Adoptive cell transfer: examining the potential of a T cell-mediated therapy for metastatic melanoma

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ADOPTIVE CELL TRANSFER: EXAMINING THE POTENTIAL OF A
T CELL-MEDIATED THERAPY FOR METASTATIC MELANOMA

by

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ABSTRACT

Adoptive cell transfer techniques identify and isolate patient anti-tumor lymphocytes *in vitro* followed by *ex vivo* expansion of these tumor specific T cells. Identification and isolation of lymphocytes from patient tumors allows for the selection of anti-tumor lymphocytes that are highly specific for individual tumor antigens. Furthermore, recombinant technology allows for engineering of chimeric antigen receptors (CARs) which allow these T cells to target multiple tumor antigens. Techniques involving *ex vivo* growth lead to a 1,000- to 5,000-fold increase in numbers of lymphocytes. Cultured lymphocytes can then be infused via IV and growth maintained with administration of exogenous IL-2. Cancer patients are then monitored for both immunological activity as well as any adverse cytokine reactions. We looked at several trial studies for the application of adoptive cell transfer in metastatic melanoma compare the efficacy of the regimen to other established melanoma treatments. Adoptive transfer has proven to be effective for patients with late stage melanoma, however, the aim of this study was to examine some of the challenges in creating an effective standard protocol for adaptation in clinical settings, including difficulty in obtaining significant cell populations from tumors, challenges in the proliferation of these tumor-infiltrating lymphocytes (TIL) and
determination of antigen-specificity, i.e. facilitation of a simplified and quicker approach to the therapy.
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<tr>
<td>ACT</td>
<td>Adoptive Cell Transfer</td>
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<tr>
<td>AE</td>
<td>Adverse Event</td>
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<td>AJCC</td>
<td>American Joint Committee On Cancer</td>
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<td>ALL</td>
<td>Acute Lymphoblastic Leukemia</td>
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<td>APC</td>
<td>Antigen Presenting Cell</td>
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<tr>
<td>BRAF</td>
<td>B Rapidly Accelerated Fibrosarcoma</td>
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<td>CAR</td>
<td>Chimeric Antigen Receptor</td>
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<td>CD</td>
<td>Cluster Of Differentiation</td>
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<tr>
<td>CDKN</td>
<td>Cyclin-Dependent Kinase Inhibitor</td>
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<tr>
<td>CLA</td>
<td>Cutaneous Leukocyte-Associated Antigen</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>CT</td>
<td>Computed Tomography</td>
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<td>CTCAE</td>
<td>Common Terminology Criteria In Adverse Events</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
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<tr>
<td>CTLA</td>
<td>Cytotoxic T lymphocyte-Associated Protein</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
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<tr>
<td>FDA</td>
<td>U.S. Food And Drug Administration</td>
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<tr>
<td>GI</td>
<td>Gastrointestinal</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage Colony-Stimulating Factor</td>
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<tr>
<td>Gp100</td>
<td>Glycoprotein 100</td>
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HLA ......................................................... Human Leukocyte Antigen
IFN ................................................................. Interferon
IL ................................................................. Interleukin
ILI .............................................................. Isolated Limb Infusion
ILP .............................................................. Isolated Limb Perfusion
LDH ............................................................. Lactate Dehydrogenase
mAb ............................................................. Monoclonal Antibody
MAPK ........................................................ Mitogen-Activated Protein Kinases
MART .......................................................... Melanoma Antigen Recognized By T cells
NCI ............................................................ National Cancer Institute
NK ............................................................... Natural Killer
NRAS ........................................................ Neuroblastoma Rat Sarcoma
PBMC ........................................................ Peripheral Blood Mononuclear Cell
PD .............................................................. Programmed Cell Death Protein
PDL ............................................................. Programmed Cell Death Protein Ligand
PET ............................................................. Positron Emission Tomography
Rag .............................................................. Recombination-Activating Gene
Rb ............................................................... Retinoblastoma
RCT ............................................................ Randomized Clinical Trial
RECIST ..................................................... Response Evaluation Criteria In Solid Tumors
REP ............................................................. Rapid Expansion Protocol
ROS ........................................................... Reactive Oxygen Species
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>SEER</td>
<td>Surveillance, Epidemiology, And End Results</td>
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<td>SOC</td>
<td>System Organ Class</td>
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<td>TBI</td>
<td>Total Body Irradiation</td>
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<td>TERT</td>
<td>Telomerase Reverse Transcriptase</td>
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<tr>
<td>TGF</td>
<td>Tumor Growth Factor</td>
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<tr>
<td>TIL</td>
<td>Tumor Infiltrating Lymphocyte</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<td>VLS</td>
<td>Vascular Leak Syndrome</td>
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INTRODUCTION

Despite being one of the most rare skin cancers, malignant melanoma accounts for the highest number of skin cancer deaths (Svane and Verdegaal 2014). The incidence of melanoma, in situ and invasive, has shown a continuous upward trend since the 1970s at an annual rate of 2.6% (Higgins II, Lee, Galan, & Leffell, 2015). Similarly, the risk of developing melanoma has gone up more than 20 fold with recent data indicating that approximately 1 in 100 people will be diagnosed with melanoma. 2001 data gathered by the American Joint Committee On Cancer (AJCC) found a significant decrease in survival rates correlated with cancer staging (See Figure 1). Additionally, 2015 Surveillance, Epidemiology, And End Results (SEER) data indicate that the prognosis for those with late stage melanoma was poor at an approximate survival rate of 16% (“Melanoma of the Skin - SEER Stat Fact Sheets” 2016).
Stratification of data indicates a disproportionate rate of melanoma in younger patients as well as in males (Higgins II et al. 2015a). As a result, mortality rates in the general population have been skewed with a higher rate of male mortality from malignant melanoma (Berwick et al. 2016). It is difficult to fully establish true incidence rates given that early screenings may play a role in the increased reported incidence. SEER studies have found that biopsy rates have doubled and have postulated that the increase in melanoma diagnoses parallels this change (Higgins II et al. 2015a).

Sun exposure is an established major environmental risk factor responsible for melanoma development in up to 90% of cases (Berwick et al. 2016). Melanoma risk has also been associated with a geographical spread encompassing larger incidence rates in Europe, Australia, and the United States (Higgins II et al. 2015a) reflecting the amount of ambient sun exposure of the population (Rommens et al. 2016). Exposure has been characterized as intermittent (short, intense exposure) or chronic. Cohort and case-control studies have found that intermittent exposure is tied to increased melanoma risk and that chronic exposure has a weaker association potentially offset by epithelial thickening (Berwick et al. 2016). Sunburn, especially in early childhood, has been strongly associated with increased skin cancer rates in adulthood. In adults, this risk and exposure is increased. In particular, an increase in tanning has been shown to contribute to increased risk (Kroon, Morton, and Thompson 2004). Other environmental exposures
associated with increased risk of melanoma include carcinogens such as pesticides, herbicides, and dyes.

Another major indicator of risk is the presence and type of nevi. Nevi are benign clusters of melanocytes and have been implicated in several studies to be associated with an increased risk of skin cancer. The size of these nevi is also correlated with melanoma risks. Finally, dysplastic nevi often serve as precursors to the development of melanoma. Today, screening and preventative public health measures rely on recognition of dysplastic phenotypes based on shape, color, and diameter (Berwick et al. 2016).

Current therapeutics for an early diagnosis of malignant melanoma include surgery and monitoring, while late stage therapeutics often involve supplemented adjuvant therapies in addition to surgical intervention. These adjuvants include chemotherapies, BRAF/MEK inhibitors, and in recent years, immunotherapy (Higgins II et al. 2015b; Eggermont, Spatz, and Robert 2014).

Multiple immunotherapeutics are currently being studied for their efficacy in treating melanoma as well as other cancers such as acute lymphoblastic leukemia (ALL). These therapeutics consist of vaccines administered after the diagnosis of melanoma as well as a number of biologics, including different types of IFNs and interleukins (Davey, van der Westhuizen, and Bowden 2016). Monoclonal antibody biologics targeting cell cycle checkpoints have been found to be effective in treatment of malignant melanoma. Currently, checkpoint blockade therapy is at the forefront of melanoma treatment with several FDA-approved drugs on the market for patients with metastatic melanoma (Susan Swetter 2016). An immunotherapy technique called adoptive cell transfer (ACT), which
focuses on harnessing tumor-infiltrating lymphocytes from patients, has shown therapeutic promise in patients who have not responded to other established treatment modalities (see Figure 2).

