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INVESTIGATION OF THE FUNCTIONAL IMPACT OF ANTI-PD-1 ON TUMOR-INFILTRATING LYMPHOCYTES (TIL) AND MAPPING OF TUMOR GENOMIC FEATURES RELEVANT FOR RESPONSE TO TIL THERAPY

Caitlin Creasy

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INVESTIGATION OF THE FUNCTIONAL IMPACT OF ANTI-PD-1 ON TUMOR-INFILTRATING LYMPHOCYTES
(TIL) AND MAPPING OF TUMOR GENOMIC FEATURES RELEVANT FOR RESPONSE TO TIL THERAPY


by

Caitlin Alane Creasy, M.S.

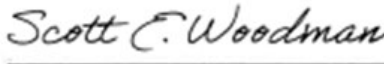
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(TIL) AND MAPPING OF TUMOR GENOMIC FEATURES RELEVANT FOR RESPONSE TO TIL THERAPY

A

DISSERTATION

Presented to the Faculty of

The University of Texas

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Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

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Houston, Texas

DECEMBER, 2020

Dedication

This work is dedicated to my family, who has been supportive of anything and everything I have ever tried doing (even soccer). Thank you to my grandparents, who have always had a listening ear and wisdom. Thank you to my dad, who imparted a love of science and encouraged me to laugh through life. Thank you to my mom, who provided me with an endless supply of freezer food and always had great, sound advice. Thank you all for your patience during my time as an eternal student. And most of all, thank you to my heavenly Father, without whom I am nothing.

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Advisory Professor: Michael Davies, M.D., Ph.D.

With the growing use of both cellular and antibody-based immunotherapies, it has become more important to uncover biomarkers to predict patient response to therapy and ideally aid in patient selection for specific therapeutics, thereby enhancing treatment outcome. At MD Anderson, we have a 43% response rate to therapy when treating metastatic melanoma patients with adoptive cell therapy (ACT) of tumor-infiltrating lymphocytes (TIL), but it is unknown what makes the other 57% of patients not respond to therapy. It is further unknown how particularly early doses of checkpoint inhibitors, like anti-PD-1, attribute to TIL functionality, adding another confounding factor in determining a unanimous response rate. Therefore, the overall goal of this study has been to identify biomarkers for patient response to TIL ACT in melanoma and to deeply study the impact of anti-PD-1 on TIL functionality, a front line therapy that a vast majority of patients now receive prior to TIL therapy. We obtained tumor tissue from a variety of rare tumor types after early exposure to anti-PD-1 on a clinical trial. We studied the functional effects of one dose of anti-PD-1 therapy on TIL by examining growth, phenotype, and soluble factor production post cellular activation, and observed enhanced CTLA-4 expression on CD4 TIL with a diminished soluble factor profile by CD8 TIL post exposure. To uncover biomarkers for TIL ACT within metastatic melanoma that are indicative of response and survival to TIL therapy, we interrogated pre-treatment samples using genomics and transcriptomics, whereby we observed neoantigen load significantly correlates with overall survival (OS) and identify particularly the gene *ELFN1* associated with unfavorable outcome to therapy. Through this research, we hope to further provide insight to understanding TIL and the tumor microenvironment and help to increase the efficacy of using

immunotherapy to treat cancer patients. Therefore, our work will provide significant impact in the usage of ACT and antibody based immunotherapies as a whole.

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Chapter 1: Introduction

1.1 Tumor immunology concepts

The Merriam-Webster dictionary defines tumor as: *“an abnormal benign or malignant new growth of tissue that possesses no physiological function and arises from uncontrolled usually rapid cellular proliferation”*. The elimination of normal cells aberrantly proliferating has proven to be a challenging task for the immune system, trained to recognize and eliminate foreign invaders.

The concept that immune cells could distinguish cells from the body (self) from exogenous pathogens (non-self) was introduced by Dr. Paul Ehrlich in 1901[1]. Dr. Ehrlich later postulated that there would be a greater frequency of cancer and tumors with age in the absence of some sort of control by the host and thus proposed that a person's own immune system could aid in tumor clearance, hypothesizing that malignant cancer cells are prevented from forming tumors through the host's natural defenses[2]. Almost fifty years later, the hypothesis gained more traction when Dr. Lewis Thomas and Sir Frank MacFarlane Burnet proposed the idea of immunosurveillance, or that the immune system recognizes and eliminates tumors based on the identification of tumor-derived genetic aberrations[3]. Cancers were considered as exceptional events, whereby rare neoplastic cells succeeded in overcoming immune system control[4]. The ability of tumor cells to progress even in the presence of the immune system gave rise to the idea of immunoediting[5]. In this concept, there are 3 phases describing the relationship between tumor cells and immune system: elimination, equilibrium, and escape[5]. The first phase, elimination, is synonymous with immunosurveillance, whereby the immune system recognizes and eliminates most tumor cells. Ideally this first phase eliminates the tumor, though some tumor cells may acquire resistance mechanisms and survive[5]. The second phase, equilibrium, describes a state where the host immune system and the remaining tumor cells enter a balancing act, where immune pressure targets tumor cell growth without reaching elimination. At this step, which may be the longest of the 3 stages, the remaining tumor cells will be suppressed by the immune cells and evolve to become resistant, often through acquiring mutations to enhance survival

and proliferative ability[5]. The final stage, escape, is when the tumor cells that have undergone mutations or selective pressure modulation for survival will now continue to propagate in the immunologically intact environment[5].

Tumor structures are not exclusively formed of tumor cells. The tumor microenvironment (TME) is composed of the cellular milieu, vasculature, soluble factors, and extracellular matrix (ECM) that surround tumor cells to comprise the tumor[6]. The TME oftentimes creates a hospitable environment for the tumor cells to flourish and can change vastly to support the tumor as it progresses and metastasizes[7]. The importance of the TME for tumor promotion was first described in the seed-soil hypothesis[8]. Based on conclusions from breast cancer data, Dr. Paget noted metastases did not occur by chance; tumors (the seed) grew preferentially in certain tissues (the soil), ultimately selecting a beneficial tumor-promoting TME[8]. Therefore, when trying to understand cancer and its potential treatment, the importance of the TME cannot be ignored.

Therefore, in the following section, I will introduce the main cellular components of the TME, and in doing so, introduce factors that the tumor utilizes during elimination, equilibrium, and evasion. Notably, some of the concepts addressed in this section, including inhibitory receptor upregulation and antigen presentation will be described in more detail later in this thesis.

1.1.1. Tumor Microenvironment

1.1.1.1. Tumor cells

Tumor cells are characterized by their uncontrolled proliferation, typically due to genetic mutation or instability, and arise from a single tumor cell clone that ultimately develops into a tumor[9, 10]. All human tumors can be defined by the 6 hallmarks of cancer, which include “self-sufficiency in growth signals, evading apoptosis, insensitivity to anti-growth signals, sustained angiogenesis, tissue evasion and metastasis, and limitless replicative potential”[11]. In 2011, these hallmarks were updated to include “deregulating cellular genetics and genome instability and mutations”[12]. Not only are these mutations helpful for the tumor cells’ proliferation and survival, they are also often useful in

allowing the immune system to identify these cells as neoplastic and lead to their elimination, particularly through the generation of tumor antigens[13]. Tumor antigens can be broadly divided in tumor-specific antigens (TSAs, also known as neoantigens) or tumor-associated antigens (TAAs)[14]. TSAs are more immunogenic than TAAs and are derived from a protein or molecule specifically expressed by the tumor (absent from normal cells)[15, 16]. TSAs are also considered essential for an immune response and have become main therapeutic targets in cancer treatment, with an added benefit of not causing off target effects; for example, there is data suggesting the efficacy of the neopeptide vaccine for the mutated IDH1 (R132H) gene in gliomas[17, 18]. Tumor-associated antigens are those antigens that are expressed on the tumor and lowly expressed on normal tissues[16]. These antigens are produced by the tumor and can fall into several categories (listed in Table 1)[14]. Tumor antigens are then displayed on the surface of the tumor via major histocompatibility complexes (MHC), and recognized by T cells to mediate an immune response (described in details in the T-cell subsection)[14]. The recognition of tumor antigens is therefore a major target of immunosuppression and immune-escape.

Table 1. Tumor Antigens

TYPE OF TUMOR ANTIGEN	DESCRIPTION
ONCOFETAL	<i>Expressed in fetal tissues and cancerous somatic cells</i>
ONCOVIRAL	<i>Expressed in tumorigenic transforming viruses</i>
OVEREXPRESSED/ACCUMULATED	<i>Expressed in high levels in cancer, but also found in normal tissue</i>
CANCER TESTIS	<i>Found in either cancer or adult reproductive tissues</i>
LINEAGE RESTRICTED	<i>Found in one cancer histotype</i>
MUTATED	<i>Expressed due to genetic mutation or translational modification</i>
POST-TRANSLATIONALLY ALTERED	<i>Associated with post-translational modifications</i>
IDIOTYPIC	<i>Associated with producing clonal cells, e.g. lymphomas</i>

Tumor cells utilize multiple mechanisms to evade detection by the immune system. Some of these mechanisms involve camouflage, with the loss of antigens presented on the tumor itself or downregulating molecules necessary for antigen recognition (MHCs- antigen presentation machinery) to avoid detection as documented in metastatic melanoma and other tumor types (described later in

this thesis)[19-21]. Other mechanisms that tumors use to evade the immune system involve directly suppressing the immune cells by upregulating receptors that bind to T cells (e.g., PD-L1 upregulation on the tumor that binds to PD-1 on the T-cell) causing impaired T-cell function or even cellular death[22]. Tumor immunosuppression also includes changing the surrounding environment, by depleting molecules such as glucose and tryptophan needed for T-cell metabolism and anti-cancer function, or by recruiting more immunosuppressive cells like regulatory T cells (T_{regs}) or myeloid derived suppressor cells (MDSCs, described later) to the surrounding environment[23, 24]. Further, the metabolism of tryptophan occurs via the molecule indoleamine 2,3 dioxygenase (IDO); IDO contributes to multiple immunosuppressive functions, including inhibition of T-cell activation and proliferation, polarization of CD4 cells to T_{regs} , and bias of macrophages and DCs to a suppressive phenotype[25-28]. Tumor cells also secrete other cytokines (like IL-10), soluble factors (like IDO), and growth factors (like platelet-derived growth factor, PDGF, or transforming growth factor beta, $TGF\beta$) to further impair immune cell function and promote tumor cell growth. For example, the secretion of PDGF by the tumor causes a feedback loop of insulin-like growth factor 1 (IGF-1) production by nearby stromal cells, contributing to enhanced tumor cell proliferation[29]. Finally, the tumor mass tends to be hypoxic, occurring due to the tumor cell's proliferative rate being greater than the ability of the vasculature to support the tumor; hypoxia in the TME contributes to immunosuppression through promotion of suppressive immune cell types (MDSCs and T_{regs}), while inhibiting cytotoxic T-cell function (all cell types will be described in detail later)[30-32]. Tumor hypoxia also leads to the overexpression of epidermal growth factor receptor (EGFR)[33, 34]. EGFR expression has been associated with promotion of tumor angiogenesis via vascular endothelial growth factor (VEGF, also known as VEGF-A), allowing for improved vasculature and promoting tumor proliferation[34-36]. VEGF is notably more highly expressed in tumors and is often associated with increased metastasis and advanced disease stage, including in prostate, breast, and colorectal cancers[37-39]. Further, tumor cells will combine several immunosuppression/tumor

promoting mechanisms to acquire the ability to remain undetected and to continue to grow and spread.

1.1.1.2. Stroma

Stroma is comprised of the non-malignant cells in the TME, including fibroblasts and immune cells, and the extracellular matrix (ECM)[40]. The ECM is defined as the non-cellular component that offers both structural support and biochemical support for the tissue[41]. Overall, the stroma is important in providing nutrients like glucose, mainly through blood flow, and waste removal of the tumor[14]. Further, the stromal cells can actually promote tumor growth[42]. The ratios of the cell types composing the stroma and their functions, described in detail below, can often determine whether the stroma is considered hospitable to tumor growth[14]. This notion is exemplified by the importance of the tumor-stroma ratio as a prognostic factor of tumors such as breast cancer, where high stromal content corresponds to significantly shorter relapse-free survival[43]. Further, in esophageal squamous cell carcinoma, it has been shown that greater amounts of stroma are associated with poorer prognosis and greater chance of relapse[44].

1.1.1.2.1. Cancer Associated Fibroblasts (CAFs)-CAFs comprise most of the stroma and differ from normal fibroblasts, as they are always in an activated state[42]. CAFs often develop through micro RNA (miRNA, small non-coding RNA that functions in transcriptional regulation) reprogramming of normal fibroblasts by the tumor cells[45]. CAFs produce growth factors like hepatocyte growth factor (HGF) and IGF-1 to promote tumor cell proliferation and VEGF to promote tumor angiogenesis[46-48]. HGF promotes tumor cell growth and metastasis through its ligation to c-met on tumor and epithelial cells, causing the epithelial to mesenchymal transition (EMT)[49]. EMT means that an epithelial cell undergoes biochemical changes that cause it to be more migratory, invasive, and resistant to apoptosis, gaining mesenchymal features that are typically associated with an aggressive

cancer phenotype[50]. Further, CAFs produce inflammatory cytokines and chemokines like IL-6 and CXCL2 (SDF-1) which recruit tumor cells and regulatory dendritic cells into the tumor bed and lead to the break-down of the ECM, promoting metastasis[51-53]. Notably, CXCL2 can also interfere with maturation of myeloid immune cells that could help in an anti-tumor response (discussed later in this section), whereas IL-6 is often associated with poor prognosis in multiple cancer types, including colorectal cancer[54, 55]. CAFs provide a hospitable environment for tumor cells partly by their active suppression of immune functions through the production of TGF β and recruitment of IDO-producing dendritic cells (DCs)[52, 56]. TGF β notably inhibits the presentation of antigen to T cells by DCs, while both IDO and TGF β inhibit proliferation of both CD4 and CD8 T-cell types, which are vital for solid tumor regression[57, 58]. TGF β has numerous other suppressive functions, and is even linked to production of IDO by DCs[59]. Further, TGF β inhibits NK cell function and contributes to the inhibitory function of regulatory T cells (T_{regs}), which will be described in detail in the next section[60, 61].

Within the next portion, I will present the main immune cells involved in the TME, including their different functions in promoting tumor suppression or progression. Due to the focus of this thesis, the T-cell will be described in the subsequent section in detail.

1.1.1.2.2. ***Dendritic cells (DCs)***-DCs are integral cells in the presentation of tumor antigen, known as antigen presenting cells (APCs), via MHCI and MHCII molecules on their cell surface, providing a crucial step in T-cell activation and response[13]. DCs are also helpful by producing cytokines like IL-12 and IL-23 that can lead to a T-cell mediated immune response by inducing T-cell proliferation and production of the effector cytokine interferon gamma (IFN γ)[62, 63]. DCs are part of the innate immune response, which also includes monocytes (macrophages) and granulocytes (including neutrophils, eosinophils, and basophils)[13].

There are multiple DC subsets, including the plasmacytoid DC (pDC), monocyte-derived DC (moDC), conventional type I DC (cDC1), and conventional type II DC (cDC2)[64]. These subsets are defined by different transcription factors (for example, IRF8 and E2-2 for pDCs) and each have unique functions in the TME[64, 65]. pDCs can elicit a strong type I IFN (IFN α and IFN β) mediated response when stimulated by their toll-like receptors (TLR), promoting a T-cell, NK cell, and B-cell mediated responses[66]. pDCs can present antigen via the MHCII molecule, but, in comparison to cDCs, they do not present antigen as efficiently[67].

However, pDCs have the ability to utilize cross-presentation (described in detail in the next section), whereby exogenous material can be presented to a CD8 T-cell via the MHCI molecule; this process occurs rapidly, allowing for a rapid T-cell mediated clearance, which could also be beneficial in the context of tumor suppression[68]. Within the TME, pDCs have been shown to help control melanoma tumors in a mouse model system through the secretion of granzyme b and upregulation of the death pathway TNF-related apoptosis-inducing ligand (TRAIL) on tumors dependent on IFN Type I secretion[69]. In contrast, pDCs also were described to be pro-tumorigenic in both breast and ovarian cancers, with the secretion of IL-10 and IDO and the upregulation of ICOS-L, enhancing T_{reg} function and infiltration[25, 67, 70, 71]. IL-10 can suppress multiple immune cell types, including cDC1s, which predominantly present antigen to cytotoxic CD8 T cells[25]. cDC1s are excellent at MHCI antigen presentation and cross-presentation, both discussed in detail in the next section, allowing for recognition of exogenous and endogenous peptides[72]. cDC1s play an important role in recruitment of T cells and NK cells to the TME via secretion of the chemokines CXCL9 and CXCL10[73]. cDC1s cells are known to produce large amounts of IL-12 in humans, which promotes a tumor suppressive CD8 response[74]. Although a minority of the DC subset, cDC1s are associated with improved prognosis in multiple cancer types, including breast cancer, metastatic melanoma, lung adenocarcinoma, and head and neck

squamous cell carcinoma[75]. The other conventional DC type, cDC2s, are the main type of DC in the body[76]. cDC2s, which predominantly are involved in presentation of antigen to CD4 T cells, are suppressed by T_{reg} cells by inhibiting their migration to tumor-draining lymph nodes[77, 78]. The last main DC subtype, moDCs, arise during time of inflammation through monocyte differentiation[79]. These cells can be involved in CD4 T-cell Th1 polarization in response to infection, which is described in detail later in this thesis[80]. Relatively little is known regarding the role of moDCs in the context of the TME, but data has suggested that these cells can aid in the suppression of melanoma in mouse models through the production of IL-12 and activation of CD8 T cells[81].

1.1.1.2.3. **Macrophages-** Macrophages are a phagocytic cell type that is found in all tissues of the body and as members of the innate response, are often some of the first cells at the site of infection[13]. These cells engulf pathogen and then can act as an APC, presenting antigen to the T-cell to induce a T-cell mediated response. Further, macrophages are important in producing inflammatory cytokines like TNF α , IL-1, IL-6, IL-8, and IL-12; however, macrophages can also produce the immunosuppressive cytokines IL-10 and TGF β [82]. Based on the type of cytokines expressed and function, macrophages are classified into subtypes and two distinct polarizations: M1 and M2[83]. In the context of the TME, I will focus on tumor-associated macrophages (TAMs). TAMs are considered to have properties resembling the M2-like polarization state, which is typically associated with an immunosuppressive response, including TGF β production; however, TAMs can be found in both an M1 and M2 polarization state[83-85]. Macrophages in the other polarization state, M1, are associated with an immune permissive response, with an increased inflammatory cytokine production like IFN γ and enhanced phagocytic activity[83, 86]. Therefore, a therapeutic target for many solid tumors is skewing the M2 polarized TAMs to an M1 polarized phenotype[87]. TAMs can induce tumor progression through various mechanisms

including release of soluble factors like growth factors (EGF) or the chemokines (CXCL8) promoting tumor growth and metastasis, supporting angiogenesis with the secretion of VEGF, and impairing T-cell function by producing IDO[88-91]. Though macrophages can present antigen for use in T-cell mediated tumor clearance, TAMs will impair T-cell function through the use of inhibitory cytokines like IL-10, upregulation of checkpoint inhibiting molecules like PD-L1 on tumor cells, and physical exclusion of T-cell infiltration into the TME[92-94].

