T Cell Mediated Combination Immunotherapy

by

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Abstract

Immunotherapy is a broad treatment strategy that harnesses the immune system to fight off a particular condition or disease. Cancer immunotherapy is the specific application of agents designed to interact or stimulate the immune system to fight off tumors. Treatments as diverse as passive antibody therapy, cytokine support, and comprehensive adoptive T cell transfer make up the broad field of immunotherapeutics. Due to the naturally complex interactions inherent in the immune system, there are many options for therapeutic intervention, however, this same complexity makes it extremely difficult to optimize treatment strategies. Because of this, research into developing new immunotherapies, optimizing existing immunotherapies, and designing new combinations of immunotherapies is still critical in the fight against cancer. Although there have been ongoing successes of individual immunotherapies in the clinic, the complexity and interdependence of the immune system suggests that any single therapeutic intervention will be insufficient to reject established malignancies. Increased interest in applying combinations of immunotherapies in the clinic requires more thorough preclinical work to guide the designs of these studies. The work presented in this thesis focuses on developing combinations of immunotherapies to treat preclinical models of cancer, as well as studying the underlying mechanism of tumor control.

T cells are potent mediators of cytotoxicity and when properly used in adoptive cell transfer (ACT) protocols, can be highly effective in the treatment of cancer. ACT consists of three steps: 1) harvesting and purifying T cells from the patient, 2) enriching or modifying the T cells to become tumor specific, and 3) reinfusing the T cells along with supporting therapies. Therapies given alongside ACT are often adjuvants designed to enhance T cell response. However, focusing therapies only on enhancing the activity of the transferred T cells may miss out on synergistic effects when other parts of the immune system are simultaneously engaged. To study the effect of adjuvant therapy on ACT, a preclinical murine model was analyzed. Large, established B16F10 tumors were controlled when pmel-1 T cells were given with a course of supportive MSA-IL2 cytokine therapy, however, no cures were observed. When a
course of TA99 antibody therapy was added alongside ACT, a high rate of cures was observed. Flow cytometry of both circulating and tumor infiltrating pmel-1 cells showed massive expansion and activation. Additionally, tumor infiltration of neutrophils, NK cells, and DCs were greatly enhanced by adjuvant therapy. DCs in the tumor draining lymph nodes were largely unchanged by the therapies. Engagement of the humoral immune response was also observed in both treatment cases. Surprisingly, antibody therapy did not substantially alter any of the mechanistic observations made in this study, despite its critical role in achieving cures of tumors.

While ACT is a highly effective therapy, its clinical applicability is hindered by the complexity of performing T cell transplants and manipulations. A more optimal solution would involve purely injectable treatments that could elicit the same level of tumor specific T cell response in conjunction with potent recruitment of the adaptive immune system against tumors. To achieve this, working in collaboration with the Irvine Lab, combinations of immunotherapy using up to four different components were tested to identify critical factors in the successful rejection of established tumors in preclinical models. The four components of tumor targeting antibody, cytokine support, checkpoint blockade, and cancer vaccine acted synergistically to reject tumors from B16F10, TC-1, and DD-Her2/neu cell lines. The cancer vaccine elicited large numbers of tumor-specific T cells, and acted as a replacement for ACT. By analyzing subset combinations of this full treatment, the roles of each therapeutic component were identified. CD8 T cells and cross-presenting DCs were critical to curing subcutaneous tumors. Cytokine therapy was indispensable for effective tumor control, promoted immune cell infiltration into the tumor, and led to an increase in DCs. In combination with the other therapies, vaccination against a tumor antigen elicited a strong immunological memory response that was able to reject subsequent tumor rechallenge, as well as promote antigen spreading to new epitopes. Successful combinations were demonstrated to be dependent on the recruitment of both the adaptive and innate branches of the immune system. Finally, the efficacy of this combination of treatments was demonstrated by controlling the growth of induced tumors in a BRaf/Pten model.
Combination immunotherapy promises a future where synergistic treatments are specifically tailored to individual cancers leading to highly effective responses. However, determining the optimal combination of therapies, the complexity of dosing strategies, and the availability of targeted treatments are all barriers that must be overcome. The analysis presented here will make a significant contribution to the body of knowledge on immunotherapy as it has shown the importance of combining orthogonal immunotherapies in order to get durable cures to established tumors. These results will hopefully encourage combinations of orthogonally acting therapies based on T cells to achieve stronger clinical responses. By determining the necessary requirements for a strong, synergistic response to tumorous growths, more effective combination immunotherapy protocols may be designed in the future.

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1 Introduction

The war on cancer was officially begun with the signing of the National Cancer Act of 1971. However, in the more than forty years since then, the mainstays of cancer treatment continue to be radiation, surgery, and chemotherapy. Although effective, these treatments have costly side effects and are often ineffective at controlling recurrent secondary tumors. Immunotherapy offers the promise of more targeted and long-lived treatments against cancer, leading to fewer side effects and more robust cures. Immunotherapy is a broad treatment strategy that hinges on the idea that the immune system can be harnessed to fight off a particular condition or disease. Cancer immunotherapy is the specific application of agents designed to interact or stimulate the immune system to fight off tumors.

Immunotherapy consists of treatments as diverse as passive antibody therapy to comprehensive adoptive T cell transfer. Some immunotherapies simply attempt to boost the overall immune system through cytokines. Others attempt to guide the immune system against specific targets through specific vaccination strategies. Due to the naturally complex interactions inherent in the immune system, there are many options for therapeutic intervention, however, this same complexity makes it extremely difficult to optimize treatment strategies. Because of this, research into developing new immunotherapies, optimizing existing immunotherapies, and designing new combinations of immunotherapies is still critical in the fight against cancer.

The idea of cancer immunotherapy has an over 100 year history of attempting to use components of the immune system, either passively or actively, to fight off and cure malignancies. William B. Coley is generally credited with the first recorded use of immunotherapy to treat inoperable tumors in 1893 (Coley, 1893). Coley mixed bacteria and bacteria lysates and injected them into the tumors of his patients in order to stimulate the immune system at the site of injection. Rather than treating the tumor directly, this treatment was revolutionary in the sense that its primary goal was to stimulate the immune system against the cancer. Although successful, the use of these “Coley’s Toxins” did not gain widespread use due to concerns about efficacy and side effects.

A pivotal moment in the development of immunotherapy was the idea of “imunosurveillance” introduced by Paul Ehrlich in 1909. The idea is that cancer cells are being formed continuously throughout a person’s life but the immune system is able to identify and eliminate those cells before they become malignant. The idea that immune cells were already naturally eliminating cancer cells lent credibility to the
thought that the immune system could be harnessed to treat more advanced forms of tumors. Cancer itself is partially an immunological disorder. Although the traditional hallmarks of cancer (Hanahan and Weinberg, 2000) describe it as primarily a disease of cells acquiring the defined set of traits that allow them to divide uncontrollably, more recently it has been recognized that enabling characteristics and emerging features of cancer directly involve the immune system (Hanahan and Weinberg, 2011).

During the 1940s through the 1960s, the use of chemotherapy and radiation, along with surgery, became the mainstays of cancer therapy and remain so to this day. Immunotherapy meanwhile did not have meaningful clinical successes during this time. Most of the advancements during this time centered on elucidating the mechanisms of the immune system, especially in the fields of antibodies and histocompatibility. Additionally, knowledge of tumor immunology made great strides and cell surface differentiation antigens were identified.

In the 1970s multiple immune cell subtypes were discovered, including NK cells (Jondal and Pross, 1975; Kiessling et al., 1975), DC cells (Steinman and Cohn, 1973), and various types of T cells (Gershon and Kondo, 1970), as well as T cell growth factor (interleukin-2 / IL-2) (Ruscetti et al., 1977). Elucidation of these various functional immune cells has proved invaluable to immunotherapy by allowing scientists to tailor their treatments for specific cell types. The discovery of IL-2 was important for two reasons, it greatly facilitated in vitro culturing of T cells and would become one of the first successful cytokine therapies. Clinically, immunotherapy also made strides as the first use of a biological therapy for cancer was performed as interferon alpha was tested at M.D. Anderson, Houston, Texas.

The 1980s saw a rapid advance in both the knowledge of the immune system and the pace of clinical studies involving immunotherapy. The T cell receptor (TCR) was discovered (Allison et al., 1982) and characterized (Kappler et al., 1983; Samelson et al., 1985). The first successful treatments of patients using monoclonal antibody therapy were performed (Miller et al., 1982). IL-2 was also studied extensively in the clinic by the Rosenberg lab, on its own (Lotze et al., 1986), or in combination with adoptive cell therapy (Rosenberg et al., 1985, 1988). Nonclinical advances were also abundant, laying the foundations for future clinical treatments. One particularly notable advancement was the first use of T-bodies (Gross et al., 1989), which would later be called chimeric antigen receptors (CARs).

With the massive expansion of genetic information and knowledge of the immune system gained in the previous decade, the 1990s heralded new clinical applications, especially in the field of cancer vaccines. The knowledge of specific
peptide sequences that could be recognized by T cells advanced first in melanoma (Anichini et al., 1993), opening up their use in therapeutic applications (Marchand et al., 1999). Whole cell vaccines were also used, most notably with the development of GVAX (Dranoff et al., 1993), where tumor cells were engineered to express granulocyte macrophage colony stimulating factor (GM-CSF). The understanding of several key negative regulators of T cell activation was also advanced during this time, with CTLA-4 (Walunas et al., 1994) and PD-1 (Nishimura et al., 1999) being shown as primary mediators immune suppression. These molecules and pathways would later become critical points of intervention in so-called checkpoint blockade therapies.

The 2000s up until today have been characterized by a steady progression of clinical studies aiming to put into practice the basic discoveries, preclinical knowledge, and clinical experience gained during the last half-century. Monoclonal antibodies such as bevacizumab and cetuximab were FDA approved. Genetically engineered T cells were used in adoptive cell transfer protocols with both TCRs (Morgan et al., 2006) and CARs (Till et al., 2008). Advances in protein engineering also allowed the design and production of new types of biologics, notably bispecific antibodies (Bargou et al., 2008) designed to redirect T cell killing by engaging the CD3 antigen and a tumor associated antigen (TAA) simultaneously. Checkpoint blockade therapy made several advancements and clinical successes (Brahmer et al., 2010; Hodi et al., 2010) leading to the approval of ipilimumab, pembrolizumab, and nivolumab. Additionally, the first cancer vaccine, sipuleucel-T, was approved by the FDA (Kantoff et al., 2010), which, although ultimately proving unsuccessful financially, showed that a cell-based immunotherapy could be successfully brought to market.

Antibody treatment is by far the most successful of all immunotherapy methods for the treatment of cancer (Scott et al., 2012). Although considered a passive immunotherapy, antibodies function in a wide variety of ways often interacting with the immune system in a complex fashion. Clinically, antibodies have utilized various mechanisms of action in order to treat the specified cancer. Traditionally, antibodies have been used to target TAAs, and the assumed mechanism of action was to elicit antibody-dependent cell-mediated cytotoxicity (ADCC) to directly kill cancer cells. However, other mechanisms include: activation of complement-dependent cytotoxicity (CDC), blocking molecules such as growth factors, activating or inhibiting cell signals, mediating phagocytosis or opsonization, and modulating receptor internalization. Although the tumor cells themselves are most often targeted, newer antibodies have been designed to target tumor vasculature and stroma. Additionally, a new class of drugs known as antibody drug conjugates (ADC) are being developed with antibodies that have been designed to deliver a specific small molecule payload leading to the possibility of dual mechanisms of action.
Although immunotherapy of cancer has been studied for several decades, the field has only recently begun to deliver on the promise of individualized and effective treatments of malignancies (Mellman et al., 2011). In the last few years, a new class of checkpoint inhibitor drugs have been FDA approved and are showing great promise and efficacy against a wide variety of cancers. Additionally, clinical applications of adoptive cell transfer protocols with CARs have had dramatic success in the treatment of hematological malignancies. Finally, antibodies have been a mainstay in the treatment of cancer for the past few decades. The number of antibodies in clinical development has consistently increased during this time period, indicating that more and more specific targets will be available for treatment.

Despite these clinical successes and widespread optimism, cancer immunotherapy has many challenges to overcome to in order to become as effective and widespread and surgery, radiation, and chemotherapy. As clinical experience with a broad range of immunotherapeutics and their dosing grows, combinations of these therapies becomes more realistic. This thesis work examines three manners of immunotherapy with T cells as the focal point. First, an antibody enhanced adoptive cell therapy protocol is described and analyzed. Second, a comprehensive injectable therapy consisting of four components was developed and analyzed to parse the necessary components for a durable immunological rejection of preclinical model tumors. Third, T cells activated by a bispecific T cell redirecting antibody were analyzed using next generation sequencing to get complex view of the cellular response to artificially induced cytotoxicity. Finally, several additional works on enabling research are described. T cells are incredibly effective mediators of tumor destruction, but the work here highlights their inability to completely cure cancer on their own. By showing how multiple immunotherapies acting with distinct mechanisms can work synergistically to create robust anti-tumor immune responses, this work hopes to inform and guide future clinical strategies.
2 Adoptive Cell Therapy

2.1 Abstract

Therapies given alongside adoptive cell transfer (ACT) are often adjuvants designed to enhance T cell response. However, focusing therapies only on enhancing the activity of the transferred T cells may miss out on synergistic effects when other parts of the immune system are simultaneously engaged. Large, established B16F10 tumors were controlled when pmel-1 T cells were given with a course of supportive MSA-IL2 therapy, however, no cures were observed. When a course of TA99 antibody therapy was added alongside ACT, a high rate of cures was observed. Several experiments were performed to determine the role of antibody treatment in enhancing ACT therapy. Flow cytometry of both circulating and tumor infiltrating pmel-1 cells showed no difference in the magnitude or phenotype between mice treated with or without antibody. Additionally, tumor infiltration of neutrophils, NK cells, and DCs were unchanged by the addition of antibody. DCs in the tumor draining lymph nodes were substantially decreased by TBI, however, their total numbers and activation state were unchanged by the addition of antibody therapy. Antibody therapy did qualitatively enhance a humoral response against tumor antigens. Antibody therapy acted independently of the T cell response, but was critical in achieving durable cures. These results will hopefully encourage combinations of orthogonally acting therapies with ACT to achieve stronger clinical responses.

2.2 Introduction

Adoptive cell therapy (ACT) is one of the most effective treatments for metastatic melanoma and, along with chimeric antigen receptor (CAR) technology, has opened up new possibilities to achieve long lasting cures of cancer (Hinrichs and Rosenberg, 2014; June, 2007; Rosenberg et al., 2008). In vitro expanded TILs and the genetic transfer of TCRs can yield potent tumor reactive T cells (Chacon et al., 2013; Hughes et al., 2005). However, in the treatment of established solid tumors, T cell transfer alone has been shown to be insufficient to induce regression. Other supportive components are necessary to overcome the immunosuppressive tumor microenvironment and reverse T cell tolerance (Overwijk et al., 2003). Several methods of enhancing transferred T cell function have been used such as non-myeloablative conditioning (Dudley et al., 2008), cytokine support (Klebanoff et al., 2011) and vaccine stimulation (Palmer et al., 2004). Much of the focus of past research has been on enhancing the T cells themselves, however, targeting multiple discrete pathways in combination immunotherapy may lead to better treatment outcomes (Spranger and Gajewski, 2013).
IL-2 is an immunostimulatory cytokine commonly given as a supporting therapy to ACT, as well as a primary therapy against metastatic melanoma and renal cancer (Rosenberg, 2014). IL-2 is a powerful growth stimulant in CD8 cells and mediates differentiation of both CD8 and CD4 T cells into a variety of subsets with different functions. IL-2 also maintains and supports T<sub>r<sub>reg</sub> homeostasis (Nelson, 2004). IL-2 was one of the first agents given to support ACT, and dramatically improved its therapeutic efficacy (Rosenberg et al., 1988). When coupled with optimized conditioning protocols, IL-2 enhanced ACT saw increasingly more effective outcomes in melanoma patients (Rosenberg et al., 2011). Targeted immunocytokines have improved ACT (Singh et al., 2007), although the improvement may be due to serum persistence (Tzeng et al., 2015). IL-2’s ability to enhance survival and function of adoptively transferred cells has also been used to support more recent clinical protocols based on T cells engineered to express CARs (Dotti et al., 2014).

Antibody treatment is the most common biologic given in the treatment of cancer (Scott et al., 2012), however it is less commonly applied in combination with ACT. Antibodies that are combined with ACT are often used with the purpose of enhancing the T cell response (John et al., 2013; Kobold et al., 2015; Watanabe et al., 2010) as opposed to specifically targeting tumor associated antigens. Tumor targeting antibodies have been shown to augment cancer vaccine response to melanoma antigens (Ly et al., 2013; Saenger et al., 2008), suggesting that they could play an important role in stimulating adoptively transferred cells as well. This has been the case in at least one study, where TA99 was shown to augment the efficacy of adoptively transferred pmel-1 cells (Saenger et al., 2008).

A combination of an extended-pharmacokinetic IL-2 (MSA-IL2), a tumor targeting antibody (TA99), and melanoma specific T cells (pmel-1) was previously shown to eradicate established B16F10 tumors (Zhu et al., 2015). Therapy without antibody effectively delayed tumor growth, but was unable to achieve any cures. The work presented here extends this combination ACT treatment to larger tumors, analyzes the transferred cell population, examines tumor infiltrating immune cells, and characterizes DC subsets in the tumor draining lymph node. Additionally, a humoral response to tumor cell lysate was identified. Analysis of multiple therapeutic indicators demonstrated a robust anti-tumor T cell response and enhanced adaptive immune cell infiltration of the tumors. Unexpectedly, no significant mechanistic differences were observed between cases treated with or without tumor targeting antibody, despite dramatically different therapeutic outcomes.
2.3 Materials and Methods

2.3.1 Cell Lines and Mice

B16F10 were purchased from ATCC and cultured in complete DMEM (DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 4 mM L-alanyl-L-glutamine). HEK293 were purchased from Life Technologies and cultured in Freestyle Media (Life Technologies). All cell lines were maintained at 37°C and 5% CO₂.

B6 (C57BL/6NTac) mice (Taconic) and pmel-1 (B6.Cg-Thylα/Cy Tg(TcraTcrb8)Rest/J) mice (The Jackson Laboratory) were aged between 6–10 weeks before tumor induction or splenocyte isolation. All animal work was conducted under the approval of the Massachusetts Institute of Technology (MIT) Division of Comparative Medicine in accordance with federal, state, and local guidelines.

2.3.2 Therapeutic Proteins

TA99 (Thomson et al., 1985) and MSA-IL2 (Zhu et al., 2015) were generated as previously described (Reuel et al., 2013; Zhu et al., 2015). Briefly, TA99 was generated by HEK-TA99LH cells, purified by Protein A, and buffer exchanged into PBS. MSA-IL2 was generated by transient transfection of HEK cells with vector gWiz-MSA-IL2 using PEI. MSA-IL2 was purified by TALON Metal Affinity Resin followed by size exclusion chromatography on a Superdex 200 pg column (GE Healthcare Life Sciences) using an ÄKTA FPLC (GE Healthcare Life Sciences) in PBS.

2.3.3 Adoptive Cell Transfer

A subcutaneous tumor model was used to examine the effect of antibody therapy on ACT (Figure 1). B16F10 is a syngeneic, poorly immunogenic cell line that forms aggressive tumors in B6 mice. TA99 is an antibody to TRP-1, expressed on the surface of B16F10 cells. MSA-IL2 is a fusion protein of mouse serum albumin and murine IL-2. Pmel-1 T cells are specific to the gp100 MHC epitope, also expressed by B16F10 cells.

CD8+ pmel-1 T-cells were prepared as described previously (Zhu et al., 2015). 48 hours before administration, splenocytes were harvested by mechanically disrupting pmel-1 mouse spleens. After hypotonic (ACK) lysis of red blood cells, splenocytes were cultured in T-cell media (RPMI supplemented with 10% heat inactivated FBS, 20 mM HEPES, 1 mM sodium pyruvate, 0.055 mM 2-mercaptoethanol, 100 I.U. penicillin, 100
μg/mL streptomycin, 2 mM L-alanyl-L-glutamine, 1X MEM non-essential amino acids; Corning Cellgro) supplemented with 10 μg/mL murine IL-7 (Peprotech) and 2 μg/mL concanavalin A (Sigma-Aldrich). 24 hours later, CD8+ T-cells were isolated using magnetic bead negative selection (Stemcell). CD8+ T cells were cultured in T-cell media supplemented with 10% T-STIM with ConA (BD Biosciences) treated with 15 mg/mL methyl-alpha-D-mannopyranoside (Sigma-Aldrich). 24 hours after isolation, the CD8+ T-cells were washed in PBS and administered to recipient mice. All cells were maintained at 37°C and 5% CO₂.