Early studies examined the efficacy of ACT using both *ex vivo* tumor tissue cultures as well as animal models. Using these models, a therapeutic protocol was established which found that treatment with chemotherapy prior to cell transfer could boost the effectiveness of cell transfer therapy (Steven A. Rosenberg and Dudley 2009). Initial studies in patients using these techniques have been limited to Phase I and II clinical trials, however, response rates in these trials have been promising. One study found that administration of high dose total body irradiation followed by cell transfer had a response rate of 72%, as measured by the Response Evaluation Criteria In Solid Tumors (RECIST), in comparison to administration of chemotherapeutic drugs followed by transfer showing a rate of 52% (Steven A. Rosenberg and Dudley 2009). Many patients were found to have responses that lasted up to, and beyond, three years with a smaller cohort showing complete responses that were ongoing at the time of the report. These responses ranged anywhere from 18 to 36 months (Steven A. Rosenberg and Dudley 2009). Regression was noted in all sites of metastases including brain and bone. (Steven A. Rosenberg and Dudley 2009)
Figure 2: Patient survival rates following ACT therapy. Comparison of survival rates following total body irradiation, chemotherapy, and control group response. Patient response of joint therapy with total body irradiation shows promising potential as a combinatorial regimen, however, further randomized trials are necessary to assess efficacy of the treatment.

Source: (Steven A. Rosenberg and Dudley 2009)
OBJECTIVES

The primary aim of this study is to provide a comprehensive review of the techniques and efficacy of ACT as a potential novel therapy for malignant melanoma and the impact of the trials on other non-melanoma cancers. In addition to this, the study will compare the new technique and its impact with previously established modalities for cancer treatment. Specifically, the study will examine the establishment of ACT in cancer treatment as a future standard of care. We believe that adoptive cell transfer therapy provides a promising avenue for cancer therapy in late stage (III and IV) melanoma cases where other therapies have failed. We will analyze trial studies on adoptive transfer methods to compare remission rates with administration of autologous lymphocytes selected for antigenic properties with standard melanoma therapies in order to evaluate survival rates of late stage melanoma patients.
HISTOLOGY OF MELANOCYTES

The skin is divided into three layers: the epidermis, dermis, and hypodermis. Melanoma is classified as a cancer of melanocytes which reside in the topmost layer of the skin, the epidermis. The epidermis itself consists of five cellular layers from the outermost layer, stratum corneum, which is exposed to the environment to the stratum basale which is adjacent to the dermis (see Figure 3). Melanocytes are found in the stratum basale and make up about 8% of epidermal cells. They produce a pigment called melanin which is derived from DOPA and bound to a protein. This melanoprotein is then transferred via vesicles called melanosomes and phagocytosed by surrounding keratinocytes (Kroon, Morton, and Thompson 2004).
Melanin serves a protective barrier to UV DNA damage by forming a barrier over the nucleus. Regardless of race, each human being has the same number of melanocytes however, variation in the amount of pigment produced, the size of melanosomes, and the aggregation of the melanin produces variation in skin color. UV exposure tends to increase the amount of melanin through mechanisms that upregulate production in response to UV damage (Kroon, Morton, and Thompson 2004).

Histologically, melanoma has two growth phases: radial or horizontal growth and vertical growth (see Figure 4). Initial growth can be classified as melanoma in situ with melanocyte proliferation being restricted to the epidermis. Radial growth is considered intraepidermal and microinvasive (Higgins II et al. 2015b). Microinvasion refers to melanocyte proliferation and invasion of the papillary dermis either as nests of cells or singularly (Kroon, Morton, and Thompson 2004). At this point, the melanoma does not have the capacity for metastasis due to lack of access to lymphatic channels. Further growth into the subsequent skin layers is classified as vertical growth at which the point the melanoma has capacity for metastasis. Histologically, this phase is characterized by presence of sheets and/or large nests of cells in the dermal layer of the skin.
Figure 4: The figure diagrams both the histological progression of melanoma and its movements through the different growth phases beginning initially as a benign nevus and moving through radial and horizontal growth to ultimately metastasize. Common DNA mutations contributing to the growth are also noted and will be discussed further under therapies.

Source: (Arrangoiz et al. 2016)
The most common histological pattern of melanoma is characterized as superficial spreading melanoma. This type of histology is seen in at least two-thirds of melanoma cases but could be as high as 75% (Higgins II et al. 2015b). In the epidermis this type of spreading is seen as poor circumscription of melanocytes, aberrant distribution of melanocytes, and the presence of melanocytes above the basal layer, i.e. pagetoid spread (Kroon, Morton, and Thompson 2004). Often spreading is haphazard and distributions can vary leading to either the presence of melanocytic nests, or the presence of single melanocytes spread over a large area. These nests often lack cohesion due to lack of desmosomal attachments. The cells themselves generally have a large eosinophilic cytoplasm with multiple nucleoli, often hyperchromatic with the presence of mitotic activity, and are characterized by lack of maturation (Higgins II et al. 2015b). As these melanocytes migrate towards the papillary dermis, they begin to shrink losing both cytoplasm and nucleoli.

A second common type of melanoma is nodular melanoma found to be more common during middle-age and often occurring around the trunk. While it shares similar cytological features with superficial melanoma, one prominent feature is that unlike superficial melanoma, nodular melanoma is well circumscribed (Higgins II et al. 2015b). This subtype lacks lateral spread and is often ulcerated. Since it bypasses lateral spread, this type of melanoma is correlated with aggressive growth and higher rates of metastasis (Higgins II et al. 2015b).
ETIOLOGY AND PATHOGENESIS

ENVIRONMENT

In 2004, Meyskens et al. proposed a pathogenesis pathway for melanoma development due to sun exposure. The team hypothesized that generation of radical oxygen species served as a key factor in the initiation and proliferation of melanoma. It is known that melanin has a variety of redox properties and Meyskens, et al. proposed that the key to melanoma pathogenesis was in the redox-recycling of this molecule (see Figure 5). Initial studies postulated that a source of exogenous oxidative stress will create a signal for radical oxygen species (ROS) development in melanocytes and melanoma cells. The study also proposed that this reaction to oxidative damage differed from typical oxidative stress in non-pigmented cells. One experiment found that the addition of superoxide dismutase, which normally serves as an antioxidant in protecting cells from reactive O\(^2\)-, to melanoma cultures resulted in an enhanced stress signal (Meyskens, Farmer, and Anton-Culver 2004). In contrast, addition of superoxide dismutase had no negative effect on melanocytes. Further examination found increased levels of intracellular superoxide anions in melanoma cells compared to melanocytes (Meyskens, Farmer, and Anton-Culver 2004). From these and previous studies, it is believed that melanin in this context functions as a pro-oxidant.

In conjunction with these, and previous other, studies on the progression of melanoma, a proposed pathway was set out as follows:
i) Oxidation of synthetic melanin due to UV damage, normal metabolism, or inflammatory responses increases its affinity for metal ions due to tautomerization of a quinone-imine

ii) Accumulation of metal ion(s) and other chemicals then promotes redox recycling

iii) This increased accumulation exhausts cellular anti-oxidant stores and machinery

iv) Depletion of anti-oxidants serves as the final step for DNA damage via free radicals and ROS.

Normally uptake of metals is regulated by enzymes known as metallothioneins. In addition to the carcinogenic role played by heavy metals such as Cu, Fe, Mn, and Co, it is believed that polymorphisms in the metallothioneins could potentiate genetic risk to cancer (Meyskens, Farmer, and Anton-Culver 2004). Additional nonmetals such as herbicides, pesticides, organic amines, and dyes have also been identified as contributors to the pathogenesis of cancer.
**Figure 5: Mechanism of Melanoma Pathogenesis**

Figure illustrates the proposed mechanism through which normal redox cycling mechanisms become overwhelmed and lead to DNA damage of melanocytes.