1.1.1.2.4. ***Myeloid-derived suppressor cells (MDSCs)***- MDSCs are an immature myeloid cellular subtype that is typically immunosuppressive, as they are found to suppress an inflammatory response and promote chronic viral infections by suppressing T cells through L-arginine depletion[95, 96]. When T cells lack L-arginine, they will lose TCR expression, and thereby be less effective functionally[97]. There are two types: polymorphonuclear- MDSCs (PMN-MDSCs) and monocytic MDSCs (mMDSCs), which are named based on their morphological and phenotypic features[98, 99]. mMDSCs are similar to monocytes (e.g., macrophages), while PMN-MDSCs are similar to polymorphonuclear cells (e.g., neutrophils, basophils, and eosinophils)[98]. PMN-MDSCs have many similarities to neutrophils, and due to this, it is difficult to decipher their individual impacts on the TME[100]. Differences in the two MDSC cell populations are indicated by comparative cellular presence/proportion in the tumor, immunosuppressive functions towards NK and T cells, and transcriptomic genotype, with studies ongoing to determine the specific effects of the two cell types in the TME[101]. In the literature, there is a predominant focus on the mMDSC subtype of MDSCs as these typically become TAMs in the TME[30, 102]. VEGF, IL-6, and IL-1 β production typically recruits MDSCs to the TME and can promote tumor progression[100, 103]. Notably, MDSCs have a survival advantage in the TME, in that they are resistant to reactive oxygen species (ROS) mediated cellular death[100]. MDSCs can thereby survive in

conditions where they and surrounding tumor cells produce ROS, effectively inhibiting cytotoxic immune cells by limiting both their activation and cytotoxic function[30, 104]. Also, IDO secreted by MDSCs can further promote ROS production, and ROS mediates apoptosis through cytochrome c activation, thereby activating a caspase cascade; notably, T cells are highly susceptible to ROS mediated cellular death[105-107]. mMDSCs also sequester essential amino acids like arginine and cysteine, which are necessary for T-cell activation and function in the TME[108, 109]. Arginine sequestration by the mMDSCs in the TME, for example, can switch the T-cell from oxidative phosphorylation to glycolytic metabolism, causing a shift in cellular phenotype, leading to less survival and less anti-tumor efficacy facilitating tumor immune escape[110].

1.1.1.2.5. **Natural Killer (NK) cells-** NK cells are innate lymphoid cells with cytotoxic function that is dictated by activating/inhibitory receptors[111, 112]. NK cells impact the immune response through their release of cytokines and chemokines, particularly the cytokine IFN γ , that can upregulate MHC expression on DCs and macrophages and polarize T cells to a Th1 phenotype (described later)[113-115]. They are also cytotoxic by their ability to release granzyme and perforin[113, 116]. Unlike T cells, which will be discussed in depth in the next section, NK cells utilize MHC-independent-mediated cytotoxicity and actually have inhibited effector function in the presence of MHC molecule signaling[111, 112, 117]. Due to this, when tumor cells try to evade T-cell mediated cytotoxicity with downregulation of their MHC molecules, NK cells are still able to recognize their killer activating receptors, causing cellular death[118]. Typically, NK cell infiltration is associated with improved patient prognosis in multiple cancer types, including breast, renal cell carcinoma, head and neck, and lung cancers[119-122]. This could be explained by NK cells recruitment of cDC1s via chemokines CCL5 and CXCL1 to the tumor to further inhibit tumor growth[73]. NK cells are inhibited in the TME by a multitude of factors, including depletion of metabolites like

glutamine and glucose for glycolysis[123]. Further, a multitude of suppressive soluble factors are inhibitory to their function, including TGF β and IDO[124, 125]. However, it has also been hypothesized that these soluble factors not only inhibit NK cells, but can also lead to a pro-angiogenic switch, causing NK cells to also aid in the propagation of the tumor by secreting VEGF[126]. In fact, in cutaneous T-cell lymphoma (CTCL), NK cell presence is associated with poor prognosis[127]. However, NK cells are not always associated with negative outcome; for example, in metastatic prostate cancer, cytotoxic NK cells in the peripheral blood of patients can predict patient survival. As such, it must be taken into account that NK cells are considered part of the innate immune system with their quick initial response and lack of MHC restriction for antigen recognition; thus, they are often some of the first cells in the TME and are often helpful in the recruitment of other cytotoxic cells like T cells via cDC1s[73]. Therefore, NK cells are often a first line of defense against cancer and are a target of suppression for immune escape by the tumor[73].

1.1.1.2.6. **B cells-** B cells are often synonymously associated with the humoral (antibody) mediated response to pathogen[13]. However, B cells also contribute to the cellular immune response through their ability to perform antigen presentation to T cells and production of cytokines that can sustain other immune cell types (IL-4 and IL-6), including macrophages, T cells, and DCs[128]. B cells are activated through either recognition of antigen via the B-cell receptor (BCR) or through toll-like receptors (TLRs), which recognize pathogen associated molecular patterns (PAMPs) typically found on microbes[129]. After activation, B cells undergo proliferation and differentiation into antibody-secreting plasma cells or memory B cells that have a lower threshold of activation to mount a response[130]. Though only a low proportion of TME cellular infiltrate, B cells play critical roles in complement-mediated immunity and antigen presentation for T cells, and the generation of tertiary lymphoid structures (TLS)[131-134]. Further, plasma cells, which derive from B-cell progenitors, play

a major role in cytokine (including effector cytokine TNF- α) and antibody production[135, 136]. In the context of the tumor, antibodies are useful in mediating antibody dependent cell cytotoxicity (ADCC), whereby antibodies will bind to the antigen on the tumor cell, NK cells will recognize these bound antibodies via their Fc receptor CD16, and NK-mediated cell lysis will ensue via cytokine or lytic granule release[137, 138]. The importance of humoral immunity in tumor control is evidenced by multiple reports showing that detection of tumor-associated antigen specific antibodies in serum, such as anti-MUC1, correlated with improved patient prognosis in multiple cancer types, including pancreatic, breast, gastric, lung, and ovarian cancers[139-142]. Also, there is a prognostic association between B-cell and CD8 T-cell co-existence in multiple cancer types, including melanoma, breast, and ovarian cancers[143-145]. B cells may form TLS within the tumor tissue It has been recently demonstrated that the presence of these TLS are associated with patient response to immune checkpoint blockade therapy (described later in this thesis) in metastatic melanoma and sarcoma[146-148]. Recently, it has also been shown that response to immune checkpoint blockade in melanoma is dependent on B cells through not only TLS formation, but promotion of PD-1 expression on T cells, leading to improved response to anti-PD-1 therapy[149]. However, due to the main inflammatory role of B cells in cancers by promoting tumor clearance through antigen presentation and recruitment of plasma cells to the TME via CCL28 secretion, they also can cause disruption by inflammation or degradation by matrix metalloproteinases (MMPs) of the ECM, thereby promoting enhanced tumorigenesis[133, 136, 150, 151]. A subtype of B-cell, known as B regulatory cells (B_{reg}), are also known suppressors of the immune component of the TME through the secretion of IL-10 and IL-35 which inhibit T cells and can cause conversion to the suppressive T_{reg} subset[152, 153]. B_{regs} are also associated with worse prognosis in some cancer types, including gastric cancer[154]. There is still relatively much unknown about

these cells and analysis is still ongoing to determine more immunosuppressive mechanisms B_{regs} utilize to promote tumor progression.

1.1.1.2.7. **Granulocytes**- There are 3 main types of granulocytes: neutrophils, basophils, and eosinophils[13]. These cell types are introduced below.

1.1.1.2.7.1. *Neutrophils* are granulocytic cells that arrive typically first to the site of infection and trigger an inflammatory response[13]. They are the most prevalent white blood cell in the blood and upon identification of a pathogen, can kill the cell via release of their granules containing proteases and antimicrobial peptides extracellularly or in a phagosome (organelle that contains phagocytosed material)[155]. Neutrophils identify pathogens not through antigen presentation, but through a process of opsonization[13]. In this process, a cell covered with antibodies or complement proteins are recognized by the receptor on the neutrophil and trigger cytotoxicity[13]. In the context of the tumor microenvironment, however, there is a current focus on the prognostic value of the neutrophil-to-lymphocyte ratio (NLR) in the pre-treatment blood of cancer patients, which is associated with poorer survival in the following cancer types: breast, colorectal, esophageal, hepatocellular, melanoma, pancreatic, and prostate[156].

In this section, I will be specifically focusing on tumor associated neutrophils (TANs). There is some data that supports the anti-tumor role of TANs, including their production of soluble TRAIL (sTRAIL), which promotes tumor cell apoptosis[157]. However, due to their strong inflammatory response, most studies support their ability to promote tumorigenesis due to disruption of the ECM and causing tumor promoting epithelial damage with the secretion of ROS[158]. Neutrophils, like many cells in the TME, release ROS inhibiting many immune cell types including T cells; they also secrete soluble factors like BV8 and MMP9 that promote angiogenesis[159-161]. TANs also play a key role in tumor metastasis, predominantly through neutrophil extracellular traps (NETs)[162]. NETs

are comprised of extracellular chromatin and antimicrobial proteins that surround the pathogen for clearance; however, data has shown that these NETs also trap circulating cancer cells, allowing for their dispersion to different tissues, ultimately leading to metastasis[162].

1.1.1.2.7.2. *Eosinophils* are a relatively minor cell type in the blood, representing only 1% of white blood cells[163]. They are known predominantly for their involvement in allergy and the cytokine IL-5 typically determines their activation and proliferation; IL-5 can be produced by tumor cells, recruiting eosinophils to the TME[164, 165]. The impact of eosinophils is mainly attributed to their ability to release cytokines (including IL-5 and TNF α) and chemokines (CXCL9 and CXCL10 recruit Th1 cells, for example) that influence the surrounding cell types, causing T-cell polarization (described later) and cellular recruitment. Eosinophils are most often associated with improved prognosis in solid cancer types, including colorectal and gastric cancers[166, 167]. However, they are associated with poor prognosis based on infiltration in cervical cancer and Hodgkin's lymphoma[168, 169].

1.1.1.2.7.3. *Basophils*, the last main granulocytic cell type, are the least prevalent, representing less than 0.5% of the immune cells of the blood. Their function is also typically associated with allergy and they are major producers of the cytokine IL-4, which is necessary for the development of a specific T-cell subtype, Th2 (described in detail later)[170]. In cancer, basophilia is often experienced in patients with hematologic malignancies, including acute myeloid leukemia and chronic myeloid leukemia[171, 172]. In mice bearing melanoma tumors, it has also been shown that basophils function to produce chemoattractants that lead to CD8 T-cell recruitment (CCL3 and CCL4), allowing for improved tumor control[173].

1.1.1.2.8. ***T cells***- T cells play a vital role in tumor regression, and are often a main target of immunosuppression by the tumor. Due to their importance and the focus of this thesis, I

will utilize the following subsection to define the subsets of T cells and address in depth the role of T cells in the immune response in the TME.

1.2 T-cell subsets, activation, and differentiation

Although the TME is made up of a milieu of cells, T cells have been shown to play an important role in anti-tumor response but are subject to immunosuppression at the tumor site[174]. These cells play a critical role in the adaptive anti-tumor immune response (described in previous section) through recognizing peptides presented by specific molecules known as MHC on tumor cell membranes, secreting cytokines and chemokines for cellular recruitment, or releasing lytic granules to trigger targeted cellular cytotoxicity[13]. A number of different T-cell subsets, performing distinct functions, have been described and will be detailed in this section.

1.2.1 *T-cell receptor and $\gamma\delta$ T cells*

T cells are classified into two major subsets; $\alpha\beta$ or $\gamma\delta$, indicative of the chains comprising the T-cell receptor (TCR) of these cells (e.g., $\alpha\beta$ T cells have an α chain and β chain)[175]. The TCR is a transmembrane receptor that contains highly variable regions (hypervariable regions), causing diversity of TCRs between T cells and allowing for specific recognition of complexed antigen presented by the MHC molecule (this is detailed later in this section)[130]. The TCR also is comprised of the signal transducing units CD3 γ , CD3 δ , CD3 ϵ , and a dimer of ζ chains, which allow for signal transduction, as shown in Figure 1[176]. $\gamma\delta$ T cells account for only 0.5-5% of all T lymphocytes[177]. Unlike $\alpha\beta$ T cells, $\gamma\delta$ T cells are typically found in epithelial tissues, particularly in the gut mucosa[178]. Even more distinctive, these cells utilize different receptors rather than MHCI or MHCII for antigen recognition; for example, recognition of lipids occurs through the MHC-like molecule CD1d[179]. Therefore, $\gamma\delta$ T cells recognize many self and non-self antigens, including phospholipids, prenyl phosphates, and sulfatides[180]. Notably, $\gamma\delta$ T cells in humans are also often responsive to molecules like heat shock

proteins via recognition by TLRs or NK receptors[181]. For example, upon recognition of phycoerythrin peptide, an algae derived B-cell antigen, $\gamma\delta$ T cells secrete IL-17 indicating antigen recognition and triggering an inflammatory response[182, 183]. Notably, there are three subtypes of $\gamma\delta$ T cells in humans, as defined by their δ chain, $\gamma\delta 1$, $\gamma\delta 2$, and $\gamma\delta 2$ negative (non $\gamma\delta 1$)[184]. Due to the nature of this thesis, I will focus on their implications in cancer. In the context of response to tumor, the role of the subtypes of $\gamma\delta$ T cells in promoting or suppressing tumor development is variable between cancer types; therefore, $\gamma\delta$ T cells can be divided into two major classes, effectors and regulators, in the context of the TME[185]. Effectors can aid in tumor cell ADCC and release cytokines like IFN γ and TNF α to promote an inflammatory response[185]. These effector $\gamma\delta$ T cells can also directly kill tumor cells via perforin and TNF α release through activation by costimulatory molecule NKG2D that recognize stress ligands upregulated on the tumor cell[186]. Regulators, however, can attract MDSCs via IL-17 secretion and release inhibitory cytokines like IL-10 and TGF β [177, 187]. Further, the secretion of IL-17A by $\gamma\delta$ T cells is correlated with tumor promotion in ovarian and hepatocellular carcinomas[188, 189].

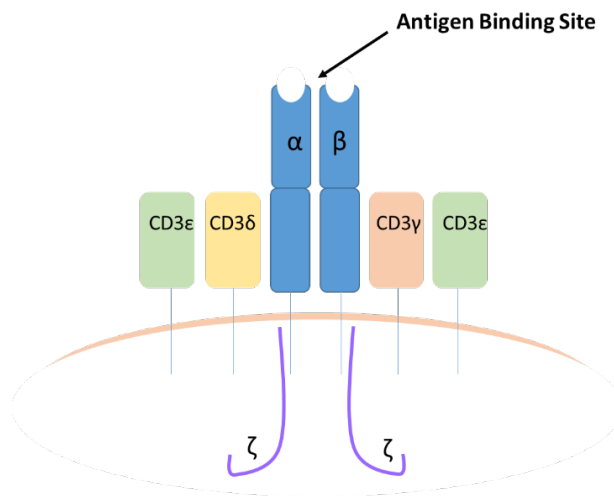


Figure 1. T-cell Receptor (TCR).

The TCR is comprised of $\alpha\beta$ or $\gamma\delta$ chains, the signal transducing units CD3 γ , CD3 δ , CD3 ϵ , and a dimer of ζ chains.

The majority of T cells express the $\alpha\beta$ TCR[13]. $\alpha\beta$ T cells can broadly be classified by either CD4 co-receptor presence or CD8 co-receptor presence[13]. The function of these co-receptors and the differences between these cells will be detailed further in the following subsections.

1.2.2 CD4 T cells

CD4 T cells are known to be the “helper” T cells. Upon activation, CD4 T cells can release cytokines to elicit an inflammatory response, aid in the recruitment of immune cells including APCs, and promote B-cell function and antibody secretion[190]. To perform a wide range of immune functions, there are multiple subtypes of CD4 T cells as shown in Figure 2[191].

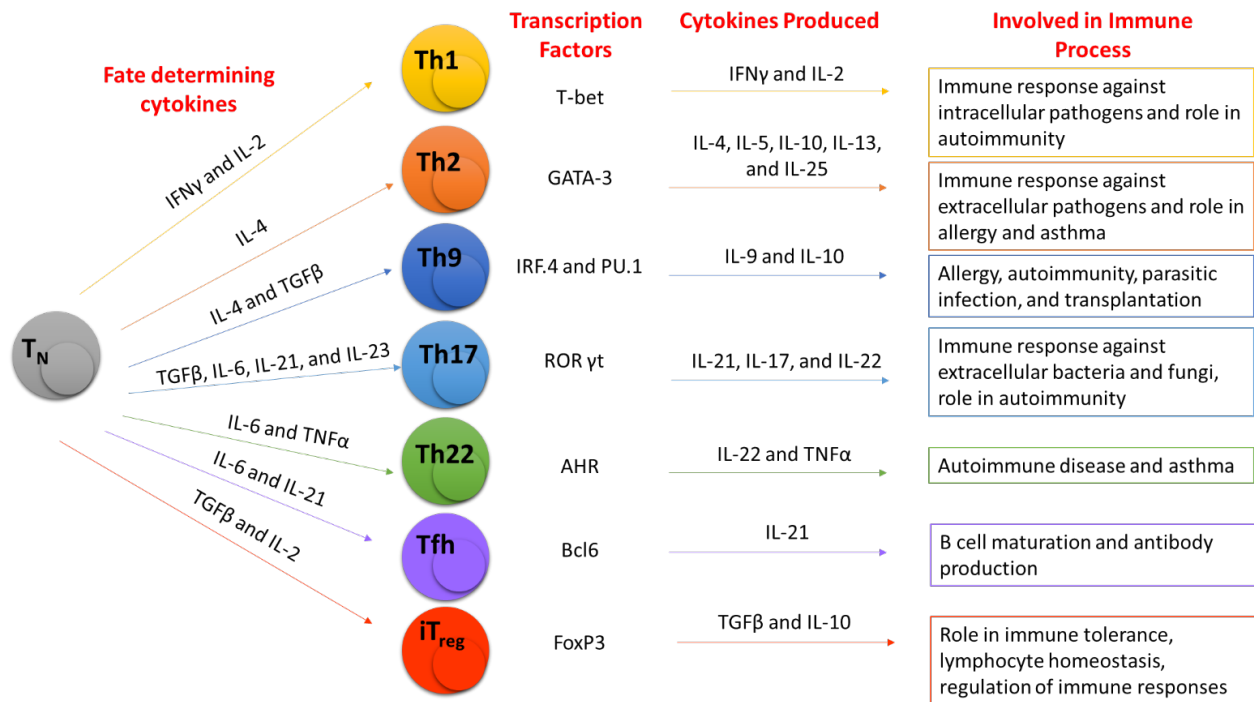


Figure 2. CD4 T-cell lineages in humans.

A naïve CD4 T-cell will polarize into different subtypes with specific immune response functions based on the cytokines present in the environment and transcription factors utilized during cellular activation.