Dosing was as follows. Tumors were inoculated with 10⁶ B16F10 cells injected s.c. into the flanks of recipient B6 mice. The day before ACT, recipient mice underwent 5 Gy of total body irradiation (TBI). Mice receiving adoptive cell transfer treatment were given a single i.p. injection (Petersen et al., 2006) of 10⁷ CD8+ pmel-1 T-cells. TA99 was administered i.p. at 100 μg per dose for 5 doses. MSA-IL2 was administered i.p. at 30 μg per dose for 5 doses. Two different treatment schedules were used. More aggressive therapy was performed after 6 days of tumor growth and treatments were given every 6 days for a total of 5 times (Figure 2). A more difficult therapy schedule treated larger tumors at day 8 and treated every 7 days for a total of 5 times (Figure 3). Control cases were either untreated B6 or pmel-1 mice as indicated. Tumor size was measured as an area (length x width).
Figure 1: Adoptive Cell Transfer (ACT) Model.
A murine model for combination ACT therapy of large, established tumor cells was used. B16F10, a syngeneic, aggressive melanoma cell line, were inoculated into B6 mice. Tumor specific T cells were from pmel-1 mice which have a monoclonal T cell population specific for the gp100 peptide MHC present on the surface of B16F10 cells. TA99 is an antibody to TRP-1, a melanoma associated antigen also expressed on the surface of B16F10 cells. MSA-IL2 is a mouse serum albumin fusion to mouse IL2 with favorable pharmacokinetic properties.
Figure 2: Aggressive Therapy Timeline.
Smaller B16F10 tumors were treated on day 6 with pmel-1 ACT. Supportive therapies were also given on day 6 and then every 6 days after for a total of 5 treatments.

Figure 3: Aggressive Tumor Timeline.
Larger B16F10 tumors were treated on day 8 with pmel-1 ACT. Supportive therapies were also given on day 8 and then every 7 days after for a total of 5 treatments.
2.3.4 Antibodies and Flow Cytometry

The following antibodies were purchased from BioLegend: Thy1.1, CD8, CD44, CD62L, IFN-γ, TNF-α, CD3ε, IgM, CD19, NK1.1, Ly-6G, F4/80, CD11b, CD25, CD69, PD-1, PDCA-1, I-A/I-E, CD86, Ly-6C, and CD64. CD4 and Foxp3 antibodies were purchased from eBioscience. Viability was assessed by Zombie Aqua (BioLegend). Cells were fixed using BD Cytofix. Foxp3 was labeled using the Transcription Factor Buffer Set from BD. Cells were analyzed using BD FACS LSR II, BD FACS LSR Fortessa, and BD FACSCanto flow cytometers. Data was analyzed using FlowJo (FlowJo, LLC).

2.3.5 Analysis of Circulating T Cells

Blood samples were taken, treated with ACK lysis buffer, and then stained for memory, activation, and exhaustion markers. Intracellular cytokine staining (ICS) was performed as previously described (Liu et al., 2014). Briefly, PBMCs were cultured with hgp100 peptide (KVPRNQDWL) for 2 hours, treated with Brefeldin A and cultured for another 4 hours. Cells were stained for CD8, fixed and permeabilized, then stained for IFN-γ and TNF-α.

2.3.6 Analysis of Tumor and Lymph Nodes

Immune cell infiltrates of tumors were measured 48 hours after the second treatment (day 17). Tumors were dissected from euthanized mice and passed through a 40 μm cell strainer. Disaggregated tumor cells were then stained and analyzed for T cells, neutrophils, NK cells, and DCs. Tumor draining lymph nodes (LN) were analyzed 48 hours after the second treatment (day 17). LNs were disaggregated and then incubated in dissociation buffer (1 mg/mL Dispase, 1 mg/mL Collagenase I, 1 mg/mL DNaseI in DMEM/F-12) before being passed through a 40 μm cell strainer. Disaggregated LNs were stained for various DC subtypes.

2.3.7 Serum Reactivity Immunoblot

Cell lysate from B16F10 cells was run on an SDS-PAGE gel, transferred to nitrocellulose membranes, blocked for 1 hr at RT, and then stained with serum from treated mice and rabbit anti-beta-actin overnight at 4°C. Membranes were then stained with goat anti-mouse-IRDye800 and anti-rabbit-IRDye680 for 1 hr at RT. Imaging was performed on an Odyssey scanner (Licor).
2.4 Results

In an aggressive treatment model, $10^6$ B16F10 tumors were injected into the flanks of B6 mice and grown for 6 days. On day 5, the mice were subjected to 5 Gy of TBI. On day 6, $10^7$ pmel-1 CD8+ T cells were injected i.p. along with MSA-IL2 alone or MSA-IL2 and TA99. MSA-IL2 with or without TA99 was given every 6 days afterwards for a total of 5 treatments (Figure 4). When all three components were given, tumors were completely controlled, leading to cures of all mice treated. Treatment by MSA-IL2 with pmel-1 led to sustained control of the tumors until treatment ended on day 30, and by approximately day 35, growth resumed. By day 80, the tumors of all mice treated without antibody had grown out.

Figure 4: ACT Treatment of Day 6 Tumors.
$10^6$ B16F10 cells were grown s.c. for 6 days and then treated with pmel-1 and MSA-IL2 or pmel-1, MSA-IL2, and TA99. MSA-IL2 and, if indicated, TA99 were given every 6 days for a total of 5 treatments. Complete regression of all tumors was achieved when TA99 was given. Without antibody, the ACT therapy was effective until therapy stopped at approximately day 35, when tumor growth resumed, and ultimately all tumors grew out by day 80. Left panel shows mean tumor size for each condition. Middle panel shows individual tumor size for each condition. Right panel shows survival curves for each condition.

Because of the success in aggressively treating smaller B16F10 tumors, a more difficult model was tested. In an aggressive tumor model, $10^6$ B16F10 tumors were injected into the flanks of B6 mice and grown for 8 days. On day 7, the mice were subjected to 5 Gy of TBI. On day 8, $10^7$ pmel-1 CD8+ T cells were injected i.p. along with MSA-IL2 alone or MSA-IL2 and TA99. MSA-IL2 with or without TA99 was given every 7 days afterwards for a total of 5 treatments (Figure 5). When all three components were given, the tumors were effectively controlled with the majority of mice clearing large, established tumors. Established tumors treated by MSA-IL2 with pmel-1 led to a delay in tumor growth, followed by a slight decrease in size. Ultimately, however, these tumors all grew out by day 70.
Figure 5: ACT Treatment of Day 8 Tumors.
10^6 B16F10 cells were grown s.c. for 8 days and then treated with pmel-1 and MSA-IL2 or pmel-1, MSA-IL2, and TA99. MSA-IL2 and, if indicated, TA99 were given every 7 days for a total of 5 treatments. Contrasted with the smaller tumors, a more significant difference between therapy with antibody and without was apparent. The addition of antibody therapy to ACT led to cures being observed in the majority of mice. Without antibody, the ACT therapy was effective in extending survival, but did not substantially decrease tumor size, and by day 70, all tumors had grown out. Left panel shows mean tumor size for each condition. Middle panel shows individual tumor size for each condition. Right panel shows survival curves for each condition.

To determine the effect of antibody therapy on ACT, circulating T cells were examined every 2 weeks (Figure 6). Transferred CD8+ T cells were identified by Thy1.1 and analyzed by flow cytometry for phenotype and activation markers. Interestingly, the overall phenotype of the two therapies was identical. Overall T cell numbers in both cases rose to a maximum after the last therapy, before dropping to lower levels by day 49. This indicates that the expansion of the transferred cells was heavily dependent on MSA-IL2. The progression to a Tcm phenotype was also the same regardless of antibody therapy. Activated and Tcm cells made up the majority of transferred T cells during the early treatment phase, before the overall shift in population to Tcm. Despite a significant difference in therapeutic outcome, the overall phenotype of the transferred T cells was unaffected by the presence of tumor targeting antibody or that fact that, by day 49, the MSA-IL2 + pmel-1 treated mice had large tumors, while those treated additionally with TA99 were largely cured by that point.
Figure 6: Phenotype of Circulating Transferred Cells.
Circulating Thy1.1+ CD8+ T cells were analyzed in ACT treated mice with or without antibody therapy. Antibody therapy appeared to be unrelated to T cell expansion and phenotype. Total numbers of transferred T cells peaked at the second measurement, before falling significantly after therapy was ended. Overall phenotype was also unaffected by TA99 therapy as Activated, Tcm, Tem, and Naive cell percentages were nearly identical between the two cases.
Functional markers for the transferred T cells were measured on day 17. Circulating T cells were measured for PD-1 and CD69 expression by flow cytometry (Figure 7). For comparison, wild type pmel-1 mice were also inoculated with B16F10 tumors and tested for exhaustion and activation. PD-1 expression in wild type mice was higher than in either ACT treatments. CD69 was higher in ACT treated mice than in wild type mice. Taken together, this suggests that ACT pmel-1 T cells are in a more activated and non-exhausted state, however, the absolute percentage of activated cells in any case was low. Pmel-1 cells in the transferred mice were also measured as a percentage of all CD8+ T cells. At this timepoint, essentially all of the T cells in ACT treated mice were pmel-1.

![Graphs showing PD-1 and CD69 expression](image)

Figure 7: State of Circulating Transferred Cells.
On day 17, circulating Thy1.1+ CD8+ T cells were analyzed for activation, exhaustion, and total numbers. For comparison, wild-type pmel-1 mice with B16F10 tumors were also measured. Transferred T cells showed lower PD-1+ T cells and higher CD69+ percentages, indicating a more activated and less exhausted phenotype. However, in all cases, the absolute percentage of exhausted and activated cells was very low. Transferred T cells also made up nearly all of the circulating CD8 T cells in recipient mice. The percentage of CD8+ T cells that are Thy1.1+ was almost 100%. There was no observed difference in any of these measurements when antibody was administered. Example FACS plots are shown below.
Differences in tumor infiltration were measured in wild type pmel-1 mice as well as ACT treated mice. 17 days after tumor inoculation, all three conditions were analyzed for T cell infiltration of their tumors. CD8+ infiltration was significantly increased in the two therapeutic cases over wild type mice (Figure 8). CD4 and Treg infiltration was equivalent for all conditions at low levels. Given the high level of CD8+ infiltration and low levels of CD4+ infiltration, it was unsurprising that the CD8+:Treg ratio was significantly increased in the ACT cases. Once again, no difference was observed between MSA-IL2 and MSA-IL2 + TA99 ACT treated mice.

The CD8+ TILs were then further analyzed for activation and exhaustion markers (Figure 9). Very few TILs were observed in wild type pmel-1 mice, and those that were had high PD-1 and low activation (CD25 and CD69), consistent with a tolerized state. ACT treated mice had both low levels of PD-1 and high activation (CD25 and CD69), suggesting an active and functional state.

Tumor infiltrating innate cells were measured at the same time as TILs (day17) for wild type pmel-1 mice and both treatment conditions (Figure 10). NK cells and DCs were unchanged in any of the cases. Neutrophils were elevated in both treatment cases.

Characterization of tumor draining lymph nodes (TDLN) was performed 17 days after tumor inoculation. TDLN were mechanically disaggregated, treated with tissue dissociation buffer, and then filtered prior to staining and analysis by flow cytometry. Conventional (CD11c+ CD11b+) and cross-presenting DCs (CD11c+ CD11b- CD8+) were measured. Plasmacytoid dendritic cells (CD11c+ CD11b- PDCA1+) were also measured, but were not found in significant numbers in any condition (data not shown). There were very few differences observed between any of the conditions. Total conventional and cross-presenting DC numbers per LN were not statistically different. CD86 levels were also not significantly different between any of the conditions. MHCII levels were slightly lower in treated cases when compared to wild type pmel-1 cells, but there was no difference when antibody therapy was administered.
Figure 8: Tumor Infiltrating T Cells.

After 17 days, tumors from wild type pmel-1 mice and both treatment cases were analyzed for T cell infiltrates. Wild type pmel-1 mice had almost no CD8+ T cell infiltration, while both treatment cases had high levels of infiltration. CD4 and Treg levels were low in all cases. The prognostic indicator CD8+/Treg ratio was markedly higher for both treatment cases. No significant differences were observed with antibody therapy.
Figure 9: Activation State of Infiltrating T Cells.
Tumor infiltrating CD8+ pmel-1 cells were characterized for exhaustion and activation markers 17 days after tumor inoculation. Of the few wild type pmel-1 TILs, the majority were of an exhausted, inactivated state. TILs from ACT treated mice were high in activation markers CD25 and CD69, as well as low in the exhaustion marker PD-1. No difference was observed with TA99 therapy.

Figure 10: Tumor Infiltrating Innate Cells.
After 17 days, tumors from wild type pmel-1 mice and both treatment cases were analyzed for innate immune cell infiltrates. NK cells and DCs were identical between all cases. Neutrophils showed increased levels. No significant differences were observed with antibody therapy. Neutrophils are Ly6G+ CD11b+. NK cells are CD3- NK1.1+. DCs are CD11c+.
Figure 11: Characterization of LN DCs.
Tumor draining lymph nodes (TDLN) were dissected on day 17 from wild type pmel-1 mice and both treatment cases. DC subtypes were characterized and measured for activation by MCHII and CD86 expression levels. No significant differences were observed with antibody therapy.
The humoral response of the immune system to B16F10 antigen was measured by immunoblot. Serum samples were taken on day 35 and used to probe SDS-PAGE gels of B16F10 tumor cell lysate. Untreated pmel-1 mice had no reactivity to the tumor lysate. ACT treated mice had reactions with multiple tumor antigens. Subjectively, the results are similar with and without antibody, indicating that tumor antigen processing and presentation to the humoral immune system was dependent only on pmel-1 and MSA-IL2 therapy.

![Figure 12: Serum Reactivity to Tumor Cell Lysate. Immunoblots were performed using serum to probe B16F10 cell lysate separated on SDS-PAGE. Untreated pmel-1 mice had no reactivity to B16F10 lysate, while the two treated cases had responses to several antigens. Beta-actin is shown as a loading and exposure control. TA99 showed the location of TRP-1 in the cell lysate. For each treated condition, four mice were tested in separate lanes.](image)

MSA-IL2 is a potent growth stimulant for T cells and was tested for its ability to generate a robust ACT response without the need for TBI. As shown in Figure 13, TBI was critical to the full efficacy of either treatment. MSA-IL2 + pmel-1 treated mice had significantly shorter median survival times when not treated with conditioning radiation. Mice additionally treated with TA99 were unable to reject any of their
tumors. TBI was well tolerated by ACT treated mice as weight gain was not affected by any of the therapies (Figure 14).

![ACT Survival Graph](image)

Figure 13: Survival of ACT Treated Mice without TBI. Mice were treated identically to the aggressive tumor model, but not given TBI prior to ACT. Survival results clearly show a decrease in treatment efficacy, and the loss of incremental benefit provided by antibody therapy.

![ACT Day 8 Tumors Weight Graph](image)

Figure 14: Weight of ACT Treated Mice. Mice receiving ACT were weighed as an indicator of overall health and treatment toxicity. All cases showed progressive weight gain from prior to treatment, through the third treatment.
2.5 Discussion

Although one of the goals of this research was to determine the mechanism by which tumor targeting antibody augmented ACT, none of the measured parameters were significantly different when TA99 was administered. It remains a possibility that the gross phenotypic measurements performed in this study were insufficient to detect subtle, but important, differences in the T cell populations. More detailed studies could be performed such as microarrays, next generation sequencing, or functional assays, which could more finely discriminate between the two treatments. However, it would be fascinating to see if those subtle differences would be sufficient enough to elicit such a strong curative response without modifying more traditional T cell activation and phenotype markers.

The transferred CD8+ pmel-1 response was very strong in this model of ACT. Circulating transferred T cells expanded rapidly and made up essentially all CD8+ T cells after the second treatment. Tumor infiltration also indicated a strong T cell response. Infiltrating CD8+ T cells were highly functional with low PD-1 expression and high CD25 and CD69 levels. Additionally, T_{reg} levels were not enhanced by MSA-IL2 treatment over wild type mice. T_{reg}s exert a strong influence on CD8 T cell functioning and their interaction with tumor antigen via APCs can have a dramatic effect on the tumor microenvironment (Bauer et al., 2014). The low numbers of T_{reg}s as well as the increased CD8+ numbers in the tumors gave highly favorable CD8+:T_{reg} ratios, indicative of favorable clinical outcomes in several types of cancer (Ibrahim et al., 2014; Sato et al., 2005; Shah et al., 2011).

Although neutrophils were a critical component to the functioning of MSA-IL2 and TA99 therapy, they were expendable when ACT was added (Zhu et al., 2015). In that study, TA99 significantly increased the infiltration of neutrophils, whereas here, there was no difference between antibody and no antibody cases. These results appear to indicate a lesser role of innate cells in the efficacy of the combination ACT therapy, however, the innate immune system has been shown to be crucial to the success of other ACT protocols. One study showed a lack of efficacy in SCID mice, where Rag deficient and wild type mice were cured (Ammori et al., 2015). The effectiveness of these combinations were also dependent on the interaction of innate and adaptive immune cells. Eosinophils have been shown to normalize tumor vasculature during effective therapies, facilitating the infiltration of CD8 T cells into the tumor space (Carretero et al., 2015). This result highlights the complexity of combination immunotherapy treatments. Critical treatments may function in very different ways when used in combination with different therapies.
The importance of DCs in successful adoptive cell therapy has not been fully studied. In this study, no difference in proliferation or activation was observed with antibody therapy, however, cross-presentation was not fully examined. Antibody therapy was shown previously to improve cross-presentation and elicit strong tumor-specific killer T cells without enhanced tumor cell uptake or DC maturation (Dhodapkar et al., 2002). DC recovery after lymphodepletion has been shown to affect the overall response of adoptively transferred mice (Salem and Cole, 2010). Lymphodepletion is also responsible for an overall immunostimulatory signal created by microbial translocation (Paulos et al., 2007), but another study showed this was not the only factor in DC stimulation (Espinosa-Carrasco et al., 2015). In this study, the addition of sustained cytokine signaling was insufficient to abrogate the requirement for TBI. Another group attempted to bypass TBI with anti-CD40 conditioning, but was similarly unable to get the same level of protection (Cozza et al., 2015).

Humoral response has been seen previously in patients treated with CARs (Beatty et al., 2014). The combination of cellular and humoral immunity to provide tumor protection has been shown previously (Orlandi et al., 2007).

Although CARs have yielded many clinical successes, it remains to be seen if the results presented here would enhance their efficacy, especially in the treatment of solid tumors. The use of CARs in combination with antibody therapy could have an advantage in that there is presumably already a tumor specific antibody available and the same tumor antigen could be targeted by both Fc-effector function and cytolytic T cells. Antibody therapy is often given before ACT (Brentjens et al., 2011; James et al., 2009; Kalos et al., 2011; Kochenderfer et al., 2012), losing any potential synergy from administering the components at the same time, such as when adoptive transfer of LAK cells improved the functioning of rituximab when given with IL2 (Berdeja et al., 2007).

In addition to genetic modifications to redirect the specificity of T cells, ACT protocols have engineered T cells to express a variety of other adjuvant molecules. T cells expressing multiple cytokines (Markley and Sadelain, 2010), IL-12 (Kerker et al., 2010), and TLR5 ligands (Geng et al., 2015) have all increased the effectiveness of the transferred cells. Safety has also been improved by silencing endogenous TCRs during genetic modification (Ochi et al., 2011) and the use of suicide systems to control overactive immune responses, such as the HSV-TK system (Ciceri et al., 2007; Maury et al., 2014). The ability of T cells to actively secrete tumor targeting antibodies adds to this body of work. This concept could be especially useful with antibodies that have more severe off target toxicities, such as anti-GITR antibodies, which were produced in a similar manner by DCs to reduce side effects (Boczkowski et al., 2009).
2.6 Conclusion

ACT promises dramatic results based on the effectiveness of T cell mediated killing. Despite success in many types of hematologic cancer, solid tumors present new and unique challenges to the efficacy of cell based therapies. This study had two major findings. First, in some cases, T cells alone cannot mediate complete tumor rejection. Second, treatments that elicit similar T cell responses can have very different outcomes when combined with orthogonally acting therapies. These results demonstrate that T cell characterization alone cannot determine therapeutic efficacy of combination ACT therapy. A strong and robust T cell response was elicited by support with MSA-IL2, but this was insufficient to cure established tumors. When TA99 was added, the overall phenotype of the T cells was unchanged, but therapy was dramatically enhanced, although the precise mechanism was unable to be established. Tumor antigen targeting antibody support of ACT showed significant improvement in therapeutic outcome in a mouse model of cancer and warrants further study.
3 Combination Immunotherapy

3.1 Abstract

With the ongoing success and approval of individual immunotherapies in the clinic, the use of combination immunotherapy in investigative trials is increasing. Immunotherapy covers a broad range of treatments that seek to manipulate the immune system to effectively mount a response against cancerous cells, however, the complexity and interdependence of the immune system suggests that any single therapeutic intervention will be insufficient to reject established malignancies. This study applied combinations of immunotherapy using up to four different components to identify critical factors in the successful rejection of established tumors in preclinical models. The four components of tumor targeting antibody, cytokine support, checkpoint blockade, and cancer vaccine acted synergistically to reject tumors from B16F10, TC-1, and DD-Her2/neu cell lines. By analyzing subset combinations of this full treatment, the roles of each therapeutic component were identified. CD8 T cells and cross-presenting DCs were critical to curing subcutaneous tumors. Cytokine therapy was indispensable for effective tumor control, promoted immune cell infiltration into the tumor, and an increase in DC proliferation. In combination with the other therapies, vaccination against a tumor antigen elicited a strong immunological memory response that was able to reject subsequent tumor rechallenge, as well as promote antigen spreading. Successful combinations were demonstrated to be dependent on the recruitment of both the adaptive and innate branches of the immune system. Finally, the efficacy of this combination of treatments was demonstrated by controlling the growth of induced tumors in a BRaf/Pten model. By determining the necessary requirements for a strong, synergistic response to tumorous growths, more effective combination immunotherapy protocols may be designed in the future.