*Source:* (Meyskens, Farmer, and Anton-Culver 2004)

**GENETIC**

Melanoma studies have found that mutations in BRAF are one of the earliest contributing factors to melanoma development. Forty to 50% of melanomas have a mutation in BRAF which activates the MAPK pathway activating telomerase and disrupting the normal cellular G1-S checkpoint (Eggermont, Spatz, and Robert 2014; Hugdahl et al. 2016). Shain et al. found that the majority of benign lesions harbored a BRAF V600E mutation. No other mutations were associated with melanoma risk in this study and it was concluded that the singular mutation was sufficient for nevus formation. Further studies found that lesions that did not have mutations in the V600E of BRAF but had other BRAF mutations or NRAS mutations were the result of multiple oncogenic alterations rather than a single event. The most common secondary mutation found was that of TERT. Other mutations common to melanoma include CDKN2A, p53, and Rb (Eggermont, Spatz, and Robert 2014).
**PROGNOSIS**

Despite being a more aggressive form of cancer, most early forms of melanoma are curable with surgery. SEER data indicated that 84% of melanomas are caught early and 5-year survival rates were around 98%. However, stage IV metastatic melanoma 10-year survival rates are less than 10% and despite increased understanding of the disease, new therapies have yielded mixed results during late stage cancer. Additionally, progression or recurrence in the future are also liable to influence these survival rates.

Studies have found that the average time for localized cancer to metastasize is about 28 months (“Melanoma of the Skin - SEER Stat Fact Sheets” 2016). Common sites for this metastasis included the bone, brain, and liver. Finally, a third of melanoma patients experience recurrence in the future.

Currently, the standard of care for most patients continues to be dacarbazine though tumor response rates have varied from 6% to 20% with a duration of 6 months (Kim et al. 2010). A primary factor influencing survival that has been studied is the site of metastasis. AJCC analysis of the melanoma database studied patient survival rates and found that patients with distant metastases had a better prognosis if metastasis was in the skin and subcutaneous tissue (Balch et al. 2009). Patients with pulmonary metastasis had an intermediate prognosis and those with metastasis of any other visceral sites had the worst prognosis (Balch et al. 2009).

One melanoma biomarker which has been noted since 1954 is elevated serum lactate dehydrogenase (LDH) which serves as an indicator for liver metastasis. Abnormal LDH levels have been associated with decreased survival and could be
significant for monitoring during late-stage malignant melanoma (Gogas et al. 2009). Other significant biomarkers include increased S100B, a calcium binding protein found in cell cytoplasms, correlating with disease progression. Subsequently, low S100B could be utilized to measure a positive response to treatment (Gogas et al. 2009).

CURRENT TREATMENTS

SURGERY

Surgery, particularly in early stage melanoma, has proven to be an effective tool for treatment of melanoma in situ, and as previously mentioned, was correlated with a high survival rate if the cancer was localized (Susan Swetter 2016). Multiple techniques for excision are used in removal depending on the staging of the cancer and the site of the lesion. Wide-local excision focuses on lower risk body site and is used for early stage cancer (Higgins II et al. 2015b; Davey, van der Westhuizen, and Bowden 2016). Skin cancer tissue and a smaller margin of healthy tissue (0.5 cm) is removed and the samples are sent for further processing. In contrast, Mohs surgery will often remove visible skin cancer along with layers of skin and adjacent soft tissue in an effort to excise the totality of the cancer in the epidermal and dermal layers (Susan Swetter 2016). Finally, a staged excision will use surgical removal along with sectioning and analysis to identify margins. This immediate section analysis is then used to determine if further removal is necessary or whether margins have cleared. Wound closure in this case is delayed until a negative margin is confirmed (Eggermont, Spatz, and Robert 2014).
TOPICAL THERAPY

Currently, FDA approval for the drug *imiquimod* is limited to superficial basal cell carcinoma, actinic keratosis, and genital warts (Ellis et al. 2012). It is unknown if the FDA is currently investigating its potential application for melanoma in situ. Nevertheless, it has been used as an off-label drug for the treatment of melanoma because of its properties as an immunotherapeutic. The drug works by activating toll-like receptor 7 which leads to an immune response at target sites. Efficacy of the drug has been variable with responses ranging from 0 clearance to 100 percent. (Fan et al. 2015; Ellis et al. 2012) In addition, since no standardized guidelines have been set for dosage, regimens have been variable. Currently, it is difficult to identify the significance of the drug in treating melanoma.

CHEMOTHERAPY

*Dacarbazine*

Dacarbazine is considered one of the primary chemotherapeutic drugs for melanoma treatment, is the only chemotherapeutic agent approved for melanoma, and functions as an alkylating agent (BHATIA, TYKODI, and THOMPSON 2009). Despite being the standard of care, response rates from studies are variable. Retrospective analyses of randomized trials compared results from patients administered dacarbazine with non-dacarbazine treatments (Lui et al. 2007). Dacarbazine administration revealed an objective response rate of 15% with the majority of patients having a partial response at 11.2% (BHATIA, TYKODI, and THOMPSON 2009) Only a small percentage of
patients were able to sustain these responses and 5-year survival rates were found to be 2% (Lui et al. 2007; BHATIA, TYKODI, and THOMPSON 2009). In addition, the drug is associated with typical chemotherapeutic toxicities such as nausea, fatigue, and myelosuppression (BHATIA, TYKODI, and THOMPSON 2009).

**Temozolomide**

An oral analog of dacarbazine, temozolomide, has the advantage of increased bioavailability as well as access to the CNS which is promising for cases of brain metastases. A phase III randomized study compared survival and response rates in patients with metastatic melanoma who received temozolomide to those receiving dacarbazine and found no statistically significant differences (Middleton et al. 2000). In the Middleton study, oral temozolomide was administered 5 days every 30 days or dacarbazine 5 days every 21 days. A second multi-center study compared dacarbazine (10%) administration response rates to temozolomide (14%) and also found no significant differences in survival rate (Patel et al. 2011). Oral temozolomide was administered for seven days every two weeks or dacarbazine administered intravenously every three weeks.

**Combination Chemotherapy**

A drug cocktail known as the Dartmouth regimen combines cisplatin, dacarbazine, BiCNU, and tamoxifen (CDBT) (BHATIA, TYKODI, and THOMPSON 2009). Initially, response rates were reported to be between 40 and 50% however, later multicenter RCTs did not show a significant different in survival when compared to control therapy with dacarbazine (P. B. Chapman et al. 1999). Patients with stage IV
melanoma were randomly assigned to either the Dartmouth regimen or standard dacarbazine and were observed for tumor response, survival time, and side effects. Other cocktails with vinblastine has shown a 40% increase in phase II trials however, many of these combinations have a greater toxicity and do not significantly alter the response or survival rates (McDermott et al. 2000; Legha et al. 1996). Stage IV patients in these phase II trials were given a mix of cisplatin, vinblastine, and dacarbazine and tumor response as well as survival measured (McDermott et al. 2000).

Intravenous Limb Perfusion

One technique, limb perfusion, have emerged as an effective way to deliver chemotherapy drugs while bypassing adverse systemic effects. The technique essentially creates a circuit between the vasculature of the tissue and an ex vivo system, similar to a dialysis or plasmapheresis machine, allowing for drugs to only travel along the vessels of the target tissue. Early trials have found response rates from 30-60% (Maverakis et al. 2015; Cumberlin et al. 1985) with increased responses of up to 80% in conjunction with TNF and IFNγ (Maverakis et al. 2015). Addition of cytokine factors like TNF and IFNγ have been found to increase response rate significantly (Maverakis et al. 2015). Patients were randomly assigned to a group administered either melphalan or the IFN/TFN combination using isolated limb perfusion and response rates were measured using RECIST criteria (Cornett et al. 2006). TNF addition yields a 68.9% response rate where melphalan alone showed a 46.5% response (Maverakis et al. 2015; Cornett et al. 2006). It has been difficult however, to assess survival rates beyond initial response without proper comparative trials and as a result the technique needs further study.
BRAF/MEK Inhibition

The BRAF gene is found mutated in 40-70% of melanomas and encodes for a Ser/Thr kinase that is part of the MAP kinase pathway (Davies et al. 2002). The mutation leads to activation of, and uncontrolled, cell growth (Hugdahl et al. 2016). Inhibitors of BRAF such as Vemurafinib and Dabrafenib are specific to the V600E and V600E/K mutations respectively. In comparison to the standard of care, dacarbazine, Vemurafinib was found to have better response rates (Paul B. Chapman et al. 2011). A multi-center study with patients in stage IIIC or IV melanoma positive for the V600E mutation was conducted. Patients were randomly assigned to either Vemurafenib or dacarbazine and tumor clearance measured using RECIST criteria (Paul B. Chapman et al. 2011). Initial studies found that 6-month response rates to BRAF inhibition were around 86% compared with 64% for dacarbazine (Paul B. Chapman et al. 2011; Maverakis et al. 2015). In addition, survival time was lengthened by three times as much. Other studies found 1-year survival rates to be around 64% for BRAF inhibitors in comparison to dacarbazine at 42% (Maverakis et al. 2015).