1.2.2.1 CD4 T-cell activation: The lineage polarization of CD4 T cells is dictated by cytokines in the surrounding environment and transcription factors during activation[190] (Figure 2). As depicted in Figure 3, CD4 T-cell activation occurs when a naïve CD4 T-cell encounters an antigen presenting cell expressing the cognate peptide in the groove of the MHCII molecule, which provides concomitant engagement of a co-activating receptor, and releases cytokines binding to receptors on T cells, leading to transcription[192]. The first step, TCR recognition of an antigen in the context of MHCII antigen presentation, triggering signal transduction

downstream of TCR as a result of antigen recognition is considered Signal 1 of 3 of CD4 T-cell activation[192]. The process of MHCII presentation is initiated when the MHCII molecule is shuttled from where it is generated in the Endoplasmic Reticulum (ER), loaded with a 13-25 amino acid extracellular protein derived peptide in an endosome containing proteases, and presented on the membrane of the APC[193]. Peptides that can elicit an immune response are referred to as antigens. The TCR recognizes the peptide in the groove of the MHCII molecule on the surface of the APC, with the CD4 co-receptor enhancing this interaction through the recruitment of TCR signaling molecule Lck[194]. TCR signal alone is not sufficient for proper T cell activation. Signal 2 potentiates the TCR signaling and is generated by the binding of activating co-receptors[195]. Most commonly, CD28 co-receptor on the surface of T cells binds to either CD80 or CD86 on the APC, but there are other co-stimulatory receptors including OX-40 and ICOS that CD4 T cells can utilize, which will be described later in this thesis[196]. The last signal, Signal 3, is cytokine signaling, whereby the cytokines released by the APCs like DCs or macrophages help to determine the fate of these CD4 T cells (see Figure 2, Fate determining cytokines)[197]. Notably, other cytokines can also play roles in CD4 T-cell function and proliferation during activation; for example, IL-1 has also been demonstrated to help in cellular expansion and differentiation[198]. Once these cytokines produced by the APCs (fate determining cytokines, Figure 2) bind to their corresponding receptors on the CD4 T-cell membrane, a signaling cascade occurs mediated by the JAK-STAT pathway, leading to the expression of specific transcription factors defining the lineage polarization (Figure 2)[199]. These transcription factors then can cause specific cytokine production by the T cell (cytokines produced, Figure 2) that thereby lead to different immune functional specialization (Involved in Immune Process, Figure 2). The importance of these three signals and their order cannot be understated; for example if signal 2 is missed, a cell will become anergic or lack functional activation[200].

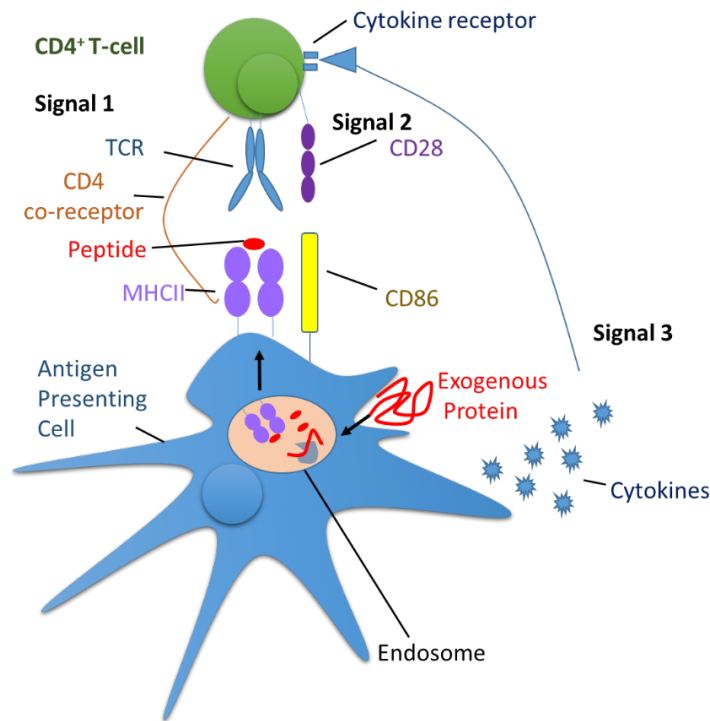


Figure 3. CD4⁺ T-cell Activation. Cellular activation is based on 3 signals: (1) TCR recognition of the MHCII complex and endogenous peptide (2) Ligation of costimulatory ligands (binding of CD28 and CD86; also could bind CD27 or CD80 which are not shown) (3) Release of cytokines by APCs impacting the T-cell.

1.2.2.2 T helper type 1 cells (Th1): Th1 cells are those that are generated by the fate determining cytokines IFN γ and IL-2, produce these same cytokines after activation, and are involved in the immune response to intracellular pathogens[201]. In the context of the TME, Th1 cells are considered to actively participate in anti-tumor response, mainly due to their secretion of IFN γ [202]. The cytokine IFN γ can cause tumor cell death particularly in synergy with TNF α , in several ways; upregulation of MHCI on tumor cells allowing for recognition by CD8 T cells leading to tumor cell death, expression of IP10 which is an inhibitory chemokine of angiogenesis, or recruitment of macrophages that provide enhanced tumor antigen presentation to T cells[203-206]. In fact, loss of IFN γ signaling in tumor has been identified as a resistance mechanism to immunotherapy[207]. However, it should be mentioned that IFN γ can actually be immunosuppressive at higher dosages, leading to the upregulation of the T-cell inhibitory molecules PD-L1 on tumors and PD-L2 on macrophages (described in depth in the Immune Checkpoint Inhibitors Section) and the production of IDO which degrades tryptophan that is essential for T cells[208].

1.2.2.3 **T follicular helper cells (Tfh)**: Another subset, T follicular helper (Tfh) cells, are typically also associated with tumor control, as they elicit a B-cell mediated response to the tumor[209]. These cells are often found in germinal centers, interacting with B cells and through this interaction, are credited with aiding to generate antibodies by plasma cells[210]. Tumors can have germinal center like-structures, known as TLS, which harbor B cells and their presence is often associated with positive prognosis in cancer (breast, ovarian, prostate, colorectal, etc.)[211]. Further, Tfh cells in colorectal cancer have been shown to help CD8 function through the production of IL-21, a cytokine that particularly functions in CD8 memory T-cell generation[212, 213]. Positive correlations between the presence of Tfh cells in tumor and survival in breast cancer has been reported[214]. Like Tfh and Th1 cells, the most recently distinguished Th9 subset is also associated with tumor control[215]. Th9 cells can generate an inflammatory response through secretion of IL-9, which can recruit immune cells into the tumor[216]. Adoptive transfer studies in mouse models with B16F10 melanoma tumors indicate that the Th9 cells were superior in inhibiting tumor growth due to the production of IL-9, as compared to Th1, Th2, and Th17 cells[217]. This could be explained further as IL-9 secreted by Th9 cells can increase the infiltration of CD8 T cells and DCs via CCL20 in a mouse melanoma model[215].

1.2.2.4 **T helper type 2 cells (Th2)**: Th2 cells are known for their function in helminth immunity and allergic reactions, particularly with their secretion of IL-4, IL-5, and IL-13 (Figure 2)[218]. Unlike Th1, Tfh, and Th9 cells, the role of Th2 cells within the TME tends to vary based on tumor type[219]. Through IL-4 secretion, Th2 cells tend to suppress the tumor via eosinophil recruitment, and in mouse models, have been shown to promote eosinophilic mediated tumor regression in B16 metastatic melanoma that was resistant to cytotoxic T lymphocyte (CTL) mediated killing [220, 221]. Further, these Th2 cells help to recruit macrophages to the tumor through the production of IL-4 and IL-13; Th2 cells also help to promote the polarization of M2

macrophages[83, 222]. However, data has also implicated Th2 cells in the promotion of tumorigenesis in pancreatic cancer and the promotion of myeloma cell growth[223-225]. Also, IL-4 secreted by Th2 cells has been shown to induce resistance to apoptosis in tumor cells[226].

1.2.2.5 **Th17 cells:** Like Th2 cells, the role of Th17 cells in the TME is a bit more difficult to distinguish, as the type of cancer often indicates whether the cytokine IL-17 is pro- or anti-tumorigenic[227]. Th17 cells are typically associated with extracellular bacteria immunity through the production of IL-17 (Figure 2)[228]. In cancer, Th17 cellular infiltration is associated with positive prognostic outcomes in ovarian cancer, which is attributed to IL-17 production and its upregulation of CXCL9 and CXCL10 to recruit cytotoxic cells[229]. In contrast, Th17 infiltration is associated with poor prognosis in hepatocellular carcinoma, pancreatic carcinoma, and colorectal carcinoma[230-232]. Further, the role of IL-17 is dualistic in the cancer setting. IL-17 is pro-angiogenic, as demonstrated by increased vasculature of IL-17 positive ovarian tumors and through its promotion of the enhanced secretion of VEGF[233, 234]. However, IL-17 has been shown to enhance IFN γ production of CD8 and CD4 T cells in ovarian cancers and, in fibrosarcoma tumor lines expressing IL-17, there is enhanced T-cell mediated memory clearance of the tumor[229, 235].

1.2.2.6 **Regulatory T cells (T_{regs}):** The final two CD4 T-cell subtypes, T_{regs} and Th22 are known to be immunosuppressive. T_{regs} are probably the most well-known immunosuppressive T-cell. There are 2 types of T_{regs} , the induced T_{regs} (iT_{regs}) and natural T_{regs} (nT_{regs})[236]. nT_{regs} develop their lineage in the thymus, while iT_{regs} polarize from a naïve CD4 T-cell during activation; both subsets have generally the same immunosuppressive mechanisms, including the expression of CTLA-4[236]. T_{regs} also suppress APC function through CTLA-4, consume IL-2 needed for other T cells for survival, and can secrete granzyme and perforin to kill effector cells[237-239]. T_{regs} constitutively express the inhibitory molecule CTLA-4, which competitively binds to CD80 and CD86 on APCs[240]. This causes a direction competition between ligation with inhibitory T_{regs} or

effector T cells that would normally lead to antigen presentation, cellular activation, and memory formation. T_{regs} also secrete IL-10 and TGF β which are conventionally considered to be immunosuppressive cytokines. For example, IL-10 is known to inhibit CD4 T-cell proliferation through the suppression of antigen presentation in macrophages and dendritic cells[241]. Similarly, TGF β secreted by the T_{regs} can inhibit the function of CD8 cytotoxic T cells, other CD4 subtypes, APCs, and NK cells; it also supports the subtype polarization of CD4 to iT $_{\text{regs}}$ [242, 243]. In CD8 T cells, TGF β inhibits the production of effector molecules, including IFN γ and granzyme B, through transcriptional repression[244]. Further, TGF β has been demonstrated to inhibit the expression of CXCR3, thereby reducing CD8 T-cell infiltration into the tumor[245].

1.2.2.7 Th22 cells: The other immunosuppressive CD4 cell subset, Th22 is often associated with autoimmune disease, specifically of the skin due to the expression of the chemokine receptors CCR4 and CCR10[246]. In cancer biology, Th22 cells, through the secretion of IL-22, have been associated with tumor progression in lung cancer, gastric cancer, hepatocellular carcinoma, and colon cancer[246]. This link to enhanced tumor progression is supported by data that shows IL-22 promotes tumor cell proliferation and cellular invasion in gastric cancer through the IL-22R/AKT signaling pathway in gastric tumor cells[247]. Further, IL-22 protects tumor cells from apoptosis through the upregulation of the BCL-XL pathway in lung cancer and pancreatic cancer[248].

Due to the ability of the CD4 T-cell subtypes to elicit immunosuppression, tumors will often hijack these mechanisms for immune escape[249]. Immunosuppressive cells like Th22 and especially T_{regs} are often recruited to the TME where they inhibit the function of the other cell types[250]. For example, pancreatic cancer will secrete the chemokine CCL5 which recruits T_{regs} via their chemokine receptor CCR5[251]. This chemokine mediated recruitment occurs in other cancer types as well, as CCL28 expression by liver and ovarian cancer recruits T_{regs} via their chemokine receptor CCR10[252].

Tumor cells also can secrete TGF β , causing the polarization of CD4 T cells to the iT_{reg} subtype[24].

Further, CAFs can help to degrade T-bet by releasing lactate, switching the predominant CD4 T-cell phenotype in the TME from an anti-tumor Th1 to an immunosuppressive T_{reg} population[253]. Other mechanisms of immune escape, particularly the upregulation of checkpoint molecules will be detailed in the next section, as CD8 cytotoxic T cells are considered the main mediators of immune effector functions in tumors.

1.2.3 CD8 T cells

The other $\alpha\beta$ T-cell phenotype, CD8 T cells, are known to be cytotoxic, as they contain lytic granules of granzymes and perforin[13]. Release of these granules occurs upon antigen recognition and activation through the TCR[13]. Perforin will then poke holes in the target cell causing cellular death, while granzyme b is a protease that triggers a signaling cascade causing DNA degradation, ultimately leading to apoptosis[13]. These cells also have the ability to cause Fas-FasL mediated apoptosis, as binding of the expressed T-cell FasL to the corresponding Fas triggers a caspase cascade in the target[254]. CD8 T cells can also use cytokine mediated killing through the production of IFN γ and TNF α [13]. Notably, the ability of CD8 T cells to kill is dependent on cellular activation.

1.2.3.1 CD8 T-cell activation: Like CD4 T cells, naïve CD8 T-cell activation requires the same 3 signals. A key difference in CD8 T-cell activation is that rather than presentation of peptide via MHCII restricted to professional APCs, the CD8 T-cell TCR recognizes the MHCI molecule found on all nucleated cells (Figure 4)[255]. MHCI also differs in the type of peptides that it presents, as in a classical model, it presents endogenous protein derived peptides that are 8-10 amino acids in length[256]. These proteins are degraded in the proteasome to become peptides, loaded onto the MHCI molecule in the ER, and then shuttled to the surface of the cell[257]. However, these cells can also recognize exogenous peptide using the process of cross-presentation, in which exogenous peptide is taken up by the cell and presented via the MHCI molecule[257]. In both

scenarios, the CD8 co-receptor helps to stabilize the interaction between the TCR and the MHC I molecule[194]. Like Signal 2 for CD4 T cells, the signal 2 for CD8 T cells is also typically CD28 ligation to CD80/CD86 found on macrophages and dendritic cells (Martin 1986). Notably, there are numerous other co-stimulatory receptors on T cells, including 4-1BB and OX40, which will be revisited later in this thesis. Signal 3 for CD8 T cells is often IL-12 or IFN- α/β [197, 258]. IL-12 is produced by CD141 DCs that are considered optimal CD8 T-cell primers, while type I IFNs are often produced during viral infections by most immune cell types[258, 259]. Similar to CD4 T cells, the duration of TCR stimulation and cytokine presence during activation will determine the fate of the CD8 T-cell, whether these cells undergo proliferation, develop memory, or become anergic[258].

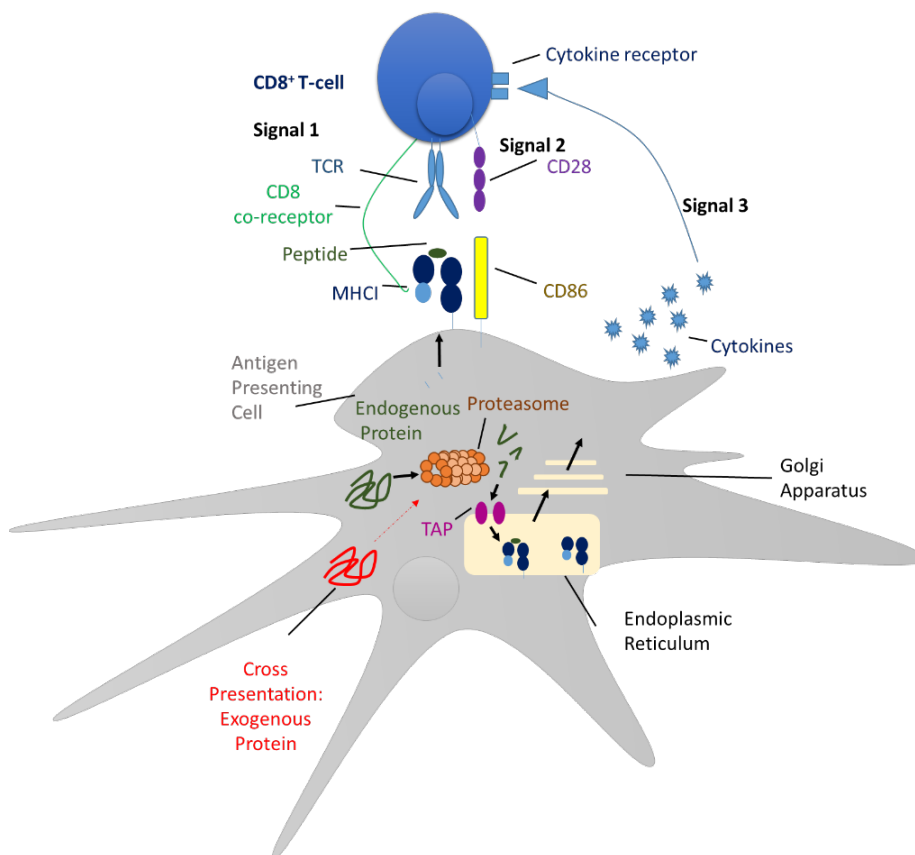


Figure 4. CD8⁺ T-cell Activation. Cellular activation is based on 3 signals: (1) TCR recognition of the MHC I complex and endogenous peptide (2) Ligation of costimulatory ligands (binding of CD28 and CD86; also could bind CD27 or CD80 which are not shown) (3) Release of cytokines by APCs impacting the T-cell. These cells also have the ability to process exogenous antigen through cross-presentation, denoted in red.

1.2.3.2 T-cell differentiation: CD8 T cells are typically divided into the 3 main subsets dependent upon activation and differentiation: naïve, effector, and memory[260]. As briefly described, naïve

cells are those that have not been activated or encountered antigen. After leaving the thymus, these cells circulate in the blood, lymph nodes, and secondary lymphoid tissues including the spleen surveying for the APC presenting the proper peptide that will stimulate their TCR[261]. Only one in 100,000-1,000,000 naïve T cells have a TCR with the proper affinity for their corresponding antigen leading to activation[261]. Once activated as described above, these cells then acquire effector functions and proliferate up to 10,000 fold in the lymph node[262]. The activated CD8 T cells then recirculate to the peripheral tissues to recognize their antigen[261]. Effector cells are highly cytotoxic, with larger cellular proportions of perforin and granzyme granules[263]. After response to antigen, the effector cells undergo a stage of contraction, whereby 95% of these cells undergo apoptosis[264]. The remaining cells differentiate into memory cells, which are long-lived cells that can pose a quick, highly specific response to antigen re-encounter[260, 264]. The ability of memory cells to elicit a strong, rapid response to a previously encountered antigen with a lower threshold of activation has significant importance and is the basis for immunization response[265]. Notably, the process of memory development also occurs in CD4 T cells in a similar fashion for each cellular subtype[266].