3.2 Introduction

Previous work done in the Irvine lab developed a highly potent vaccine by targeting tumor associated antigen peptides and CpG DNAs to sentinel lymph nodes (Liu et al., 2014). By covalently linking these two components to lipophilic albumin-binding domains, both the peptide and adjuvant were trafficked to the lymph node, where a robust CD8 T cell response was developed (Figure 15).
Figure 15: Lymph Node Targeting Amphiphilic Vaccine.
The amph-vaccine system consists of two components. The first is a peptide antigen fused to a lipophilic albumin-binding domain. The peptide contains a CD8 epitope to a tumor associated antigen. The second components is a CpG DNA sequence fused to a lipophilic albumin-binding domain which provides an adjuvant danger signal as the immune system associates CpG DNA with a bacterial threat. Together, these components traffic to the lymph node and raise a specific T cell response against the peptide antigen. Diagram courtesy of the Irvine lab.

The CD8⁺ response was further boosted when a checkpoint blocking αPD-1 antibody was added. Despite a high number of circulating tumor specific T cells, only modest control of B16F10 melanoma cells was observed (Figure 16). This result is common among cancer vaccines and indicates the complexity of treating tumors, where a complex, suppressive microenvironment can resist systemically strong responses.

Figure 16: Vaccine Response and Tumor Control.
A strong tumor specific T cell response was achieved after addition of αPD-1 to the vaccine therapy. Intracellular cytokine staining showed over 10% of circulating T cells were antigen specific after the second vaccination. Despite this strong systemic response, tumor control remained relatively modest, with only a delay in growth being achieved when treating small tumor at early times. Right panel reproduced from (Liu et al., 2014).
Studies done in the Wittrup lab showed that a potent combination of tumor specific T cells, cytokine support, and tumor targeting antibody could cure large, established B16F10 tumors (Zhu et al., 2015). However, when ACT of tumor specific T cells was not performed, these tumors were not cured despite significant extension of survival over untreated controls. Additionally, ACT on its own has shown similar results to those seen in the cancer vaccine study in Figure 16. ACT is a powerful immunotherapy with positive progress in clinical trials, however its cost and complexity make alternative, non-cell based approaches particularly attractive. Given the Irvine lab vaccine’s ability to elicit an ACT level response and the Wittrup lab treatment’s ability to support ACT, the combination of these two approaches was expected to generate a robust, synergistic response to large, established tumors in murine models.

Given the complexity of the immune system, and the various checks and balances that allow a focused and controlled response, it is unsurprising that most monotherapies are ineffective in treating cancer. The benefit therefore of using multiple immunotherapies at the same time in order to engender a more robust anticancer response has been recognized for a long time. By combining immunotherapies possessing different and complementary mechanisms of action, such as targeted therapies, immunostimulatory molecules, and checkpoint blockade, long-lasting clinical remissions could be possible (Vanneman and Dranoff, 2012). As the number of approved immunotherapies increases and dosing strategies for those drugs are more mature, the clinical feasibility of combining multiple agents becomes more realistic. Due to synergistic effects, the use of combinations of immunotherapies is highly complex. While individual drugs may have intolerable side effects, when used in combination new toxicities may manifest themselves.

The success of single immunotherapeutics can often be related to pre-existing features such as genetics (Snyder et al., 2014) or the presence of lymphocytosis (Phan et al., 2001) which are beyond the control of most clinicians. Combinations of therapies designed to overcome these limitations, or create more favorable prognostic indicators can extend the value of each individual component. Preclinical models with more therapeutic components have shown success in controlling large, established tumors. Four immunomodulatory antibodies were used to control and cure a variety of tumors (Dai et al., 2015), however, intratumoral injections were required.

This study sought to define the critical attributes necessary for a successful immune response against established malignancies. Rational combinations of immunotherapies have been suggested previously (Spranger and Gajewski, 2013).
Using these criteria, as well as previous results (Liu et al., 2014; Zhu et al., 2015), as guides, a combination of tumor targeting agents and supportive therapies was tested. In doing so, a highly effective treatment was developed, which relied on the systemic administration of well-established immunotherapeutic agents.

### 3.3 Materials and Methods

#### 3.3.1 Cell Lines

B16F10 were purchased from ATCC. TC-1 cells were kindly provided by Dr. T. C. Wu at John Hopkins University. B16-OVA cells were kindly provided by Dr. Glenn Dranoff at Dana-Farber. B16-GFP-Luc cells were generated as previously described (Tzeng et al., 2015). DD-Her2/neu cells were made by isolating a tumor cell line from Balb/c mice and then transfecting with rat Her2/neu. A stable cell line was then isolated and designated DD-Her2/neu. HEK293 were purchased from Life Technologies.

B16-TRP2-KO were generated using a CRISPR-Cas9 system. B16-GFP-Cas9 cells were generated by transduction of B16F10 cells with lentivirus expressing humanized SpCas9-P2A-EGFP from an EFS promoter (unpublished, kindly provided by Tim Wang in Dr. David Sabatini’s Lab at the Whitehead Institute). Clones were isolated by single cell FACS for GFP positive cells. A clone stably expressing Cas9 was designated B16-GFP-Cas9. GuideRNA expression vectors were created by cloning a human U6 promoter and sgRNA sequences into a minimal vector with an ampicillin-selectable marker and a ColE1 replication origin. We used the optimized sgRNA sequence described previously (Chen et al., 2013). GuideRNA targeting sequences (Table 1) were designed using tools provided by the Zhang Lab at the Broad Institute (available at [http://crispr.mit.edu/](http://crispr.mit.edu/)). B16-GFP-Cas9 cells were co-transfected with 2 GuideRNA expression vectors for TRP2 knockout and a plasmid expressing EGFP and a puromycin selectable marker from a CAG promoter using Xfect (Clontech) according to manufacturer’s instructions. Stable cells were selected for with 2 µg/mL puromycin followed by single cell FACS for GFP positive cells. Clones were grown for 3 weeks before being analyzed for knockout by PCR amplification of the TRP2 gene (Primers shown in (Table 1). The final clone was then selected and designated B16-TRP2-KO. PCR amplification of genomic DNA showed a smaller fragment (Figure 17) and sequencing confirmed deletion of the TRP2 peptide sequence.
Figure 17: PCR of B16-TRP2-KO.
Genomic DNA from either wild type B16F10 cells (TRP2-wt) or B16-TRP2-KO cells (TRP2-KO) was isolated and used to amplify a subsection of the TRP2 gene by PCR. The PCR products were then run on an agarose gel and imaged. The TRP2-KO band showed clear deletions. Sequencing of the band showed that the TRP2 vaccine epitope was not present in B16-TRP2-KO cells.

Tumor cell lines were cultured in complete DMEM (DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 4 mM L-alanyl-L-glutamine). T cells and splenocytes were cultured in RPMI with 10% heat inactivated FBS, 20 mM HEPES, 1 mM sodium pyruvate, 0.055 mM beta-mercaptoethanol, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-alanyl-L-glutamine, and 1X MEM non-essential amino acids. Hybridomas were cultured in CD Hybridoma AGT Medium (Life Technologies). HEK293 cells were cultured in Freestyle Media (Life Technologies). All cell lines and assay cultures were maintained at 37°C and 5% CO₂.

3.3.2 Mice

B6 mice (C57BL/6NTac) were purchased from Taconic. Balb/c mice (BALB/cj), Batf3⁻/⁻ mice (B6.129S(C)-Batf3tm1Kmm/J), BRaf/Pten mice (B6.Cg-Braf<sup>tm1Mmcm</sup> Pten<sup>tm1Hwu</sup> Tg(Tyr-cre/ERT2)13Bos/BosJ), and mT/mG mice (B6.129(Cg)-Gt(Rosa)26Sor<sup>tm4(Actb-tatm1Ros-</sup>tdTomato,-Egfp)Luo<sup>+/+</sup>) were purchased from The Jackson Laboratory. Mice used in the inducible cancer model (BRaf/Pten-TG) were crosses of BRaf/Pten and mT/mG bred in-house and having the following genotype: Braf<sup>tm1Mmcm</sup> +/−, Pten<sup>tm1Hwu</sup> +/+ , Tg(Tyr-cre/ERT2)13Bos + , Gt(Rosa)26Sor<sup>tm4(Actb-tatm1Ros-tdTomato,-Egfp)Luo</sup>+/-, where "+" indicates presence of the mutant/transgenic allele. All mice were genotyped via Transnetyx. Mice were used in studies when 6-8 weeks old. All animal work was conducted under the approval of
the Massachusetts Institute of Technology (MIT) Division of Comparative Medicine in accordance with federal, state, and local guidelines.

3.3.3 Vaccines and Therapeutic Proteins

Lipophilic vaccines were produced as previously described (Liu et al., 2014). Vaccine peptide sequences are shown in Table 1. TA99 (Thomson et al., 1985), Fc-IL2, and MSA-IL2 (Zhu et al., 2015) were generated as previously described (Reuel et al., 2013; Zhu et al., 2015). Trident-Fc was generated by fusing an integrin (αvβ3, αvβ5, and α5β1) binding domain to a murine IgG2a Fc region to create an antibody-like tumor targeting molecule (Kimura et al., 2009; Moore et al., 2013). Trident-Fc was produced by transient transfection of HEK293 cells and purified by Protein A. An antibody to rat Her2/neu was generated from hybridoma 7.16.4 (Park et al., 2010) purchased from ATCC. 7.16.4 and was purified using Protein A. Anti-PD-1 (RMP1-14) and anti-CTLA-4 (9H10) were purchased from BioXCell.

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<th>Item</th>
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<td>(Liu et al., 2014)</td>
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<td>Modified Trp1 peptide</td>
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<td>p66 (Her2/neu) peptide</td>
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</table>

Table 1: Sequences. Peptide sequences for vaccines / ICS stimulation, guide sequences for CRISPR TRP2 knockout, and PCR primers for knockout verification are listed.
A subcutaneous tumor model of large, established syngenic cells was used (Figure 18). Tumors were grown for 8 days followed by 5 weekly treatments and a second tumor challenge on day 75 (Figure 19). For the primary challenge, $10^6$ tumor cells were inoculated s.c. into the flanks of mice. For the rechallenge, $10^5$ tumor cells were inoculated s.c. into the opposite flank. Tumor specific antibody was administered i.p. at 100 µg per dose for 5 doses. Trident-Fc was administered i.p. at 500 µg per dose for 5 doses. MSA-IL2 was administered i.p. at 30 µg per dose for 5 doses. Anti-PD-1 and anti-CTLA-4 antibody were administered i.p. at 200 µg per dose for 3 doses. Vaccine was administered s.c. at base of the tail, half the dose given on each side. 1.24 nmol amph-CpG and 20 µg of amph-peptide were administered per dose for 3 doses. The dosing schedule for each component was consistent across all cases. Tumor size was measured as an area (length x width). Different combinations of treatments were used, and for simplicity are referred to using the abbreviations: A=Antigen Targeting (Antibody or Trident-Fc); I=MSA-IL2; P=PD-1 Checkpoint blockade ($\alpha$PD-1); V=Vaccine. In this document, TETRIS is equivalent to the full combination or AIPV treatment case.
The main in vivo tumor model components are outlined. Unless otherwise noted, the following components were used. B16F10 is a syngenic melanoma cell line which establishes aggressive tumors after transplant into C57BL/6 mice. The amphiphile vaccine consists of two amphiphilic molecules, amph-CpG and amph-peptide. The peptide for Trp-2 was used and causes a robust response to the Trp-2 epitope, which is presented by B16F10 via MHC. TA99 is a murine IgG2a antibody to Trp1, a melanoma marker expressed by B16F10 cells. The cytokine fusion of murine serum albumin and interleukin-2 (MSA-IL2) has an extended pharmacokinetic profile and leads to an activation of multiple immune pathways. Checkpoint blockade was administered in the form of αPD-1 antibodies. A=Antigen Targeting; I=MSA-IL2; P=αPD-1; V=Vaccine.
Figure 19: Tumor Model Timeline.
A tumor model of large, established subcutaneous melanoma cells was used. $10^6$ B16F10 cells were inoculated into the flanks of B6 mice and allowed to establish for 8 days. On day 8, treatment was initiated on a weekly basis. The first three treatments consisted of all four components on days 8, 15, and 22. The final two treatments consisted of only cytokine and tumor targeting antibody on days 29 and 35. On day 75, surviving mice were rechallenged with $10^5$ B16F10 cells to test for immunological memory.

3.3.5 Immune Cell Depletion Studies

Cytotoxic Lymphocytes were depleted by Anti-CD8α (clone 2.43). Natural killer cells were depleted by anti-NK1.1 (clone PK136), and anti-Ly-6G (clone 1A8). Starting one day prior to the first treatment, depletion antibodies were administered twice a week at 400 µg per dose. Depletions were confirmed by flow cytometry of PBMC. All depletion antibodies were purchased from BioXCell.

3.3.6 Antibodies and Flow Cytometry

Antibodies to CD8, CCR7, CD11a, CD44, CD62L, KLRG1, CD127, IFN-γ, TNF-α, CD3ε, IgM, CD19, NK1.1, Ly-6G, F4/80, CD11b, CD25, CD69, PD-1, Lag-3, Tim-3, PDCA-1, I-A/I-E, CD86, Ly-6C, and CD64 were purchased from BioLegend. Antibodies to CD4 and Foxp3 were purchased from eBioscience. Tetramers complexed with TRP-2 or OVA peptides were purchased from MBL. Tetramer staining was performed in buffer containing 50 nM dasatinib. Viability was assessed by LIVE/DEAD Fixable Aqua purchased from Life Technologies. TA99 was labeled with Alexa Fluor 647 NHS Ester (Life Technologies) to generate TA99-647. After staining, cells were fixed using BD Cytofix. Foxp3 was labeled using the Transcription Factor Buffer Set from BD. Cells
were analyzed using BD FACS LSR II, BD FACS LSR Fortessa, and BD FACSCanto flow cytometers. Data was analyzed using FlowJo.

3.3.7 Tumor, ICS, and Lymph Node Analysis

Intracellular Cytokine Staining (ICS) was performed as described previously (Liu et al., 2014). Peptides used for stimulation were identical to those used to immunize (Table 1). Immune cell infiltrates of tumors were analyzed as previously described (Zhu et al., 2015). Lymph nodes were stained as previously described (Tzeng et al., 2015).

3.3.8 ELISPOT

Target B16F10, TC-1, or B16-TRP2-KO cells were treated with 500 U/mL mIFN-γ (Peprotech) overnight, then irradiated (120 Gy). Effector cells were splenocytes isolated from cured AIPV treated mice 6 days after rechallenge with 10⁶ B16F10 cells. A Mouse IFN-γ ELISPOT Kit (BD) was used. Targets cells were seeded at 25,000 cells per well. Effector cells were seeded at 10⁶ cells per well. Plates were wrapped in foil and cultured for 24 hours then developed according to manufacturer’s protocol. Plates were scanned using a CTL-ImmunoSpot Plate Reader and data was analyzed using CTL ImmunoSpot Software.

3.3.9 Serum Reactivity Western

B16F10 cell lysate was run on an SDS-PAGE gel, transferred to nitrocellulose membranes, blocked for 1 hr at RT, and then stained with serum from treated mice and rabbit anti-beta-actin overnight at 4°C. Membranes were then washed and stained with goat anti-mouse-IRDye800 and anti-rabbit-IRDye680 for 1 hr at RT. Imaging was performed on an Odyssey scanner (Licor). As a control, TA99 was used in place of mouse serum in the protocol above to show the presence of TRP1 in the B16F10 lysate.

3.3.10 Inducible Tumor Model

BRaf/Pten mice (Dankort et al., 2009) were crossed with mT/mG (Muzumdar et al., 2007) in order to generate BRaf/Pten-TG mice, which were used in treatment studies. To induce tumors, 2 μL of 5 mg/mL tamoxifen was administered to the left ear on three consecutive days. 24-26 days later, when visible tumor lesions were present, treatment was begun and executed as described for the subcutaneous tumor model (Figure 20). The vaccine used in this model was a combination of three amph-peptides (15 μg amph-gp100, 15 μg amph-Trp1, and 15 μg amph-Trp2) and 1.24 nmol amph-CpG.
administered as a single dose. Mice were euthanized when pigmented lesions covered the induced ear or when apigmented tumors reached 10 mm in diameter.

BRAF / PTEN Timeline

Figure 20: BRaf/pten Inducible Model Timeline. Tumors were induced by applying tamoxifen for three consecutive days. 21-24 days later, visible pigmented lesions were visible and therapy was started. The vaccine used in this model combined three different melanoma antigens, Trp1, Trp2, and gp100 to achieve the broadest reactivity to the heterogeneous tumors. Treatments were applied as in the subcutaneous model at weekly intervals. Vaccine and checkpoint blockade were given for the first 3 weeks. Antibody and cytokine therapy was given for 5 weeks.

3.4 Results

A combination immunotherapy treatment regimen was performed on B6 mice inoculated with $10^6$ B16F10 cells s.c. and grown for 8 days. Combinations of up to four types of treatments were evaluated for their ability to control these tumors (Figure 21). TA99 antibody (A) targets the Trp1 protein which is expressed on the surface of B16F10 cells. MSA-IL2 (I) is an extended pharmacokinetic cytokine fusion providing a long-lasting immunostimulatory signal. Checkpoint blockade (P) was administered as an αPD-1 antibody. Finally, Amph-TRP2 Vaccine (V) elicits a strong CD8 T cell response to the TRP2 peptide displayed on the MHC of B16F10 cells. The combination of all four agents (AIPV) showed the strongest tumor control and led to cures of established tumors in over 60% of cases. Two three-agent combinations, AIP and AIV, also showed strong tumor control with several cures. Two treatment combinations, AI and IPV, delayed tumor growth, but failed to cure any of the tumors. Combinations without MSA-IL2 performed similarly to untreated mice, indicating the importance of overall immune stimulation to the efficacy of immunotherapy in this model.
The previous results were grouped by overall efficacy (Figure 22). Conditions with no cytokine support were indistinguishable from untreated mice. Without supportive cytokine therapy, it appeared that none of the other therapies were able to function against a large, established, and aggressive tumor burden. Combinations that controlled tumor growth but ultimately failed to cure them were missing a critical component. AI lacked direct T cell support in the form of vaccination or checkpoint blockade, while IPV failed to directly engage the innate immune system with a targeted antibody therapy. The effective three-component treatments were missing a supportive component in each case. Checkpoint blockade had a strong effect on the vaccine.
response and without it, AIV was only able to cure a small number of mice. AIP was a strong combination in this model, indicating that a monoclonal T cell population provided by vaccination was not critical to the response against a primary tumor challenge. Finally, the strongest tumor control was observed when all of the components were applied. These groupings demonstrated the high degree of synergy that immunotherapy combinations required to be successful.

Tumor Control: By Efficacy

Figure 22: Grouped Tumor Control and Survival.
Tumor growth and survival curves shown in Figure 21 were grouped according to therapeutic efficacy to visualize broad trends. A critical factor for any kind of tumor control was the presence of cytokine support. Even the APV combination offered no significant benefit over untreated mice. Two therapies which controlled, but did not cure tumors, were missing critical components. All lacked T cells support in the form of vaccination or checkpoint blockade. IPV lacked a targeted antibody therapy. The two effective three component treatments, AIV and AIP, missed a supportive component which enhanced the cure rate of the therapies. AIV significantly prolonged survival and cured a small fraction of mice, indicating the importance of inhibiting immunosuppressive signals using αPD-1. AIP cured the majority of mice, but did so without a widespread vaccine elicited T cell response. Although AIPV had a marginal improvement in survival over AIP, future studies demonstrated the benefit of vaccine therapy.
Mice surviving their primary tumors were rechallenged at day 75 with $10^5$ B16F10 cells s.c. into their opposite flanks. Additionally, a cohort of mice lacking primary tumors were treated with AIPV and rechallenged at day 75. Figure 23 shows that only mice surviving a primary tumor challenge treated with AIPV were able to reliably reject subsequent tumor cell injections. AIV demonstrated no immunological memory, as secondary tumors grew progressively. Although AIP showed comparable primary tumor control with AIPV, its inability to elicit significant rejections of secondary tumors showed the therapeutic benefit of the vaccine treatment.

Robust Memory achieved only with full combo and antigen exposure

Figure 23: Immunological Memory Demonstrated by Rechallenge.
Mice cured of their initial tumors at 75 days after inoculation were given a secondary rechallenge of $10^5$ B16F10 cells. Despite the modest increase in primary survival seen in Figure 21, AIPV demonstrated strong immunological memory with over 75% of mice rejecting rechallenge. AIP was able to reject a minority of secondary challenges, while AIV offered insufficient protection to reject any rechallenge. Tumor antigen played a critical role in the establishment of immunological memory, as non-tumor bearing mice treated with the full course of AIPV were unable to reject tumor challenge on day 75. Survival fractions are: AIPV 9/11; AIP 2/7; AIV 0/3; AIPV no tumor 0/10.
Interestingly, unlike equivalently treated mice that were inoculated with primary tumors, mice treated with AIPV and no primary tumors were unable to reject subsequent tumor challenge. Despite this lack of protection from rechallenge, these mice underwent strong vitiligo responses, normally associated with successful immunotherapies (Figure 24). The dissociation between vitiligo and successful immunotherapies has been observed previously (Byrne et al., 2014). These results indicated the importance of tumor-derived antigen and suggested that cross-presentation of those antigens may have led to antigen spreading. They also showed that vitiligo may indicate a strong immunological response to targeted melanoma differentiation markers, but a complex, suppressive tumor microenvironment may overcome this response.

