tumor antigen-specific $CD8^+$ T cells, but the culture conditions used result in the final cell product being dominated by terminally differentiated effector $CD8^+$ T cells. These cells demonstrate poor

engraftment, survival, and effector function, which translates into suboptimal clinical responses upon infusion into patients.

Consequently a great deal of effort has been made by investigators to generate higher-quality, less-differentiated memory $CD8^+$ T cell subsets (e.g. central memory $CD8^+$ T cells) for use in ACT. These efforts have been largely successful, but they revealed another issue: because proliferation and differentiation are intertwined in $CD8^+$ T cells, culture conditions generating central memory $CD8^+$ T cells compromise the final yield of the product [22, 62, 69]. Additionally central memory $CD8^+$ T cells require activation *in vivo* in order for them to acquire effector function. Optimal ACT appears to require a balance of $CD8^+$ T cell number and quality, and it is in this respect that we believe rhabdoviral oncolytic vaccination offers the opportunity for synergy. We cultured central memory DUC18 $CD8^+$ T cells using IL-15 and IL-21, both of which have been observed to impart a more "memory-like" differentiation program to $CD8^+$ T cells [92]. After a brief evaluation of culture conditions (Appendix Figure 1), we also included the mTORC1 inhibitor rapamycin in our final central memory $CD8^+$ T cell culture condition. Inhibition of mTORC1 has been demonstrated to skew $CD8^+$ T cell differentiation towards a central memory phenotype, and this effect is in part due to the inhibition of glycolytic metabolism [107]. It has been demonstrated by others that a metabolic shift from fatty acid oxidation to glycolysis is one of the major changes which $CD8^+$ T cells undergo as they undergo terminal effector differentiation and that metabolic stress may be one of the key mechanisms which limit the survival of effector $CD8^+$ T cells [63, 108].

Upon *in vivo* activation by oncolytic rhabdoviral vaccination, 1×10^6 *ex vivo* cultured central memory $CD8^+$ T cells underwent rapid expansion (making up close to 50% of the total circulating $CD8^+$ T cell pool at the peak of the response) and eliminated established tumors. For

comparison, 1 x 10^7 of the same cells without rhabdoviral oncolytic vaccination had no impact on tumor growth (Appendix Figure 2). We also observed that rhabdoviral oncolytic vaccination improved the quality (as measured by TNFα and IFNγ co-production) of adoptively transferred DUC18 $CD8^+$ T cells which translated into the complete elimination of established CMS5 tumors. The ability of rhabdoviral oncolytic vaccines to facilitate the *in vivo* activation, expansion, and improvement of effector function in adoptively transferred anti-tumor central memory $CD8^+$ T cells offers the possibility to addressing the issue of balancing cell number and functional quality by allowing for the infusion of a much smaller (and much more quickly prepared) dose of higher-quality anti-tumor central memory $CD8^+$ T cells. This should allow for a far greater number of patients to receive ACT as the time required to culture a therapeutically beneficial number of anti-tumor central memory $CD8^+$ T cells will be significantly reduced if combined with rhabdoviral oncolytic vaccination; simultaneously, this will unlock the option of directly infusing central memory $CD8^+$ T cells for ACT which we speculate may confer superior engraftment compared to *ex vivo* activated cells.

Another issue facing ACT is long-term persistence; a goal of ACT is to not only eliminate a patient's current tumor, but also to provide long-term surveillance against recurrence of that tumor by long-lived anti-tumor memory cells. In this study we were unable to detect adoptively transferred DUC18 $CD8^+$ T cells after the first week following rhabdoviral oncolytic vaccination which contradicts current literature about the expected *in vivo* persistence of central memory $CD8^+$ T cells. One possible explanation for this observation is that, somehow, the *ex vivo* conditioned DUC18 $CD8^+$ T cells are more susceptible to tumor-mediated mechanisms of T cell exhaustion despite their demonstrably potent effector function. The central role of PD-L1 as a "master regulator" of $CD8^+$ T cell exhaustion in the TME is well-established[109]. Thus, it is