The states of cellular differentiation are often distinguished by surface markers (CD45RA, CD45RO, CCR7, CD28, and CD27), activation state, and function[263]. CCR7 is constitutively expressed on naïve cells and allows for recirculation of T cells into lymphoid organs. CD45RO is representative of antigen experience, while CD45RA is representative of antigen inexperience[267]. The initial distinction of cells using CD45RA/CD45RO and CCR7 can distinguish 4 main cell types: terminally differentiated cells (T_{emra} ; CD45RA⁺CCR7⁻), effector memory cells (T_{em} ; CD45RA⁻CCR7⁻), central memory cells (T_{cm} ; CD45RA⁻CCR7⁺), and naïve cells (T_N ; CD45RA⁺CCR7⁺)[268]. The characterization of these cell types are described in Figure 5. T_{cm} cells are often found within the lymphoid tissues due to their expression of CCR7 and act as a

reserve of cells rather than serving an effector function[269]. They are useful in that they generate IL-2 and after proliferation, can produce large amounts of IL-4 and IFN γ [269]. T_{em} cells are typically found within peripheral tissues and have perforin to elicit a quick effector response[269]. Further divisions of T_{em} function can be made based on the expression of the costimulatory molecules CD27 and CD28 (Figure 5)[268]. T_{emra} cells have experienced antigen and undergo homing to the peripheral tissues, but are highly sensitive to apoptosis; these cells are often called terminally differentiated[270]. T_{emra} cells are often found in the blood and have the ability to secrete Th1 type cytokines, but quickly die after[271]. As we age, we accumulate these T_{emra} cells, which could attribute to the susceptibility of the elderly to common infections[272]. Two other main divisions of memory cells, stem cell like memory (T_{scm}) and resident memory cells (T_{rm}), can also be classified based on receptor expression. T_{scm} cells express the same receptors as T_N cells (CD45RA⁺CCR7⁺), but express additional receptors for tissue trafficking like CXCR3 and cytokine signaling like IL2R β (others markers also expressed include: CD95, CD11a, CD58)[273]. These cells have the distinct ability to self-renew and can develop into all memory subtypes and effector cells[273]. T_{rm} cells functionally resemble T_{em}; however due to their tissue residence, often lead to a more rapid immune response[274]. Thus, as their name indicates, they are non-circulating T cells involved with memory responses generated in specific tissues; however, recent data has suggested that these cells do have the ability to circulate and regain a T_{rm} phenotype[275]. T_{rm} cells are often defined by CD69, depicting activation, and CD103, indicating tissue specificity[274]. T_{rm} cells develop from the same precursor cells as T_{em} and T_{cm} cells, but become T_{rm} due to their migration to their periphery, gaining CD69 and CD103 expression, combined with exposure to the cytokines TGF β and IL-15[276]. Notably, T_{rm} cells may be a novel target for tumor immunotherapy, as their presence in tumor tissue has been linked with improved prognosis in breast and lung cancers[277, 278]. Further, T_{rm} cells as defined by Dr. Weinberg's group as CD39⁺CD103⁺ T cells

(often also co-express CD69; confirmed phenotype through microarray analysis) were identified in the following cancers: melanoma, lung cancer, ovarian cancer, colon cancer, rectal cancer, and head and neck squamous cell carcinoma (HNSCC)[279]. T_{rm} cells are also shown to have enriched tumor reactivity in melanoma and HNSCC; thus, harnessing the power of these T_{rm} cells could provide the next step in immunotherapy[279].

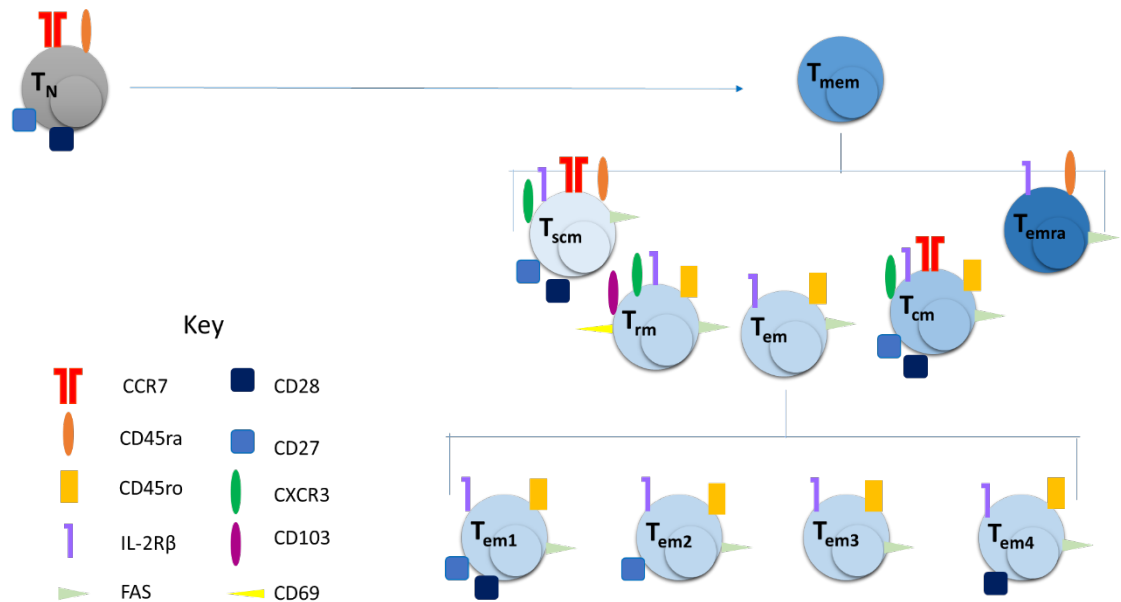


Figure 5. Memory subsets in CD8 T cells.

Receptor expression helps to differentiate the status of memory CD8 T-cell subsets. Minimal molecules are depicted as others can be used to identify these subsets, including IL-7Rα, CXCR3, CD95, CD11a, CD58, and CD57.

As described above, co-stimulatory molecules can also help to differentiate the status of T-cell subsets. Like the CD27 and CD28 activating receptors, there are also inhibitory receptors on the T-cell, which include but are not restricted to PD-1, Tim-3, Lag-3, and CTLA-4. Inhibitory receptors can outcompete binding to the activating ligand or can contain inhibitory domains (ITSM or ITIM) that effect the early downstream signaling events in T-cell activation[280]. These inhibitory receptors are upregulated on the T-cell surface during cellular differentiation and activation, with specific receptors upregulated at different time points post-

activation[281]. For example, CTLA-4 (within the first 4 hours, but cycling expression after) and PD-1 (within the first 24 hours, but sustained expression after) are upregulated in the early stages of activation, whereas Tim-3 and Lag-3 are upregulated slightly later (within 48 hours)[282, 283]. Initially, this upregulation was thought to be due to exhaustion, which was first illustrated in chronic viral infection models[284]. Rather than depicting exhaustion, the upregulation of these molecules may naturally occur to balance the upregulation of activating receptors like 4-1BB occurring concurrently or to induce contraction after pathogen clearance with memory established[281]. The immune system therefore has a naturally developed system whereby inhibition occurs when a response needs to be stopped after antigen clearance; otherwise, there would be a potential for autoimmune activity[285]. As most T cells within the tumor have encountered antigen, they have some type of upregulation of these inhibitory receptors. Due to the ability of these markers to either promote or inhibit T-cell function, these checkpoint molecules on CD4 and CD8 T cells are essential targets for tumor immune evasion. I will revisit and describe some of these inhibitory receptors in more depth in the subsequent section of this thesis.

The importance of T cells in solid tumor immunity cannot be overlooked, with the correlation of infiltration associated with improved prognosis in most solid tumor types[286]. As reviewed above, T cells come in different flavors. While the role of T cells in clearing infections is well understood, there is much to learn about how the tumor microenvironment affects T-cell function and on how to overcome these functional impairments. Understanding the fundamental functions and the interactions of T cells with components of the TME is necessary to harness their ability to be used as a curative mechanism for cancer therapeutics. This knowledge can thereby be applied to the ever-growing field of tumor immunotherapy, which will be introduced in the next section.

1.3 Immunotherapy of Cancer

Immunotherapy is defined by the National Cancer Institute (NCI) as a substance that inhibits or stimulates the immune response to fight cancer, infection, or other disease[16]. The rise in the field of immunotherapy for the treatment of cancer has continued since the pioneering work by Dr. William Bradley Coley in 1891, whereby he injected pathogenic bacteria (later termed Coley's toxins) into patients with long bone sarcomas, yielding regression by activating their immune system[287]. The use of these toxins as a non-specific method to promote tumor regression through activation of a generalized immune response, were based on the observation of infections being beneficial to cancer patients and the initial work of Dr. Bruns in 1888, who injected the bacteria causing erysipelas and demonstrated tumor shrinkage in his patient[287]. The use of Coley's Toxins have continued over the centuries, with a Phase I trial occurring as recently as 2012 for the treatment of NY-ESO-1 expressing cancers[288]. Work and discovery was almost stymied until the 1950s until the discovery of IFNs and the proposed hypothesis of cancer immunosurveillance (described in the TME section)[3, 289]. The idea of immunotherapy then was given more traction combined with new discovery and novel therapies, including the finding that the infusion of a T-cell growth factor, IL-2, could lead to tumor eradication in 1976 and its continued use as a cancer treatment since the 1980s[290]. Further, studies utilizing the infusion of a patient's expanded *ex vivo* autologous T cells began in 1988, which has evolved into other iterations of therapy, including chimeric antigen receptor (CAR) T cells and endogenous T-cell (ETC) therapy (described in the Cellular Based Therapy section in detail later)[291]. A series of discoveries on T-cell function and the demonstration of the regulation of T-cell activation through costimulatory and co-inhibitory signals have recently culminated in the work by Drs. James Allison and Tasuku Honjo in specifically immune checkpoint blockade (described later) earning the Nobel Prize in Physiology and Medicine in 2018[291]. In this next section, I will describe three main types of immunotherapies for cancer treatment: checkpoint inhibitors, agonistic antibodies, and cellular based therapies. Notably, these sections will be introduced in the order of their clinical resurgence rather than discovery, as checkpoint inhibitors will be presented before agonistic antibodies.

1.3.1 Immune checkpoint Inhibitors (ICIs)

The term “immune checkpoint inhibitors” (ICIs) encompasses antagonist antibodies for negative checkpoint molecules expressed on immune cells, including T cells (like PD-1, CTLA-4, TIGIT, Tim-3, etc.), and tumor cells (like PD-L1; see Figure 6 for examples that will be discussed in detail later). When targeted, ICIs can release the optimal killing potential of the T-cell[282]. However, in the context of viral infection, ICIs are often upregulated upon cellular activation, effectively shutting down the immune response once antigen is recognized and cleared[292]. In disease-free conditions, negative molecules play an important role in preventing autoimmunity or aberrant self-recognition; for example, the first discovered ICI, CTLA-4, prevents the Signal 2 of activation of self-reactive T cells that could cause autoimmune disease[293, 294]. However, CTLA-4 was initially reported to be a Signal 2 molecule, improving T-cell activation, due to its distinctly higher affinity to CD80/CD86 expressed on APCs than its homologous activating receptor CD28[295, 296]. Ultimately, work by Dr. Jim Allison disproved this observation by demonstrating an inhibitory function of the molecule through the use of an anti-CTLA-4 antagonist antibody, which led to improved T-cell proliferation and IL-2 cytokine production[296, 297]. However, following Dr. Allison’s discovery, efforts were deployed to understand the expression and kinetics of CTLA-4 on T cells to determine by what mechanism CTLA-4 was impacting the T-cell function.

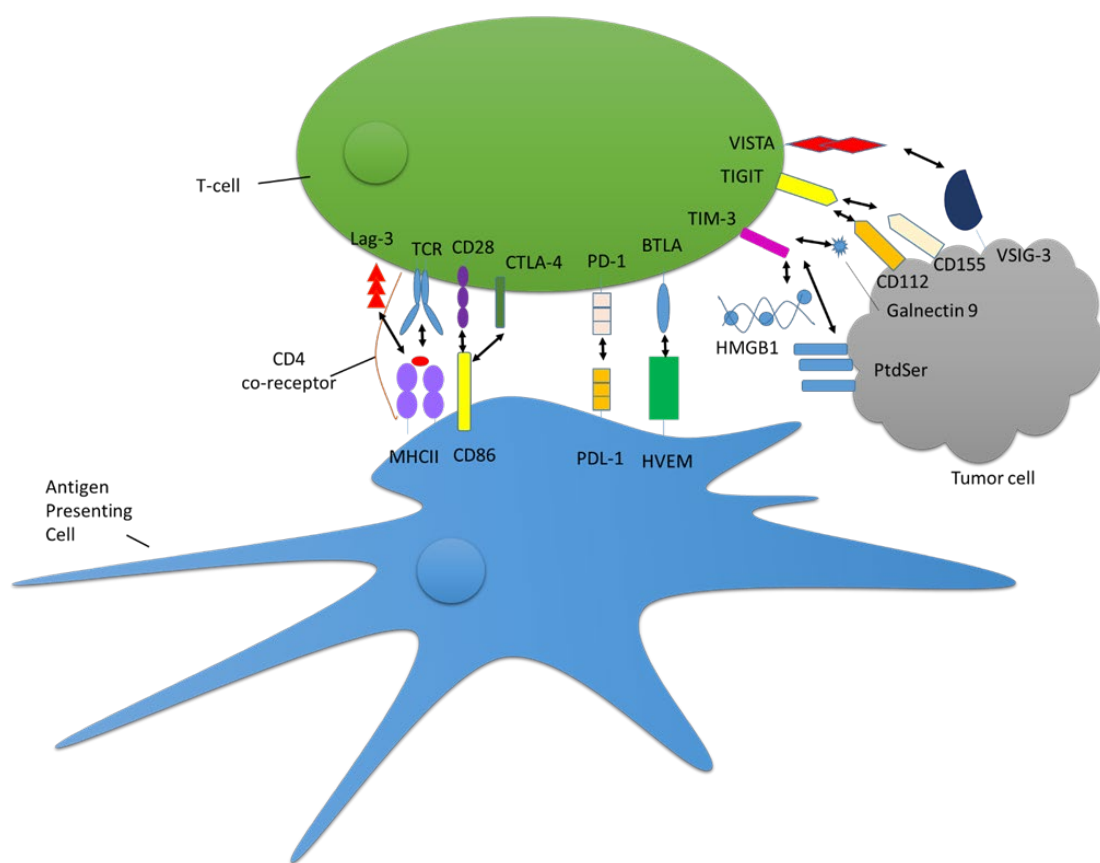


Figure 6. Checkpoint inhibitor molecules. T-cell checkpoint inhibitory receptors are upregulated to the surface of T cells, yielding inhibitory functions upon ligation with their corresponding ligands designated with arrows found on typically APC cells or tumor cells. Notably, all ligand/receptor combinations are represented in this figure.

1.3.1.1. Cytotoxic T-Lymphocyte-Associated Protein 4 (CTLA-4)

CTLA-4 is found to be mainly on T cells (also found on B cells) and is expressed on T-cell subtypes to varying degrees[298, 299]. In CD8⁺ T cells, specifically when investigating the CD8⁺ TIL, the highest surface expression of CTLA-4 is found on T_{em} cells, with under 20% observed on the surface of T_N (less than 2%), T_{cm}, or T_{emra} by flow cytometry staining[300, 301]. This is somewhat expected, as CTLA-4 surface expression on T cells is induced upon activation[296]. Not only can CTLA-4 have differential surface expression, but it also has expression in intracellular pools that must be taken into consideration[295]. CTLA-4 expression is typically intracellular post-activation with low expression on the surface (only 12% of activated cells expressing), but has a quick recycling (2 hours) mechanism to the surface, thereby allowing for pools of the molecule to quickly upregulate to the surface and provide

inhibition; this cycling of CTLA-4 occurs similarly in secondary activation[295, 302, 303]. Further, the variability of CTLA-4 surface expression and amount of expression is correlative with intensity of TCR activation, as a strong stimulus allows for improved trafficking of the intracellular CTLA-4 to the immunologic synapse[304]. CTLA-4 surface expression on T cells is also induced by presence of the cytokine IL-2 and the cytokine IFN γ produced by independently of TCR activation[300].

In comparison to CD8 $^{+}$ T cells, there is higher CTLA-4 total protein expression and specifically surface expression on CD4 $^{+}$ T cells[305]. Most naïve CD4 $^{+}$ T cells express low levels of intracellular and extracellular CTLA-4 with high levels of CTLA-4 (both intracellular and extracellularly expressed) in memory CD4 $^{+}$ T cells post-stimulation[299]. CTLA-4 expression is downregulated after 4 hours in naïve cells, but remains upregulated in memory cells[299]. Notably, the CD4 $^{+}$ T cell subset of conventional T $_{\text{regs}}$ have constitutive CTLA-4 expression due to FoxP3 mediated control of transcription[306]. These T $_{\text{regs}}$ utilizing their CTLA-4 can perform trans-endocytosis, thereby removing and degrading CD80 and CD86 off of the APC surface, leading to further inhibition of other T-cell subtypes with the lack of ligation to CD28 though lack of signal 2 co-stimulation[307]. Thus, targeting the inhibitory CTLA-4 can be beneficial two-fold: 1) allowing for CD8 $^{+}$ and CD4 $^{+}$ post-activated cells to have improved efficacy and 2) inhibiting one of the suppressive functions of T $_{\text{regs}}$, allowing for increased expression of CD80 and CD86 on APCs for ligation to CD28.

It was hypothesized that abrogation of CTLA-4 signaling may in turn allow for improved T-cell activation[297]. However, this hypothesis was challenged, as CD28 KO mice and mice with CTLA-4 blockade have very different phenotypes[295]. The blockade of CTLA-4 led to mice with severe lymphoproliferative disease and high serum immunoglobulin levels, whereas mice lacking the CD28 co-stimulatory receptor have relatively small defects in the peripheral immune response; thus, it was concluded that CTLA-4 plays a role in T-cell function rather than activation[295]. Further investigating the role of CTLA-4, in his pioneering work on the subject, Dr. Allison utilized a CTLA-4 blocking antibody on lymph node T cells from BALB/c mice and demonstrated improved T-cell proliferation and IL-2

production[297]. Dr. Allison performed a follow-up study whereby he utilized the CTLA-4 inhibiting antibody in two cancer mouse models of colon carcinoma and fibrosarcoma and witnessed reduced tumor growth and even durable tumor regression (particularly in the colon carcinoma model)[308]. These findings were eventually translated to the clinic with use of the humanized version of the antibody for treatment of metastatic melanoma cancer patients[309]. The initial studies with ipilimumab (the first anti-CTLA-4 antibody, developed by Bristol-Myers Squibb) compared patients treated with ipilimumab with or without a gp100 peptide vaccine (gp100 is a melanoma tumor antigen; for reference, tumor antigens are discussed in Section 1.1), which was used as the active control for the trial as no standard of care existed[310]. Results indicated that patient's median overall survival (OS) was significantly increased by 32 to 34% when treated with ipilimumab (10.1 months with ipilimumab vs. 6.4 months with gp100 vaccine), which has shown to be durable in patients, with a survival curve plateau occurring after 3 years of treatment[310, 311]. Further, some of these patients that did respond even survived ten years post-treatment[311]. Due to the significant increase in survival in melanoma, ipilimumab was tried as a treatment in other cancer types including renal cell carcinoma (RCC) yielding a total of 1 and 5 partial responses in two cohorts of 61 patients receiving different doses of antibody (n=21 low dose and n=40 high dose, respectively)[312]. However, with the success of anti-CTLA-4 eliciting a potent immune response, a major side effect occurred: significant immune related adverse events (irAEs) due to an over active immune system[313]. By removing one of the major inherent negative regulatory mechanisms of the T-cell, the immune system loses its ability to keep homeostasis and can thereby lead to significant lymphoproliferative disease and autoimmunity[314]. These irAEs associated with anti-CTLA-4 treatment were also mainly related to breaking self-tolerance, including severe cases of colitis, which could even lead to death[315]. Also, though successful in melanoma for improving patient survival and response, it was not ubiquitous, as there was lack of success in other cancer types including metastatic pancreatic cancer (no response; mean OS of 3 months) and squamous non-small cell lung cancer (NSCLC) (44% response and 15.4 mean OS in

ipilimumab and chemotherapy treated vs. 47% response and 12.4 mean OS in chemotherapy treated)[316, 317]. Reasons for differential response rates between cancer types and individual patients will be revisited in this thesis in the Biomarker section.