Vitiligo

![Vitiligo in Combination Treated Mice](image)

Figure 24: Vitiligo in Combination Treated Mice.
Loss of pigment, or vitiligo is a promising indicator of autoimmunity and is often a positive indicator in the treatment of melanoma. Mice undergoing AIPV treatment consistently displayed widespread vitiligo regardless of the presence of primary tumor. This indicates that despite a strong response against the differentiation markers, the immunosuppressive tumor microenvironment is able to prevent mice inexperienced with other tumor antigens from preventing the formation of subcutaneous tumors.
As a first study of therapeutic mechanism, various immune cell depletions were performed on the AIPV treatment case (Figure 25). These studies identified two critical cell types contributing to the potency of the AIPV therapy: CD8 T cells and cross-presenting DCs. CD8 cells were depleted by administering antibodies twice weekly for the duration of the treatment cycle. Cross-presenting DCs were depleted by using Batf3−/− mice. A primary result of these depletions is that the vaccine and checkpoint blockade therapies are marginalized since they primarily act through T cell mediated pathways. More generally, it showed that the adaptive immune system is a critical part of this combination immunotherapy. NK cells and neutrophils were not essential for some cures to be observed, but their depletion led to significant reductions in overall survival rates. Both NK cells and neutrophils were depleted using antibodies administered twice weekly for the duration of the treatment cycle. This showed that the adaptive immune system was also critical to realize the full potency of the combination immunotherapy.

**Depletions**

![Depletion Survival Graph](image)

Figure 25: Combination Therapy Depletion Survival.
Cells critical to the efficacy of these immunotherapy combinations were broadly identified by performing antibody-mediated depletions. Batf3−/− mice have no functional cross-presenting DCs and were used as a “depletion” for that cell type. The adaptive immune cells, CD8 T cells and cross-presenting DCs, were the most critical for the performance of the AIPV treatment. Without either of those cell types, no cures were achieved. The innate immune system was also important for the full therapeutic efficacy of AIPV. Survival significantly decreased when either Neutrophils or NK cells were depleted.
The vaccine established a large number of tumor reactive T cells, and that response was significantly boosted by the other components in the AIPV treatment (Figure 26). Circulating Trp2 reactive T cells were measured using IFN-γ ICS of PBMCs stimulated with Trp2 peptide. Although both the vaccine alone and AIPV treatment conditions reached peak levels of approximately 12% at day 21, only the full combination therapy generated sustained responses until 60 days after tumor inoculation. Furthermore, that T cell response was tumor antigen dependent and boosted after subsequent rechallenge (Figure 27). Despite a full course of AIPV, mice without primary tumors did not sustain high levels of Trp2 reactive T cells. AIPV treated mice also showed a stronger rechallenge response than equivalently treated mice without primary tumors. This showed that tumor antigen was necessary to establish a strong T cell response and helped to explain why mice without primary tumors were unable to reject subsequent tumor challenges.

![IFN-g response over time](image)

Figure 26: TRP-2 T cell Response.
ICS was used to track systemic CD8 T cell response to the Trp2 vaccine. The vaccine alone gave a strong peak response after the second injection, but quickly fell and by day 40 was under 2%. In contrast, AIPV treated mice showed a prolonged response lasting well after treatment was stopped at day 35.
Figure 27: Vaccine Response after Rechallenge.

B6 mice were treated with AIPV with or without tumor and their antigen response was tracked over time using ICS. The presence of tumor antigen did not change the peak response seen after the second treatment, but significantly enhanced the response after day 35. Additionally, these mice were given secondary tumors at day 75. A strong secondary response was observed in the mice which rejected primary tumors, leading to protection from growth of secondary tumors. Mice without prior exposure to tumor antigens had a comparatively weak secondary response and were unable to reject secondary tumor growth.

Tumor cell infiltrates were examined by disaggregating the tumors and staining them for immune cell markers. Several cell types were highly enriched in the tumor, suggesting their importance to the mechanism of the therapy combinations. CD8 T cells were critical to the efficacy of the treatments as shown during the depletions studies, and their role in tumor control was further validated by having high levels of infiltrates in effective combinations (Figure 28). Although critical to the success of these immunotherapy combinations, MSA-IL2, is a potent stimulator of T\textsubscript{reg} cells which were highly increased in potent therapies (Figure 29). However, the CD8/T\textsubscript{reg} ratio is often considered an accurate indicator of an effective immune response and an increased ratio was correlated with successful therapies (Figure 30). Finally, infiltrating neutrophils were significantly increased in the most effective AIPV, AIP, and AIV cases (Figure 31). This further demonstrated the necessity of engaging both the adaptive and innate immune systems in effective immunotherapy combinations.
Tumor Infiltration: CD8 T Cells

Figure 28: CD8 T Cell Tumor Infiltration.
Tumor infiltrating CD8 T cells were measured on day 14 by flow cytometry. Ineffective therapies not using MSA-IL2 showed insignificant increase over untreated tumors. Although AIPV showed the highest levels of CD8 T cell infiltration corresponding to the greatest therapeutic efficacy, CD8 T cell infiltration was consistent among the other therapies despite a disparate therapeutic response, especially between AIP and IPV.
Figure 29: T<sub>reg</sub> Tumor Infiltration.
Tumor infiltrating T<sub>reg</sub>s were measured on day 14 by flow cytometry. Like IL2, MSA-IL2 is a potent stimulator of T<sub>reg</sub>s. Therapies which saw higher levels of CD8 T cell infiltration also saw higher levels of T<sub>reg</sub> infiltration.
Tumor Infiltration: CD8/Treg Ratio

Figure 30: CD8/Treg Ratio.
The CD8/Treg ratio was calculated from the previous data. A high CD8/Treg ratio is more representative of an effective immune response against a tumor. Despite effective combinations increasing Treg infiltration, in general, even more CD8 T cells infiltrated the tumor leading to a higher CD8/Treg ratio. This trend was not completely predictive, as the AI ratio was higher than more effective treatments such as AIV. Additionally, even the ineffective therapies had progressively higher ratios than untreated mice, despite little improvement in overall survival. A high CD8/Treg ratio was necessary but insufficient to achieve durable cures of established tumors. Bonferroni corrected p-values: *<0.05, ****<0.0001.
Figure 31: Neutrophil Tumor Infiltration.
Tumor infiltrating neutrophils were measured on day 14 by flow cytometry. As with CD8 T cells, innate tumor infiltrates were correlated with more effective therapies. Neutrophils were significantly increased in therapies which were able to cure established tumors and were markedly increased in the AIPV treatment. Bonferroni corrected p-values: **<0.01, ***<0.001.

Dendritic cells and their role in antigen uptake, processing, and presentation are critically important for effective combination immunotherapy, as the Batf3+ survival rates demonstrated. Total numbers of DCs were enhanced by administration of MSA-IL2 (Figure 32). This may have been a contributing factor when non-cytokine treated combinations failed to provide any tumor control, however this did not explain the variety of treatment outcomes among the MSA-IL2 treated conditions. To study tumor antigen uptake and processing, B16F10-GFP-Luc cells were used instead of B16F10 in the standard tumor model. Additionally, labelled TA99 antibody was administered during the second treatment. Two days after the second treatment, tumor draining lymph nodes were stained and analyzed by flow cytometry (Figure 33). Cross-presentation DCs (CD8+CD11b-) showed higher levels of GFP when treated with antibody, indicating that they effectively took up tumor antigen associated with opsonized antibody targets. Cross-presentation was also critical to the functioning of the vaccine. In Batf3−/− Mice, ICS showed levels of antigen response equivalent to unvaccinated mice (Figure 34).
Numbers of DCs Increased by IL2

Figure 32: MSA-IL2 Mediated Proliferation of DCs.
Various DC subpopulations were enumerated by flow cytometry. Treatment with MSA-IL2 was enough to boost all DC subtype levels to approximately the same value over untreated mice. Although DC numbers did not correlate with survival outcomes, the presence of more DCs was necessary for effective therapies to work. This result highlights the dependence of immunotherapy combinations on synergistic mechanisms. Despite a high number of DCs, the IPV treatment delivers insufficient tumor antigen mediated by antibody therapy.

Figure 33: Antibody-Mediated Antigen Uptake by LN DCs.
B6 mice were inoculated with B16-GFP-Luc cells and treated with AIPV or IPV using 647 labeled antibody. On day 17, tumor draining lymph nodes were analyzed for the presence of GFP as a surrogate marker for tumor antigen. Antibody therapy significantly increased the number of GFP positive cross presenting DCs, indicating antibody opsonization of tumor derived antigens enhanced processing of tumor antigens in general. Despite the high levels of activated DCs shown in the previous figure, their functioning on processing tumor antigen was impaired without antibody therapy.
Batf3 KO Mice: Vaccine Antigen Response

Figure 34: Vaccine Response in Batf3⁺/⁻ Mice.
ICS was used to measure antigen response in wild type and Batf3⁺/⁻ mice. The necessity of cross-presenting DCs for vaccine response was clear, as Batf3⁺/⁻ mice had essentially no response. In addition to eliminating a vaccine response, cross-presentation was essential for the functioning of as seen in the decrease in survival of AIP treated mice (data not shown).

Antigen spreading facilitated by cross-presentation of tumor-derived antigen was tested using OVA as a surrogate. Tetramers complexed with OVA peptides were purchased from MBL. Tetramer staining was performed in buffer containing 50 nM dasatinib. B16F10-OVA cells were used to inoculate tumors in B6 mice, which were then treated with AIPV. After 21 days, T cells were analyzed for OVA-specific TCR expression by tetramer staining and flow cytometry. A significant response to OVA peptide was observed by flow cytometry, despite specifically not targeting the OVA antigen (Figure 35). Because none of the therapies targeted OVA or were specifically engineered to elicit an OVA-specific response, the immune system itself developed this response. This indicated that cross-presentation of tumor derived antigens was occurring as a consequence of the combination immunotherapy.
Antigen spreading is when the immune system identifies novel epitopes against the targeted tumor and raises an adaptive response against them. Antigen spreading is highly effective in curing established tumors and preventing recurrence (Corbière et al., 2011). To test for antigen spreading in the AIPV case, splenocytes from AIPV treated mice after rechallenge were incubated with irradiated B16F10 as a positive control, B16-TRP2-KO as a test case for antigen spreading, or TC-1 as a negative control in an ELISPOT assay. Additional negative controls were provided by testing splenocytes from naïve mice. SFU were counted and normalized to determine relative immune responses to antigen positive cells. Figure 36 shows the results of the ELISPOT. As expected from the ICS results, the AIPV case showed a strong response to B16F10 cells, the majority of which could be assumed to be TRP2 specific because of the strong vaccine response. Interestingly, when TRP2 was knocked out, there was still a significant increase in response when compared to AIPV splenocytes alone or with an irrelevant control cell line. This indicated that there was a tumor specific, but not TRP2 specific response that was elicited by the therapy.
Antigen spreading was further tested by rechallenging cured mice treated with AIPV, waiting six days, and then using their splenocytes in an ELISpot assay. Antigen was provided by B16F10 cells, B16-TRP2-KO cells, or irrelevant antigen control TC-1 cells. Naïve splenocytes were also used as negative controls. AIPV treated splenocytes are more activated than naïve splenocytes under any circumstances, but show a significantly higher response when cultured with B16F10 cells. This result was expected due to the large number of TRP-2 specific cells caused by the vaccine. To observe the response of T cells against non-TRP2 antigens, B16-TRP2-KO cells were used. The response to these cells was significantly lower than to B16F10 cells, but higher than the response to irrelevant control TC-1 cells. This observed difference indicates specificity to B16F10 cells, but not to the TRP-2 antigen, suggesting that the antigen spreading has occurred and the immune system has created T cells specific to non-therapy targeted antigens.
Humoral immune response was also tested by a Western against B16F10 cell lysate (Figure 37). B16F10 cell lysate was run on an SDS-PAGE gel. Serum from AIPV treated mice was used to probe the cell lysate for binding. Untreated serum from naïve mice was used as a control. After secondary binding and imaging, a robust humoral response was observed in the treated mice when compared to the untreated. Serum was collected pre- and post- secondary challenge. A clear increase in response was observed after rechallenge, consistent with the CD8 T cell response boost. Although not fully characterized, these initial findings showed the engagement of the humoral immune system against tumor antigens.

**Antibody Response to B16 Cell Lysate**

**Pre and Post Re-challenge**

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Figure 37: Combination Immunotherapy Induces Humoral Response.

The involvement of the humoral immune response was tested by using serum from AIPV treated mice against B16F10 cell lysate in a Western blot. Serum was taken before and after rechallenge to observe a reaction to antigen stimulus. The paired lanes were from a specific mouse. Serum from untreated mice showed no reactivity to B16F10 lysate. AIPV treated mice showed a strong response to multiple antigens in the B16F10 cell lysate. Furthermore, this response was boosted by rechallenge.

In order to test the combination immunotherapy regimen in a more realistic model, the BRaf/Pten inducible tumor system was used. Tumors were induced on the
ears of BRaf/Pten-TG mice and when visible lesions were present approximately 25 days later, treatment was initiated. The AIPV therapy was effective at controlling the initial pigmented lesions as shown in Figure 38. These examples are representative of the level of response achieved during the first 60 days of tumor establishment and treatment. By day 60, almost all of the untreated mice had complete coverage of their ears by the pigmented tumor cells. In contrast, lesions on the full combination treated mice can be observed to shrink and in many cases disappear. Overall survival of the BRaf/Pten-TG mice was significantly improved by the combination treatment (Figure 39). Eventually, however, apigmented tumors appeared in approximately half of the treated mice and grew progressively until euthanasia criteria were met. This may indicate that in more complex tumor pathologies, escape variants can emerge which the immune system is unable to contain.

Figure 38: Combination Immunotherapy Control of BRaf/pten Tumors. BRaf/Pten-TG mice were induced on the ear and treated with AIPV after pigmented lesions were clearly visible. Representative images from untreated control mice and mice treated with AIPV are shown above. Pigmented lesions on untreated mice grow progressively until virtually the entire surface of the ear is covered and the mouse is euthanized. AIPV treated mice see decreased in size and pigmentation of the lesions.
3.5 Discussion

The role of T cells in the rejection of tumors is multifaceted. The fact that the no vaccine case (AIP) was able to dramatically control and cure established tumors may seem to obviate the necessity of an antigen specific T cell response. Indeed, other researchers have shown that non-specific T cells were potent mediators of tumor control after cytokine-based immunotherapy (Tietze et al., 2012).

Although the analysis presented here focused on TDLNs, Batf3/- mice are developmentally deficient in cross-presenting DCs throughout their bodies (Edelson et al., 2010). Cross-presenting DCs are critical for an effective T cell response (Broz et al., 2014) and effective immunotherapies, including the ones presented here, enhance both their numbers and functioning.
The need for tumor antigen targeting antibodies is often overlooked when combination immunotherapies are developed. Although classically thought of as a method of direct killing of tumors through ADCC, antibody opsonization of tumor antigens can have a powerful vaccinal effect (DiLillo and Ravetch, 2015), recruiting other arms of the immune system. This is further supported by the efficacy of antibodies targeting intracellular antigens (Guo et al., 2011), which can be opsonized by the antibodies after tumor cell lysis and elicit responses through adaptive immune cell pathways. The enhancement of DC presentation of tumor antigens by antibody therapy observed here is similar to other studies where T cell tolerance to self-antigens were overcome by antibody therapy (Furlan et al., 2014; Harbers et al., 2007). Antibody mediated augmentation of vaccines and eliciting of antigen spreading has been previously observed in the B16F10 model (Saenger et al., 2008). DC cross presentation has also been shown previously to be enhanced by antitumor antibodies (Dhodapkar et al., 2002).

Another underappreciated element in effective immunotherapy is the humoral response against tumor antigens. The strong serum response observed in this model is similar to clinical findings associating tumor antigen specific serum responses with positive clinical outcomes (Yuan et al., 2011). The success of vaccine of virus-like replicon particles in treating B16F10 tumors was also attributed to engaging both a humoral and T cell response (Avogadri et al., 2010). With B cells being implicated in not only directly targeting tumors through serum responses, but also enhancing T cell responses (DiLillo et al., 2010), further study on their involvement with successful combination immunotherapies such as the one presented here are warranted. The combination of cellular and humoral immunity to provide tumor protection has been shown previously (Orlandi et al., 2007). CAR T cells also caused a humoral response against tumor antigen (Beatty et al., 2014).

The effectiveness of these combinations were also dependent on the interaction of innate and adaptive immune cells. Eosinophils have been shown to normalize tumor vasculature during effective therapies, facilitating the infiltration of CD8 T cells into the tumor space (Carretero et al., 2015). Tregs exert a strong influence on CD8 T cell functioning and their interaction with tumor antigen via APCs can have a dramatic effect on the tumor microenvironment (Bauer et al., 2014).

Inducible tumor models, such as the BRaf/Pten model used here, are often more difficult to treat due to their complex morphology that more accurately matches
clinically observed cancer (Hooijkaas et al., 2012b). Often these models have a specific mutation that drives malignancy, that when specifically targeted lead to control of these tumors. When combined with other immunotherapy treatments, these results can be even more dramatic (Hooijkaas et al., 2012a). In the therapies presented here, only melanoma markers were used as targets and strong tumor control was achieved. As seen in other studies (Carmi et al., 2015), DCs were a critical factor in achieving this result.

Checkpoint blockade is already a successful treatment clinically, however its complete mechanism of action is still being elucidated. Despite initially being thought of as restoring function to exhausted T cells, various other immunostimulatory functions are being revealed. PD-1 blockade was shown to enhance infiltration of T cells at the tumor site through increasing IFN- inducible chemokines (Peng et al., 2012b). In this study, infiltration of T cells was primarily enhanced by MSA-IL2 treatment. Additionally, the enhancement of cancer vaccines by checkpoint blockade has been shown previously in several studies (Fu et al., 2014; Tan et al., 2014).

3.6 Conclusion

After decades of research and clinical study, a wide variety of immunotherapeutics are now available for clinicians to treat patients with cancer. As a complex and evolving disease, cancer is unlikely to be successfully treated with single agents. Additionally, many courses of therapy tend to take place sequentially, and in doing so lose any synergies from combining complementary mechanisms of action. This study presented a highly efficacious, four component combination immunotherapy in a preclinical model of cancer. Successful rejection of tumors relied on an orchestrated response from both the adaptive and innate branches of the immune system. The results of this work will hopefully help guide clinicians in developing more effective combination immunotherapies.
4 Bispecific Antibodies

4.1 Abstract

The efficacy of bispecific T cell engagers (BiTEs) has been well studied in vivo and in vitro. These bispecific antibodies are potent mediators of tumor cell lysis, as they redirect lymphocytes via the CD3 signaling complex to tumor associated antigens. However, the detailed biological mechanism of BiTE mediated cytotoxicity has not been thoroughly examined. This study analyzed the transcriptomes of CD8+ T cells engaged in BiTE mediated tumor cell lysis at several timepoints to map out the specific biological response. RNA-seq was used to track expression changes and GSEA was performed showing a high degree of similarity between BiTE activated T cells, and traditional T cell activation pathways. Finally, upregulated membrane proteins were analyzed and Mpzl2 was shown to be co-expressed with exhaustion markers PD-1 and Tim-3 on CD8+ T cells after treatment with a BiTE antibody.

4.2 Introduction

Bispecific T cell engagers (BiTEs) are molecules that seek to treat cancer by redirecting T cells to specific tumor associated antigens (TAAs). A bispecific scFv is made by adding a linker between two scFv sequences. BiTEs are specific for tumors on one scFv and CD3 on the other, linking T cells to their targets in an MHC independent fashion. Recruitment of both CD8 and CD4 T cells is considered advantageous due to the CD4 release of granzyme B in the presence of BiTE binding (Baeuerle and Reinhardt, 2009; Huehls et al., 2015). Studies of the functional mechanisms of this class of bispecific antibodies are becoming more important as BiTEs become increasingly relevant in the treatment of cancer. Blinatumomab, a CD3/CD19 BiTE has had success in clinical studies and is approved for treatment of Philadelphia chromosome-negative B-precursor relapsed/refractory acute lymphoblastic leukemia (Zugmaier et al., 2015).

Carcinoembryonic antigen (CEA, CD66e) is a widely expressed TAA in colorectal, breast, and lung cancers and represents a clinically relevant target for BiTE therapy. CEA is a validated target used for imaging, vaccine (Hörig et al., 2000), and antibody-based therapeutic approaches. Expression is normally limited to the lumen of adult colonic epithelial tissues, however, during malignancy CEA is overexpressed (Hammarström, 1999). CEA is also atypical in its expression due to phospholipase cleavage causing soluble antigen to be shed from the cell surface, a phenomenon that has been used to track the presence and resurgence of malignancies (Rother, 2007), as
well as a potential hindrance to anti-CEA antibody based therapy as a source of competitive inhibition.