possible that the expression of PD-L1 and other immune checkpoint molecules within the TME were selectively killing off the adoptively-transferred DUC18 $CD8^+$ T cells. Indeed, experiments using antibody-mediated blockade of PD-L1 and other immune checkpoint molecules (e.g. CTLA4 blockade in melanoma) within the TME demonstrated that such treatments significantly enhance the effector function, survival, and proliferation of anti-tumor $CD8^+$ T cells [110, 111]. Despite the lack of persistence of adoptively transferred DUC18 $CD8^+$ T cells established CMS5 tumors were eliminated and, to our great surprise, we were able to detect a continuing anti-ErkM response as far as 60 days post-treatment. Interestingly, the Balb/c hosts were able to generate an endogenous anti-ErkM response (which did not occur in animals treated without both ACT and oncolytic rhabdoviral vaccination) during the course of treatment, and endogenous anti-ErkM $CD8^+$ T cells were capable of persisting long-term. Presently, we do not know why endogenous anti-ErkM $CD8^+$ T cells do not undergo the same dramatic contraction that adoptively transferred DUC18 $CD8^+$ T cells do. It is possible that their differential kinetics of expansion result in them encountering a far more accommodating TME in which the majority of the tumor has been killed by the adoptively-transferred DUC18 $CD8^+$ T cells, thus limiting their exposure to immunosuppressive factors. Upon rechallenge with CMS5 these circulating endogenous anti-ErkM $CD8^+$ T cells were capable of expanding and rejecting the tumor. It appears (at least in the immunogenic CMS5 model) that the persistence of adoptively transferred $CD8^+$ T cells is dispensable for the establishment of long-term protective anti-tumor memory, but their presence in the initial attack on the tumor is vital to therapeutic success as evidenced by a decrease in the dose of adoptively transferred cells resulting in a decrease in tumor control (Appendix Figure 3).

In the context of conventional ACT protocols, lymphodepletion of patients prior to infusion of the *ex vivo* cultured $CD8^+$ T cell product is widely accepted by the field as standard

practice in pre-clinical models as well as clinical trials [105]. Regulatory $CD4^+$ T cells play a demonstrably detrimental role in the adaptive anti-tumor immune responses ACT seeks to elicit. Similarly, endogenous anti-tumor $CD8^+$ T cells have been demonstrated to be functionally impaired *in vivo* due to the phenomenon of T cell exhaustion [105]. Endogenous lymphocytes (including endogenous anti-tumor $CD8^+$ T cells) are also thought to be a detrimental sink for homeostatic cytokines such as IL-7 and IL-15 which deprives adoptively-transferred anti-tumor $CD8^+$ T cells of vital pro-survival signals, which leads to a severe impairment in their *in vivo* survival [58]. Due to these reasons the consensus in the ACT field is that pre-infusion lymphodepletion is required to optimize the engraftment and survival of the transferred $CD8^+$ T cells.

Combining ACT and rhabdoviral oncolytic vaccination appears to remove pre-infusion lymphodepletion and exogenous IL-2 infusion as prerequisites for optimal therapeutic outcome, because combination therapy-treated animals uniformly experienced complete and durable responses. The likely reason for this observation is a side-effect of the massive induction of type I IFNs and subsequent inflammatory responses that characterize rhabdoviral infection, thus resulting in an abundance of available cytokines for the adoptively transferred cells without the need to deplete competing endogenous lymphocytes [94, 112]. Our interpretation is that this phenomenon allows the adoptively-transferred $CD8^+$ Tm to expand without the gruelling side-effects of current lymphodepletion protocols and IL-2 infusion. In turn, this property of rhabdoviral oncolytic vaccines should provide more patients with access to ACT by removing the limitation of patients being able to withstand chemotherapy and/or radiation preconditioning treatments.

Motivated by the unexpected endogenous anti-ErkM response which was generated in combination treated animals we decided to investigate if our combination therapy approach would demonstrate the same efficacy in a lymphodepleted environment. We predicted that therapeutic efficacy would be maintained while simultaneously enhancing the survival of the transferred DUC 18 $CD8^+$ T cells. Interestingly, despite the widely reported benefits of lymphodepletion/lymphopenia in ACT, CMS5-bearing NRG mice treated with a combination of ACT and rhabdoviral oncolytic vaccination uniformly experienced relapse following an initially complete response to treatment. Additionally, the lack of DUC18 $CD8^+$ T cell persistence confirmed our initial suspicion that there is some factor within the TME which is responsible for causing the rapid contraction of the *ex vivo* conditioned central memory DUC18 $CD8^+$ T cells, and that it was not due to competition from endogenous lymphocytes. Ultimately, tumor relapse in NRG animals suggested that endogenous lymphocytes were playing a larger than expected role in the elimination of established tumors.