MEK is another signaling protein found downstream of the BRAF pathway. Inhibitors of MEK (cobimetinib or trametinib) in conjunction with BRAF inhibitors have proven to be more effective than BRAF inhibition alone (Maverakis et al. 2015). Comparison of the joint therapy with other immunology-based therapeutics such as monoclonal antibody administration found that BRAF/MEK inhibitor regimens had higher overall and progression-free survival (Long et al. 2014). Patients with stage IIIC or
stage IV melanoma with BRAF V600E or V600K mutations were enrolled and randomized into two groups. One group was administered BRAF/MEK combination drugs while the second was administered BRAF and a placebo. Efficacy of treatment was gauged with progression-free survival as well tumor response rates. Overall response rate was 67% in the combination group versus 51% (Long et al. 2014).

**CTLA4 Inhibitors**

The T-cell CTLA4 receptor normally functions to prevent or down regulate the immune response. Binding of the B7 receptor on antigen-presenting cells to the CTLA-4 receptor of T-cells is a specific mechanism that serves as a checkpoint for the immune system. Cancer cells can take advantage of this process by inhibiting T-cell proliferation by expressing B7 receptors and binding T-cell CTLA-4 receptors thereby initiating a false down regulation response. Administration of antibodies to CTLA4 allows for unopposed T-cell activation and proliferation against tumor antigens (Maverakis et al. 2015). These free antibodies serve to bind the CTLA-4 without activating a down regulating response. Antibodies bound to CTLA-4 receptors block tumor cell B7 receptors from inhibiting the immune response. *Tremelimumab, a first generation monoclonal antibody*, was found to demonstrate anti-tumor activity in late stage (IIIC or IV) melanoma patients (Camacho et al. 2009) though later trials comparing the drug to standard of care did not demonstrate significant difference in survival rates (Ribas et al. 2013). However, a similar drug *ipilimumab*, was found to prolong survival rates when administered alone or in conjunction with a peptide vaccine (Hodi et al. 2010). A cancer vaccine comprised of HLA-0201 restricted peptides from the gp100 protein individually
does not have strong anti-tumor effects, however, Hodi et al examined the effects of gp100 in conjunction with ipilimumab. Stage III and IV patients were separated into three groups with one group receiving gp100 vaccine with ipilimumab, a second group administered ipilimumab along with a gp100 placebo, and a third group given gp100 with an ipilimumab placebo. Risk of progression was reduced by 19% in the group co-administered ipilimumab with gp100 and the group administered ipilimumab alone was found to have a 36% overall response based on RECIST (Hodi et al. 2010). One side effect of these medications is adverse immune cytokine-related events such as rashes, vitiligo, as well as gastrointestinal events such as diarrhea.

**PD1/PDL1 Inhibitors**

PD1 is a receptor found on activated T-cells, B-cells, and myeloid cells and is involved in modulating the T-cell response. Generally, binding of the PD1 of the T-cell with its ligands on cells such as antigen-presenting cells is considered inhibitory. Several cancers have mutations that allow for expression of PD1 ligands. This helps cancers evade the natural immune response by binding PD1 ligand with the PD1 receptor of T-cells. Therefore, antibodies against either ligand can block inhibition of T-cell and serve to increase T-cell activation and proliferation. Therapies combining nivolumab (anti-PD1) and ipilimumab (anti-CTLA4) show therapeutic promise however, trials are still ongoing and efficacy is difficult to assess (Wolchok et al. 2013; Tsai and Daud 2015). Nivolumab is a monoclonal antibody that blocks the PD1 receptor and prevents T-cell inhibition by the cancer (Maverakis et al. 2015). A second drug, pembrolizumab (anti-
PD1) was given FDA approval after a recent study found that response rates were around 26% (Robert et al. 2014). Phase I trials conducted by Robert et. al selected for patients (n=173) who had undergone previous therapy with ipilimumab and had disease progression. Pembrolizumab was intravenously administered and response rates were measured based on RECIST.

**IL-2 Therapy**

Administration of intravenous IL-2 promotes T-cell proliferation (Yamamoto, Ueta, and Osaki 2003). Data from several clinical trials found that a IL-2 could produce responses in patients who had failed to respond to previous therapies (Atkins et al. 1999; Weide et al. 2010). Patients with metastatic melanoma were administered intravenous infusions and monitored through multiple cycles. In metastatic melanoma, IL-2 delivery has a moderate response of 16% (Atkins et al. 1999), however, the side effects of IL-2 therapy can often be a barrier to effective treatment. Intravenous IL-2 can lead to vascular leak syndrome (VLS) and toxicities with symptoms resembling septic shock (Atkins et al. 1999). Therefore, it is limited to a small population of individuals (Maverakis et al. 2015). Intralesional administration of IL-2 has also been studied and has shown responses ranging from 40-70% (Radny et al. 2003; Boyd, Wehrli, and Temple 2011). Radny et. al enrolled patients with stage III or IV melanoma and monitored response rates to weekly intralesional injections of IL-2. Boyd et. al utilized a similar method and evaluated patients using RECIST criteria. Both studies found significant clearance rates. Multiple studies have shown strong response rates with biweekly administration of high dose IL-2 in combination with imiquimod. Green et. al recruited patients who were at stage III and
IV of melanoma where the cancer had failed to respond to other treatment modalities. 5% imiquimod was applied topically for 4 weeks and was followed by three regimens of intravenous IL-2 (Green et al. 2007). A total of 182 lesions was treated and responses seen in approximately 50% with a complete response in 40.7% of lesions (Green et al. 2007).

**IMMUNOLOGICAL MECHANISMS OF MELANOMA**

It has been noted that a subset of melanomas undergo spontaneous regression, partial or full (Kroon, Morton, and Thompson 2004). In up to 10% of patients with metastasis, no evidence of a primary tumor was found (Requena et al. 2009). Given that there is a possibility of regression before the melanoma is diagnosed, regression may be underreported. Partial regression is more common and can be found in up to 50% of melanoma patients although it is likely underreported as well (Kroon, Morton, and Thompson 2004).

Histological features that can be used to identify regression rely on tumor cell “clumps” found in the dermis that result from lymphocyte infiltrates (Smoller 2006). Specifically, the immune response causes tumor cell areas to become less cohesive and disparate. The events that mediate spontaneous regression are immunological; a direct result of increased T-cell response. Specifically, it was found that tumors with regression events had an increase in the number of activated CD4+ T cells (Printz 2001). Early stage melanoma had a higher concentration of CD4+ T cells whereas advanced melanoma had a higher concentration of CD8+T cells.
Studies using RT-PCR found that a large majority of melanomas showed increased IL-2 and Th1 cell concentrations. Classification of 285 patients into subsets based on the magnitude of T cell infiltration found that increased infiltration of tumor tissue was correlated with survival rates (Clemente et al. 1996). Histological sections were obtained from patients enrolled in the WHO Melanoma Study Group. Clemente et al. used Elder and Clark criteria to classify infiltrates into brisk, non-brisk, and absent categories. “Brisk” infiltrate was defined by lymphocyte presence throughout the vertical growth phase whereas “non-brisk” infiltrate was identified if lymphocytes were located as foci in the vertical growth phase. Five-year survival rates for higher infiltration was 77% as compared to a 53% survival rate for “non-brisk” infiltration (Clemente et al. 1996). Therefore, the concentration of immune lymphocytes can be used a factor to measure prognosis of melanoma.

Laboratory studies in the early 1980s noted the presence of immune cells, lymphokine-activated killer (LAK) cells, that targeted malignant cells and hypothesized a future application of these CTLs for in vitro development of a strengthened immune response (Yang and Rosenberg 2016). IL-2 administration in conjunction with culturing lymphocytes with their autologous tumor stimulated peripheral lymphocyte proliferation (Topalian, Solomon, and Rosenberg 1989). Rosenberg et. al noted that IL-2 induction of peripheral blood mononuclear cells (PBMC) could generate LAK cells that targeted tumor cells. These initial cells were hypothesized to be related to NK cells (Steven A. Rosenberg 2014). These initial trials removed NK cell precursors from patients with melanoma at stage III and IV following an intravenous course of IL-2. Cells were then
further proliferated with IL-2 and administered to patients. These lymphocytes were shown to lyse tumor lines and more specifically, were found to be prevalent in melanoma patients. Another benefit was that these lymphocytes could potentially be harvested from patient surgical specimens. These studies using NK cells led to alternative therapeutic strategies utilizing tumor antigen specific T lymphocytes. This approach takes advantage of the fact that tumors will contain anti-tumor T-cells which, even if weakly response to tumor antigens, can potentially be harnessed and modified to provide stronger therapeutic potential.