CEA-targeting BiTEs have shown activity against a wide variety of CEA-expressing cell lines in vitro as well as against xenograft tumor in vivo (Lutterbuese et al., 2009). CEA-BiTEs have also been shown to be highly active against otherwise intractable tumor samples (Osada et al., 2010) and have been specifically engineered to overcome competition by soluble CEA (Peng et al., 2012a). BiTE design has primarily focused on in vitro cytotoxicity (Nazarian et al., 2014) and a determining factor for the success of a BiTE antibody is the relationship between the epitope and the cell membranes for both the target and effector cells. The distance may affect the formation of the cytolytic synapse, a structure which facilitates the controlled delivery of perforin and granzymes, which lead to cell lysis (Bluemel et al., 2010).

Next generation whole transcriptome sequencing (RNA-seq) allows for detailed examination of expression changes or differences between cell types. Expression profiling of immune cells has given great insight into their regulation and activity. RNA-seq and ChIP-seq were used to track a variety of transcriptional and regulatory elements in the development of T cell subsets (Äijö et al., 2014; Simeoni et al., 2015; Zhang et al., 2012). Detailed analysis of differences between regulatory and helper CD4 T cells revealed previously undescribed transcriptional regulation events (Birzele et al., 2011). RNA-seq was also shown to be superior to microarray in profiling the transcriptome of activated T cells (Zhao et al., 2014) and used to profile cytotoxic lymphocytes treated with rapamycin (Mattson et al., 2014).

An area of BiTE research that has been less studied is the specific mode of action through which BiTEs function. It has been largely assumed that BiTE mediated lysis is directly analogous to TCR mediated cytotoxicity. Confocal microscopy studies have shown a TCR-like cytotoxic synapse is formed upon BiTE engagement (Offner et al., 2006). However, few mechanistic studies have been done to link BiTE treatment with changes in cell phenotype and expression profiles. In this study, RNA-seq analysis of in vitro BiTE mediated cell lysis showed direct pathway linkages to traditional lymphocyte activation and TCR mediated cytotoxicity. Additionally, the exhaustive state of BiTE activated T cells was analyzed and compared to isolated tumor infiltrating lymphocytes from lung cancer tissue samples. This analysis also led to the finding that Mpzl2 is expressed on a subset of PD-1+ and Tim-3+ after activation by BiTE antibodies and may be a marker for T cell activation or exhaustion.
4.3 Materials and Methods

4.3.1 Cell Lines and Media

HT-1080 cells were acquired from ATCC and cultured in complete DMEM (DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 4 mM L-alanyl-L-glutamine). Wild type HT-1080 cells do not express CEA. HT-1080-CEA cells were generated as previously described (Schmidt et al., 2008) and cultured in complete DMEM with 0.75 mg/mL G418. G418 was only used during routine maintenance and not during any assay. Suspension adapted HEK-293 cells were purchased from Invitrogen and cultured in Freestyle media. T cells and assays were cultured in RPMI with 10% heat inactivated FBS, 20 mM HEPES, 1 mM sodium pyruvate, 0.055 mM beta-mercaptoethanol, 100 units/mL penicillin, 100 µg/mL streptomycin, 2 mM L-alanyl-L-glutamine, and 1X MEM non-essential amino acids. All cell lines and assay cultures were maintained at 37°C and 5% CO₂.

4.3.2 Clinical Sample Processing

Whole blood from healthy donors was purchased from Research Blood Components. Untouched CD8 T cells were isolated by Ficoll-Paque (GE) density separation followed by magnetic bead selection (StemCell). Clinical samples were acquired from the Lahey Clinic during VAT resection of lung tumors. Clinical samples consisted of peripheral blood, tumor tissue, and adjacent normal tissue. Peripheral blood cells were prepared by Ficoll-Paque (GE) density separation followed by ACK lysis. Cells from tissue samples were prepared as follows: Tissues were mechanically dissociated by dissection with scalpels and then transferred to tubes and incubated with digestion media (DMEM/F-12 with 1 mg/mL dispase, 1 mg/mL collagenase I, and 1 mg/mL DNase I) for 1 hr at 37°C. Digested tissue and media were then passed through a cell dissociation sieve (Sigma) followed by ACK lysis.

4.3.3 Flow Cytometry and Fluorescence-Activated Cell Sorting

Fluorescently labeled antibodies were used to analyze various samples and isolate cells for RNA extraction. Antibodies to the following antigens were purchased from Biolegend: CD8-AF647, CD8-BV421, CD8-AF488, CD4-PE, CD127-AF488, PD-1-PerCP/Cy5.5, Tim-3-PE/Cy7, CD57-PE. Polyclonal rabbit anti-Mpzl2-AF647 was purchased from Bioss. Live cells were identified in all assays and sorts by Calcein Violet AM (Invitrogen). Samples were sorted on a BD FACSAria. Flow cytometry was performed on a BD LSR II. Analysis was performed using FlowJo. Samples were rinsed
and stained in ice cold FACS buffer (PBS with 1 mM EDTA, 25 mM HEPES, 1% HI-FBS, 100 units/mL penicillin, 100 µg/mL streptomycin) and passed through a 40 µm cell strainer prior to sorting or analysis. For certain assays, CD3/CD28 (Invitrogen) activation beads were used as activated T cell controls.

4.3.4 Expression and Purification of CEA-BiTE

The CEA-BiTE (Lutterbuese et al., 2009) expression cassette (Figure 40) was synthesized (Genscript) and cloned into the gWiz vector (Genlantis). A C-terminal 6xHis tag was added for purification and an N-terminal FLAG tag was added to facilitate detection by flow cytometry binding assays. Endotoxin-free plasmids were expressed and purified by maxi-prep according to manufacturer's instructions (Macherey-Nagel). CEA-BiTE was expressed using transient transfection of suspension adapted HEK-293 cells with polyethylenimine followed by an 8 day production culture. Two step purification was performed after centrifugation and sterile filtration using immobilized-metal affinity chromatography (IMAC) and size-exclusion chromatography (SEC). Supernatant was passed over TALON resin (Clontech), which was then washed with phosphate-buffered saline (PBS, pH 7.4). Protein was eluted with PBS with 0.5 M imidazole (Figure 41). The eluted fractions were then concentrated and purified by SEC over a Superdex 200 column (GE Healthcare Life Sciences) on an AKTA FPLC (Figure 42).

Figure 40: CEA-BiTE Expression Cassette.
A BiTE targeting CEA was expressed using the diagramed vector. Two scFv domains were linked in the order indicated. A C-terminal 6xHis tag was added for purification and an N-terminal FLAG tag was added to facilitate detection by flow cytometry binding assays.
Figure 41: Purification of CEA-BiTE by TALON Resin. The CEA-BiTE was produced by transient transfection and then purified by TALON resin. The resulting elution fraction was run on an SDS-PAGE gel and visualized by Coomassie blue stain. The major purified component was seen at the expected size of ~60 kD.

Figure 42: Purification of CEA-BiTE by SEC. The CEA-BiTE was purified by SEC and fractions were run on an SDS-PAGE and visualized by Coomassie blue stain. As shown, the BiTE elution is a mix of highly aggregated product. Monomer fractions can be isolated from C2 onward to ensure proper product quality and prevent non-specific activation cause by aggregate-induced CD3 clustering.
Figure 43: Stability of Purified CEA-BiTE.
Purified CEA-BiTE was stored at 4°C for 2 months isolated from either a new column (black line) or old column (red line). The new column was far superior to the old column and showed that the BiTE remained 97% monomer over the 2 months of storage. The red line indicates that the old column did not purify monomer effectively.

Binding of the CEA-BiTE was confirmed by flow cytometry (Figure 44). Naïve CD8 T cells were stained with CEA-BiTE then stained with an anti-FLAG-PE secondary antibody and co-stained with an anti-CD8-Alexa 647 antibody. Staining showed a complete double positive population indicating the anti-CD3 portion of the BiTE was binding properly. Binding of the anti-CEA domain of the BiTE was tested by staining a CEA-expressing target cell line with the BiTE followed by an anti-FLAG-PE secondary antibody. The cell line was clearly stained with the BiTE indicating that the anti-CEA domain was also functioning properly.
Figure 44: Binding Characteristics of CEA-BiTE.
Flow cytometry was performed to ensure appropriate CEA-BiTE binding. Successful binding of the BiTE was confirmed by secondary staining with an anti-FLAG-PE antibody. CD3 binding was confirmed by binding of the BiTE to isolated CD8 T cells from healthy human donors. Co-staining with CD8 confirmed binding of the appropriate cell type. CEA binding was confirmed by staining of a CEA expressing target cell line.

4.3.5 Cytotoxicity Assay

Target cells (HT-1080 or HT-1080-CEA) were incubated with 250 μCi/mL of Chromium-51 (Perkin-Elmer) in complete media for 1 hr at 37°C and 5% CO₂. Target cells were then washed 3 times in media. Labeled target cells (20,000 cells/well) and CD8 T cells (200,000 cells/well) were incubated in 96-well flat bottom plates with the indicated amount of CEA-BiTE for the indicated amount of time. After incubation, supernatant was harvested, added to LumaPlates (Perkin-Elmer) and allowed to dry. Chromium release cpm was measured by a TopCount NXT (Perkin-Elmer). Percent Cytotoxicity was calculated as (Experimental - (Spontaneous Target + Spontaneous Effector))/(Max Target - Spontaneous Target). Spontaneous release was measured by
samples treated with CEA-BiTE but without co-culture. Maximum release was calculated from wells treated with 1% Triton X-100. Samples were measured in triplicate, and error bars represent standard deviation.

4.3.6 RNA-Seq Analysis

RNA was isolated from sorted cells and cDNA libraries were made from RNA kits by Clontech. Libraries were sequenced on an Illumina HiSeq. Sequencing results were mapped to a reference genome and transcript amounts were quantified. Finally, differential expression was calculated with significance levels. Gene set enrichment analysis (GSEA) was performed as previously described (Subramanian et al., 2005).

4.4 Results

4.4.1 Cytotoxicity Assays

CEA-BiTE activity was established with the HT-1080 and HT-1080-CEA cell lines by using a standard chromium release cytotoxicity assay. Healthy donor CD8 T Cells were isolated by negative bead selection and cultured at an effector to target ratio of 10:1. A dose response curve of the CEA-BiTE was generated at 48 hours showing effective and specific killing of HT-1080-CEA cells (Figure 45). The half maximal effective concentration (EC50) for the CEA-BiTE was approximately 0.1 ng/mL while maximum killing was seen at 10 ng/mL. Off target killing was observed with antigen negative HT-1080 cells at concentrations above 1000 ng/mL. The objective was to achieve maximum killing at 48 hours for analysis so the minimum concentration of BiTE was used that still achieved this, 10 ng/mL, for all further assays.
Healthy donor CD8 T cells were co-cultured with either antigen expressing HT-1080-CEA cells, or HT-1080 cells which have no detectable CEA surface expression. The E:T ratio was 10:1. After 48 hours, supernatant samples were assayed for chromium release. The EC50 of the CEA-BiTE was observed at approximately 0.1 ng/mL and maximum killing was seen at approximately 10 ng/mL. Off-target killing of HT-1080 cells was negligible until levels of BiTE exceeded 1000 ng/mL.

The time-dependence of CEA-BiTE killing was established by assaying chromium 51 release at 2, 6, 24, and 48 hours of co-culture (Figure 46). As previously determined, CEA-BiTE was used at a concentration of 10 ng/mL as the lowest dose that gave maximum efficacy at 48 hours. After 2 hours, very little killing had occurred, half of maximum killing occurring by 6 hours, and maximum levels of cytotoxicity were observed by 24 hours. Off target killing of HT-1080 cells was low throughout the assay’s duration.
Figure 46: Time Dependence of CEA-BiTE Cytotoxicity.
Healthy donor CD8 T cells were co-cultured with either antigen expressing HT-1080-CEA cells, or HT-1080 cells which have no detectable CEA surface expression. The E:T ratio was 10:1. The lowest concentration of CEA-BiTE necessary to give maximum killing at 48 hours was used (10 ng/mL). Cytotoxicity was measured at 2, 6, 24, and 48 hours after co-culture. Maximum killing occurred after 24 hours. Off-target killing of HT-1080 cells gradually increased, but was small in absolute terms.

4.4.2 Individual Gene Expression Analysis

To begin to analyze the RNA-seq data, individual transcripts commonly associated with traditional T cell activation (Malissen et al., 2014; Reddy et al., 2004; Russell and Ley, 2002; Shipkova and Wieland, 2012) were analyzed (Figure 47). Expression levels of these markers closely followed those expected from TCR mediated T cell activation. CD69, a minimally-expressed marker of early cell activation peaked at 2 hours and stayed constant at low levels until 48 hours. The alpha chain of the IL-2 receptor (IL2-Rα/CD25) is a highly expressed marker prominent during the middle stages of activation. Consistent with this, IL2-Rα transcripts rise consistently until 24 hours, then begin to fall off. Growth signals and cell cycle transcripts were up during BiTE mediated activation. GM-CSF quickly rose to a maximum level at 2 hours after antigen exposure. IFN-γ peaked at 2 hours before also falling to baseline at 24 hours. Active production of IFN-γ closely matches the data shown in Figure 46, where after 24 hours all of the target cells have been killed. Granzyme B had an interesting expression pattern in that it progressively rose over the course of the entire experiment, despite being rapidly released during T cell mediated cell killing. This can be explained by the fact that Granzyme B is stored in secretory granules and released upon engagement of
the cytolytic synapse with target cells. In order to restore these depots, cells upregulated transcription of Granzyme B mRNA. Finally, transcripts of exhaustion markers were analyzed. CTLA-4 transcription rose progressively over the course of the experiment.

![Expression Levels of RNA by CD8 T Cells Treated with BiTE](image)

Figure 47: Expression of Individual RNA Transcripts. Log2 fold change was calculated for each timepoint. Transcripts commonly associated with TCR mediated T cell activation were plotted and matched expected results.

4.4.3 GSEA Analysis

Although useful, single transcript analysis does not capture the full complexity of the changes in the transcriptome of T cells engaged in BiTE mediated tumor cell killing. To more thoroughly analyze the data, GSEA analysis was used. A manually curated list of publically available gene sets related to T cell activation was used with the BiTE transcriptome data. Normalized Enrichment Scores were calculated for each set at each timepoint and a selection of significant sets were plotted in Figure 48. As expected, gene sets containing groups of individual transcripts that were significantly upregulated at certain times were also significantly enriched.
Cytokine expression was rapid and early. Gene sets for cytokines were significantly enriched at 2 and 6 hours, before dropping off at 24 and 48 hours. TCR and NFAT signaling also followed similar trajectories, falling off after 6 hours. These findings match the cytotoxicity levels shown in Figure 46. Surface markers showed delayed enrichment, peaking at 24 hours before falling to baseline at 48 hours indicating a lag between transcription and expression of protein that is maintained in the cell membrane. Cell cycle analysis showed a rapid increase in enrichment at 2 hours, before falling off at 6 and 24 hours, and then rising again at 48 hours. This may be due to two different phases of growth, a rapid period of cell division in response to a primary antigen stimulus, and a second one after a period of recovery. Importantly, exhaustion analysis showed how gene set analysis can reveal biological mechanisms that could be missed during individual transcript analysis. Although the PD-1 transcript levels themselves were not significantly altered during BiTE activation (data not shown), the PD-1 signaling pathway gene set was significantly enriched after 6 hours before falling at 24 and 48 hours. This showed the potential disconnect between protein levels, which are a function of not only transcription, but also trafficking, secretion, and degradation, and transcript levels which are a function of transcription rates as well as degradation.

![GSEA Enrichment](image)

**Figure 48: GSEA Analysis of CEA-BiTE Mediated Cytotoxicity.**
GSEA sets related to T cell activation were tested against the transcriptome data generated from T cells co-cultured with either HT-1080 or HT-1080-CEA cells.
4.4.4 Exhaustion Markers

After 48 hours CEA-BiTE treated T cells were analyzed for the expression of various signatures of exhaustion. T cell senescence was measured by the CD127(-)CD57(+) phenotype (Brenchley et al., 2003), as well as exhaustion markers PD-1 (Riley, 2009) and Tim-3 (Ferris et al., 2014). Mpzl2 (EVA1) (Guttinger et al., 1998) was also measured as an example of a poorly studied transcript that emerged from the analysis of the clinical samples (Figure 49). Untreated T cells cultured for 48 hours were used as an inactivated case and CD3/CD28 activation beads were used as a positive control for T cell activation. A senescent phenotype was upregulated by CEA-BiTE mediated killing. CD127(-)CD57(+) cells increased slightly. PD-1 and Tim-3 were highly expressed on the surface of T cells incubated with antigen positive cells and BiTE. All three of these markers indicate that the T cells went through full activation and were progressing to an exhausted state. The results with Mpzl2 confirm the RNA-seq findings and closely match the expression patterns seen in the exhaustion markers.

Staining of Mpzl2 alongside the exhaustion markers showed patterns of co-expression (Figure 50). As with the expression of individual exhaustion markers, the co-expression of these markers with Mpzl2 was increased by T cell activation. Mpzl2 expression did not significantly correlate with T cell senescence. Expression of Tim-3 and Mpzl2 double positive cells increased during BiTE mediated cytotoxicity and the majority of Mpzl2+ cells was also Tim-3+. Expression of PD-1 and Mpzl2 double positive cells was also increased with the presence of antigen, and was remarkable in that virtually all Mpzl2 positive cells were also PD-1 positive.
Figure 49: BiTE Mediated T cell Exhaustion.
48 hour co-cultures were analyzed for the expression of exhaustion markers. Cells were harvested and stained for CD8 and an exhaustion marker. All three markers measured showed increased expression by activated T cells and T cells incubated with antigen positive target cells. PD-1 showed the largest overall expression upon activation. Tim-3 was not expressed on T cells incubated with antigen negative target cells, but expressed on ~20% of T cells incubated with antigen positive target cells. Mpzl2+ was expressed at higher levels upon T cell activation.
Figure 50: Mpz12 is Expressed on a Subset of PD-1+ T Cells.
Co-expression of Mpz12 with various exhaustion markers was examined on T cells co-cultured with BiTE and target cells after 48 hours. Double positive cells were more frequent for each set of markers when antigen positive cells were present. Mpz12 was not significantly correlated with T cell senescence markers. Tim3+ Mpz12+ cells were significantly increased with antigen positive cells, and co-expression was particularly strong with Mpz12 and PD-1, where virtually all Mpz12+ cells were also PD-1 positive.
4.5 Discussion

BiTEs were designed to mimic TCR mediated killing by directly clustering CD3ε domains and activating the ITAM signaling cascade (Smith-Garvin et al., 2009). However, other signals, notably co-stimulatory signals such as from CD28, are absent from the BiTE mechanism of action. Despite this, the transcriptome profile of BiTE activated T cells closely matched recognized pathways of T cell activation. Activation from various chemical agents showed similar expression patterns as shown here with BiTEs (Reddy et al., 2004). TCR mediated signaling is believed to be rapid, with cytoskeletal reorganization and phosphorylation of TCR-proximal components such as CD3ε happening within seconds (Malissen et al., 2014). The time series analysis performed here was sensitive enough to differentiate early and late markers of activation such as CD69 and CD25 between 2 and 6 hours, but it is possible even more sensitive analysis of earlier timepoints could show further nuances of BiTE mediated activation.

The generic and highly potent gene expression profile that has been shown here supports findings that BiTE mediated killing is a function of the TAA and not any subsequent genetic mutations (Oberst et al., 2014). The differences shown here were from a modified cell line with its parent line, keeping genetic variation to a minimum. Any differences in activity can be implicated directly by the action of the BiTE on creating a cytolytic synapse and not on any effect from genetic variability between cell lines or on target-independent activation by CD3-only ligation.

The role of exhaustion and its relationship with BiTEs has been studied previously. One report showed a lack of T cell anergy in a long term study of high dose BiTE in mice (Amann et al., 2009). Another study showed serial killing of tumor cells, with sustained T cell motility (Hoffmann et al., 2005). However, BiTE activity in vitro was enhanced by anti-PD-1 and anti-PD-L1 blockade (Osada et al., 2015), indicating that at least the early mechanisms of exhaustion are activated by BiTE mediated cytotoxicity. The analysis here shows that after 48 hours, BiTEs are not significantly functionally senescent, but express high levels of PD-1 and Tim-3. This may indicate that many of the T cells are functionally impaired, or on the path to senescence. Given this, studies of BiTEs in combination with checkpoint blockade or T cell agonist therapy are warranted to possibly improve the therapeutic efficacy of the treatment.

Mpzl2 (EVA1) emerged from our analysis as a little studied transcript that correlated with common T cell activation indicators. The protein has been studied
primarily for its role in thymus development (Guttinger et al., 1998). It is highly upregulated during progenitor cell entry into the T lymphocyte developmental pathway, but is then downregulated during the maturation process (DeMonte et al., 2007; Tydell et al., 2007). It is critical for lymphocyte development and stromal differentiation (Iacovelli et al., 2009). In normal thymic tissue, Mpzl2 expression is restricted to CD4-CD8- double negative cells (Garabatos et al., 2014), indicating an early stage of thymocyte development. Mpzl2 has also been implicated in oligodendrocyte differentiation (Letzen et al., 2010), spermatogenesis (Nakata et al., 2012), and the functioning of Sertoli cells (Willems et al., 2010).