Through the present study it became clear to us that the endogenous $CD8^+$ T cells were instrumental in the earliest phases of tumor regression, and that the possibly beneficial role of endogenous lymphocytes may currently be overlooked by the ACT field. Indeed, there is ample evidence in the literature which demonstrates that surgical resection of tumors can restore adaptive anti-tumor immunity *in vivo*. Additionally, there is the interesting possibility proposed by some groups that the success of conventional cancer therapies (i.e. chemotherapy and radiation) may be dependent on the successful generation of adaptive anti-tumor immunity following, or as a consequence of, treatment [95, 113, 114, 115]. We propose that in ACT and rhabdoviral vaccination combination immunotherapy the rapid tumor debulking mediated by the adoptively-transferred anti-tumor $CD8^+$ T cells may act in a similar fashion to lift the

immunosuppressive conditions restraining endogenous adaptive anti-tumor immunity. The unrestrained, endogenous anti-tumor immune response (likely mediated by $CD8^+$ T cells) is then able to ensure tumor elimination, and provide long-term protection from re-challenge.

Overall in this study we have demonstrated the powerful synergy of ACT and rhabdoviral oncolytic vaccination to overcome several limitations associated with ACT. Using a rhabdoviral oncolytic vaccine to activate and expand adoptively transferred central memory $CD8^+$ T cells *in vivo* provides a reliable tool for the use of less-differentiated memory $CD8^+$ T cell subsets in ACT which may augment therapeutic outcomes. Rhabdoviral oncolytic vaccines can significantly streamline the currently labour-intensive process of *ex vivo* culture for ACT by enabling the use of smaller numbers of higher-quality cells in the adoptive transfer, and this could allow staff at cell production facilities to handle a greater number of orders in a more timely fashion. A possible benefit of this streamlining is an increase in patient access to ACT and a reduction in cost as fewer cytokines, small-molecule drugs, media, and man-hours would have to be invested in order to produce the cells required for one infusion. Because the combination of ACT and rhabdoviral oncolytic vaccination does not require lymphodepletion, using this approach may greatly expand the number of patients with advanced disease able to enrol in clinical trials and receive this potentially curative treatment. However, our most important finding was the generation of long-term endogenous protective adaptive anti-tumor immunity in the absence of lymphodepletion. This phenomenon, while still requiring validation in other less immunogenic models, addresses one of the goals of ACT which is to provide patients with life-long protection from tumor relapse by providing ongoing immunosurveillance. This finding suggests that the current standard in ACT of pre-infusion lymphodepletion may in fact be a detriment to therapeutic outcome as it may inadvertently deprive patients of beneficial