Studies in the mid-1980s found that culturing IL-2 with tumor infiltrating lymphocytes had an anti-tumor effect which was tumor specific and had an efficacy that was 50-100 greater than previous NK cell therapeutic responses (Spiess, Yang, and Rosenberg 1987). Tumor cell suspensions were cultured with IL-2 and cells demonstrating anti-tumor activity were expanded, transferred into tumor-bearing mice, and metastases was measured for reduction.

One of the most notable findings from this study was that there was a stronger response observed in advanced melanoma tumors. Primary phase I trials done by Rosenberg et al. found a correlation with previous animal studies in terms of regression of metastases. The initial trial included 20 patients with pulmonary metastases who were administered cyclophosphamide followed by TIL and IL-2. Of the 20, 11 patients had complete or partial tumor responses (S. A. Rosenberg et al. 1988). A later follow-up trial with 86 patients found a similar response rate in patients with metastatic melanoma (S. A. Rosenberg et al. 1994). In the follow-up trial, TIL with IL-2 was administered in two
cycles separated by 2 weeks. Of these 86 patients, 57 received cyclophosphamide before the initial infusion of TILs. Objective response rates in patients receiving TIL/IL-2 was 31% while those receiving cyclophosphamide prior to transfer had a response rate of 35% based on RECIST (S. A. Rosenberg et al. 1994).

MATERIALS AND METHODS

Although the idea of adoptive cell transfer therapy showed promise, several issues needed to be evaluated before it could be classified as a widespread and efficient standard treatment. The primary necessity was the establishment of a standard protocol for administration. First, this required trials that could create a standard and successful method for extracting, establishing, and expanding cultures. Secondly, the treatment modality had to be able to identify and evaluate the specificity of cultured cells in targeting tumor antigens. Finally, it required assessment of toxic side effects common to many immunological treatment methods. In the case of adoptive transfer, the major factor would be toxic effects of IL-2 or other adverse cytokine events.

Standard treatment protocols at this point follow a linear progression that is as follows:

1. Extraction of autologous tumor-infiltrating lymphocytes from patients
2. Lymphodepletion with chemotherapeutic agents
3. *In vitro* cultivation and expansion of tumor-infiltrating lymphocytes specific for tumor antigen
4. Transfer of TIL to patients with subsequent systemic IL-2 administration
The following analysis examines the studies that evaluated the feasibility of each step and the efficacy of each step in treatment of metastatic melanoma.

**Melanoma-associated antigens**

MART-1 and gp100 are two of the most common melanoma proteins that can be targeted during therapy. Melan-A, also labeled as MART-1, is a protein normally expressed in melanocytes as well as in a large majority of melanomas (Khammari et al. 2009). This marker is especially useful for distinguishing melanocytic tumors from non-melanocytic neoplasms and has both high immunogenicity and expression on melanoma cells (Gogas et al. 2009). The MART-1 protein is a melanocyte specific marker that serves as an antigen for CTLs particularly for metastatic melanoma and is a useful target of immunotherapy. This type of immunotherapy, however, has a potential autoimmune effect which can lead to the destruction of healthy melanocytes resulting in vitiligo.

**HLA-A2**

HLA is involved in the presentation of antigenic peptides to CD8+ CTLs. A large number of human tumors have been found to have reduced expression of HLA-I antigens (Pandolfi et al. 1991). A variant of HLA, HLA-A2 (HLA-*02), has been associated with a stronger immune response towards melanoma cell lines in animal studies (Pandolfi et al. 1991). This variant, HLA-A2, was originally identified by Wolfel et. al., and studies by Darrow et al. confirmed that HLA variants play a role as a restricting element for lysis of several melanoma-associated antigens (Wölfel et al. 1994; Darrow, Slingluff, and Seigler 1989). Specifically, HLA-A2 variants contain restriction elements that
preferentially generate CTLs that are more reactive against autologous melanoma antigens.

Changes in the expression of HLA-A2 have also been observed in several cancers. It is hypothesized that loss of HLA-A2 expression contributes to metastatic progression by allowing cells to escape immune surveillance specifically via bypassing CTL cytotoxicity (Pandolfi et al. 1991). Specifically, expression of the HLA-*0201 allele has been noted to be significant for ACT. Several studies have found that expression of HLA-A2 is crucial for T-cells to recognize melanoma tumor antigens, though it has been posited that HLA variants may be key in other cancer therapies as well. Experiments done by Pandolfi et al. derived TIL and melanoma lines from 4 HLA-A2+ patients. Of these, HLA-A2 expression was lost in 2 of the 4 melanoma lines and this loss of expression was correlated with decreased immunological recognition by lymphocytes. Additionally, it was noted that TIL derived from patients whose melanoma lines were HLA-A2+ had a CD8 phenotype and had the capacity to lyse the melanoma cells (Pandolfi et al. 1991). It was found that patients whose melanoma lines had lost HLA-A2 expression had a CD4 phenotype and did not have cytotoxic activity (Pandolfi et al. 1991).

**T2 cell lines**

In order to introduce a specific antigen prior to cell culture expansion, T2 cell lines can be used. Normally, MHC I antigen presentation relies on peptide transport from the cytoplasm to the endoplasmic reticulum or Golgi via the transport-associated with antigen presentation transporter (TAP). T2 cell lines are TAP-deficient, however absence
of ER peptides in these deficient cells leads to a greater than normal amount of MHC I complexes lacking peptides in the ER and Golgi as well as on cell surfaces (Luft et al. 2001). Not only can these MHC I complexes on the surface be loaded with available peptides, but studies by Day et al. found that there is a TAP-independent route which allows cells to internalize exogenous peptides (Day et al. 1997).

Additionally, Luft et al. found that certain specific peptides have greater binding specificity for MHC I complexes in melanoma patients. An HLA-A*0201-restricted MelanA peptide can have either the AAG sequence (AAGIGILTV), which has low affinity for MHC I complexes, or the EAA sequence (EAAGIGILTV) which has high affinity. The AAG sequence required MHC synthesis and transport for antigen presentation, but the ELA does not. Instead, the ELA sequence can replace peptides from preformed complexes. Finally, it was also noted that ELA sequences had a longer duration of antigen presentation (Luft et al. 2001).

Anti-tumor response was also noted for both peptide sequences. Peptide-specific CTLs generated using either the AAG sequence or ELA sequence were tested for specificity and cytotoxicity against melanoma cell lines. Both lines were capable of recognizing and killing HLA-A2 Melan-A+ tumor lines but did not recognize Melan-A- cell lines. As before, tumor lysis was higher for ELA peptide (Luft et al. 2001).

**Tetramer Technology**

The application of tetramer technology to ACT methods allows for quantification of the number of tumor antigen-specific lymphocytes. A tetramer assay can be used to detect and measure the number of T cells directed against a given antigen. The assay
takes into account that each TCR is specific for a select peptide and will bind with high affinity to the chosen peptide (Constantin et al. 2002). The tetrameric complex is generated using a central streptavidin molecule. The streptavidin molecule has the ability to form homotetramer complexes and has a high affinity for biotin. Genetically engineered MHC molecules with a biotinylated domain are mixed with the target peptide resulting in a central streptavidin molecule with four bound peptide-MHC complexes (Constantin et al. 2002). Once these complexes are added to the T cell culture, the T cell receptor specific for the peptide will bind. Unbound cells can be washed and removed and T-cells stained to be quantified.

**Cytokine release assays**

A large majority of the studies analyzed in this paper utilized a cytokine affinity matrix to select for T cells that had specific effector functions and therefore were secreting specific cytokines. This method, developed by Manz et al., allows separation and sorting of cells based on the products they secrete. Previously, this was not a selection method that could be used as it was difficult to assign secreted products to a specific cell. In this case, the secreted product is retained on the cell surface allowing for easier detection. An affinity matrix specific for the product of interest is attached to the cell surface and cells are then stimulated in our case, with antigen. Cell secretion is allowed in a medium of low permeability for the secreted product, and these products are then stained as an artificial surface molecule. In several ACT studies, induction of IFNγ secretion was done by stimulating T cell lines with T2 cells that had been pulsed with a
specific peptide (Manz et al. 1995). IFNγ-positive T cells were then separated using magnetic cell sorting.