Mpzl2 was suggested as T cell activation marker in previous studies as a result of increased expression following immunization (Wright et al., 2013). In our experiments, Mpzl2 was upregulated on the surface of T cells after activation by BiTE mediated cell lysis. It has been implicated in developed T lymphocytes in a number of other studies, which suggest a role in T cell regulation. CD4+ T lymphocyte adhesion to human choroid plexus epithelial cells was regulated by Mpzl2 as was calcium flux in the epithelial cells (Wojcik et al., 2011). Calcium flux is a key feature of T cell activation (Joseph et al., 2014) and Mpzl2’s suggested role in modifying it in epithelial cells may have a similar role in the T cells themselves. The implications for this upregulation are still unclear and the function of Mpzl2, if any, in T cell activation have yet to be determined. However, the confirmation of co-expression with exhaustion markers by flow cytometry validates the method of searching for potentially new by RNA-seq.

4.6 Conclusion

BiTE mediated killing was analyzed by RNA-seq and found to be highly similar to TCR mediated killing. Although the formation of a cytolytic synapse differs between the two methods of cell killing, the transcription changes were similar enough to support the use of many of the same treatment strategies to enhance therapies that rely on T cell support, such as checkpoint inhibitors. Furthermore, BiTE mediated killing caused T cell exhaustion markers to be upregulated and therapies relying on BiTEs may benefit from being combined with supportive measurements to overcome T cell senescence. Finally, by cross-referencing RNA-seq data and expression location databases, proteins with potentially novel implications in T cell biology can be identified for further testing and analysis.
5 Supplementary Information

5.1 Polycistronic Expression

5.1.1 Introduction

Polycistronic expression refers to the production of multiple polypeptides from a single open reading frame (ORF) (de Felipe, 2002). This is a very useful technique when used in protein engineering for several reasons. For example, a reporter gene such as GFP can be linked in a stoichiometric fashion to another protein of interest. Also, polycistronic vectors allow guaranteed expression of multiple peptides in the production of proteins with quaternary structures, such as antibodies (Kunes et al., 2009). Many methods for polycistronic expression come from natural expression strategies from viruses. Viral genomes have evolved to efficiently translate multiple genes from very small sequence lengths. They do this using a variety of methods from extremely compact promoter regions to alternative splicing. These methods developed naturally by viruses have been repurposed as powerful tools for protein expression using compact vector designs.

One of these vector designs is the so-called “2A” skip peptide sequences which are used by a variety of viruses, and first discovered in the Picornaviridae family. These approximately 20 amino acid long sequences form an extremely rigid alpha helix structure which results in the ribosome failing to create a peptide bond during translation but not stopping translation thus skipping a bond and creating 2 distinct polypeptides (Donnelly et al., 2001). A diagram of the 2A expression system is shown in Figure 51. 2A skip peptides are often identified by a letter preceding “2A” indicating the virus from which that specific sequence was derived. For example, the P2A sequence is from the porcine teschovirus-1 and the T2A sequence is from thoseaasigna virus 2A.

Multiple 2A sequences can be daisy chained together allowing for more than 2 polypeptides to be expressed from a single ORF. Additionally, signal peptides can be used optionally, thus allowing combinations of cytoplasmic, secreted, and transmembrane proteins to be expressed in the same cassette. A potentially major downside of the 2A expression system is that residual “tags” are added to the C-terminus of the first protein and the N-terminus of the second protein. The N-terminal tags can be removed if the protein is transmembrane or secreted, as the tag will be lost during signal peptide processing. However the C-terminal tags are more problematic. One way of eliminating the tag is to add a furin cleavage site (R-X-R/K-R) to the
sequence (Fang et al., 2005). Furin is a ubiquitous protease that many different cell types express, which cleaves after positive amino acids near the C-terminal end of proteins.

![Diagram of cleavage sites]

Figure 51: General Schematic for a 2A Bicistronic Expression Vector.
This figure shows a typical 2A expression vector to express a secreted protein with 2 chains and quaternary structure, such as in antibody. The first cleavage site is a result of the furin protease. The same cleavage site is the result of the 2A sequence. Both peptide sequences have a leader domain which is essential for having both secreted. The lack of a stop codon after the first peptide is also essential for the second peptide to be expressed. Finally the furin cleavage site was added to cleave the residual skip peptide sequence.

5.1.2 Antibody Expression with 2A Vector Design

To test the 2A expression system, the antibody TA99 was expressed in several different formats using transient expression in HEK cells. All expression was tested using the murine IgG2a isotype. The standard way of expressing TA99 using two different vectors, one expressing the heavy chain and another expressing light chain, was compared to two different 2A vectors which differed in the order of peptide expression (i.e. LC-HC or HC-LC). HEK-293 cells were transfected using PEI in Freestyle media and cultured for 8 days. Standard expression was achieved using two plasmids, gWiz-TA99-HC and gWiz-TA99-LC, in a 1:1 ratio by weight. Polycistronic expression was achieved using the plasmids XXXX and XXX to test how the order of peptide expression in the cassette affected yields and product quality. After 8 days, cells were separated by centrifugation and IgG was purified using Protein A purification followed by buffer exchange into PBS. The resulting protein was visualized on an SDS-PAGE gel stained with Coomassie blue (Figure 52).
Figure 52: Bicistronic Vector Expression of TA99.
TA99 was expressed via transient transfection of HEK-293 cells, run on an SDS-PAGE gel, and stained with Coomassie blue. All three protein samples were run in both reducing and non-reducing conditions.

All three expression formats showed expected TA99 banding patterns, however, the light chain first format differed more significantly than the other two. Under non-reducing conditions, TA99 has a typical antibody band at the expected molecular weight of approximately 150 kD, along with 2 or more smaller bands that represents fragmented protein. This is most likely an artifact of the SDS gel itself, since size exclusion chromatography (SEC) and dynamic light scattering (DLS) show a relatively homogenous non-aggregated profile of TA99. A prominent additional band at approximately 125 kD can be seen in the light chain first format. However, in other assays (SEC and DLS) this discrepancy is not seen. The reduced lanes show the effect of the 2A skip peptide more clearly. The residual furin peptide sequence can be clearly seen in the light chain first lane. Overall, the three formats yielded roughly equivalent protein by this method.

A more dramatic difference between the three formats was seen in overall expression yields (Figure 53). By this metric, the light chain first construct was far
superior to the other two, with the heavy chain first construct yielding approximately the same amount of protein as separate vectors. This result is not entirely unexpected, as sequences flanking the 2A peptides have been shown to affect the cleavage efficiency and would therefore possibly have an effect on overall protein expression (Szymczak et al., 2004).

Figure 53: Comparison of Bicistronic Vector Yields.
Total purified antibody from three different expression formats of 1 L of cultures were measured by A280. H-L is the heavy chain first bicistronic vector, L-H is the light chain first bicistronic vector, and H+L is chains on separate vectors.

The final test required to determine the overall utility of bicistronic expression was to determine if the TA99 produced using those vectors was functionally equivalent to TA99 expressed using traditional vectors. To determine this, the binding properties of all three antibodies was measured by flow cytometry. B16F10 cells were harvested and rinsed in FACS buffer then labeled with the different TA99 antibodies. After 30 minutes on ice, the cells were rinsed and then incubated with a fluorescently labeled secondary antibody to mouse IgG. After another 30 minutes on ice the cells were rinsed twice and analyzed on a flow cytometer. The results showed no discernible difference between any of the antibodies (Figure 54).
Overall, the above results demonstrate that bicistronic expression of antibodies can yield high levels of protein that is functionally equivalent to antibodies expressed using traditional methods. One critical element not tested in this section of work was any kind of functional impairment caused by the additional 2A or furin peptides. Specifically, for antibodies this could manifest itself in some kind of impairments of the functioning of the Fc domain. Although not specifically tested here, the in vivo results described earlier were unchanged by the use of TA99 produced from either separate vectors or bicistronic ones. Additionally, the 2A system described above was used in several projects as outlined in the following sections, and no abnormal effects were observed by the presence of the additional amino acids.

5.2 Transfection

5.2.1 Introduction

In order to generate several stable cell lines and optimize foreign gene expression, multiple transfection reagents and protocols were tested. The results of different transfection screening experiments are described below, as well as the process to create a stable B16F10 cell line expressing carcinoembryonic antigen (CEA).
The marketplace is crowded with many options for transfection reagents. Vendors often promise high transfection performance in easy-to-use packages. However, often the reported results are achieved with easy to transfect cell lines under optimum conditions. These results are often not indicative of the true performance of the reagents when more difficult cell lines are used. One of the more difficult cell lines to transfect is the B16F10 melanoma line and one of the most difficult proteins to transfect is CEA, due to its large size, the fact that it is membrane-bound, and furthermore that binding is GPI-linked. These factors required a robust and effective transfection protocol.

Due to the difficulty of transfection with CEA, a bicistronic vector was cloned with both CEA and GFP expressed as separate polypeptides using the 2A sequence. In this way, GFP could be used as a surrogate marker for CEA expression. Additionally, in order to make a stable cell line, a vector with resistance to the selection marker G418 was used, pDisplay. The resulting vector was called pDisplay-CEA-P2A-eGFP.

5.2.2 Transfection Reagent Evaluation

Several transfection reagents were tested using B16 cells and the CEA expression vector, the results of which are shown in Figure 55. Unfortunately in this difficult test case, none of the transfection reagents yielded results above roughly 15%. The best-performing methods were lipofectamine and PEI (Liu et al., 2008) which both yielded 15.3% CEA positive cells. Several electroporation protocols were also tested, however none of these yielded better results than the transfection reagents (data not shown). Although it may have been possible to isolate stable clones from these low transfection pools, due to the difficulty of expressing CEA continued work was done on improving transfection efficiencies.
Several transfection reagents were evaluated according to the manufacturer's instructions. CEA expression was evaluated by staining with an Alexa Fluor 647-linked sm3E antibody.

After testing several reagents, far superior transfection rates were achieved by using the Xfect reagent from Clontech (Figure 56). Over 50% of cells were transfected using Xfect, and a clear GFP/AF647 double positive population can be seen. The manufacturer's instructions required no optimization and after 48 hours, high levels of transgene expression were detected.
5.2.3 Generation of B16-GFP-CEA

Once the robust transfection protocol was established using Xfect, the clonal selection process was started. Initial results using G418 showed that B16F10 cells were highly resistant to the selection agent. Additionally, it is possible that the B16F10 cells decoupled expression of the selection resistance marker and the transgene, by epigenetic effects, as often transfected cells would continue to be resistant to G418, but lose CEA expression. Finally, due to the high levels of melanin expressed by B16F10 cells, false positive cells can be detected through the GFP channel of flow cytometers. Given all these complicated factors, a very aggressive chemical and flow selection process was used to isolate stable cell lines.
To generate stable B16F10-GFP-CEA cell lines, transfection was performed using Xfect according to the manufacturer’s instructions. 48 hours after transfection, 5 mg/mL G418 was added to the culture media. From that point on, cells were cultured in the selection media. 5 days after G418 selection was started, bulk sorts of double positive cells, stained with anti-CEA-AF647 and co-expressing GFP, were performed and a polyclonal mix of positive cells was collected. Six bulk sorts were performed followed by a single cell sort into 96-well plates, all one week apart. Single cell clones were allowed to grow until visible colonies were formed. At this point individual wells were assayed for GFP and CEA expression. Clones showing the most homogenous expression levels were selected for expansion and banked as shown in Figure 57.

Figure 57: Stable B16F10-GFP-CEA Clones.
Six clones were isolated and expanded from single cell sorting and expansion. These clones show a diversity of transgene expression levels, with Clone A being a low expressing variant, Clone F having medium level expression, and Clone B expressing a high level of CEA.
5.3 Viral Transduction

5.3.1 Introduction

The introduction of transgenes into a target cell is performed in a variety of ways. As described in the last section, chemical transfection is a very simple and common way to achieve this. However, there are significant drawbacks to many transfection methods, namely the requirement of selection agents to generate stable cell lines, low efficiency, and the existence of difficult to transfect cell types. Given these drawbacks, it is sometimes advantageous to use alternative genetic engineering techniques, one of which is viral transduction. Viral transduction is the repurposing of natural viral systems with the goal of introducing new genetic elements to a target cell. In a simplified view, viral vectors are created by replacing functional viral genomes with genetically modified ones that introduce genes of interest.

This section describes the optimization process for a retroviral vector system, specifically the genetic modification of primary murine T cells. Three examples of this genetic modification are also demonstrated: the creation of a stable B16F10 cell line expressing GFP and luciferase, the development of a fully murine chimeric antigen receptor (CAR), and production of antibodies by primary mouse T cells. Additionally, a lentiviral system is described along with an example stable cell line producing TA99.

The initial retroviral system that was used is based off of the Phoenix Eco (Pear et al., 1993) and pCL-Eco (Naviaux et al., 1996) systems developed in the early 1990s. These systems use packaging and envelope proteins from the Moloney murine leukemia virus. The viral particles have been rendered non-replicative via several deletions and mutations. The murine retrovirus system can efficiently transduce many different primary cell types, however the ectopic pseudo-type renders it relatively safe to work with as it does not infect human cells. One disadvantage of these retroviral systems is that they do not infect nondividing cells, necessitating the use of various activating agents in the transduction process. Phoenix Eco are HEK293 cells that have been stably transfected with both the packaging and envelope proteins necessary to produce competent viral vectors. The pCL-Eco vector system also produces these same packaging and envelope proteins. By transfecting Phoenix Eco cells with a suitable expression vector, viral particles are produced that can stably transduce target cells.

The initial protocol was based on work by Riviere (Riviere and Sadelain, 1996). However, more optimal components were utilized where necessary, coming from work by Engels (Engels et al., 2003). Retrovirus is a good way for stable integration of
transgenes, but transduction is limited to dividing cells, and random integration biased towards sites of active chromatin is prone to causing insertional mutagenesis (Rohdewohld et al., 1987; Scherdin et al., 1990). Native virus replicates by infecting the cell via membrane fusion, ssRNA is reverse transcribed into dsDNA, transportation to the nucleus, integration into host DNA, native polymerase transcribes viral mRNA which is either directly processed generating the gag-pol polypeptides and the packaged RNA or spliced generating the env proteins, the virus is finally assembled and acquire the env glycoprotein upon budding. The non-replicative portion of this process is carried out by the viral vectors, and is responsible for the genetic modification of the target cells.

Several aspects of the viral genome are critical for the functioning of viral vectors. Long terminal repeats (LTRs) are essential sequences that promote reverse transcription and DNA integration. After the 5’ LTR are three essential elements: primer binding site, psi (packaging signal), and gag sequence (with low homology, promotes packaging, sometimes called psi+ or the extended packaging region). A splice donor site 5’ of the psi is usually also included. Before the 3’ LTR are viral sequence past the env stop. The 3’ U3 region can be modified to decrease transcription or for the construction of internal promoter vectors. Other viral sequences can also be inserted here to generate novel LTRs. The U3 region is reverse transcribed and incorporated into both LTRs during transduction. It is comprised of an enhancer and promoter that drive strong transcription.

A key feature of viral vectors is the ability to customize infection and expression by modifying the pseudotype and promoter of the virion. Pseudotyping is replacing the envelope (env) protein that is originally expressed by virus with one from another virus that can give the vector different species and tissue selectivity. Additionally, the promoter structure of the virus’ expression cassette can be modified to allow for specific expression. Specific env or promoters can be used for tissue selective expression. Ecotropic particles bind to mouse and rat receptors, amphotropic and GaLV particles bind to receptors present on many different types of mammalian cells.

Broadly, there are two main steps in a viral transduction protocol: the production of the virus and the infection of the target cells. The first step is commonly carried out by traditional transfection of a suitable target virus-producing cell line. Transfection efficiency, viral concentration, viral purity, and harvest timing are the critical optimization points. The second step is where cell-free viral vector particles are added to the target cells and allowed to infect and therefore genetically alter them. This step can be optimized by appropriately activating the target cells, adding adjuvants to the virus, and optimizing the physical conditions of the transduction.
5.3.2 Optimization of Viral Titer

The most critical factor in any transduction protocol is the level of viral titer achieved during the viral production phase. Viral titer can be measured in different ways. Specific viral proteins can be measured by techniques such as ELISA, however, these methods may overestimate the quality of the virus because inactive virus is counted. Functional virus can be measured and expressed as infectious units per milliliter (IU/mL). This is done by serial dilutions of virus onto a model cell line. This has the advantage of giving a more realistic readout of vector quality, but is problematic in that a very specific protocol must be used and may not be applicable or reproducible across labs. The importance of viral titer is shown in Figure 58. Virus was produced by transfecting Phoenix Eco cells with a GFP expression vector and then concentrated using a 100,000 kD centrifugal filter concentrator to different factors. The plot clearly shows the dependence of transduction efficiency as a function of viral titer.

![%GFP Cells 48 hrs After Transduction](image)

Figure 58: Effect of Viral Concentration on Transduction Rate.
GFP coding virus was produced in Phoenix Eco cells and then added to a target cell line. The virus was concentrated or diluted to several different levels using a 100,000 kD centrifugal column concentrator. Polybrene was added to facilitate the transduction of the target cell line.
Another key factor in the delivery of transgenes via viral vector is the physical limitations determined by the size of the viral capsid itself. Unlike linear or plasmid DNA, which has a large range of sizes possible, the genetic “payload” of the viral vector must fit inside the viral protein capsid. Furthermore, the efficiency of viral production is dependent on how large or small this payload is. The packaging particle constrains the expression cassette to 7-8 kb, which includes all sequences between the 5’ and 3’ LTR regions, not just the gene of interest. As shown in Figure 59, the size of the expression cassette can have varying effects on transduction efficiency, even between cell lines. 3T3 cells are often used as controls in viral transduction experiments due to the ease in which they are genetically modified. This was confirmed by the relatively weak dependence of transduction efficiency on insert size. More difficult cells, such as primary mouse T cells, required higher viral titers to transduce and thus had a greater dependence on the size of the genetic payload. These results also showed that when testing viral constructs it is important to use the target cell line in addition to test cell lines to determine the expected real performance of the vector system.

Figure 59: Transduction Efficiency as a Function of Insert Size.
The same viral expression system was used with expression inserts of three different sizes. The inserts were all tracked by expression of GFP. The easy to transduce 3T3 cell line was relatively unaffected by the increase in size, however, a significant decrease in transduction efficiency was observed with primary mouse T cells.
Optimization of the transduction process was first focused on improving viral titer. The first step in that optimization was the improvement of the transfection process. Unless otherwise noted, optimization experiments were performed using the vector pMP71-eGFP-P2A-FLuc2 and efficiency was measured by GFP expression by flow cytometry. Transfection was performed using calcium phosphate precipitation. Figure 60 shows the results of modifying the transfection procedure on the transfection efficiency of the Phoenix Eco cells themselves. This can serve as an initial predictor of transduction efficiency, as only transfected Phoenix Eco cells produce active viruses. Chloroquine is a reagent known to enhance the transfection efficiency of calcium phosphate transfection (Sambrook and Russell, 2006) and in this test showed a 10-20% improvement over expression DNA alone.

![Phoenix Eco Transfection Optimization](image)

Figure 60: Transfection Optimization of Virus Producing Cells.
Transfection of Phoenix Eco cells was optimized by three factors: adding chloroquine (CQ), adding a viral protein support plasmid (pCL-Eco), and producing the virus at different temperatures. The GFP expression vector (DNA) allowed for tracking of transfection efficiency by flow cytometry. As expected, temperature and pCL-Eco did not significantly affect the transfection efficiency, since these factors are virus specific. Also as expected, chloroquine increased the transfection efficiency of the calcium phosphate method.
Figure 61 shows the direct impact each optimized transfection parameter had on the final transduction efficiency of primary murine T cells. Retroviral particles are quite fragile, and can be damaged by freeze/thaw cycles, ultracentrifugation, and heat. As a result, it has been shown that culturing virus at lower temperatures can improve viral titer (Kaptein et al., 1997). For each case, virus cultured at 32°C had higher transduction efficiencies than virus cultured at 37°C. Viral accessory proteins are also key to producing high levels of functional virus. These include the products from the gag, pol, and env genes that have been stably transfected into Phoenix Eco cells. Despite robust stable generation of these proteins, high levels of expression vector production may outpace the cells’ ability to package and secrete virus. Therefore, viral packaging component expression was boosted by co-transfecting with the pCL-Eco vector. Although no difference in the transfection of the Phoenix Eco cells was seen (Figure 60), a direct improvement in transduction was observed (Figure 61), indicating virus titer was limited by the expression of packaging proteins by the Phoenix Eco cells. That bottleneck was removed by the transient enhancement of proteins in the pCL-Eco vector. Overall, by enhancing transfection efficiency, boosting viral component expression, and lowering production temperature, large gains in transduction efficiency were achieved.

Figure 61: Impact of Transfection Optimization on Transduction.
Transfection of Phoenix Eco cells was optimized by three factors: adding chloroquine (CQ), adding a viral protein support plasmid (pCL-Eco), and producing the virus at different temperatures. The GFP expression vector (DNA) allowed for tracking of transfection efficiency by flow cytometry. Viral supernatant from the different transfection conditions was used to transduce murine T cells. Progressive improvements in transduction efficiency were seen, with the highest levels achieved when all three parameters were optimized.
The next factor optimized was the virus producing cell line. The Platinum Eco (Plat-E) cell line reportedly produced higher viral titers than Phoenix Eco cells (Morita et al., 2000). Figure 62 shows the results of calcium phosphate transfection for the two different cell lines using two different expression vectors, one producing GFP and one producing a transgenic TCR to the 2C antigen. Since both Plat-E and Phoenix Eco cells are based on HEK293 cells, there transfection efficiencies were similar, as expected.