1.3.1.2. Programmed cell death protein 1 (PD-1)

With investigation ongoing regarding CTLA-4, concurrent investigations were occurring to identify and understand other T-cell co-receptors. Through this work, the second checkpoint receptor discovered on the T-cell was PD-1 by Dr. Tasuku Honjo in 1992[318, 319]. Like CTLA-4, PD-1 is also expressed on myeloid cells and B cells, but is also constitutively expressed in some organs including the heart and kidney; due to the nature of this thesis, I will focus on its expression on T cells[320].

PD-1 is expressed on the surface of T cells after activation, with extremely low levels of expression (around 100 MFI) in T_N cells[321]. Further, PD-1 is upregulated in late stage effector cells and in cells that are chronically stimulated[322]. For example, in a chronic viral infection model, PD-1 is expressed in T_{em} cells at a greater level than T_N cells and most highly expressed in T_{emra} cells[321, 323]. In fact, there is currently a contentious debate whether PD-1 expression is synonymous with functional exhaustion, as characterization of exhausted $CD8^+$ T cells from chronic viral infection through both genetic analysis and functional assays have demonstrated high expression of PD-1[323]. However, some believe that PD-1 expression just demonstrates an activated, effector T-cell[324]. To support the expression of PD-1 on activated cells, when investigating the expression of surface PD-1 in CD8 TIL from gastric cancer patients, there was lower expression in T_N as compared to T_{cn} (500 vs. 750 MFI, respectively)[325]. Further, PD-1 surface expression was also found to be roughly equivalent in T_{cm} , T_{em} , and T_{emra} cell types [325].

PD-1 is also found to be expressed on the T_{reg} population[326]. In healthy individuals, PD-1 plays a major role in peripheral tolerance and autoimmunity, by promoting T_{reg} development (through its ligand's induction of FoxP3) and by inducing inhibition of self-reactive T cells in the periphery[326, 327]. Therefore, similar to CTLA-4, mice lacking PD-1 have enhanced autoimmune disease and T-cell

proliferation[328]. However, unlike CTLA-4, PD-1 lacks the clathrin cycling motif, causing its continued expression on the surface rather than cycling between intracellular and surface expression[329]. This difference between CTLA-4 and PD-1 will be addressed further later in this subsection.

PD-1 has two ligands: PD-L1 found predominantly on tumor cells and APCs (also found on other cell types like microvascular endothelial cells) and PD-L2 typically expressed on macrophages and DCs; notably, PD-1 can bind with a higher affinity to the lower expressed PD-L2[330-333]. PD-L2 surface expression is enhanced on macrophages and DCs after exposure IFN γ , IL-4, and GM-CSF, but also is expressed on T cells due to exposure to IL-2, IL-7, IL-15, and IL-21[331, 334]. Unlike PD-L2, PD-L1 expression can be induced by IL-17 and TNF α on tumor cells[335]. PD-L1 was also found to be expressed by the T-cell following engagement of the TCR, as well as exposure to cytokines such as IFN γ , IL-4, and GM-CSF[330, 331].

Upon binding of PD-1 to its ligand, an inhibitory signal is transduced through PD-1 via its ITIM and ITSM inhibitory cytoplasmic domains[336]. Ligation causes the ITSM domain to phosphorylate, recruiting SHP-1 and SHP-2 to ITSM, causing de-phosphorylation and inactivation of Zap70, a component of the TCR complex (See Figure 7)[17]. Notably, the recruited SHP-2 can also dephosphorylate the costimulatory molecule CD28, further inhibiting T-cell activation[337]. This signaling cascade can also inhibit the PI3K pathway leading to cellular apoptosis with downregulation of the pro-survival gene BCL-XL or decreased cytokine expression (including IL-2, TNF α , and IFN γ)[17, 338]. Further, the RAS- ERK1/2 signaling pathway can also be inhibited through the ITIM/ITSM domains of PD-1 causing diminished T-cell proliferation[339]. Thus, the significant T-cell inhibitory functions of PD-1, similar to the demonstrated ability of CTLA-4 to hinder T-cell mediated toxicity, positioned PD-1 to become a primary candidate for checkpoint blockade therapy.

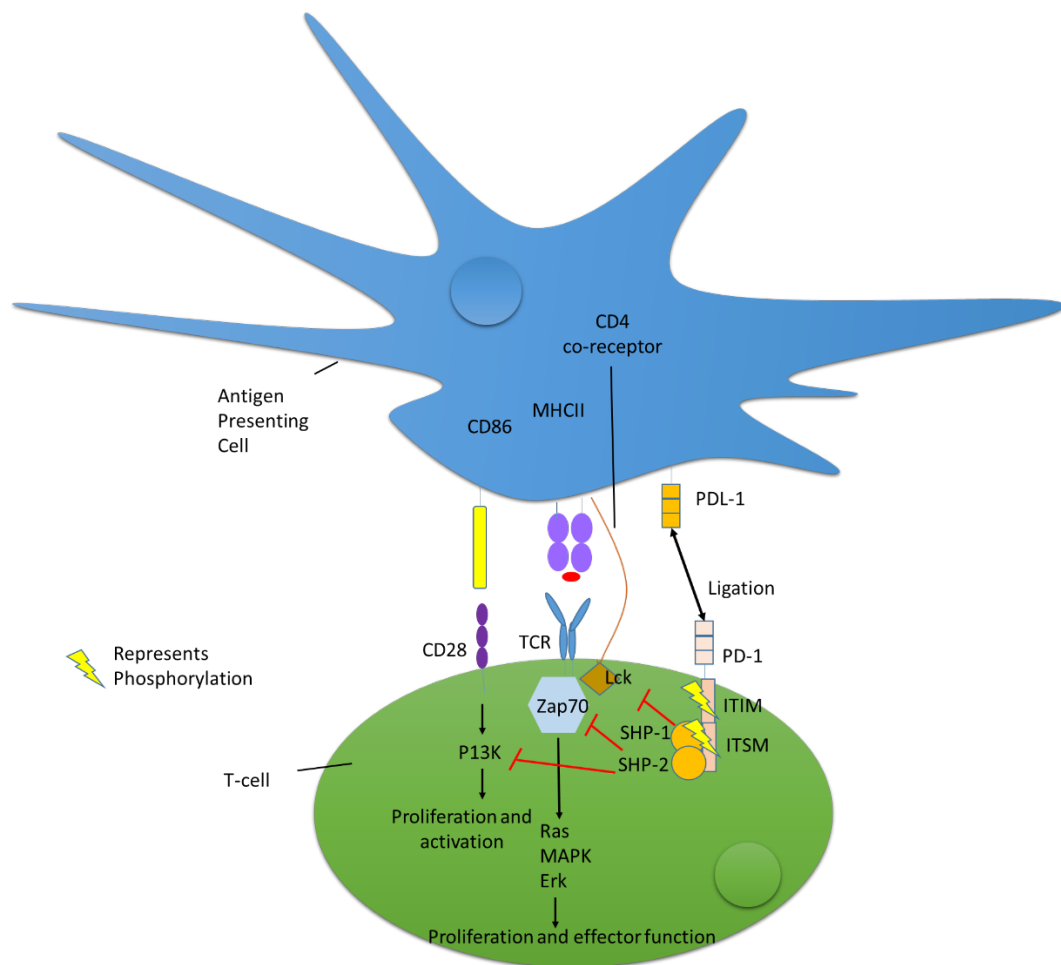


Figure 7. Inhibitory mechanism of PD-1 intracellular domains. Ligation of PD-1 and PD-L1 (or PD-L2) results in an inhibitory signaling cascade. The ITSM motif of PD-1 will phosphorylate, recruiting SHP-1 and SHP-2. Dephosphorylation and inactivation of Zap70 occurs. Further, dephosphorylation of CD28 can occur. Altogether, this leads to decreased cytokine expression, survival, and inhibition of T-cell activation

As with the CTLA-4 studies, it was observed that a PD-1 deficient mouse has hindered tumor progression in melanoma and myeloma[22]. Further, the use of either a PD-1 KO mice or anti-PD-1 targeting antibodies in mouse models with metastatic melanoma or colorectal carcinoma demonstrate diminished dissemination of tumor cells[340]. Based on preclinical experiments showing efficacy and potentially improved tolerability of anti-PD-1 therapy, the approach was translated to human patients, with an initial clinical trial demonstrating objective responses in melanoma, RCC, and NSCLC[341]. Further, an initial trial of anti-PD-1 in melanoma demonstrated a 38% response rate, durable median

OS of 11 months, durable median progression free survival (PFS) of greater than 7 months, and with critical findings showing low toxicity of the regimen[342]. These results ultimately led to the Food and Drug Administration (FDA) approval of the anti-PD-1 antibody (pembrolizumab (Merck) and nivolumab (Bristol-Myers Squibb)) for the treatment of metastatic melanoma patients in 2014[343]. Clinical trials were then conducted comparing the use of anti-PD-1 and anti-CTLA-4 in metastatic melanoma, whereby pembrolizumab treated patients demonstrated an OS and PFS rate double to that of the ipilimumab treated patients, with lower amounts of more severe irAEs (Results summarized in Table 2)[344]. It is hypothesized that there are less irAEs associated with PD-1 blockade due to the different kinetics of expression of PD-1, CTLA-4, or their receptors, whereas upregulation of PD-L1 and PD-L2 occurs in response to inflammatory cytokine exposure like IFN γ , as opposed to CTLA-4 being upregulated at early stages of T-cell activation and constantly replenished by intracellular pools for cycling back to the surface[341]. Further, the overall response rates were 43% in the nivolumab treated patients compared to 11% in the ipilimumab treated group, with a reported 27% response rate in patients' refractory to ipilimumab treatment before receiving pembrolizumab[310, 345, 346].

Due to the success initially observed in metastatic melanoma, anti-PD-1 was tried as a treatment in other cancers. This led to improved overall response, OS, or PFS in cancer types where anti-CTLA-4 therapy was less effective, including in NSCLC[347, 348]. As of 2019, there were current ongoing phase II/III trials using anti-PD-1 in the following cancer types: Hodgkin's lymphoma, primary mediastinal large B-cell lymphoma (PMBCL), head and neck squamous cell carcinoma (HNSCC), cervical cancer, gastro-intestinal cancer, hepatocellular carcinoma (HCC), RCC, urothelial carcinoma, and DNA mismatch repair or microsatellite instability (MSI)-high colorectal cancers [349]. Those cancer types and individuals who did not respond, including those responders who eventually progressed, on anti-PD-1 therapy will further be discussed in the Biomarker section later in this thesis.

Notably, due to the different mechanism of action, there has been much interest in combining anti-CTLA-4 and anti-PD-1 to use as a novel treatment regimen. In a clinical trial comparing this

combination versus monotherapy in melanoma, median PFS is 11.5 months in the combination treatment group, while only 2.9 months with anti-CTLA-4 monotherapy or 6.9 months with anti-PD-1 monotherapy[350]. Further, response rates of melanoma patients were shown to be 57.6% in the combination group, with 43.7% in the anti-PD-1 and 19% in the ipilimumab monotherapy groups; a subsequent study also found similar results in melanoma treated patients with response rates of 61% in the combination treatment group as compared to 11% in the anti-CTLA-4 treatment group[350, 351]. In a report of 5 year outcomes of the trial, the median OS in the combination group was still not reached at 60 months, as compared to the 19.9 months in the ipilimumab monotherapy or 36.9 months in the nivolumab treatment alone group[352]. These promising results also have been witnessed in other cancer types, including RCC and NSCLC[353, 354]. Thus, with this success in combining ICIs, the focus of the field has now been directed to developing novel combination treatment regimens for the treatment of cancer. A new area of interest has become the combination of use of PD-1 and PD-L1 blocking antibodies concurrently. Through there has been much focus on the inhibition of PD-1, other groups have targeted its ligand, particularly PD-L1, as a therapeutic cancer treatment. Initially, there was greater popularity within the field of blocking anti-PD-1 as this would in turn block ligation to both PD-L1 and PD-L2[355]. Also, blocking PD-L1 would be focused on improving APC interaction with the T-cell rather than affecting the T-cell directly[331]. Notably, however, blockade of PD-L1 also prevents the ability of PD-L1 to also ligate CD80, providing another mechanism of T-cell inhibition[356]. PD-L1 clinical trials have shown the efficacy of targeting this receptor, as in small cell lung cancer, both OS and PFS were improved with anti-PD-L1 therapy as compared to receiving chemotherapy alone[357]. Further, recent data has depicted that PD-L1 antibodies may be more efficacious in preventing ligation with PD-1, as measured by drug concentrations utilized for blockade[358]. Thus, when in combination, preliminary data has shown promising results in mouse models of pancreatic cancer through the increased generation of tumor antigen specific T cells with memory phenotype[359].

1.3.1.3. Second and third generation inhibitors

Due to the nature of this thesis, I have predominantly focused on the first generation ICIs, anti-CTLA-4 and anti-PD-1[360]. Since their discovery, other ICIs have been identified as second generation therapeutics (Lag-3, Tim-3, and TIGIT) and third generation therapeutics (VISTA and BTLA) for use in cancer treatment[360, 361]. Table 2 present for each molecules their ligand, mechanism of action, and cellular expression. Because of the scope of this thesis, I have only focused on those ICIs related to T cells, which will also apply to the next section whereby cellular agonists are discussed.

Table 2. Second and Third Generation ICIs[360, 362-366].

<i>ICIs</i>	<i>Ligand(s) and cells expressing the ligand</i>	<i>Intracellular Signaling Domain</i>	<i>Mechanisms of Inhibition</i>	<i>Expression on T cells</i>	<i>Clinical Trials</i>
<i>Lag-3</i>	MHCII on APCs; Galectin 3 on epithelial or CD8 T cells; LSEctin on tumor cells	KIEELE Motif	Binding to MHCII; inhibits TCR signaling	Activated CD4, activated CD8, and T _{regs}	Phase I: Metastatic RCC Phase I: MBC Phase I: Metastatic Melanoma Phase I: Advanced pancreatic cancer Phase I: Advanced Solid Tumors (12 trials) Phase I: GBM Phase I: TnBC Phase I: Gastro/esophageal cancer Phase I: NSCLC and HNSCC Phase I/II: Disease-free melanoma Phase I/II: Advanced melanoma Phase I/II: Advanced Solid Tumors (4 trials) Phase I/li: Advanced hematological malignancies (2 trials) Phase I/II: Virus-associated Cancers Phase II: Advanced hematological malignancies Phase II: Advanced TNBC Phase II: NSCLC (2 trials) Phase II: Advanced chordoma Phase II: Metastatic melanoma

					Phase II: Colorectal carcinoma Phase II: MSI-high solid tumors Phase II: Advanced RCC Phase II: Advanced GC Phase II: Advanced CRC Phase II: Melanoma (2 trials)
<i>Tim-3</i>	Ceacam-1 on tumor cells; soluble Gal9 produced by APCs, T cells, tumor cells, and endothelial cells; soluble HMGB1 on dying cell DNA; PtdSer on dying tumor cells	Tyrosine Residues	Gal9 ligation inhibits CD45 and Lck in TCR; suppression particularly of IFN γ producing T cells	Activated CD4, activated CD8, and T _{regs}	Phase I: Advanced Solid Tumors (9 Trials) Phase I: Glioblastoma Phase I: AML or high risk MDS Phase I/II: Advanced Solid Tumors (2 trials) Phase II: Liver cancer
<i>TIGIT</i>	CD112 and CD155 expressed on APCs and tumor cells	ITIM Motif and ITT motif	Contains ITIM motif; Competes for binding with activating CD96/CD226	Activated, effector, and memory T cells (CD4 and CD8) and T _{regs}	Phase I: Advanced Solid Tumors (6 trials) Phase I/II: Advanced Solid Tumors Phase II: Advanced NSCLC
<i>VISTA</i>	VSIG-3 on tumor cells and PSGL-1 at acidic pH	SH2/SH3 binding domain in cytoplasmic tail	Suppress T-cell activation (measured by IFN γ and IL-2 secretion) and induce FoxP3; homologous to PD-1	Low expression on T cells; expression on activated T _{regs}	Phase I: Advanced Solid Tumors (3 trials)
<i>BTLA</i>	HVEM on T cells, B cells, DCs, NK, and myeloid cells	ITIM and ITSM Motifs	Contains ITIM and ITSM motifs; inhibits production of IL-2 and inhibits $\gamma\delta$ T cell proliferation	Mature T cells and T _{regs}	Phase I: Advanced or metastatic solid tumors

Delving deeper into the most common clinical trial for secondary inhibitors, Lag-3, improved survival was observed with its use in breast cancer in combination with chemotherapy, however little benefit was demonstrated in the RCC, pancreatic, or melanoma studies[367-370]. Most recently, the combination of anti-Lag-3 and anti-PD-1 has shown promise in melanoma, improving response rates and survival of patients, particularly those that failed initial ICI monotherapy of PD-1 or PD-L1[371]. Like Lag-3, these other checkpoint inhibitors listed above have poor efficacy as a monotherapy but are in current trials in combination with anti-PD-1 for use in solid tumor types[362, 366, 372, 373]. Notably, the most promising combination still remains the blockade of both CTLA-4 and PD-1[350, 352].

Taken together, removing the cell's natural mechanism for inhibition through the use of ICIs is effective and shows promise in multiple cancer types, including metastatic melanoma and NSCLC, with the ability to combine ICIs for even greater efficacy[374]. The success of ICIs then refocused the field of cancer therapy particularly back to immunotherapy. Due to this refocus, there has been a resurgence in not only targeting the inhibitory molecules on the surface of T cells, but the costimulatory molecules, like 4-1BB or ICOS, often utilized to enhance T-cell mediated killing and efficacy in generating an anti-tumor response[375]. Therefore, another potential therapeutic target utilizing particularly monoclonal antibodies are these activating receptors, which will be described in the following subsection.