**Response Evaluation Criteria In Solid Tumors (RECIST)**

RECIST is considered an internationally defined set of guidelines that can be used to standardize the response of a tumor to any treatment in patients. Specific criteria classify tumor response, stabilization, or progression. The criteria were spearheaded by a collaboration of the National Cancer Institute of the U.S. and that of Canada, as well as the European Organization for Research and Treatment of Cancer.

In order for researchers performing studies measuring responses to treatment to use RECIST guidelines, eligibility must first be met. Eligibility is classified as patients who have measurable disease at baseline, wherein the term ‘measurable disease’ refers to the presence of at least one measurable lesion. A measurable lesion defined as one that can be accurately measured in one dimension with diameter at least 20mm by X-ray or diameter of 10mm by CT scan or caliper measurement. Criteria for tumor response evaluation will be presented but other criteria will not be mentioned further.

Tumor response evaluation criteria are separated into four response categories:

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Complete Response (CR)</td>
<td>Disappearance of target lesions and reduction of pathological lymph nodes (&lt;10mm)</td>
</tr>
<tr>
<td>Partial Response (PR)</td>
<td>A minimum decrease of 30% in the sum of diameters (based on baseline sum measurement)</td>
</tr>
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</table>
**Stable Disease (SD)**
No shrinkage or increase (using smallest sum as reference)

**Progressive Disease (PD)**
A 20% increase in the sum of diameters of target lesions (using the smallest sum as a reference point) AND an increase of at least 5 mm

Finally, studies also use RECIST guidelines to evaluate overall response to treatment. This response takes into account the total patient response from the beginning to the end of treatment, the findings on both target and non-target disease, and the presence and appearance of new lesions. Determination of best overall response for studies that require classification of CR or PR is based on criteria being met at a subsequent point in time. For example, if at an initial point in time, the response was noted as CR and at a later point in time is noted as SD, then the best overall response will be classified as SD. Duration of overall response is defined as the time from when criteria for CR/PR is met until the first date of recurrence or progression.

**Common Terminology Criteria In Adverse Events (CTCAE)**

The CTCAE are national criteria published by the National Cancer Institute which standardize any adverse reactions to drugs or therapies with most the recent version (4.03) published in 2010. CTCAE classifies an adverse event (AE) as “any unfavorable and unintended sign, symptoms, or disease temporarily associated with the use of a medical treatment or procedure…” The grading scale is from 1 – 5 with 1 being the least severe and 5 representing any death related to an AE. Grading criteria are listed separately for each physiological or anatomical system listed as System Organ Class.
A sample CTCAE for classification of cytokine AE under Immune Disorders is shown below.

<table>
<thead>
<tr>
<th>Adverse Event</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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</thead>
<tbody>
<tr>
<td>Cytokine Release Syndrome</td>
<td>Mild reaction; no infusion interruption; intervention not indicated</td>
<td>Therapy/Infusion interruption indicated, etc.</td>
<td>Prolonged recurrence of symptoms following initial improvement</td>
<td>Life-threatening consequences, etc.</td>
<td>Death</td>
</tr>
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**A. ESTABLISHING AND EXPANDING CELL CULTURES**

Developing a method to identify and culture tumor-specific T cells has been a difficult process due to the smaller *in vivo* pool of T cells with tumor-specific antigens, but also because methods used to generate larger pools of T cells have been variably successful.

The previous discussion on regression and data regarding immune reactions at tumor sites establishes that therapeutic techniques utilizing tumor-infiltrating lymphocytes hold promise as a target-specific regimen for melanoma. The next step required was measurement of the pool of available T cells within tumor sites.

Generation of tumor-infiltrating lymphocytes begins with the resection of melanoma tumor lesions and establishing cultures *in vitro* from digested tissue fragments. Figure 6 illustrates the methods used to culture lymphocyte cell lines. Multiple lines per patient are cultured in media with IL-2 to stimulate initial growth (Yang and Rosenberg
2016). The general growth period for this phase is 2 to 3 weeks at which point TIL cultures reach cell populations of several millions. The range of cells varies from $10^8$ to $10^9$. Cultures can then be further expanded using a rapid expansion protocol (REP) with increases up to a 1000 fold (Yang and Rosenberg 2016). Stimulation via REP uses anti-CD3 (more common) and/or anti-CD28 monoclonal antibodies with IL-2. These methods help offset the initial low number of tumor antigen-specific T-cells since melanoma cells are poorly antigen-presenting and are not strongly antigenic. Once cultures are complete, cytokine assays can then be used to determine activity and specificity of lymphocytic lines.
Figure 6: Illustration of adoptive cell transfer (culture to infusion). The figure above represents two techniques, autologous cell transfer or genetically modified transfer that are currently under study for the treatment of multiple cancers. 
Source: (Phan and Rosenberg 2013)

Generation of cell cultures

Dudley et al. examined three different methods of deriving tumor-infiltrating lymphocyte cell cultures and the efficacy in generating cell populations of each technique. Tumor-infiltrating lymphocytes can be derived from tumor fragments, single-cell digests, or physical disaggregation of melanoma tissue fragments. The tumor fragment method cuts chunks of specimen into 1-2 cm pieces and each fragment is then placed in a well and cultured. Single-cell digests go a step further and incubate the
fragments in a slurry of enzymes such as collagenases, hyaluronidases, and DNases. Finally, physical disaggregation involves mechanical shear force generated by a device called a Medi-machine to break up tumor tissue. Cultures can then be examined for specificity and activity using cytokine assays. Many lymphocytes were stimulated with tumor cell lines or an aliquot of single-cell tumor digests in order to identify and isolate tumor antigen-specific lymphocytes. T2 cells that lacked the TAP transporter, were pulsed with melanoma antigens that could be internalized and presented. These T2 lines were measured for IFNγ secretion measured with cytokine assays to sort effector function specific cells.

PBMC feeder cells were obtained from normal donors, treated with anti-CD3 antibodies and IL-2 to activate the cultures (REP). These PBMC feeder cells are important in generating mononuclear cells (Raulf-Heimsoth 2008). In this case, the REP creates an environment where these mononuclear cells can be exposed to a selected antigen to generate specificity (Raulf-Heimsoth 2008). After 14 days, cells were harvested and prepared for patient treatment. In all three cases, cultures were obtained from greater than 90% of patients, however, in examining the expansion rate of cultures per method, we find that tumor fragments resulted in expansions of 69.9% whereas single-cell digests and physical disaggregation yielded greater than 90% expansion (Dudley et al. 2003).

Analysis of antigen activity and specificity was performed on these cell cultures after stimulation by HLA-A2 tumor cells and analysis of respective cytokine secretion. TILs were screened from 36 patients to measure specificity against tumor antigens and
83% were found to have detectable activity against melanoma tumor cell targets (Dudley et al. 2003). Additionally, analysis of TILs from a single tumor found that there is a varying pattern of antigen recognition within the cells. Further examination of multiple cell cultures from a single tumor specimen also found significant variation in the frequency of CD4+ T cells with CD4+ cells ranging anywhere from 19% to 70% of total cells (Dudley et al. 2003). Once cultures with tumor-reactive TILs were identified, cells were expanded by administration of IL-2 which led to an average cell population of $3 \times 10^7$ (Dudley et al. 2003). These expanded cells served as the preliminary cell lines for patient treatment. Subsequent application of REP expanded the cell population 1320 fold ($4 \times 10^{10}$) in 14 days (Dudley et al. 2003). Thus, 13 TIL cultures from 8 patients were expanded for the purpose of patient treatment.

Sporn et al. combined chemotherapy and adoptive immunotherapy by administering cyclophosphamide previous to adoptive transfer, however, these early results were variable. (Sporn et al. 1993). Blood lymphocytes were isolated from 14 patients, cultured with irradiated autologous tumor cells for the purpose of antigen-recognition and IL-2 to stimulate growth and then returned to the respective patients along with administration of IL-2 intravenously. Lymphocytes were primarily CD4+ cells and although they exhibited variable degrees of cytotoxicity, therapeutic responses were low despite previous NCI trials exhibiting a 20% response in melanoma cases in similar studies (Sporn et al. 1993). Lack of T cell specificity for tumor antigen, as well as difficulty in assessing the strength of lymphocyte response, were noted as primary hurdles in achieving strong therapeutic responses.
Although adoptive cell transfer shows promise, it has been hindered by difficulty in extraction and isolation of antigen-specific T cell populations. Early techniques utilized polyclonal T cells (tumor infiltrating lymphocytes) after IL-2 expansion. Once melanoma-specific antigens were able to be identified and specifically targeted, T cells that were antigen-specific could be specifically selected. This technique is particularly promising for late stage metastatic melanoma.