![Virus Producing Cells Transfection](image)

Figure 62: Transfection Comparison of Virus Producing Cells. Platinum Eco cells (Plat-E) were compared to Phoenix Eco cells for transfection efficiency by calcium phosphate precipitation. As another comparison a vector encoding for a transgenic TCR (2C-TCR) was included and measured by flow cytometry. Both cell lines were transfected with similar efficiencies.

Although both cell lines were transfected with similar efficiencies, their viral production was different, as shown in Figure 63. When viral supernatant from the two different cell lines were used to transduce primary murine T cells, Plat-E cells showed superior results. This is most likely due to improved expression of packaging protein by the Plat-E cells. The results of Figure 62 and Figure 63 mimic the results shown in co-transfection of pCL-Eco during the transfection of Phoenix Eco cells, where transfection
rates were not improved, but viral titers were increased. In fact, when co-transfected with pCL-Eco, Phoenix Eco cells perform equivalently to Plat-E cells (data not shown), confirming the bottleneck of viral protein production.

Figure 63: Transduction Comparison of Virus Producing Cells.
T cells were transduced with vectors from two different virus producing cell lines using identical transfection and transduction protocols. The Plat-E cells clearly outperformed the Phoenix Eco cells with both vectors showing higher expression of the transgene.

As with any transient system, viral expression starts out at a low level, increases with time to a peak, and then drops off as the plasmids are diluted by cell growth and degradation. Additionally, since retrovirus is not stable over long periods of time, optimal harvest windows were determined during which the highest titer was achieved. To determine when peak titers were achieved, viral vectors were harvested at several times and tested for transduction efficiency (Figure 64). Phoenix Eco cells were transfected by calcium phosphate for 8 hours and then rinsed and plated with fresh media. 24 hours later, this media was harvested and new media was added to the Phoenix Eco cells. This was repeated at 48, 72, and 96 hour timepoints. At each timepoint, 3T3 cells were immediately transduced with the harvested viral supernatant. The results show viral titers ramp up to a maximum at 72 hours after transfection, then
begin to decline. 48 and 96 hour timepoints show roughly equivalent viral titers, while 24 hour titers were the lowest.

![Graph showing viral transduction optimization over time](image)

Figure 64: Impact of Viral Harvest Timing on Transduction. Phoenix Eco cells were transfected and then sequentially harvested to determine viral titer during several consecutive 24 hour segments. 3T3 cells were transduced with viral supernatants and assayed for gene expression 48 hours later. Peak virus titer was achieved at 72 hours after transfection. Roughly equivalent viral titers were seen at 48 and 96 hours after transfection.

Using the optimized transfection protocol derived from the described experiments, serial dilutions of viral supernatant with different numbers of 3T3 target cells were performed in order to calculate viral titer (Figure 65). In order to accurately calculate viral titer, low levels of transduction must be achieved. This is due to the fact that at higher transduction levels each cell begins to be infected with more than one viral particle. This can be seen in the results above due to the sigmoidal curve of the dilutions. Most viral titers are calculated using transduction efficiencies of below 30%. Using the two highest cell densities at the two highest dilutions, viral titer was calculated as $9 \times 10^5$ IU/mL.
Figure 65: Viral Titer Measurements.
Several densities of 3T3 cells were plated and treated with serial dilutions of viral titer using the optimized protocol. As can be seen, undiluted virus is at saturating levels, however at dilutions in the 1:100 range with higher numbers of cells, a lower saturation point starts to appear.

5.3.3 Optimization of Transduction Protocol

Once high viral titers were achieved, optimization experiments moved on to the infection phase of the transduction protocol. Two factors were optimized, additives that help to facilitate viral infection and the activation protocol for the cells of interest. The end result of these two optimized phases were then combined to generate a complete transduction protocol. For this phase of experiments, a standard transfection protocol was used with a GFP expressing vector and applied to all conditions.

Spinoculation is a common technique used to enhance transduction efficiencies (Lee et al., 2009). For all conditions, target cells were resuspended in viral supernatant and then added to retronectin coated plates. The plates were then spun in a centrifuge at 2000 xg for 1 hr. Some spinoculation procedures call for the addition of cationic polymers to further facilitate infection, however, this may be unnecessary because the maximum transduction rates are already achieved by the spinoculation process. To test
the marginal benefit of adding cationic polymers, primary murine T cells were tested with either protamine sulfate or polybrene added to the spinoculation process (Figure 66). The results show that the addition of these polymers did not improve transduction efficiencies. The spinoculation process along with the retronectin coated plates were sufficient to drive maximum infection levels.

Active division is required for retroviral vectors to stably transduce the target cells. This is because during division, the nuclear membrane is dissolved and the viral proteins and nucleic acids have direct access to the genome. Resting T cells do not actively divide and must therefore be activated in order to be infected by retroviral vectors. Thus, the activation protocol for T cells is a critical part of an optimized transduction procedure (Lee et al., 2009). Several standard methods of T cell activation were tested for their ability to produce transduction-ready cultures (Figure 67). For this experiment, pmel-1 cells were used in order to test specific peptide stimulation as an activation method. As described, pmel-1 T cells have a TCR recognizing a fragment of the gp100 melanoma differentiation marker. This peptide fragment was added to
splenocytes from pmel-1 mice for 24 hours before CD8 T cells were isolated by negative magnetic bead selection. CD28/CD3 activation beads are also sold as artificial APC-like elements that promote T cell division. The beads are magnetic dextran particles coated with antibodies to CD28 and CD3. These beads were added at a 1:1 ratio with isolated CD8 T cells for 48 hours prior to transduction. Another common method to use antibody activation of T cells is to coat plates with antibodies to CD28 and CD3. These plates were generated and then isolated T cells were incubated on them for 48 hours before transduction. The final method of T cell activation that was tested was general mitogenic stimulation by Concanavalin A (ConA) and IL-7. Splenocytes were treated with these two agents for 24 hours before CD8 T cells were isolated as before. All four of these methods led to enough stimulation to cause some levels of T cell transduction. The activation beads proved to be the most superior, with peptide and ConA leading to slightly lower levels of transduction. Plate activation did not induce significant proliferation which was observed by the lowest transduction efficiencies.

![T Cell Transduction Optimization](image)

**Figure 67: T Cell Activation Methods.**
Transduction of primary murine T cells was performed by a standard spinoculation. Prior to infection, the cells were expanded using different methods for 48 hours. Pmel-1 cells were used in this experiment to test the use of specific peptide expansion. Four different activation methods were used, the human gp100 fragment recognized by pmel-1 cells (Peptide), CD3/C28 activation beads (Beads), CD3/CD28 antibody coated plates (Plate), and Concanavalin A with IL-7 (ConA). The activation beads show the highest level of transduction. ConA and peptide showed equal amounts of medium transduction levels. The coated plates showed the lowest level of activation.
Given that beads showed the best initial stimulation of T cells, further optimizations were performed using them only. The ratio of beads to cells is an important factor in the activation of T cell cultures. Additionally, given the expense of the beads, it was important to determine a balance between transduction efficiency and costs. Cytokines are potent stimulators of T cell expansion and are often added to activation protocols. The most common cytokine used to activate T cell cultures is IL-2. Alternatively, more complex cytokine mixtures can be used to give a variety of growth signals to the T cell culture, an example of which is T Stim by BD. T Stim (or T Stem) is filtered supernatant from ConA Activated rat splenocyte cultures, with Methyl-alpha-D-mannopyranoside added to scavenge the ConA. This culture additive is used in concentrations between 2 and 10% and provides a host of cytokines and other growth stimulant molecules to the T cell culture. Overstimulation is another concern during the activation protocol, as very high levels of IL-2 have been shown to cause premature T cell apoptosis. These factors were tested and the results are shown in Figure 68. Cell to bead ratios were also tested at 1:1 and 1:3. For the cases with IL-2 stimulation, the higher bead ratios led to significant increases in transduction efficiency. With T Stim support, the ratio of beads did not lead to meaningful difference in T cell transduction. At this point, however, at almost 90% transduction efficiency with GFP, further gains would be difficult to achieve.

After these extensive optimization experiments were performed, a consensus protocol for retroviral vector production was reached (Figure 69), utilizing the results shown above as well as information from other protocols. The step by step protocol can be seen at the end of this document. Several key optimization points resulted in significant improvements in the overall efficiency of the transduction procedure. First, the use of the pMP-71 vector was critical to high level expression of the transgene. Second, either boosting production of viral helper proteins using pCL-Eco, or using better cell lines (e.g. Plat-E) led to higher transduction rates. Finally, optimal simulation using T Stim and activation beads gave the best transduction results.
Figure 68: Optimization of Bead Activation.

T cells were activated with beads at different ratios, as well as different levels of cytokine support. Standard spinoculation protocols were used for all cases. 1:1 and 1:3 refer to the bead to cell ratio used in that experiment. Low IL-2 levels were 20 U/ML while High IL-2 levels were 100 u/mL. T Stim (T Stem) was used at a concentration of 10%. In each case, a 1:1 ratio of beads to cells was superior to a 1:3 ratio, however the difference was negligible in the case of T Stim. Low levels of IL-2 were slightly better at generating transduction ready T cells than higher levels of IL-2, but T Stim was significantly higher than either IL-2 concentration.
Figure 69: Summary Results of Transduction Improvements.
The improvements of each stage of the optimization are shown. The improvements are progressive, i.e. the T cell activation optimized protocol builds on the viral production optimized protocol. The initial infection protocol utilizing retroenetcin-coated plates and spinoculation was already optimized. Viral production and T cell activation optimization both generated significant gains in transduction efficiency.

5.3.4 Evaluation of a Murine Chimeric Antigen Receptor

Given an optimum transduction protocol for murine T cells, a practical application was tested in the form of a chimeric antigen receptor (CAR). CARs are hybrid molecules designed to redirect T cell specific killing to target antigens by using antibody fragments and have experienced recent clinical successes (Levine, 2015). In its most basic form a CAR consists of an extracellular targeting domain (usually an scFv antibody), a transmembrane domain, and one or more intracellular activating domains (usually the intracellular CD3ζ domain and a costimulatory domain such as from
CD28). This fusion protein is able to mimic natural signal 1 and 2, except that instead of targeting peptide antigens displayed by MHC molecules through a TCR, extracellular epitopes are directly targeted by antibody CDRs. For this study, a CAR with a TA99 scFv was used with CD28 and CD3ζ intracellular domains produced by the vector pMP71-CAR-TA99-CD28-CD3ζ. As a control, the 237 scFv was used with identical signaling domains, produced by the vector pMP71-CAR-237-CD28-CD3ζ.

The optimized transduction protocol was repeated with primary murine T cells to determine if CARs could be expressed efficiently (Figure 70). Expression was evaluated by waiting 48 hours after transduction, then staining for the presence of the extracellular scFv by using a labeled anti-mouse antibody and analyzing by flow cytometry. Expression was high for both CARs. Control expression of the GFP vector was 87%, while both CARs was approximately 70%. CAR expression was expectedly lower, due to the difficulty of producing transmembrane proteins over cytoplasmic ones.

![Figure 70: CAR Transduction Efficiency.](image)

Transduction of primary murine T cells was performed using the optimal protocol described earlier. 48 hours after transduction, T cells were stained for the presence of the extracellular domain of the CAR. GFP was included as a positive control. Both the TA99 and 237 CARs transduced at approximately 70% efficiency.
Once high level expression of the CARs was achieved, functional tests were performed. 48 hours after transduction, the T cells were used in cytotoxicity assays. Target B16F10 cells were labeled with Chromium 51 and seeded in 96-well v-bottom plates at 10,000 cells per well. Varying numbers of effector T cells were then added to achieve effector to target cell ratios of 1:1 to 81:1. After 5 hours of co-incubation, the plates were centrifuged and supernatant samples were taken and tested for chromium release. Percent cytotoxicity was then calculated as \[\frac{(\text{Sample cpm} - \text{Spontaneous Lysis Control cpm})}{(\text{Maximum Lysis Control cpm} - \text{Spontaneous Lysis Control cpm})} \times 100\%\].

The results show that the TA99 CAR was highly specific for B16F10 cells (Figure 71). All 3 negative controls, irrelevant target (237-CAR), irrelevant transduction (GFP-Luc), and untransduced (Control), showed negligible killing at any E/T ratio. The TA99-CAR however only achieved modest killing at very high E/T ratios.

![5 Hour Release Assay](image)

Figure 71: Cytotoxicity Assay of CAR Expressing T Cells.
Primary murine T cells were transduced with CARs and tested for their ability to specifically lyse target cells. Transduced T cells were incubated with chromium labeled B16F10 cells in v-bottom plates for 5 hours. Supernatant samples were then taken and measured for chromium release. Effector to Target ratio (i.e. T cells to B16F10) was increased to determine killing potency. TA99-CAR is specific for B16F10 cells, and showed moderate killing, but only at higher E/T ratios. 237-CAR targets an irrelevant antigen and, along with T cells transduced to express GFP and luciferase (GFP-Luc) and untransduced T cells (Control), showed no appreciable killing at any level.
Although initial results showed promising cytotoxicity, the level was insufficient for in vivo efficacy (data not shown). Additionally, other studies showed that TA99’s affinity is dramatically reduced after conversion to the scFv format. In order to improve a B16F10 targeting CAR, affinity maturation would have to be performed on the TA99 scFv. Additionally, successful in vivo testing would require a range of CARs to be generated with various spacer regions and optimized signaling domains in order to determine an effective molecule.

5.3.5 Production of TA99 by Primary Murine T Cells

Previous results showed that antibody support of an ACT protocol could lead to permanent cures of established melanoma tumors. Additionally, although TA99 is well tolerated, other antibodies, especially immunomodulatory ones, have higher off-target effects, which can limit their dosing to the point of ineffectiveness. By having T cells deliver the antibody specifically to the tumor, these dose limitations and side-effects may be ameliorate to some extent.

As previously described, the 2A expression cassette was used to produce both the heavy and light chains of TA99 from a single ORF. The resulting vector, pMP71-TA99LCv1-F-T2A-TA99HCv3-v1, was used in the optimized transduction protocol as described above. Microwell expression analysis of antibodies (Ogunniyi et al., 2009) was performed to determine production levels from the transduced T cells (Figure 1). Total transduction percentage was quite low at 37%, indicating both the difficulty T cells have in expressing the antibody and the larger size of the expression cassette when compared to GFP. However, the average specific productivity for the transduced cells was adequate at 0.13 pg/cell/day when compared to 1-100 pg/cell/day achieved by industrially optimized CHO cells (Chusainow et al., 2009).
Figure 72: Microwell Analysis of Antibody Expression by T Cells.
Primary murine T cells were transduced to express

This proof of concept study shows that T cells can be used to express molecules that do not necessarily support ACT directly, but enhance the therapeutic outcome. There are many additional applications and improvements that could be made to this idea. For example, CARs already have antibody specificity and are transduced during cell preparation. The vectors could be modified to express both the CAR and an equivalent antibody with the same specificity. In this way, the cancer would be attacked using two different modalities, thus linking the innate and adaptive immune system in a single treatment. This is especially convenient, since the same specificity is used, obviating the need for determining multiple unique targets in a single patient’s cancer. Specificity of expression could also be achieved by using more precise promoter structures. For example, the expression of antibody could be linked to the activation of the CAR, thereby delivering the antibody only when the T cell is actively engaged in killing cancer cells. In this way, systemic delivery of the antibody would be limited and subsequent side effects could be lessened. Similar strategies have been used to control the delivery of IL-12 (Chmielewski et al., 2011) and to specifically control T cell killing through dual targeting CAR (Kloss et al., 2013).
5.3.6 Generation of Stable Cell Line Producing TA99

The final demonstration of viral transduction was to utilize a lentiviral system to create a stable antibody producing cell line from HEK293 cells. Not only did this serve as a control example for the development of a lentiviral transduction protocol, but it generated a valuable research tool facilitating quick and easy production of TA99. To perform the transduction, published protocols were used (Kuroda et al., 2009; Oberbek et al., 2010). The system used was a second generation lentiviral vector using the packaging plasmid pCMV-dR8.2-dvpr, the envelope plasmid pCMV-VSV-G (Stewart et al., 2003), and the expression plasmid pLB2 (Stern et al., 2008).

Viral titers were performed using the control vector pLB2-CMV-eGFP-F2A-CBRLuc-v1 and measured using flow cytometry (Figure 73). PEI transfection of HEK293FT cells was performed using the three vectors and viral supernatant was harvested 48 hours after transfection. HEK293 were resuspended in the viral supernatant with protamine sulfate added to facilitate infections. 48 hours after the transduction, the cells were analyzed for expression of GFP. Virus was also concentrated using a 100 kD centrifugal concentrator column. Dilutions of the virus showed a clear linear dependence of transduction efficacy on viral titer. Although overall transduction rates were quite low, the ability to sort the positive cells to a homogenous population was planned.
Figure 73: Lentivirus Titering.
A standard lentivirus production and transduction protocol was followed. Virus was concentrated or diluted as shown in the figure. A clear linear dependence on viral titer was shown.

To express functional antibody, a multi-cistronic expression cassette was cloned into the pLB2 backbone generating GFP and both the heavy and light chains of TA99. The resulting vector was called pLB2-CMV-eGFP-F2A-TA99LCv1-F-T2A-TA99HCv3-v2 (Figure 74). The GFP was added so that flow sorting of transduced cells could be performed easily. A CMV promoter was used to drive the ORF translation and a furin cleavage site was used to minimize the effect of the 2A tag. Two different 2A sequences were used to achieve the expression of three distinct proteins. The use of different 2A sequences is essential in viral vectors to avoid homologous recombination events that could silence parts of the expression cassette. Secretion signals were added to the heavy and light chain proteins to ensure proper processing. GFP was not secreted to ensure adequate cell signal during sorting.
Three separate proteins were expressed using two 2A skip peptides. The GFP was added in order to determine transduction efficiency as well as for use as a marker during flow sorting. Secretion signals were placed before both the heavy and light chains to ensure proper processing. With no secretion signal, GFP would be expressed in the cytoplasm.

The expression cell line HEK293-GFP-TA99LH was generated as follows. PEI transfection of HEK293FT cells was performed using the three vectors described above. Due to the low transduction rates observed during viral titer measurements, an additional round of infection was performed. Viral supernatant was harvested 48 hours after transfection for 2 consecutive days. HEK293 were resuspended in the viral supernatant with protamine sulfate added to facilitate infections. 48 hours after the second transduction, the cells were analyzed for expression of GFP (Figure 75). The additional infection cycle yielded much higher transduction rates at around 50%. This gave an ample initial pool to begin sorting for stable clones. GFP positive cells were collected every week for 6 rounds of selection before a stable >95% positive population was established. Lentiviral vectors are designed to create stable cell lines, however, pseudo-transduction can still be an issue, where viral particles never integrate into the target cell’s genome, but their expression cassettes are still transiently expressed in the cytoplasm. This was most likely a factor during this selection process, and necessitated the multiple rounds of sorting, especially since no other selection agent was used.
Figure 75: Generation of HEK293-GFP-TA99LH Cells. Transduced HEK293 cells show clear GFP expression. Improvements in transduction efficacy were made by performing an additional round of infection on the HEK293 cells.

Once a stable polyclonal population of TA99LH cells were established, production cultures were started. TA99LH cells were seeded at 1e6 cells/mL in Freestyle media. After 8 days, supernatant was generated by centrifugation and the antibody was purified by Protein A. The resulting purified antibody was buffer exchanged into PBS and run on an SDS-PAGE gel stained with Coomassie blue (Figure 76). In parallel, TA99 was produced by the standard two vector transient expression method. Overall product quality by this measurement showed the two products were quite similar. The unreduced lanes showed additional bands produced by the stable cell line in addition to the main product at ~150 kD. The reduced bands show clearly the effect of the 2A and furin amino acids on the light chain. These differences were not expected to impact the binding or function of the antibody. Consequently, binding assays to B16F10 cells via flow cytometry and in vivo study results were identical between the two products (data not shown).
Figure 76: Analysis of TA99 Produced by HEK293-GFP-TA99LH Cells. TA99 produced by both transient, two vector expression, as well as by stable TA99LH cells. The non-reduced lanes show comparable product quality, with the stable line producing slightly more additional bands to the main product at ~150 kD. Additionally, the LC of the stable line is larger as shown in the reduced lanes due to the additional amino acids from the Furin/2A sequence.

Finally, overall yield of TA99 was calculated from the purified cultures (Figure 77). Antibody was generated either by standard 2 vector transient transfection or by stable cell line. Both cultures were seeded at 1e6 cells/mL in Freestyle media. 8 day cultures were purified via centrifugation, Protein A, and buffer exchange. Final protein concentration was measured using A280. The results clearly showed a significant boost in productivity by the stable cell line.
Figure 77: Yield of TA99 Produced by HEK293-GFP-TA99LH Cells.