1.3.2 Agonistic antibodies

Co-stimulatory receptors are utilized for Signal 2 of activation of T cells, as previously described in the T cells section of this thesis, and similarly to checkpoint inhibitory molecules, these receptors and their ligands can also be targeted to modulate T-cell activity; in this case engagement of co-stimulatory receptors would enhance T-cell survival, proliferation, or function for the treatment of cancer[295, 376-

382]. In the context of this thesis, I will focus on agonistic antibodies, or those antibodies that bind and thereby activate or stimulate their target and their effect on T cells[16]. One of the earliest trials utilizing a CD28 agonistic antibody almost stifled development of the field completely (described in detail shortly), but with the success of the ICIs for cancer treatment, there has been a renewed interest in targeting activating receptors for improved T-cell mediated killing[383, 384]. In this section, I will describe common T-cell co-stimulatory receptors and give an overview of select agonistic antibodies being evaluated for cancer treatment (See Figure 8).

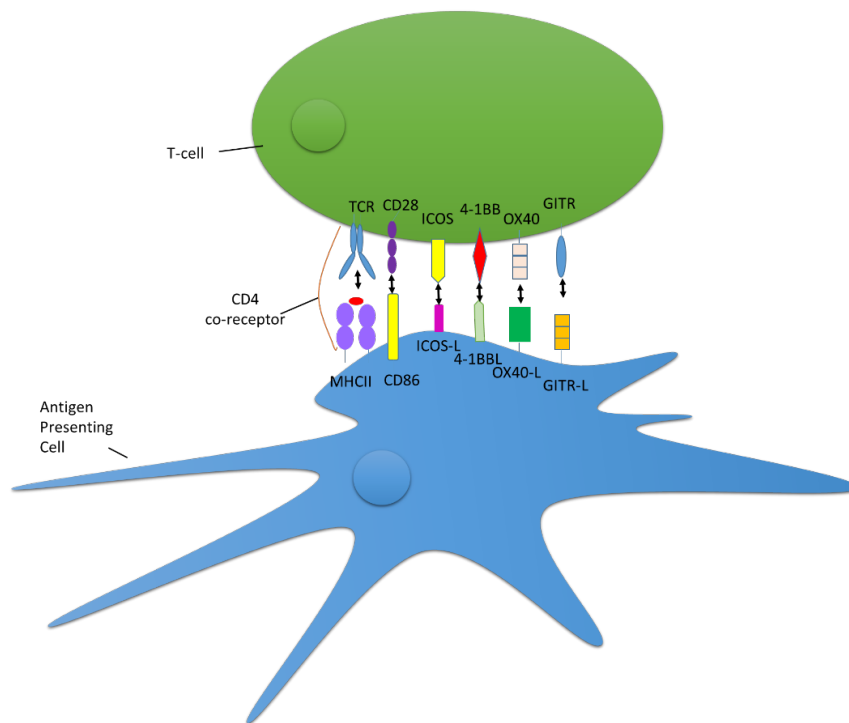


Figure 8. Checkpoint co-stimulatory molecules. T-cell checkpoint co-stimulatory receptors are upregulated to the surface of T cells, leading to improved proliferation, cell survival, or effector function upon ligation with their corresponding ligands designated with arrows found on typically APC cells. Notably, CD28 can bind to CD80 as well.

1.3.2.1 CD28

As aforementioned, CD28 is a co-stimulatory receptor found on T cells that ligates CD80/86 on APCs, causing increased IL-2 production, cell proliferation, and anergy prevention[376, 377, 385]. Co-stimulation by CD28 is the primary and strongest method of co-stimulation, but CD28 competes with

the inhibitory molecule CTLA-4 for binding to its ligand CD80/86[386]. The constitutive expression of CD28 allows T cells to benefit from a strong costimulatory signal when encountering TCR stimulation, while the induced expression of CTLA-4 a few days later, which has a 10 times greater affinity for CD80/86 and outcompetes CD28 binding, serves to terminate the activation of the T cells and prevent over-activation[387]. However, CD28 ligation on the surface of T_{regs} also can inhibit cytotoxic T cells by promoting the immunosuppressive T_{regs} ' thymic generation and peripheral homeostasis (demonstrated using CD28 knock-out mice)[388].

CD28 is constitutively expressed on T cells, but upon TCR activation, CD28 is transiently downregulated[389]. CD28 helps to determine CD4 T-cell polarization, as cells lacking CD28 expression fail to produce Th1 and Tfh subtypes[390]. Further, CD28 ligation is necessary for generation of maximum pathogen clearance or anti-tumor immunity and an effective response in memory CD4 and CD8 T cells[391]. In terms of CD28 expression in the memory subsets, its surface level expression has been demonstrated in naïve and central memory CD8 T cells, whereas T_{emra} cells typically have low expression[268]. The T_{em} subtypes have variable expression of CD28 (EM1 and EM4 express, while EM2 and EM3 do not express)[268]. CD28 expression in CD8 memory cells has also been linked to higher perforin and IFN γ expression[392].

CD28 is a member of the immunoglobulin receptor family, which includes the ICIs CTLA-4, PD-1, BTLA, and TIGIT (and co-stimulator ICOS, described below)[393]. The cytoplasmic tail of CD28 contains tyrosine signaling motifs that bind to SH2 and/or SH3 domains in protein and protein kinases upon phosphorylation triggered by TCR activation or CD28 ligation.[394, 395] Engagement of CD28 and the TCR leads to the activation of JNK, PKC θ , and the PI3K pathway (activating Akt, a molecule important in cellular survival and proliferation), culminating in the transcription of NF κ B, NFAT, and/or AP-1[396, 397] [398]. In general, these transcription factors are influential in IL-2 secretion and cellular

proliferation, but the more precise signaling effects observed is dependent upon the T-cell subset and exposure to other cellular factors[399].

Due to the co-stimulatory effects of ligation with CD28, an agonistic antibody was initially generated, which was termed a “superagonist,” as it showed that cellular proliferation occurred even without TCR stimulation[400]. This led to the use of the superagonist in preclinical rodent models to stimulate specifically T_{regs} for the prevention of autoimmune disease[401]. Due to the success experienced in animal models, the use of the superagonist for CD28 was tested in human patients for the intended treatment of autoimmune disease and B-cell lymphoma, but was met with disastrous consequences. Six healthy human males received the superagonist CD28 antibody, TGN1412, which resulted in cytokine release syndrome that almost proved fatal[384]. Unlike the rodent model systems housed in clean conditions, humans have greater exposure to infectious agents, thus leading to a larger proportion of T_{em} cells[402]. Therefore, in humans versus rodents, these T_{em} cells were activated disproportionately to T_{regs} , causing enhanced IFN γ , TNF α , and IL-2 production[402]. Overall, much knowledge was gained regarding the safety and efficacy targeting of CD28 with superagonists; with lessons learned and the promise using ICIs, the field continued to pursue other costimulatory molecules to improve T-cell function.

1.3.2.2 Inducible co-stimulatory molecule (ICOS)

Another popular agonist is inducible co-stimulatory molecule (ICOS), which is of the same immunoglobulin type receptor superfamily as CD28[403]. ICOS on activated T cells only has affinity for its ligand, B7H/B7RP-1 (also known as ICOS-L), which is expressed on B cells, macrophages, and DCs[378]. The expression of ICOS-L can be induced on other cell types including epithelial cells, tumor cells, and other non-immune cells, including fibroblasts following stimulation with lipopolysaccharide (LPS) or exposure to TNF α [404, 405]. Activated CD4 and CD8 T cells both express ICOS, but CD4 T cells typically have greater expression of ICOS; surface expression typically peaks within 12 hours post-

stimulation[404, 406, 407]. Ligation of ICOS with ICOS-L is involved with CD4 T-cell polarization, as defective ICOS signaling results in lacking Th1 and Th2 mediated responses to bacterial, parasitic, and viral infection[408-410]. Notably, data has been somewhat controversial regarding the role of ICOS in the Th1 response, as cases of *Mycobacteria tuberculosis* and *Chlamydia muridarum* in mice have demonstrated that absence or blockade of ICOS ligation demonstrates an enhanced Th1 response[411, 412]. Further, ICOS is also involved in Th17 and Tfh polarization and optimal function; for example, ICOS stimulated Th17 cells lead to enhanced mesothelioma control in mouse models as compared to CD28 stimulated Th17 cells[413, 414]. In terms of cytokine production, due to its impact on Th1 polarization, ICOS ligation is associated with IFN γ production; however, as stated above, this production is context dependent, with some mouse models demonstrating improved production after blockade[411, 412, 415-418]. Also, cytokine production by other cell types is further influenced by ICOS, as cells with intermediate levels of ICOS expression are associated with the production of Th2 cytokines (IL-4, IL-5, and IL-13)[419]. T_{regs} express high levels of ICOS, and its expression is associated with co-induction of IL-10 [403, 419]. Therefore, somewhat unsurprisingly, upon blockade of ICOS ligation, T_{reg} induction, expansion, and function, including IL-10 production, are inhibited[420]. In CD8 T cells, lack of ICOS does not affect the function of cytolytic CD8 T cells[409]. However, it has been demonstrated that ICOS expression is associated with a T_{rm} CD8 phenotype[421].

Similarly to CD28, engagement of ICOS contributes to cell survival and proliferation through Akt signaling, which is important in cellular survival and proliferation[394]. In contrast to CD28, it can also signal through the mitogen-activated protein kinase (MAPK) pathways, resulting in cellular differentiation as well, influencing cellular function[422].

In examination of ICOS function *in vivo*, it was observed that T and B cells lose their ability to recognize antigens in ICOS-L knock-out mice[423]. Then, based on data from a pre-clinical fibrosarcoma mouse tumor model showing that ligation of ICOS can enhance CD8 T-cell mediated clearance,

agonistic antibodies directed against ICOS made their debut for the treatment of cancer in human clinical trials[424]. Initial trials with use of an agonistic ICOS antibody as a monotherapy in multiple solid tumor types showed one partial response out of 67 treated patients[425]. Efforts were thus redirected to the use of ICOS in combination with ICIs and new trials have begun in combination with anti-CTLA-4 or anti-PD-1[426]. Ipilimumab treatment yields upregulation of ICOS on the T cells, thus giving a potential therapeutic target and rationale for this combination[427]. The use of anti-PD-1 combined with ICOS agonism has led to a 24% response rate in head and neck cell carcinoma, though there was no single agent anti-PD-1 arm used as a comparator (previous overall response rate published as 13.3% with single agent anti-PD-1)[426, 428]. Continued testing is needed to determine the efficacy of these combinations. There is still much to be understood regarding the mechanisms of ICOS and its potential as a novel therapeutic target to be used as a combination treatment for immunotherapy.

1.3.2.3 Glucocorticoid-induced tumor necrosis factor receptor (GITR)

GITR is another costimulatory molecule, expressed on most immune cell types, including T cells, post-activation[429]. Interestingly, its name is a misnomer, as glucocorticoid was shown to actually have no influence on its expression[429]. Its ligand, GITR ligand (GITR-L), is expressed on APCs (macrophages, DCs, and B cells), with expression upregulated upon cellular activation[430, 431]. GITR is expressed most highly on T_{regs}, particularly antigen experienced and memory T_{regs}[432, 433]. It has also been shown to polarize CD4 T cells to a Th2 phenotype in the presence of infection, with GITR KO mice showing a greater Th1 population[434]. In the context of TIL, surface level GITR is higher in CD4 than CD8 TIL[432]. However, GITR has been shown to be important in CD8 T-cell proliferation and survival during influenza infection[435]. GITR is also necessary for CD8 memory T-cell persistence. For example, antigen specific memory T cells from GITR KO mice persisted longer than wild type mice T cells; this further may be attributed to GITR's regulation of another costimulatory receptor 4-1BB, described in

detail later[436]. In terms of memory status, unlike ICOS, GITR expression does not change based on CD8 T_{rm} status (described in melanoma)[437].

GITR, among other costimulatory receptors to be discussed like OX40 and 4-1BB, is a member of the TNF receptor (TNFR) superfamily[380]. In this family, upon T-cell activation, binding of the receptor by the ligand causes a signaling cascade through its recruitment of signaling molecules dictated by the cytoplasmic tails[379, 380]. Overall, in general, ligation of GITR leads to signaling mediated by TRAFs through both NF κ B and MAPK pathways to improve T-cell survival with protection from AICD, proliferation supported by improved IL-2 production, and effector function; specifically, GITR impacts the proliferation of T_{regs} without hindering their suppressive ability[438, 439].

Agonist antibodies targeting GITR thus have been utilized in pre-clinical mouse models, yielding success in controlling tumor growth in melanoma, colon carcinoma, and colorectal carcinoma[440-443]. GITR agonistic antibodies in mice have been shown to reduce the infiltration of T_{regs} into the tumor and cause repolarization of intratumoral T_{regs} into an effector T-cell-like phenotype[444, 445]. These studies led to the first clinical trials - utilizing GITR agonists in humans. Unfortunately, GITR as a monotherapeutic agent in advanced solid tumors yielded poor response rates[446, 447]. However, pre-clinical work in melanoma had shown the potential of GITR to be effective in combination with anti-PD-1, demonstrating improved tumor control by yielding enhanced tumor-specific, highly cytotoxic CD8 T cells[446]. Unfortunately, a recent publication has also demonstrated that GITR agonism does not enhance anti-PD-1 efficacy in a clinical trial of patients with advanced solid tumors[448].

1.3.2.4 OX40

OX40 is a costimulatory molecule that was first identified on activated CD4 T cells, but has been found to be expressed on activated CD8 T cells, B cells, DCs, NK cells, and granulocytes[449-453]. OX40-Ligand (OX40-L), much like the name implies, is the ligand for OX40 and its expression can be induced on APCs and T cells[454]. OX40-L expressed on the surface of activated T cells, leads to co-stimulation

of the other T cells expressing OX40[455]. OX40 is typically induced upon T-cell activation within 12 to 24 hours; therefore, there is low expression of OX40 on naïve T cells and resting memory T cells[449, 456]. In terms of the kinetics of its expression, OX40 surface expression reaches its peak within two to three days of activation; however, memory T cells and antigen experienced T effector cells can re-express OX40 within 4 hours of stimulation[456]. In both CD4 and CD8 T cells in mice, OX40 is associated with generation of memory response and survival, with enhanced recall response also noted for human T cells expressing OX40[453, 457]. In CD4 T cells, OX40 helps to mediate Th2 and Th9 polarization particularly during airway inflammation, though data has also shown that rather than polarizing to a Th2 phenotype, ligation of OX40 by OX40-L on APCs may be actually be providing optimal Th2 priming, memory generation, and maintaining Th2 polarization[458-460]. OX40 also can help to promote Th1 responses in the presence of IL-12, thus losing its ability to polarize to a Th2[461]. Similarly to GITR, OX40 has also been shown to be expressed on both iT_{regs} and nT_{regs}, and stimulation of OX40 inhibits their suppressive function[462].

As aforementioned, OX40 belongs to the TNFR superfamily and mediates its signaling primarily through recruitment of TRAF-2 or -3 to its cytoplasmic tail[463]. After OX40 engagement, TRAF-2 signaling has been shown to mediate the generation of antigen specific T-cell memory cells (demonstrated by TRAF-2 dominant negative receptor mice with OVA specific recognition)[464]. Overall, OX40 ligation ultimately causes activation of the PI3K pathway, further impacting AKT activity downstream, and thereby promoting OX40 mediated survival through the expression of pro-survival proteins BCL-2 and BCL-XL[465]. Further, the expression of OX40 in CD8 TILs correlated with enhanced cytolytic function in transgenic tumor models of melanoma[466].

Because of its positive impact on survival and proliferation for T cells and negative impact on T_{regs}, preclinical mouse models were used to test the efficacy of agonistic antibodies for OX40. In a mouse model of sarcoma, mice receiving an agonistic OX40 antibody had curative control of tumors

and were resistant to re-challenge; OX40 agonists have also been shown to control glioma, melanoma, and breast cancer tumors[467, 468]. This success was translated to a Phase I clinical trial, whereby advanced stage cancer patients were treated with an OX40 agonist, in which 12 of 30 patients had reduction in a least one lesion, but with no responders by RECIST observed[469]. To improve the modest results achieved as a monotherapy, the combination of anti-PD-1 and OX40 agonists was investigated. Initially, concurrent administration demonstrated that anti-PD-1 therapy actually negated the protection offered by OX40 agonism in HPV mouse models, but recent publications have demonstrated that the combination could prove effective in pancreatic cancer mouse model systems, emphasizing the importance of cancer type on the efficacy of these treatments[470, 471]. Thus, there are currently a number of trials investigating potential implications and mechanisms of combinations with OX40 for the treatment of patients with cancer.

1.3.2.5 4-1BB

The final TNF co-stimulatory receptor member that will be reviewed in this thesis section is 4-1BB and is particularly of note in the context of this thesis, as it was utilized in the first study highlighted in Section 3. 4-1BB expressed on activated T cells binds to its ligand 4-1BB ligand (4-1BBL), which is expressed typically on APCs[472]. Similarly to other costimulatory molecules introduced, ligation of 4-1BB and 4-1BBL triggers T-cell proliferation, improves effector function, leads to cytokine production, and prevents cellular death[381, 382]. 4-1BB is predominantly expressed on T cells, but is also found on many other cell types, including APCs, NK cells, and even neuronal cells[473-475]. Like most other costimulatory receptors, 4-1BB is upregulated upon activation in T cells, except it is constitutively expressed in a small subpopulation of nT_{regs} and iT_{regs}[476, 477]. Agonism of 4-1BB has been shown to inhibit the suppressive function of iT_{regs} through the diminished production of IL-9 and use of an agonist 4-1BB antibody has been shown to deplete intratumoral T_{regs}[478, 479]. However, in terms of CD4 T cells, 4-1BB ligation also influences cellular polarization, favoring a Th1 phenotype[480]. 4-1BB also

preferentially activates CD8 T cells as compared to CD4 T cells and its engagement further promotes CD8 T-cell effector function, increasing IFN γ production[381, 479]. Not only does 4-1BB effect the cellular subtype, but it also effects memory generation. In comparison to CD27 stimulation which generates T cells that are highly proliferative but are more susceptible to death, 4-1BB ligation has demonstrated the ability to generate long-lived memory cells that are more resistant to cellular death[481]. Similarly, engagement of 4-1BB during activation generates CD8 antigen specific memory T cells, whereas CD28 co-stimulation expands a naïve CD8 T-cell repertoire that is less cytolytic comparatively[482].

Molecular signaling of 4-1BB is similar to that of OX-40 and GITR, previously discussed above. The cytoplasmic tail associates with a heterotrimer of binding with a single, TRAF-1 molecule and two, TRAF-2 molecules to facilitate signaling through the ERK, JNK, and PI3K pathways to cause transcriptional regulation, particularly of NF κ B[116, 483, 484]. NF κ B is of particular importance as it leads to the increased expression of anti-apoptotic genes BCL-XL and BCL-2 and cell cycle progression[485, 486]. Further, the engagement of 4-1BB causes the delayed utilization of the PI3K/ERK/AKT pathways, indirectly activating the T-cell factor 1 (TCF1)/beta-catenin pathway, which has been demonstrated to be associated with CD8 T-cell specific proliferation and potentially a more cytotoxic, persistent phenotype (determined through use of an TCF1 inhibitor)[484, 487].