Later, Oelke et al. and Maidenbauer et al. attempted to quantify T cells that were tumor antigen specific by utilizing tetrameric technology and flow cytometry cytokine analysis. Oelke et al. used CD8+ T-cells from healthy donors and stimulated them with Melan-A autologous dendritic cells in order to generate tumor antigen-specific T cells. The study attempted to devise a method for expansion of T cells that were specific and cytotoxic, and that could generate large concentrations in a reasonable time frame. *In vitro* generation was done using monocytes along with granulocyte macrophage colony-stimulating factor (GM-CSF) with addition of IL-4 and TGFβ1. After 3 to 4 weekly stimulation cycles, *in vitro*, CTL responses were determined. The frequency of CD8+/Melan-A T-cells was measured using tetramer analysis and found to be 3.5% (Oelke et al. 2000). Cells were subsequently stimulated with monoclonal antibodies against CD3 and CD28, and with IL-2 which led to a 600-fold expansion (Oelke et al. 2000). Cell testing confirmed specificity for Melan-A and ability to lyse Melan-A positive melanoma cells (Oelke et al. 2000).

Further studies were done to establish a protocol for the number of stimulation cycles necessary for optimal T cell population counts. Cycle numbers were tested using
influenza antigen and the results of the study applied when generating Melan-A specific T-cells. Results found that T cells undergoing three cycles had high toxicity for target antigen, but did not lyse unlabeled T2 cells or T2 cells with irrelevant peptides (Oelke et al. 2000). Those that had undergone cycles below three were often cytotoxic, but lacked specificity. Initial response resulted in a 40-fold increase in populations (Oelke et al. 2000).

A second set of conditions where cells, after four cycles of stimulation, were subjected to the rapid expansion protocol (REP). Addition of anti-CD3 and anti-CD28 monoclonal antibodies supplemented with IL-2 resulted in a further 15-fold increase in cell concentrations (Oelke et al. 2000). Cell lines were either directly obtained from PBMC or after 5 weeks in vitro and their specificity was measured using TM technology. Cell lines that were not stimulated using the REP had lower T-cell frequencies (0.01% to 0.48%) while the range for those T-cells that were cultured increased from to 3.42% (Oelke et al. 2000).

Oelke’s study showed that antigen-specific CTLs can expand up to 600-fold with initial antigen-specific and later nonspecific stimulation. In terms of timing, induction of CTLs using this method might have better implications for treatment time since cultures take about 4 to 5 weeks to be sufficient in number. In contrast, previous methods of expansion using CTL clones often required up to 2 months or more of in vitro culturing (Oelke et al. 2000). While the study showed promise in quantifying and creating a methodology for cell cultures, it did not address the lack of CD4+ helper cells to produce an antitumor immune response. Oelke hypothesizes that this could be overcome with
further IL-2 and IL-12 administration, however these cytokines also generate cytotoxic responses and no study has measured their effectiveness in CD4+ tumor responses. A second solution would require co-administration of melanoma specific CD4+ T-cell lines. In this study, a second limiting factor is that the selection for only one antigen can be detrimental if tumors mutate so that the target antigen is no longer recognized by T cells or “hide” target antigen. It is thought that polyclonal antigen-specific T cells could overcome this detriment but at the expense of longer cell culture timing.

This poses a separate issue of culture timings and its effect on responses to ACT. A 2010 study compared TILs that had been cultured for a shorter time respective to the usual 6 to 8 week period and found that patient responses were better. Besser et al. notes these TIL as “Young-TIL” to classify them separately and stated that unlike previous studies, there was no restriction for HLA-A2. Normally, this restriction is important as previous studies have noted the importance of HLA-A2 expression for the tumor recognition by autologous lymphocytes. Besser et al. however, noted that this restriction, in conjunction with longer incubation times and IFNγ selectivity, often leads to high exclusion rates of enrolled patients. Specifically, a fair amount of patients who are initially enrolled lack HLA-A2 expression and are subsequently removed from trial data. They hypothesize that other criteria such as culture time and telomere length of the T-cells may be more useful for predicting therapeutic response while simultaneously allowing a larger patient population to participate in experimental trials.

Previous studies have found a correlation between telomere length and response efficacy (Shen et al. 2007). Generally, longer telomeres are found in Young-TIL cultures
which can be generated in two weeks and often require simpler procedures than those mentioned above. Young-TIL can be established using one bulk T cell culture, therefore cells spend less time in culture and can be expanded in a shorter amount of time. In this case however, cytokine assays and selection was removed from protocol. Initial studies with eight patients found that there was one complete response, two partial responses, and four patients had disease stabilization (Besser et al. 2009). A subsequent phase II trial was initiated with 20 patients with stage IV melanoma, who underwent lymphodepletion followed by transfer. These patients were administered cyclophosphamide and fludarabine a week in advance of TIL infusion. Young-TIL were administered followed by IL-2. 10 of the 20 experienced an objective response as classified by RECIST with 2 complete and 8 partial remissions (Besser et al. 2010). Those with complete response had ongoing remission at four and 20 months (Besser et al. 2010). Of those who did not respond, four experienced disease stabilization and six had disease progression. Young-TIL were generated in 90% of patients and patients who responded to treatment had a median survival increase of 10 months (Besser et al. 2010).

B. LYMPHODEPLETION PRIOR TO ADOPTIVE TRANSFER

Several studies have found that lymphodepletion via either chemotherapy or radiation facilitates a stronger response to ACT in melanoma patients. Phase I initial studies at the NCI measured patient response rates following a lymphodepletive pre-treatment regimen along with ACT generated a 47% response in an early trial of 13 patients (Dudley et al. 2005). Follow-up trials conducted by Dudley et al. with 35 patients achieved an objective
response rate of 51% (Dudley et al. 2005). The results showed three complete responses and fifteen partial responses. Regression was seen at multiple sites including cutaneous sites, liver metastasis, and brain. Standard dosages of cyclophosphamide (60mg/kg) were given for 2 days followed by cell infusion with IL-2. Development of autoimmune events was noted in a small subset of patients who were diagnosed with vitiligo or uveitis.

Later studies by Rosenberg et al. compared response rates to ACT with prior chemotherapy administration or with prior chemotherapy accompanied by radiation (2 Gy or 12 Gy). In this case, chemotherapy consisted of cyclophosphamide and fludarabine. Radiation therapy was delivered in 2-Gy fractions with 15-MV photons at a dose of 0.11Gy/min (Dudley et al. 2008). Objective response rates were found to be highest in cases where high radiation was administered in conjunction with chemotherapy at 72% (Steven A. Rosenberg and Dudley 2009). 93 patients were enrolled and three consecutive protocols were established. The first protocol, consisting of 43 patients, was administered chemotherapeutic drugs as mentioned above followed by ACT. The second group of 25 patients was given chemotherapy followed by 200cGy TBI before ACT. A final group of 25 was administered TBI of 1200cGy with all other variables remaining the same. Response rates were 49%, 52%, and 72% respectively and many patients had ongoing responses at the three year mark (Steven A. Rosenberg and Dudley 2009). Since these trials were consecutive however, it is difficult to positively conclude if a direct increase in TBI leads to an increased response rate. Despite this, lymphodepletion by addition of total body irradiation (TBI) was found to increase anti-tumor effects. Studies
in mouse models have suggested that the reason for this response is a result of two immunological mechanisms:

i) Elimination of regulatory T cells

Regulatory T cells can develop in the thymus or peripheral tissues and inhibit the activation of lymphocytes that target self antigens. Most of these regulatory T cells are of the CD4 variety and express increased levels of CD25. It has been found that a decrease in the total number of CD4+ T cells enhances adoptive transfer therapy against tumor or self antigen (Antony and Restifo 2005). Physiologically, CD4+ T cells facilitate the response of CD8+ T cells against viral or foreign antigens (Antony and Restifo 2005). The subset of CD4 cells (CD4+ CD25+ T regulatory cells) work to suppress T cells and control tolerance to self-antigens (Gattinoni et al. 2005). Removal of these regulatory T cells often correlates with increased autoimmune destruction (Gattinoni et al. 2005). Therefore it is hypothesized that removal of T regulatory subsets while inherently problematic due to development of autoimmunity, may also help facilitate a stronger response to adoptive transfer. This autoimmune response was found in the previously mentioned patient populations where a small group of individuals developed antibodies to melanocytes and later developed vitiligo.