TA99 was produced by both a stable cell line generated by lentiviral vector transduction and by standard 2 vector transient transfection. Both cultures were seeded at identical densities and used the same Freestyle media. The stable cell line produced significantly higher amounts of TA99.
6 Conclusions

The work described in this thesis covered a broad segment of immunotherapies with a focus of eliciting a T cell response and augmenting it with supporting therapies against cancer. In the first case, ACT was used. T cells were directly injected into the recipient mice and supported by cytokines and tumor targeting antibodies. In the second case, T cells were elicited by a cancer vaccine, and their function was enhanced by the additional therapies of checkpoint blockade, cytokines, and tumor targeting antibodies. Finally, in the third case, BiTEs were used to redirect the action of T cells and their functional characteristics were analyzed by next generation sequencing. Although critical in the cases where in vivo efficacy was tested, T cells alone were unable to effect meaningful therapeutic benefit on their own in any of the cases tested. This fact supports the idea that effective immunotherapy regimens engage multiple facets of the immune system to form a synergistic response to tumors.

ACT is currently generating a large amount of interest and its early clinical successes have been dramatic, however, there are many barriers to the widespread use of this technology. The first cell therapy, sipuleucel-T (sold as Provenge by Dendreon), was similarly widely touted as a blockbuster drug and was expected to bring in huge profits for Dendreon. These expectations proved wildly overblown as the drug failed to gain significant market share and Dendreon eventually went bankrupt. The major culprits for the failure of sipuleucel-T were insufficient efficacy, high costs, as well as effectively marketing a completely new type of therapy to doctors, not to mention the difficulty of coordinating Medicare reimbursements. Because of the complexity, costs, and risks associated with cell-based therapies, their results must be particularly compelling to justify their use. The moderate effectiveness of sipuleucel-T clearly did not meet this high standard and the drug failed. The story of Dendreon and Provenge is clearly a cautionary tale to the ACT community and requires those therapies to be dramatically better than the current standard of care.

The clear need for optimal ACT treatment regimens supports continued research into combinations with ACT such as the ones described in this thesis. Although very effective therapies were developed, it is interesting that despite the multitude of factors tested, it is still unclear why exactly the combination of tumor specific T cells, MSA-IL2, and tumor targeting antibody therapy was able to cure mice, while the combination of T cells and MSA-IL2 was not. This is obviously where continued research would be warranted. This line of continued research would, unfortunately, be quite complicated due to the fact that many of the obvious variables to look for differences between the therapies were already examined. One place to start would be to examine the T cells in
more detail. Although their gross phenotype was unchanged, functional tests or more detailed tests, such as next generation sequencing, could be performed to further discriminate finer differences between the two populations. Another possible avenue of study would be focused on genetically modified mouse models. These would include various forms of immunocompromised mice, such as NSG and nude, which could elucidate the roles of the other cells in the immune system on the efficacy of the treatments. Mice with specific knockouts related to antibody effector function, such as Fc-receptor, and cytokine functioning, would also be helpful in determining mechanistic differences. Assays examining antibody-mediated toxicity by the innate branch of the immune system may also be helpful, especially if they can be done in vivo. Finally, it is possible that the functioning of the T cells becomes deficient in some way over time. The timepoints examined were all early enough that both therapies were in their highly effective stages. Examining the tumors of T cell and MSA-IL2 treated mice after they start to grow out may provide clues as to why they are no longer effectively controlled.

The combination immunotherapy project using vaccine generated T cells is a highly complicated treatment regimen that has several limitations in clinical applicability and great potential for further study. The clinical study of combinations of immunotherapies is still in its infancy, with usually only two therapies being used at the same time. With human studies, many complicating factors are introduced. A primary concern of practical importance is managing multiple dosing strategies. Because of the synergistic nature of immunotherapies, maximum tolerated doses of individual drugs may be significantly higher than those same drugs when used in combinations. This concern must be balanced with the need for sufficient levels of treatments to adequately treat the tumor. Additionally, progress must be made on rapid analytics in order to properly diagnose the cancer and generate appropriate reagents specific for the tumor antigens.

Future studies can be made in extending the types of therapies used as well as developing a deeper understanding of the mechanism of the combination components. One interesting avenue that could be explored is the use of fine needle aspirates or dual tumors with resection to make non-terminal measurements that could be used later with survival data for building predictive models. Additional studies using other cell types could also be performed to see similarities and differences between various forms of cancers, including humanized mouse models. Finally, this combination of immunotherapeutics is not unique, and swapping out one or more components could lead to tumor control of varying efficacy. Knowing the types of components which are effective or ineffective could contribute to the mechanistic understanding of combination therapy in general.
Finally, the BiTE project has many opportunities for continued avenues of exploration. BiTEs have not been as widely publicized as ACT, but offer similar avenues of treatment options with the advantage of being an injectable protein product. BiTEs were originally developed by Micromet, but are now being developed by Amgen. They are also part of a larger class of bispecific molecules that function by bringing target and effector cells together. Several drugs from this class of molecules are expected to be clinically relevant in the next five to ten years. With an increase in clinical relevance comes the need for more detailed mechanistic studies such as the ones described here.

The research presented here revealed much on the underlying function of T cells when undergoing BiTE-mediated cell lysis, however, much of it depended purely on transcriptional information alone. A major next step in this research would be to confirm many of the findings using functional studies such as knockouts or blockade to see if the components varying at a transcriptional level are indeed relevant on a functional level. Additionally, protein based evidence would be warranted to determine more precisely the relationship between protein expression and transcript expression during cytolytic functioning. Finally, more research should be performed on the findings related to Mpzl2. The data presented here was purely circumstantial and did not validate any of the implied functional relationships between Mpzl2 and other exhaustion markers. Follow-up studies could involve blocking Mpzl2 to determine any change in cytotoxicity, isolating Mpzl2+ cells and doing further studies on them for function, and determining downstream signaling pathways, if any, of Mpzl2 ligation.

Combination immunotherapy promises a future where synergistic treatments are specifically tailored to individual cancers leading to highly effective responses. However, determining the optimal combination of therapies, the complexity of dosing strategies, and the availability of targeted treatments are all barriers that must be overcome. The analysis presented here will make a significant contribution to the body of knowledge on immunotherapy as it has shown the importance of combining orthogonal immunotherapies in order to get durable cures to established tumors. These results will hopefully encourage combinations of orthogonally acting therapies based on T cells to achieve stronger clinical responses. By determining the necessary requirements for a strong, synergistic response to tumorous growths, more effective combination immunotherapy protocols may be designed in the future.
7 Protocols

T Cell Retrovirus Transduction Protocol

<table>
<thead>
<tr>
<th>Action</th>
<th>Check</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 1 PM</strong></td>
<td>-</td>
</tr>
<tr>
<td>Coat plates with 10 mL Poly-L-lysine solution (8 mL 0.1% Gelatin / 2 mL 0.01% poly-L-lysine), reusing the solution for each plate.</td>
<td></td>
</tr>
<tr>
<td>Seed Phoenix Eco cells at 6e6 per 10 cm dish (~80% confluent on day of transfection).</td>
<td></td>
</tr>
<tr>
<td><strong>Day 2 AM</strong></td>
<td>-</td>
</tr>
<tr>
<td>Transfect Phoenix Eco cells.</td>
<td></td>
</tr>
<tr>
<td>Incubate at 32°C for 36 hours.</td>
<td></td>
</tr>
<tr>
<td><strong>Day 4 AM</strong></td>
<td>-</td>
</tr>
<tr>
<td>Exchange 4 mL T cell media onto Phoenix Eco cells.</td>
<td></td>
</tr>
<tr>
<td>Incubate at 32°C for 24 hours.</td>
<td></td>
</tr>
<tr>
<td>Harvest mouse spleens. Homogenize tissue and pass through a 40 μm cell strainer into 50 mL conical tube using PBS.</td>
<td></td>
</tr>
<tr>
<td>Top off w/ PBS, centrifuge at 1600 rpm for 10 min.</td>
<td></td>
</tr>
<tr>
<td>Wash cells with PBS, centrifuge at 1600 rpm for 10 min.</td>
<td></td>
</tr>
<tr>
<td>Resuspend in 5 mL PBS per Ficoll tube.</td>
<td></td>
</tr>
<tr>
<td>Add 5 mL Ficoll slowly to the bottom of the tube to create a layer of Ficoll underneath the PBS cell suspension.</td>
<td></td>
</tr>
<tr>
<td>Centrifuge at 1500 rpm for 15 min at 20°C with the brake off.</td>
<td></td>
</tr>
<tr>
<td>Remove cells from centrifuge and carefully extract to a new tube. The T cells are the middle colored portion between the PBS and Ficoll.</td>
<td></td>
</tr>
<tr>
<td>Add PBS to fill tubes.</td>
<td></td>
</tr>
<tr>
<td><strong>Cell Count</strong> = ( \text{Dilution Factor} \times \text{# cells counted} \times 10,000 \times \frac{\text{Total Volume}}{\text{# squares counted}} )</td>
<td></td>
</tr>
<tr>
<td>Centrifuge at 1600 rpm for 5 min.</td>
<td></td>
</tr>
<tr>
<td>Aspirate supernatant.</td>
<td></td>
</tr>
<tr>
<td>Resuspend pellet in ACK Lysing Buffer:</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solution</th>
<th>Amount per Spleen (mL)</th>
<th># of Spleens</th>
<th>Total to Add (mL)</th>
<th>Check</th>
</tr>
</thead>
</table>

119
| ACK Lysing Buffer | 1 | x | = |

Incubate for 7 min.

Add PBS to fill tube.

Centrifuge at 1600 rpms for 5 min.

Resuspend cells in PBS.

Cell Count = \( \frac{\text{Dilution Factor} \times \text{# cells counted} \times 10,000}{\text{# squares counted}} \times \text{Total Volume} = \)

Centrifuge at 1600 rpms for 5 min.

Isolate CD8+ cells with EasySep Kit.

Prepare an expander bead solution:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Cells Required (cells)</th>
<th>Ratio (cells/bead)</th>
<th>Bead Concentration (beads/μL)</th>
<th>Total to Add (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beads</td>
<td>/</td>
<td>1</td>
<td>4e4</td>
<td></td>
</tr>
</tbody>
</table>

Dilute required beads in 3 mL of PBS with 2% HI FBS and place in a magnet.

Aspirate PBS with 2% HI FBS, and repeat 2 times for a total of 3 washes.

Select appropriate culture dish:

<table>
<thead>
<tr>
<th># of Cells (cells x e6)</th>
<th>Culture Dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1-0.5</td>
<td>24 well plate</td>
</tr>
<tr>
<td>0.5-1</td>
<td>12 well plate</td>
</tr>
<tr>
<td>1-3</td>
<td>6 well plate</td>
</tr>
<tr>
<td>7-15</td>
<td>10 cm dish</td>
</tr>
</tbody>
</table>

Resuspend beads in T cell media with T Stim:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Cells Required (cells)</th>
<th>Cell Density (cells/mL)</th>
<th>Total to Add (mL)</th>
<th>Check</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell Media</td>
<td>/</td>
<td>1e6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solution</th>
<th>T cell Media (mL)</th>
<th>Target T Stim Fraction</th>
<th>Total to Add (mL)</th>
<th>Check</th>
</tr>
</thead>
<tbody>
<tr>
<td>T Stim</td>
<td>*</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Resuspend T cells in prepared media and transfer to TC plate. Incubate 24 hrs.

**Day 4 PM**

Coat non-TC plates with 15 µg/mL Retronectin in PBS:

For 24 well plates:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Wells</th>
<th>Volume per Well (µL)</th>
<th>Volume to Add (µL)</th>
<th>Check</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retronectin (1 µg/µL)</td>
<td>*</td>
<td>3</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>*</td>
<td>200</td>
<td>=</td>
<td></td>
</tr>
</tbody>
</table>

For 12 well plates:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Wells</th>
<th>Volume per Well (µL)</th>
<th>Volume to Add (µL)</th>
<th>Check</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retronectin (1 µg/µL)</td>
<td>*</td>
<td>9</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>*</td>
<td>600</td>
<td>=</td>
<td></td>
</tr>
</tbody>
</table>

For 6 well plates:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Wells</th>
<th>Volume per Well (µL)</th>
<th>Volume to Add (µL)</th>
<th>Check</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retronectin (1 µg/µL)</td>
<td>*</td>
<td>15</td>
<td>=</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>*</td>
<td>1000</td>
<td>=</td>
<td></td>
</tr>
</tbody>
</table>

Wrap in parafilm and incubate overnight at 4°C.

**Day 5**

Aspirate retronectin (keep for reuse). Add 1 mL PBS with 2% HI FBS. Do not let wells dry out. Incubate 30 min at RT.

Harvest T cells from plates.

\[ \text{Cell Count} = \frac{\text{Dilution Factor} \times \text{# cells counted} \times 10,000}{\text{# squares counted}} \times \text{Total Volume} = \]

Centrifuge 1600 rpms for 5 min.

Remove beads by resuspending cells and pipetting cells in magnet and rinsing with PBS with 2% HI FBS.

Centrifuge 1600 rpms for 5 min.

Harvest viral supernatant.

Filter through 0.45 µm membrane.

Add 4 mL T cell media to Phoenix Eco cells.

Incubate Phoenix Eco cells for 24 hrs at 32°C.

Aspirate PBS from retronectin coated plates.
Select appropriate culture dish:

<table>
<thead>
<tr>
<th># of Cells (cells x e6)</th>
<th>Culture Dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1-0.5</td>
<td>24 well plate</td>
</tr>
<tr>
<td>0.5-1</td>
<td>12 well plate</td>
</tr>
<tr>
<td>1-3</td>
<td>6 well plate</td>
</tr>
</tbody>
</table>

Resuspend cells in viral supernatant with T Stim and add to wells:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Cells Required (cells)</th>
<th>Cell Density (cells/mL)</th>
<th>Total to Add (mL)</th>
<th>Check</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral Supernatant</td>
<td>/</td>
<td>1e6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solution</th>
<th>T cell Media (mL)</th>
<th>Target T Stim Fraction</th>
<th>Total to Add (mL)</th>
<th>Check</th>
</tr>
</thead>
<tbody>
<tr>
<td>T Stim</td>
<td>*</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Wrap plates in parafilm.

Centrifuge at 3400 rpm for 1 hour at 30°C.

Incubate overnight at 37°C.

Day 6

Harvest viral supernatant.

Filter through 0.45 μm membrane.

Dilute cells 1:1 in fresh viral supernatant with T Stim:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Viral Supernatant per Well (mL/well)</th>
<th>Check</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral Supernatant</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solution</th>
<th>Viral Supernatant (mL)</th>
<th>Target T Stim Fraction</th>
<th>Total to Add (mL)</th>
<th>Check</th>
</tr>
</thead>
<tbody>
<tr>
<td>T Stim</td>
<td>*</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Wrap plates in parafilm.

Centrifuge at 3400 rpm for 1 hour at 30°C.

Incubate overnight at 37°C.

Day 7

Harvest T cells and centrifuge 1600 rpms for 5 min.

122
Resuspend T Cells in fresh media with T Stim at 1e6 cells/mL:

<table>
<thead>
<tr>
<th>Solution</th>
<th>T Cell Media per Well (mL/well)</th>
<th>Check</th>
</tr>
</thead>
<tbody>
<tr>
<td>T Cell Media</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solution</th>
<th>Viral Supernatant (mL)</th>
<th>Target T Stim Fraction</th>
<th>Total to Add (mL)</th>
<th>Check</th>
</tr>
</thead>
<tbody>
<tr>
<td>T Stim</td>
<td>*</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Incubate overnight at 37°C.

**Day 8**
Check Infection Rate
Dilute cells to 1e6 cells/mL.

**Days 9-13**
Dilute cells to 1e6 cells/mL.

**Day 14**
Harvest cells.
Chromium Release Protocol

<table>
<thead>
<tr>
<th>Action</th>
<th>Check</th>
</tr>
</thead>
<tbody>
<tr>
<td>Label Target Cells</td>
<td>-</td>
</tr>
<tr>
<td>Resuspend target cells in media:</td>
<td></td>
</tr>
<tr>
<td>Number of Cells (cells)</td>
<td>Concentration (cells/mL)</td>
</tr>
<tr>
<td>5e6</td>
<td>5e6</td>
</tr>
<tr>
<td>Add chromium (5 mCi/mL) and gently mix:</td>
<td></td>
</tr>
<tr>
<td>Total Media Volume (mL)</td>
<td>Chromium Dilution (µL/mL)</td>
</tr>
<tr>
<td>x</td>
<td>50</td>
</tr>
<tr>
<td>Incubate at 37°C for 1 hour.</td>
<td></td>
</tr>
<tr>
<td>Wash with 10 mL media.</td>
<td></td>
</tr>
<tr>
<td>Wash with 10 mL media.</td>
<td></td>
</tr>
<tr>
<td>Wash with 10 mL media.</td>
<td></td>
</tr>
<tr>
<td>Resuspend target cells at required density and add to assay plate.</td>
<td></td>
</tr>
<tr>
<td>Assay</td>
<td></td>
</tr>
<tr>
<td>Briefly centrifuge cells.</td>
<td></td>
</tr>
<tr>
<td>Transfer 30 µL of each well’s supernatant into a Luma plate.</td>
<td></td>
</tr>
<tr>
<td>Allow plate to dry overnight.</td>
<td></td>
</tr>
<tr>
<td>Add 30 µL of LSC to each well.</td>
<td></td>
</tr>
<tr>
<td>Read the Luma plate on a scintillation counter.</td>
<td></td>
</tr>
</tbody>
</table>

Cell Ratio Cytotoxic Assay Protocol

<table>
<thead>
<tr>
<th>Action</th>
<th>Check</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resuspend effector cells in T cell media at 8.1e6 cells/mL (8.1e5 cells/initial well).</td>
<td></td>
</tr>
<tr>
<td>Resuspend target cells in T cell media:</td>
<td></td>
</tr>
<tr>
<td>Target Cells in First Well (cells)</td>
<td>Target Cell Volume per Well (µL)</td>
</tr>
</tbody>
</table>
Resuspend effector cells in T cell media:

<table>
<thead>
<tr>
<th>Effector Cells per Well (cells)</th>
<th>Effector Cell Volume per Well (µL)</th>
<th>µL/mL</th>
<th>Effector Cell Concentration (cells/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1e4</td>
<td>/ 100</td>
<td>* 1000</td>
<td></td>
</tr>
</tbody>
</table>

Perform serial dilutions of effector cells in T cell media and add to v-bottomed 96 well plates:

Initial cell density: 8.1e6 cells/mL

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Volume to Transfer</th>
<th>Volume to Add</th>
<th>Check</th>
</tr>
</thead>
<tbody>
<tr>
<td>81</td>
<td>0</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>50</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>50</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>50</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Add 100 µL of target cells to each well.
Reagent Dose Cytotoxic Assay Protocol

<table>
<thead>
<tr>
<th>Action</th>
<th>Check</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resuspend target cells in T cell media:</td>
<td></td>
</tr>
<tr>
<td><strong>Target Cells per Well (cells)</strong></td>
<td><strong>Target Cell Volume per Well (µL)</strong></td>
</tr>
<tr>
<td>2e4</td>
<td>/</td>
</tr>
</tbody>
</table>

| Resuspend effector cells in T cell media: | |
| **Effector Cells per Well (cells)** | **Effector Cell Volume per Well (µL)** | **µL/mL** | **Effector Cell Concentration (cells/mL)** |
| 2e5 | / | 50 | *1000 = 4e6 |

| Prepare initial concentration of Reagent (120 µL per lane to provide extra): | |
| **Target Concentration (ng/mL)** | **Volume per Lane (µL)** | **Number of Lanes** | **Reagent Concentration (mg/mL)** | **ng/mg** | **Final Well Volume (µL)** | **Reagent Volume per Well (µL)** | **Volume of Reagent to Add (µL)** |
| 10000 | *120 | *12 | / | 1 | / | 1e6 | *200 | / | 100 | = 28.8 |

<table>
<thead>
<tr>
<th>Number of Lanes</th>
<th><strong>Volume per Lane (µL)</strong></th>
<th><strong>Total Volume (µL)</strong></th>
<th><strong>Volume of Reagent Added (µL)</strong></th>
<th><strong>Volume of Media to Add (µL)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>x120</td>
<td>=1440</td>
<td>-28.8</td>
<td>=1411</td>
</tr>
</tbody>
</table>

| Perform serial dilutions of Reagent in T cell media and add to flat-bottomed 96 well plates: | |
| **Dilution** | **Volume to Transfer** | **Volume to Add** |
| 10000 | 0 | 111 |
| 1000 | 11 | 100 |
| 100 | 11 | 100 |
| 10 | 11 | 100 |
| 1 | 11 | 100 |
| 0.1 | 11 | 100 |
| 0.01 | 11 | 100 |
Add 50 µL of effector cells to each well.

Add 50 µL of target cells to each well.

Add 20 µL of 10% Triton X-100 cells to each Maximum release well.

Bring all wells to a total of 200 µL as required with media.

Briefly centrifuge plates.

Incubate 4 hours at 37°C.

Assay plate.
8 Citations


activation of T cells and subsequent killing of human tumors is independent of mutations commonly found in colorectal adenocarcinomas. mAbs 6, 1571–1584.


Watanabe, A., Hara, M., Chosa, E., Nakamura, K., Sekiya, R., Shimizu, T., and Onitsuka, T. (2010). Combination of adoptive cell transfer and antibody injection can eradicate established tumors in mice—an in vivo study using anti-OX40mAb, anti-CD25mAb and anti-CTLA4mAb-. Immunopharmacol. Immunotoxicol. 32, 238–245.