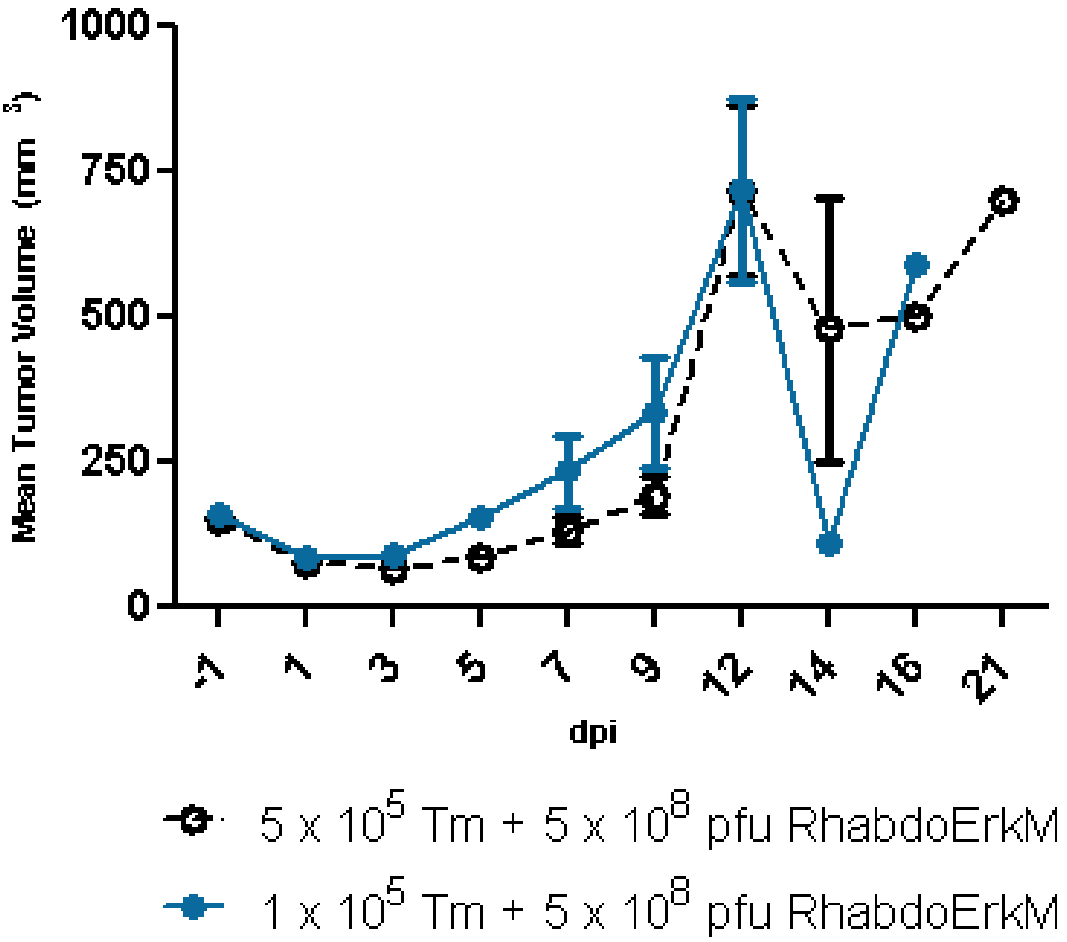
lymphocyte populations which can play critical roles in tumor elimination and long-term protection.