ii) Cytokine competition for lymphocyte growth

Initially, elimination of CD4+CD25+ T regulatory cells was believed to be the main reason that lymphodepletion helped facilitate anti-tumor responses however, Gattinoni et al. found that patients with genetic loss of regulatory T cells still saw benefit with
lymphodepletion prior to ACT. Follow-up studies found that chemotherapeutic regimens helped to remove cytokine competition for transferred lymphocytes (Gattinoni et al. 2005). IL-7 and IL-15 normally help enhance T-cell function and removal of other competing cells that would “use up” cytokines helped to keep these interleukins available for growth of transferred cells (Gattinoni et al. 2005). It had been hypothesized that NK cells may serve as sinks for IL-15. Mouse models with melanoma were used to measure the response to ACT. Tumor area and size measurement was used to gauge strength of response. Experiments with Rag1/- mice which lack both B and T cells, but have NK cells, found that irradiation improved TIL response against tumor cells (Gattinoni et al. 2005). Therefore, irradiation removed NK cells which would normally utilize IL-15 and increased availability of the cytokine for TILs. Additionally, administration of monoclonal antibodies towards NK IL-15 receptors confirmed this theory as it led to an increased response similar to that of irradiation treatment. A second experimental study using Rag2/-/γc mice which lack B, T, and NK cells was done. In this case, mice that were lacking these cells did not differ in response with mice that were subjected total body irradiation since they had no immune cells to compete for TIL cytokines (Gattinoni et al. 2005).

C. INTRAVENOUS ADMINISTRATION WITH IL-2

Yee, et al. also compared the efficacy of autologous T cell injections with varying levels of IL-2. Patients with stage IV melanoma were injected with TIL and no initial IL-2 and IL-2 infusions administered were slowly increased from 0.25x10^6 units/m^2 to 1x10^6
units/m² twice daily for 14 days (Yee et al. 2002). Therefore, IL-2 dosage was increased every 2 weeks. 10 patients were administered therapy and no serious toxic events were noted. Infusions given without IL-2 were noted to have significantly lower median survival and frequency of T cells was on average less than 0.01% by day 14 as measured by PCR and tetramer technology. In contrast, infusions that were given with low dose IL-2 had a frequency that did not go below 0.01% until day 21 (Yee et al. 2002). IL-2 dosage varied consecutively from 0.5, 1, and 2 million units/m², however, after 0.5 the responses to a higher IL-2 dosage did not show a significant change in frequency or median survival, defined as the day on which one-half maximal frequency of T-cell during tetramer analysis (Yee et al. 2002). Median survival of T cell clones with zero IL-2 was found to be on average 6.68 whereas those with IL-2 had a median survival of 16.92 (Yee et al. 2002).

Studies like the one above described the use of interleukin-2 as a potent cytokine that can be utilized for expansion of T cells in several cancer regimens. Combination of IL-2 regimens with adoptive cell transfer is now standard as it was proven effective in both mouse models and human trials. Major toxic side effects were mentioned previously but will be elaborated here as well. Patient symptoms included malaise, nausea, vomiting, hypotension, fluid retention, and organ dysfunction.

A pilot trial by Ellabaek studied the importance of IL-2 dosage and postulated that low dose IL-2, as opposed to levels normally administered during ACT, can be as effective and sidestep cytokine related adverse events. Lymphodepletion was administered approximately a week before TIL were intravenously infused and was
followed by 14 days of IL-2 injections of 6000 IU/mL. Responses were monitored using CT and PET scans and assessment was done using RECIST guidelines.

Six patients with metastatic melanoma were treated using this new protocol. Of these, two patients had a complete response and two patients had stable disease (Ellebaek et al. 2012). One patient had brain metastasis and disease had spread to lymph nodes of the pelvis. Prior to therapy, brain metastasis and one pelvic nodule were surgically removed. Therapy slowed the regression and after 1.5 years, PET scanning was negative in both sites. The patient continued to be in remission at the 30-month mark (Ellebaek et al. 2012). The second patient with complete response had metastases to the neck and cheek which were removed prior to therapy. Post-therapy the patient continued to be in remission after 10 months (Ellebaek et al. 2012).

IL-2 toxicity was labeled at grade 3-4 (CTCAE) and was mainly related in this study to the lymphodepletion chemotherapy and consisted of GI symptoms, fatigue, and low blood cell counts. Patients were noted to have grade 2 anemia and received blood transfusions, and exhibited leucopenia, neutropenia, and lymphopenia as well (Ellebaek et al. 2012). TIL infusion symptoms manifested mainly as fever and chills. A smaller subset of patients had high blood pressure and tachycardia. IL-2 administration resulted in grade 2 nausea and fatigue as well as chills in the initial hours after injection.

**DISCUSSION**

Metastatic melanoma, particularly Stage IV melanoma, has a low 1-year survival rate and is one of the most common causes of cancer death, especially in the West.
Normally, T cells that are tumor antigen-specific are low, however, adoptive transfer allows *ex vivo* proliferation and manipulation of autologous lymphocytes to significantly improve specificity and response.

Peptide targets have been useful in maintaining T cell specificity however, expression of MART-1 and gp100 can also be found in some normal tissues leading to autoimmune reactivity. This, coupled with removal of regulatory cells, could lead to serious immunological consequences if not monitored and resolved. Additionally, T cell targeting of normal tissue cells takes away from a total anti-tumor response.

Another new technology is utilizing genetic modification of the T cell receptor or T cell characteristics to produce tumor-specific T cells. The majority of this thesis has focused on tumor-specific T cells found in the blood and tumor tissue, however, as briefly mentioned, the introduction of peptide antigens via dendritic cells or endocytotic uptake and presentation has also shown promise.

In terms of ACT expansion, which has been one of the major hindrances to wider applications, technological advances have now reached a point where sorting and selecting for T cell specificity is faster than in the past. Bernatchez elucidates new cell sorting technology such as the MACSQuant Tyto and Miltenyi which uses tetramer technology to separate cells. Further effectiveness of ACT as a therapeutic requires T cell specificity and differentiation that is both quick and efficient to meet patient demands. Though ACT responses have been more significant than any previous therapeutic, neither culture growth nor response is guaranteed for all patients.
More than 400 melanoma patients alone have received TIL therapy and responses have been promising (Bernatchez et al. 2016). In addition, ACT has been used for the treatment of other cancers such as ALL, ovarian, and colorectal cancer. A major limitation, as alluded to previously, is the ability to expand cells rapidly and with the necessary specificity. A method or machine to help eliminate the laborious process will be crucial to the use of ACT in hospitals nationally and internationally. Studies by Besser et al. addressed this issue by reducing culture time of TIL and streamlining the procedure. However, this comes at the expense of removing HLA restriction and although the study found a clinical response, the patient sample size was small and further studies will be needed before its efficacy can be established.

Next steps in the advancement of these types of regimens will require further knowledge about the characteristics of tumor cells, the phenotypes of immune cells, and the ability to manipulate and modify these cells to generate effective and long-standing responses. This process includes identifying and isolating new tumor biomarkers and the generation of T cells with specificity for multiple tumor antigens. Administration of a combination of both immunotherapy with standard therapy could play a significant role in preventing tumor evasion of immune response and improvement of therapeutic response.

Despite the technical and physiological hurdles of adoptive transfer, the therapy is promising not only for metastatic melanoma but also for its potential applications in the treatment of other cancers. Current therapeutics in melanoma have had variable success rates with responses in late stage cancer. Currently checkpoint blockade and other
immunotherapies are at the forefront of melanoma research. Numerous studies have found greater responses for late stage cancer in adoptive cell transfer in comparison to other available therapies. Adoptive cell transfer therapy is one more step in the movement of medicine towards a more targeted and personalized forms of therapy. As this demand for personalized medicine increases, technology will increase, and the availability of such treatments has the potential to become more mainstream.
REFERENCES


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Pre-Calculus & SAT Math Tutor (April – May 2012)
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