Due to the ability of 4-1BB to aid in the generation of memory and prevention of apoptosis particularly in CD8 T cells as mediated by its unique signaling methodology, pre-clinical models were developed to investigate the role of 4-1BB agonism for cancer treatment. Mouse models of sarcoma and mastocytoma demonstrated that the use of a 4-1BB agonizing antibody leads to tumor control, with immune memory generated as demonstrated by the mice being resistant to tumor re-challenge[474]. Due to the success of these initial studies in mice, 4-1BB was then translated for use in human patients. There are two main clinical trials for 4-1BB agonists, urelumab (Bristol-Meyers Squibb)

and utomilumab (Pfizer)[488, 489]. The first reports using urelumab showed promise with 3 PRs in melanoma, but also generated an unfortunate side effect that led to the study being placed on pause for six years: high liver toxicity[488, 490]. This toxicity is probably due to the activation of Kupffer cells and liver monocytes, causing the expression of IL-27, ultimately inducing CD8 autoreactive T-cell activation and infiltration, while also potentially inhibiting suppressive T_{regs} [491]. Though detrimental side effects were observed utilizing urelumab, utomilumab yielded a response rate of 13.3% in merkel cell carcinoma, (no response in other solid tumor types) and did not cause liver toxicities[489, 492]. The lack of toxicity could be explained by the different binding sites to 4-1BB between the two antibodies, causing less potency of utomilumab comparatively[493]. To improve the efficacy of these agonists as cancer therapeutics, it was proposed to utilize these in combination with other ICIs [or co-stimulators (including OX-40)], and pre-clinical tumor models demonstrated tumor control utilizing anti-CTLA-4 (in melanoma, colon cancer, etc.) or anti-PD-1 (in lung cancer, ovarian cancer, etc.) in combination with 4-1BB agonist[493-497].

Use of agonist 4-1BB antibodies can also be used to enhance other immunotherapeutics, including adoptive cell therapy (ACT) of tumor-infiltrating lymphocytes (TIL), which will be discussed in detail in the following section of this thesis. Addition of 4-1BB agonist (BMS urelumab) to media containing IL-2 used to culture T cells from pancreatic cancer tumor fragments demonstrated a repertoire skewed towards CD8 T cells with a clonal repertoire as compared to media supplemented with IL-2 alone[498]. Further, the addition of 4-1BB to initial culture media containing IL-2 and anti-CD3 (OKT3) to melanoma tumor fragments triggers signals 1, 2, and 3 of T-cell activation, leading to improved T-cell growth rates within a shorter time period[499]. For example, uveal melanoma T cells expanded in 100% of culture with this method where previously there was poor growth rate (15%) using IL-2 conditioned media alone[499]. Not solely aiding in the initial stages of TIL cell growth, addition of 4-1BB into the culture media into the phase whereby millions of TIL are expanded to billions of cells (rapid expansion phase, REP) protects CD8 T cells from AICD and enhances cytolytic

potential[500, 501]. The importance of these attributes, including improved CD8/CD4 ratio, shorter growth rates, and T- cell clonality in ACT will be addressed in the following section.

Overall, the use of agonist antibodies for costimulatory molecules on T cells to cause increased cellular proliferation, resistance to apoptosis, or enhanced effector function cannot be overlooked as a potential combination therapy[502]. There are currently a number of preclinical and clinical trials ongoing for those agonists addressed in this section, but notably there are multiple other co-stimulatory molecules on the T-cell that can also be targets for an improved response, including CD27, CD30, and LIGHT[379]. Other promising uses for agonism of costimulatory molecules have been in the use of cell therapies, including ACT of TIL as described above and CAR T-cell therapy, which will be described in the following subsection.

1.3.3 Cellular Based Immunotherapy

Adoptive cell therapy (ACT) encompasses therapeutic treatments that utilize immune cells which are transferred into patients for treatment of cancer and other disease[503]. Due to the nature of this thesis, I will mainly focus on ACT of T cells with a particular attention to tumor-infiltrating lymphocytes (TIL) for the treatment of cancer. The T-cell ACT revolution began in the 1980s through the work of Dr. Steven Rosenberg at the National Cancer Institute, who treated metastatic melanoma patients with *ex vivo* grown TIL and high dose IL-2, whereby 60% of patients experienced tumor regression[504]. From this groundbreaking work, the field of cellular therapy has continued to flourish, with the development of chimeric antigen receptor (CAR) T-cell therapy and endogenous T-cell (ETC) therapy. In this sub-section of my thesis, I will thus introduce these three main therapies that are currently in use for the treatment of a wide array of cancers; details regarding potential biomarkers associated with response to these therapies, however, will be discussed in the following biomarker section.

1.3.3.1 Chimeric Antigen Receptor (CAR) T-cell Therapy

CARs are engineered receptors comprised of the extracellular binding portion of a modified antibody that binds with specificity to a targeted protein, coupled to a transmembrane and intracellular tail triggering a cytotoxic response[505]. CARs are introduced into immune cells, most commonly T cells (See Figure 9A)[505]. CAR-modified T cells are infused into patients for treatment, leading to cellular elimination based on surface protein recognition of the single target recognized by the CAR (without pre-processing, MHC independent)[506].

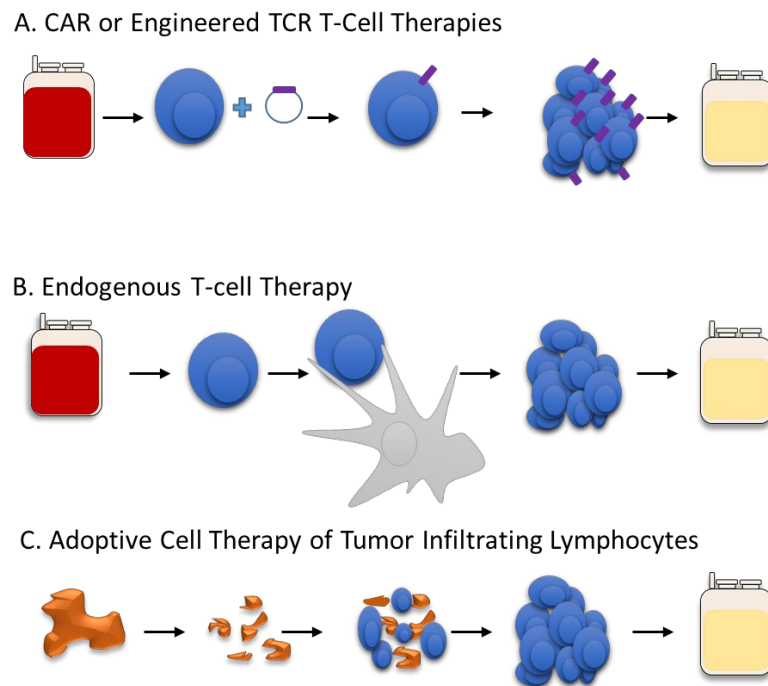


Figure 9. Adoptive Cellular Therapies. Depicted above are the 3 main methods of ACT. (A) Chimeric antigen receptor (CAR) T-cell therapy is performed through the use of patient blood, transduction of a viral vector containing the CAR that recognizes the TAA on the surface of the tumor into T cells, and expansion of CAR T cells into large numbers for infusion back into the patient. Engineered TCR T-cell therapy is performed in a similar manner, with viral transduction of the TCR recognizing specific antigens or neoantigens. **(B)** Endogenous T-cell (ETC) therapy begins with the blood of the patient as a source of T cells, peptide stimulation typically using DCs, and ends with cells expanded to large numbers to reinfuse into the patient. **(C)** TIL ACT is performed utilizing excised tumor fragments, whereby the TIL (no in vitro antigen stimulation) are cultured for infusion into the patient.

CARs are historically comprised of three characteristic regions- an ectodomain containing the single chain fragment variable (scFv) that recognizes the peptide, a transmembrane domain, and an intracellular domain that activates T cells[506-508]. Initially, CARs were built with a CD3 ζ chain for T-cell activation; these are known as the first generation CAR T cells[509]. In 1989, the first CAR T-cell was developed and demonstrated specific binding to the peptide 2,4,6-trinitrophenyl (TNP) in solid tumors across different species[506]. The first CARs contained a double chain ectodomain, with the use of single chain ectodomains adopted in 1993[510]. Pre-clinical *in vitro* studies of CAR T cells also demonstrated success, as shown by cells targeting Neu/Her-2 and TNP in these TAA overexpressing cancers and MOv18 for ovarian cancer[510-512]. Subsequently, these CAR T cells were taken into the first human clinical trials for RCC and ovarian cancer[513, 514]. Unfortunately results in human trials were not comparable to pre-clinical results, with lack of both tumor regression and CAR T-cell persistence, and ultimately led to the next generation of CAR T cells development[513, 514].

The second generation CAR T cells included an intracellular co-stimulatory domain (signal 2) in addition to the CD3 ζ chain (signal 1), which together allowed for repeated antigen exposure, increased persistence, and enhanced proliferation, ultimately leading to their improved functionality against cancer[515, 516]. The first costimulatory molecule investigated in mouse models of leukemia with CAR targeting the antigen CD19 was CD28[517, 518]. In a side by side comparison of first generation and second generation CD19 CAR with or without CD28 demonstrated advantages over first generation CARs with improved proliferation, but with still fairly low persistence that would not aid in improving clinical benefit[519]. By comparison, an initial study of CAR T cells with a 4-1BB costimulatory domain in three patients with advanced chronic lymphoblastic leukemia (CLL) CAR T cells demonstrate cellular persistence for several months and generated memory[520]. This difference in persistence reinforced the idea that the type of costimulatory molecule incorporated into the CAR will dictate attributes of the T cells. Because of the success initially reported with the second generation CAR T cells, other clinical trials began for more patients with CLL and acute lymphoblastic leukemia (ALL), demonstrating the

ability of these cells to induce tumor control[519-522]. Due to efforts notably by Dr. Carl June with work in CLL and ALL, ultimately this therapy was approved by the FDA in 2017 for the treatment of ALL in younger patients with tisaenlecleucel (Novartis), followed soon after with approval for treatment in adults with B-cell lymphomas with axicabtagene ciloleucel (Kite); both CAR T cell therapies yielded impressive results, demonstrating CRs of 70-90% and 55%, respectively[523, 524].

CD19 is a major antigen to target for successful therapy as it is expressed by all cancer cells in leukemias and lymphomas. Targeting CD19 also leads to depletion of normal B cells, but this side effect can be overcome through the use of immunoglobulin infusions[525, 526]. The most severe adverse related event associated with CAR T-cell therapy is cytokine release syndrome, causing reactions like nausea, fever, hypotension and can lead to multi-organ system failure[527]. Insertional oncogenesis, or causing mutations through viral transfection, was a notable fear, potentially causing life threatening events related to treatment[528]. This phenomenon has been documented once in a patient receiving CD19 CAR T cells, whereby these cells acquired a *TET2* lentiviral induced mutation; however, this led to a T_{cm} phenotype with no negative impact on patient clinical outcome[529].

Based on the success of CD19 CAR T cells for the treatment of blood cancers, other CARs have been explored, including CD20 and CD22 for B-cell lymphomas[530-532]. As 7-33% of patients responding to B-cell ALL CD19 CAR therapy relapse due to antigen loss, predominant use of these CARs have been to circumvent this issue[533-535]. Use of these CAR T cells have shown promise in other cancer types, as CD20 CAR T cells demonstrate efficacy (2 CR, 1 PR, 4 SD) in 7 patients with non-Hodgkin lymphoma and mantle cell lymphoma[536]. Recently, CD22 CAR T cells have demonstrated the ability to generate curative responses (80% CRs) in patients either relapsed or refractory in B-cell ALL, but required a complementary follow up treatment (typically transplantation) to ensure remission[537]. Current therapeutics have now looked to combine the power of these CARs by targeting antigens simultaneously (i.e., using tandem CARs) to generate long term response[538].

Building from these successes, further modifications to the CAR itself to include the addition of another co-stimulatory domain (known as third generation CAR T cells) have been developed to improve cellular function, proliferation, or survival[539]. This third generation contains a combination of two of the following: CD28, 4-1BB, and/or OX-40 costimulatory domains and has demonstrated efficacy in improving proliferation, memory generation, and tumor control compared to second generation as shown in MC38 CARs[539, 540].

The success of CAR therapy in blood cancers, led to efforts to use this method in treatment for solid tumors. Preclinical trials showed promise; for example, CAR T cells for EGFR demonstrated tumor control in xenograft models for glioblastoma[541]. Unfortunately, CAR T-cell therapy had poor efficacy in solid tumor clinical trials; for example, the corresponding EGFRvIII targeting CAR T-cell clinical trial yielded poor results, with only one of ten patients experiencing stable disease at 18 months[542, 543]. This could be due to multiple reasons. One is that the heterogeneity found in solid tumors is causing immune-escape of tumor cells that do not express the targeted TAA[544]. The immune-pressure caused by targeting a sole TAA can also lead to antigen loss, a mechanism often employed by tumor cells to evade immune destruction and also reported in CD19 CAR treated patients[544]. Other mechanisms leading to potential failure also include immunosuppression/in-hospitality of the TME and inability of cells to traffic to the tumor[539, 544-546]. Multiple strategies have been utilized to overcome these obstacles, including the use of bispecific CAR T cells also known as tandem CARs (TanCARs) that expresses scFvs of more than one antigen (e.g., CD19 and CD20) that are often used to combat antigen loss[544, 547]. In solid tumors, TanCAR T cells for B7-H3 and CD70 have demonstrated improved control of lung cancer and melanoma xenograft mouse models[548]. The use of checkpoint inhibitors like anti-PD-1 and the co-expression of TGF β dominant negative receptor (DNR) with a CAR are also being used to overcome the immunosuppressive factors of the TME[545, 546]. Finally, fourth generation CAR T cells, also known as T cells redirected for antigen-unrestricted cytokine-initiated killing (TRUCKS), are gaining traction in the field through their ability to combine the power of CAR T

cells and the delivery of pro-inflammatory cytokines to the tumor (predominantly IL-12 and IL-18 in colon, lung, and pancreatic carcinoma preclinical models)[549, 550].

1.3.3.2 Antigen specific T-cell Therapy

There are two main types of antigen specific T-cell therapies that will be introduced below, genetically engineered TCR-transduced T-cell therapy and endogenous T-cell (ETC) therapy. Unlike CAR T-cell therapy, both antigen specific T-cell therapies can target both surface antigens and antigens that require presentation by an MHC molecule, including cancer-testis antigens and neoantigens, giving a wider range of targets to select from for treatment[426].

1.3.3.2.1. TCR-transduced T-cell therapy

Genetically engineered TCR therapy involves the use of T cells derived from peripheral blood lymphocytes (PBL) that are then retrovirally transduced with cloned TCR vectors with specific tumor antigen reactivity (See Figure 9A)[551]. Most commonly, these TCRs target TAAs to prevent potential off target effects. In this process, TCRs are derived from patient tumors that are digested and cultured for a 14 day period, then co-stained for TCRVB and peptide tetramer to selectively undergo TIL clonal expansion; notably, TIL grown from specific wells with greater reactivity can be preferentially selected for these clones[552]. These cells are then co-cultured with APCs pulsed with peptide to determine reactivity, and RNA from reactive clones with high avidity are used to generate primers for transduction into PBL[552]. The lymphodepleted patient is then infused with these cells with high dose IL-2 (importance of these will be described in TIL section)[553]. Overall, the goal of this therapy is to generate a cellular product that can be utilized by more than one patient; however, due to the TCR restriction, patients must have the same HLA type that the TCR recognizes[554]. Thus, a product that is able to treat 100% of the patient population is still elusive, but use of $\gamma\delta$ T cells that do not face this limitation may provide the next evolution of therapy[554].

This therapy first was used initially in the treatment of melanoma by targeting MART-1, and demonstrated complete, long-term regression in 2 of 15 patients with metastatic melanoma[555]. Due to the limited response rate observed, it was hypothesized that increasing the avidity of the TCR for the MART-1 peptide would improve clinical benefit[553]. However, the use of these more reactive T cells caused off-target responses to normal melanocytes found in the skin and eye, causing vitiligo and hearing loss[553]. Further, some cases without affinity enhancement of the TCR even caused death as well[556]. Similar complications were witnessed in a trial targeting MAGE-A3 in the treatment of metastatic melanoma, whereby the TCR modified T cells cross-reacted with MAGE-A12 found in the heart, causing death in 2 of 9 patients[557]. Thus, great care must be taken to choose appropriate antigen targets and the binding affinity of TCRs, as serious off-target effects may occur[558, 559].

Subsequent trials have targeted different TAAs generating tumor regression in melanoma and other solid tumor types. For example, targeting gp100 in tumors yielded responses in 3 of 16 of metastatic melanoma patients, while the use of engineered TCR T cells for CEA yielded tumor regression in one of three CRC patients[553, 560]. Further, TCR transduced T cells for the antigen NY-ESO-1 yielded response in 5 of 11 patients with melanoma and 4 of 6 patients with synovial cell carcinoma; this response rate has led NY-ESO-1 to be the most prevalent target for engineered TCR T-cell therapy[561, 562].

Another iteration of TCR T-cell therapy is to identify antigens that arise from shared mutations (e.g., driver mutations) that would allow for treatment of multiple patients with the same TCR or TCR library[563]. For example, there are currently ongoing clinical trials of ACT with genetically engineered TCRs recognizing mutations in *KRAS* and *TP53*, which are common in multiple cancer types (melanoma, ovarian, epithelial cancers, etc.); further, preliminary data has demonstrated their efficacy, as a patient with metastatic colorectal cancer experienced a partial regression after treatment of T cells targeting the G12D mutation in *KRAS*[564, 565]. Uncovering these shared mutations or specific neoantigens in a

patient population may provide further insight on how we understand current ACT therapies; this will be revisited in the next section.

1.3.3.2.2 *Endogeneous T-cell therapy (ETC)*

The second antigen specific T-cell therapy, ETC, utilizes PBL that react to specific antigen, typically found at low frequency in circulation (often less than 1%)[566]. PBL are co-cultured with autologous DCs or artificial APCs that have been pulsed with peptide for the desired target to promote expansion of antigen specific T cells (See Figure 9B)[566, 567]. These cells undergo multiple rounds of peptide stimulation (2-4 cycles) and subsequently, T cells that demonstrate peptide reactivity are expanded[566, 567]. Patients are then infused with billions of T cells in a single or multi-dose regimen (e.g., every 3 weeks)[568, 569].

Initial clinical trials of ETC therapy utilized CD8 T cells specific for melanoma antigens MART-1 and gp100, and demonstrated clinical benefit in 8 of 10 treated patients[568]. Similar to other ACT therapies, including those discussed here, persistence of T cells was a determining factor for response[568, 570, 571]. To improve persistence, the administration IL-2 was included to the treatment regimen[568, 572, 573]. A further evolution included expanding use of not solely CD8 T-cell subtype, but rather CD4 T cells (particularly Th1)[574]. For example, a study whereby CD4 T cells recognizing the melanoma tumor antigen, NY-ESO-1, were infused into a patient, demonstrated complete tumor regression with cells persisting over 80 days; notably, this occurred in one of eight treated patients[574]. Further studying the phenotype of cells used in ETC demonstrated that there was greater persistence of T_{cm} cells compared to T_{em} cells[575]. Based on these results, a clinical trial using T_{cm} cells for ETC therapy (polarized by IL-21 during expansion) in combination with anti-CTLA-4 demonstrated persistence of T cells greater than 200 days and yielded 2 CR, 2 PR, and 3 SD in 10 metastatic melanoma patients treated[576, 577]. Notably, responses to therapy have been observed in multiple patients that have differential levels of antigen expression; this occurs through the process of epitope spreading,

whereby antigens released by dying tumor cells can activate an immune response, leading to recognition of tumor by other T cells[574]. With this cumulative knowledge, studies are still ongoing to continue identify other ICIs that may compliment ETC therapy[578].