Chapter 5: Appendix

5.1 Open questions and Future Work

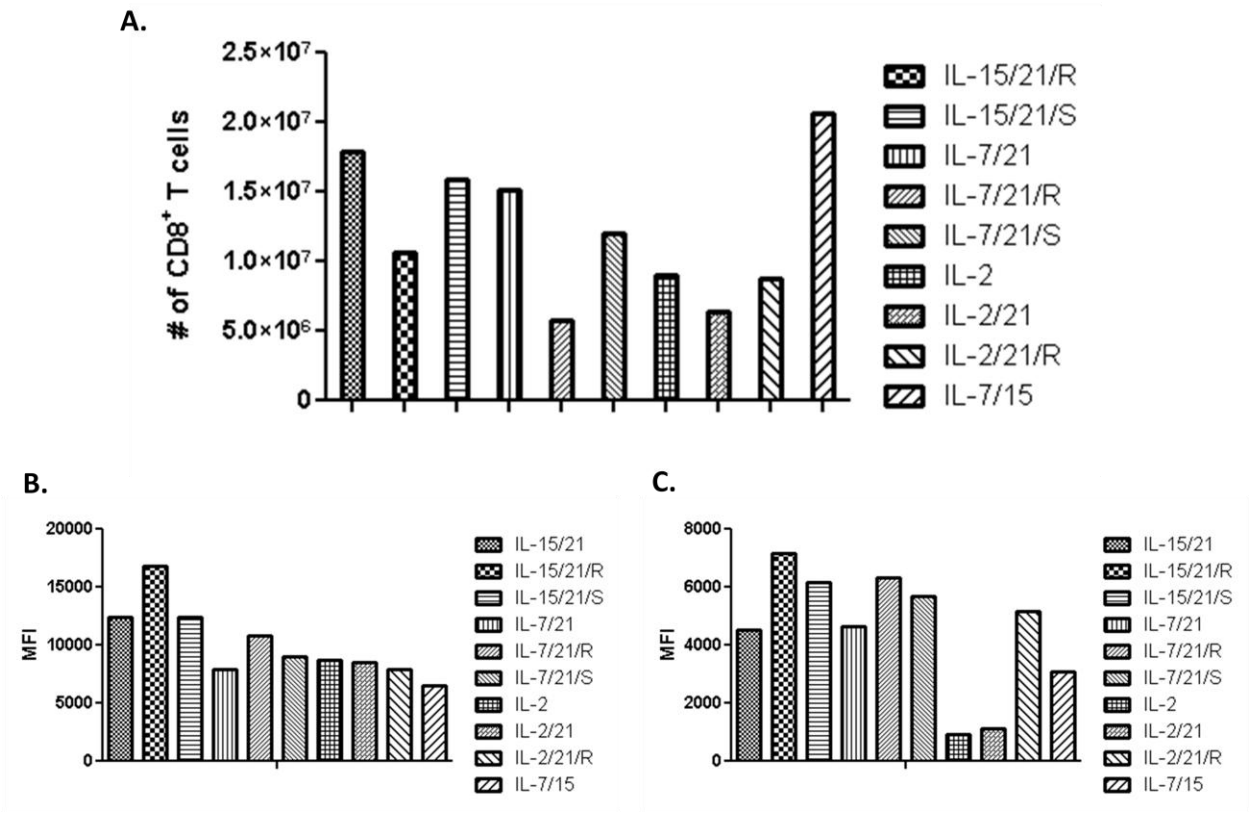
Despite our findings in this study there remain several pertinent questions to be answered. While it is evident that endogenous lymphocytes were required for complete elimination of established CMS5 tumors, it is not known which lymphocyte populations are indispensible for this phenomenon. We will repeat our tumor challenge and combination therapy experiments in Balb/c animals depleted of various endogenous lymphocyte populations (e.g. groups which are depleted of one of the following: $CD8^+$ T cells, $CD4^+$ T cells, NK cells, or all $Thy1.2^+$ endogenous lymphocytes) in order to elucidate the role (or lack of role) played by said cells in tumor elimination. Presently, we do not know why *ex vivo* cultured adoptively transferred DUC18 $CD8^+$ T cells fail to persist long-term in tumor-bearing animals. Our data in tumor-free animals suggests that there is some factor intrinsic to the tumor (or recruited by the tumor) which is responsible for the dramatic contraction of the DUC18 T cells we had observed. We will attempt to home in on the mechanism governing the contraction of the DUC18 $CD8^+$ T cell response by performing a detailed analysis of the kinetics of the T cell response within the first 9 days post rhabdoviral oncolytic vaccination in multiple compartments (blood, spleen, and tumor if applicable/feasible). We believe that the results of such an experiment, which would outline the timing of the contraction of the DUC18 $CD8^+$ T cells, will provide guidance in the design of follow-up experiments to elucidate the mechanism responsible (i.e. if the contraction begins after tumor clearance, then it is less likely to be directly mediated by the tumor).

5.2 Appendix Figures



Appendix Figure 1. Adoptively transferred DUC18 CD8$^+$ T cells are the principle contributor to tumor regression

CMS5-bearing Balb/c mice were treated with decreased doses of DUC18 CD8$^+$ T cells (5 x 10^5 and 1 x 10^5 cells) in combination with rhabdoviral oncolytic vaccination (5 x 10^8 pfu/mouse) in lieu of the standard cell dose used in this study. A trend of decreasing tumor control with reduced DUC18 CD8$^+$ T cell doses was observed, thus supporting our claim that DUC18 CD8$^+$ cells are the primary mediators of tumor regression.



Appendix Figure 2. Evaluation of central memory CD8$^+$ T cell culture conditions in order to strike a balance between cell yield and quality

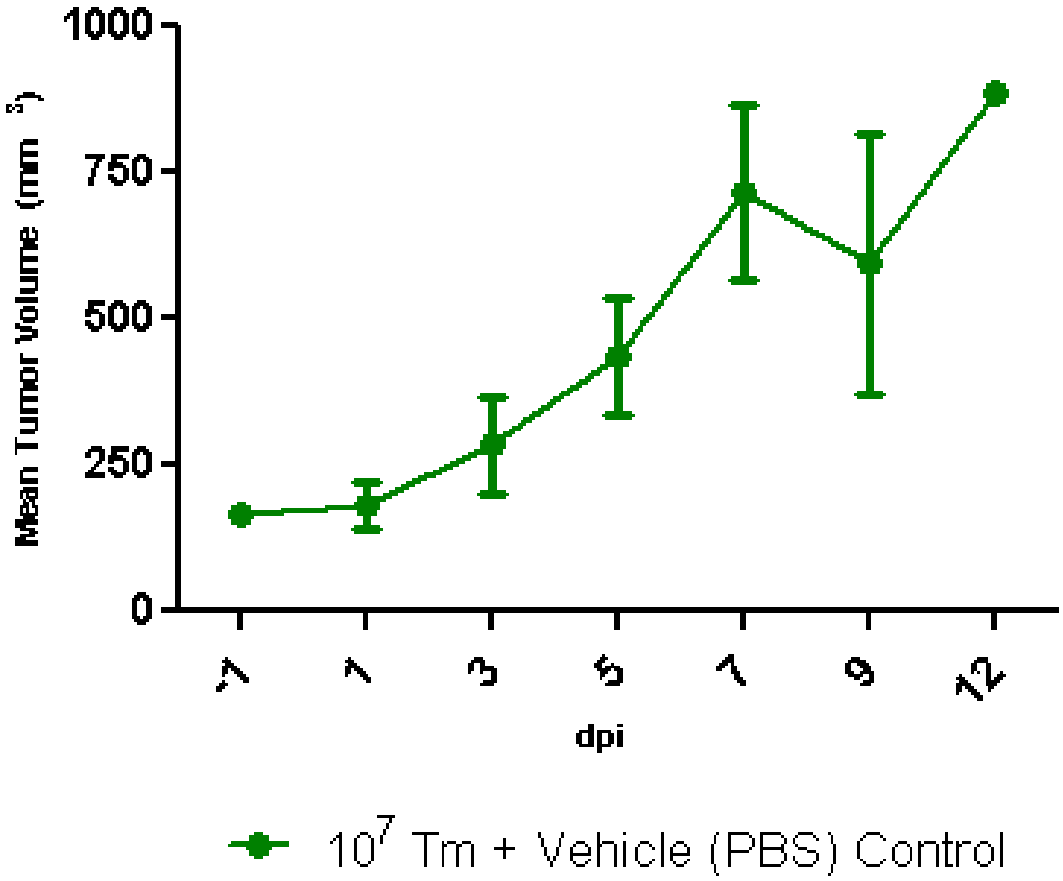
(A) Final DUC18 central memory CD8$^+$ T cell yields following 7 days of culture in the indicated culture conditions. All cultures were started from 2 x 10^6 bulk DUC18 splenocytes. Data shown are representative of two independent trials.

(B) Mean fluorescence intensity (MFI) of CD44 was used as an indicator of antigen experience and, when combined with CD62L MFI, memory differentiation. A higher MFI was interpreted as more desirable. Data shown are representative of two independent trials.

(C) Mean fluorescence intensity (MFI) of CD62L was used as an indicator of central memory CD8$^+$ T cell differentiation. A higher MFI value was indicative of a greater proportion of

high-quality central memory $CD8^+$ T cells in a particular culture condition. Data shown are representative of two independent trials.

Abbreviations: R = rapamycin, S = SD208 (a TGFβR1 signalling inhibitor)



Appendix Figure 3. A high dose of DUC18 central memory $CD8^+$ T cells does not offset the exclusion of rhabdoviral oncolytic vaccination

CMS5-bearing Balb/c mice were treated with an adoptive transfer of 1 x 10^7 central memory DUC18 $CD8^+$ T cells grown in the presence of IL-15, IL-21, and rapamycin. Adoptive transfer was followed with injection with a vehicle control (sterile PBS), and tumor size was

monitored. In the absence of rhabdoviral oncolytic vaccination, no anti-tumor effect was observed in treated animals, which highlights the utility of our combination treatment.

Chapter 6: References

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