1.3.3.3 ACT of Tumor-Infiltrating Lymphocytes (TIL)

The concept of TIL therapy is to utilize the T cells found in the tumor, expand them into large numbers (billions of cells), and reinfuse them back into the patient to overwhelm their tumor cells and ultimately cause tumor regression (See Figure 9C)[504]. The initial method to isolate and expand TIL included an initial tumor enzymatic digestion (including melanoma, sarcomas, adenocarcinomas, etc.), followed by a Ficoll separation for lymphocyte isolation and subsequent growth in 24 well plates with 1000 IU/mL of IL-2 for between 23 to 100 days[504]. Using this method, the first clinical trial conducted in metastatic melanoma patients generated an impressive 34% regression rate that lasted for one patient greater than 46 months, with little adverse events besides those associated with high dose IL-2 administration[579]. Notably, the high dose IL-2 administration was included in therapy as it also supports TIL persistence, as demonstrated by early preclinical models in lymphoma[580]. Initially, cyclophosphamide was used as lymphodepleting regimen for TIL therapy, as this had previously proven to be a curative combination in ACT mouse models of lung metastases; however, in clinical trials, the addition demonstrated no difference in response as compared to receiving IL-2 alone (35% vs. 31% response rates, respectively)[579, 581]. The method of lymphodepletion to further improve both persistence and response to therapy was revisited in later trials.

As mentioned above, TIL persistence and survival *in vivo* has been shown to correlate with response to therapy[571]. To improve the hypothesized short-lived persistence of TIL and thereby improve the duration of response, a subsequent study by the NCI characterized the most beneficial treatment regimen that is currently in use by most centers for TIL therapy today: lymphodepletion prior to therapy utilizing cyclophosphamide and fludarabine, followed by sequential dosage of HD IL-2 after

therapy[582]. Also at this time, a new method of TIL expansion was introduced, allowing for a shortened time frame for TIL growth. This process incorporated a 2 phase expansion procedure, later referred to as pre-rapid expansion protocol (pre-REP) and rapid expansion protocol (REP) phases[582]. The method of digesting the tumor for the pre-REP was subsequently replaced by cutting the tumor into 1-3 mm³ fragments that were then placed in individual wells of a 24 well plates (4-24 plates) with high dose IL-2, yielding a lymphocytic growth covering the bottom of the wells typically 1-2 weeks after culture initiation[582, 583]. This was followed by the REP, whereby cells from the pre-REP phase were used to expand to an even greater product of cells (billions)[582, 584]. Irradiated allogenic PBMCs (“feeders”) were added to the REP TIL to support TIL activation and growth[582, 584]. These cells were grown in specialized enriched cellular media (AIMV) containing anti-CD3 and high dose IL-2 for 7 days in a culture flask; cells were then transferred to a gas permeable bag for the remaining 7 days of expansion until maximal growth was reached[582, 584]. Whereas a previous trial using enzymatic digestion for TIL generation and cyclophosphamide prior to TIL infusion an IL-2 led to a 35% response rate and 4 month median duration of response, incorporation of this new treatment regimen altering both lymphodepletion and culturing technique yielded response rates of 51% and 11 month mean duration of response in a subsequent metastatic melanoma cohort[579, 585].

Further alterations have been made to generate the optimal protocol for TIL growth and overall patient treatment regimen. Shortly after identifying the need to include fludarabine to the lymphodepletion treatment, total body irradiation (TBI) was also tested for possible incorporation; although, generating a 70% response rate in metastatic melanoma patients at the highest dose tested of 12 (Gray) Gy, this was not significantly different than the other treatment groups (response rates of 48% with no TBI, 52% of 2 Gy TBI)[586]. Lack of difference with response with or without TBI was confirmed in a follow-up study in 2016[587]. Notably in the trial in 2008, however, it was observed that patients that responded to therapy were infused with TIL with longer telomeres; prior studies have also indicated the importance of CD27 and CD28 expression in TIL persistence (discussed in greater detail in

the biomarker section)[586, 588, 589]. Thus, there evolved a new goal to ultimately shorten the time frame of TIL growth to allow for quicker treatment of the patients and allow TIL to have a “young” phenotype[586]. The “young” TIL protocol removed the step in the process testing the reactivity of TIL prior to expansion, which ultimately led to TIL having a phenotype that resembled more persistent clones[590]. To confirm this, a trial using the “young” TIL protocol noted improved persistence of metastatic melanoma TIL, as measured at one month in circulating PBMCs; a response rate of 55% was observed, which was comparable to previous TIL trials[591].

Similarly to what was observed with correlations with a young TIL phenotype, it was hypothesized that modifications to the final infused TIL product could also influence patient outcome. Initially, it was suggested that CD8 TIL in the infusion product correlated with response to therapy, an idea later confirmed in multiple trials[592-595]. A trial was developed to determine whether infusion of CD8 TIL alone (through magnetic bead sorting) would thus improve patient response; retroactive comparison to prior studies showed no difference in patient response in this trial, but did still suggested high CD8 TIL numbers correlated with tumor regression[591, 596]. Further details regarding CD8 TIL and other differences in phenotype associated with response to therapy will be detailed in the following biomarker section. Other modifications also applied to the use of growing containers, initially performed in flasks or gas permeable bags, but expanded into the use of bioreactors, including the use of the Wave (GE Healthcare) or G-Rex flasks (Wilson Wolf)[597-600]. Both of these bioreactors were utilized to improve gas permeable exchange while growing TIL, thereby enhancing the proliferative capacity of these TIL[597-600].

To improve the TIL product by enhancing cellular growth of TIL and TIL phenotype that is correlative with response to therapy, there have also been improvements in culture conditions with the use of different antibodies. As previously mentioned, phenotypic correlation to therapeutic response has included proportion of CD8⁺ TIL infused and telomere length of TIL infused; this will further be

described in detail in the next section[586, 595, 601]. One such example has been the use of the addition of 4-1BB agonist (BMS urelumab) to media containing IL-2 in the initial expansion phase of TIL growth in melanoma, which yielded improved expansion of memory CD8 TIL comparatively[602]. Further, the addition of anti-CD3 (OKT3) to initial culture media containing IL-2 and 4-1BB to melanoma tumor fragments triggers signals 1, 2, and 3 of T-cell activation, leading to improved T-cell growth rates within a shorter time period[499]. For example, this method led to 100% TIL culture expansion in uveal melanoma, where only 15% of cultures grew using IL-2 conditioned media alone[499]. Addition of 4-1BB was also shown to protect CD8 TIL from AICD and enhances their cytolytic potential[500, 501]. Thus, the culturing method can impact the growth rate and ultimately contribute potentially to response of the patient.

After establishment of the cyclophosphamide/fludarabine lymphodepletion treatment regimen, TIL therapy expanded to other centers and response rates to TIL therapy have stayed relatively stagnant at the NCI in larger cohorts of metastatic melanoma patients, with 56% of metastatic melanoma patients experiencing regression and with 19 patients surviving over 3 years with complete regression at the NCI out of 93 patients[601]. A slightly lower response to therapy is observed worldwide, with response rates ranging from 38-42%, but with complete response rates between 10-20% [594, 603-605]. As TIL therapy is often the last line of treatment for patients and with more patients becoming refractory to ICI treatment, questions have been raised regarding whether ICI exposure makes this population more resistant to TIL therapy. Initial studies investigating this question determined that exposure to anti-CTLA-4 prior to ACT did not impact the response of patients to TIL therapy[601, 603, 606]. However, in our own cohort of metastatic melanoma patients at MD Anderson, it has been observed that patients refractory to anti-CTLA-4 treatment that subsequently receive TIL therapy have worse response rates (38% anti-CTLA-4 exposed vs. 47% in naïve) and shorter OS (8.6 months anti-CTLA-4 exposed vs. 24.6 months in naïve)[604]. Further, a recent publication has noted that patients refractory to anti-PD-1 treatment have similar response rates, but significantly shorter

duration of responses[606]. The impact on growth and TIL phenotype that these checkpoint inhibitors, including anti-PD-1, may have on patient TIL have not been extensively studied; this will be addressed in chapter three of my thesis.

Although predominantly used as a treatment for metastatic melanoma, TIL therapy has also been utilized in multiple other cancer types, including cervical and ovarian cancers with varying levels of clinical benefit[607, 608]. For example, HPV derived cervical cancer has a 28% response rate in a cohort of 18 patients and non-cervical HPV-driven cancers have an 18% response rate in a cohort of 11 patients; however, responses are still durable in these cancers, with 2 patients responding for over 53 and 67 months[609]. Results of TIL therapy in metastatic melanoma and cervical cancer have been reproduced in industry; lovance Biotherapeutics has announced 36.4% response rate in melanoma and 44.4% response rate in cervical cancer, and has completed accrual on a registrational cohort for metastatic melanoma[610, 611]. Thus, TIL therapy may be on the verge of being commercialized.

Promising results have been witnessed with TIL therapy in ovarian cancer, yielding tumor regression in 3 of 4 patients[608]. Thus, TIL therapy may still be a viable option for treatment for tumor types that have limited responses to other therapies, including malignancies that have been associated with poor infiltration due to improvements in the culturing method. One such example is pancreatic cancer. When culturing TIL from pancreatic cancer tumor fragments, the importance of agonist stimulation is demonstrated, as the inclusion of IL-2 and 4-1BB in the TIL culture media in the pre-REP phase of two different studies demonstrated a cellular population skewed towards cytotoxic CD8 T cells[498, 612]. Further, there was a more clonal repertoire in these TIL as compared to TIL grown in media supplemented with IL-2 alone, suggesting these cells may elicit more tumor control than the IL-2 grown cells[498]. However, the use of this culture method also improved the ability to culture CD8 TIL in ovarian cancer, a highly infiltrated cancer type that responds poorly to checkpoint blockade. With the addition of 4-1BB and anti-CD3 (with IL-2) triggering all 3 signals of activation to the pre-REP TIL

fragments, cells could be grown in a faster time frame (2-3 weeks vs. 3-5 weeks) from 95% of cultures as compared to 41% set up with IL-2 alone; further, these cells showed recognition to an HLA-matched tumor line[613]. This ability to expand TIL was further put to the test, utilizing core needle biopsies in rare tumor types, which will be described in chapter three of this thesis.

Different approaches have been attempted to increase the potency of the TIL product. One of them involves increasing the frequency of anti-tumor TIL by selective enrichment for neoantigen-reactive TIL. A methodology was developed to identify novel tumor-specific mutated antigens (neoantigens) using analysis of TIL generated for therapy[614]. Utilizing a tumor or tumor cell line, next generation sequencing (NGS) is used to identify candidate immunogenic HLA class I peptides; TIL are co-cultured with peptide pulsed APCs to determine reactivity through typically an IFN γ Elispot[614]. The identified reactive TCRs can then be selected during TIL culture (e.g., selecting TIL in a well coming from a specific fragment), skewing the population to have a greater enrichment of these clones[615]. Ultimately, however, this method of targeting neoantigens has been found to be most appropriate to treat individual patients, as most neoantigens identified are often not shared with other patients[616]. For example, using this method, TIL enriched for neoantigen reactivity have generated durable tumor regression (greater than 22 months) in a patient with metastatic breast cancer[615].

To further enhance the efficacy of TIL therapy, another approach involves the genetic modification of TIL. Genetic modification is generally used to impart TIL with better function. Genetically modified TIL are modified cells to include domains that improve their function/survival (like TGF β dominant negative receptors or CXCR2 expressing engineered TIL[617, 618]. This methodology is similar to that of normal TIL therapy, except TIL are transduced by a retrovirus containing the vector that specifies the modifications to be made to the TIL (i.e., receptor)[619]. In preclinical mouse studies, transduced pmel T cells expressing CXCR2 demonstrated improved trafficking to melanoma tumors and tumor control[618]. In a clinical study of TIL transduced to secrete IL-2 in an attempt to negate the need

of supportive IL-2 regimen, one response was observed in seven patients treated, but unfortunately no improvement in cellular persistence was noted[620]. In a similar study, inducible IL-12 transduced TIL, under the control of the NFAT promoter for targeted release of IL-12 at the tumor site upon TCR engagement, demonstrated short-lived response within metastatic melanoma patients, but suffered from poor persistence as well[621]. This study remains important since it is the only study to date demonstrating high objective response rate (63%) in 16 patients receiving low TIL doses (0.3 to 3.0 billion). It is unfortunate that IL-12 “leaking” from the cells became toxic at a dose of 3 billion TIL forcing to stop the dose escalation. There are more ongoing clinical trials of genetically modified TIL therapy, which will hopefully yield insights in elements regulating TIL function and result in improved TIL persistence and improved response rates. Other methods to improve response rates, including the identification and use of biomarkers will be discussed in the next subsection.

1.4 Biomarkers of Response to Immunotherapy

The definition of “biomarker” has gone through many iterations. In broader terms, the NCI defines a biomarker as a biological molecule that can indicate how well a body responds to treatment[16, 622]. This includes reliably measurable medical indications spanning basic clinical readouts such as blood pressure measurements to more complex laboratory discoveries like genetic markers[622]. The timing of assessment of biomarkers is also important; biomarkers assessed prior to therapy can have predictive value, while on treatment and post-treatment measurements describe attributes of potential success or failure. In this section of my thesis, I will focus on four major categories of pertinent immunotherapy biomarkers: T-cell infiltrate, tumor genomic alterations, soluble factors, and transcriptomic profiling. In doing so, I will investigate their impact on two main types of immunotherapy, antibody based (focusing on first generation ICIs) and cellular based immunotherapies (specifically TIL therapy).

1.4.1 Biomarkers for antibody based immunotherapy

1.4.1.1. T-cell Infiltrate

In general, presence of TIL is often associated with improved prognosis in multiple cancer types, including ovarian cancer, CRC, and melanoma[623-625]. The degree of TIL infiltration has been associated with therapeutic response to ICI therapy due to its mechanism of action which relies upon T cells[345, 626]. Not only is mere T-cell presence important, but localization and clonality aid in delineating response and/or survival to therapy[310, 313, 626]. For example, the presence of CD8 TIL in the invasive margin before treatment, particularly those expressing PD-1, is significantly correlated with response to therapy and tumor regression when treated with pembrolizumab in melanoma, especially when localized near PD-L1 expressing cells[626]. Further, clonal CD8 TIL (those cells expressing the same TCR) are enriched in pre-treatment samples of patients that responded to PD-1 blockade in melanoma[626]. Infiltration of CD8 TIL is also witnessed in regressing melanoma lesions in response to anti-CTLA-4 therapy[627, 628]. Differing from anti-PD-1 therapy, longer OS after blockade of CTLA-4 can be correlated to a greater infiltration of ICOS^{hi} expressing CD4 TIL in metastatic melanoma[629]. Further unlike PD-1 blockade, TIL TCR clonality at pre-treatment or on-treatment timepoints does not correlate with response or improved survival to ipilimumab treatment[630]. Overall, it is important to note that these are not predictors of response, meaning having CD8 TIL presence or a clonal population cannot be used as a determinant for whether a person should or should not receive therapy, but is only suggestive of outcome, as a threshold of guaranteed response is not applicable.

Infiltration of other cell types, including B cells and DCs, have shown to be correlative with response to checkpoint blockade[148, 149, 631]. For example, by stratifying melanoma patient samples by their presence of T cells (high vs. low), there was an observed correlation between activation of the β -catenin pathway and lack of CD3 expression; further investigation of this finding using melanoma mouse models show lack of CD103⁺ DCs cells due to the tumor intrinsic β -catenin signaling pathway are associated with poor prognosis to anti-CTLA-4 and anti-PD-1 therapy in preclinical models [631]. Further, the presence of tertiary lymphoid structures (TLS) in pre-treatment tumors is associated with the highest response rate cohort to pembrolizumab in soft tissue sarcomas (STS)[148]. The importance

of infiltration of both B cells and DCs could also be tied to their ability to present antigen to CD8⁺ TIL and generate a T-cell mediated response[148, 631].

The correlation with response to ICIs and T-cell infiltrate, phenotype, and clonality does not hold true for all cancer types, with no association of level of infiltration of TIL found in the more highly infiltrated tumor type NSCLC in pre-treatment tumors[632]. In addition to the importance of the degree of T-cell infiltration in outcome to therapy, tumors with highly suppressive TMEs, which tend to not be well infiltrated, do not respond well to checkpoint inhibitors; such cancer types include pancreatic cancer and castration-resistant prostate cancer[316, 633, 634]. Therefore, much remains to be elucidated to identify biomarkers to improve therapeutic responses in these patient cohorts.

Many studies have utilized immunohistochemistry (IHC) to determine localization and cellular infiltrate in the tumor, but this technology's main limitation is that it allows for only a handful of markers to be evaluated. Further phenotyping on dissociated tumor samples can be performed through the use of high-throughput flow cytometry or mass cytometry (CyTOF). For example, flow cytometry demonstrated the freshly resected melanoma tumors of anti-PD-1 responding patients and those with long PFS had high expression of PD-1 and CTLA-4 on CD8⁺ TIL[635]. However, there has been mostly characterization of circulating cells (PBMCs) rather than tumor cellular infiltrate after exposure to ICIs. This gap in knowledge will be addressed in the third chapter of this thesis.

1.4.1.2. Tumor Genomic Alterations

The tumor mutation burden (TMB) is the number of non-synonymous mutations (DNA change that alters amino acid protein sequence) within the tumor cell[16]. A greater TMB has been shown to correlate with clinical benefit to both anti-CTLA-4 and anti-PD-1 therapy in NSCLC and melanoma (defined as response or SD greater than 6 months)[636, 637]. For example, patients that have greater PFS and respond to pembrolizumab in NSCLC have a greater TMB; in metastatic melanoma, patients with a high TMB had greater survival after anti-PD-1 therapy[637, 638]. Also, patients with metastatic

melanoma that derived clinical benefit (response or SD for greater than 6 months or 1 year) from CTLA-4 therapy had a greater TMB[636, 639]. The relationship between greater TMB and benefit to ICI therapy has subsequently been demonstrated to hold true for other cancer types, including urothelial cancer and head and neck cancer, but is not predictive for all, as is the case for glioma, Hodgkin's lymphoma, and RCC[640-644].

The biological importance of the TMB is linked to the generation of neoantigens, which can improve tumor immunogenicity as these antigens originally derived from "self proteins" are now seen by the immune-system as foreign[645]. Therefore, neoantigens can ultimately lead to a more robust T-cell mediated response, which may be enhanced with immunotherapy; similarly to TMB, the number of predicted neoantigens have also been associated with response to ICIs[639, 646]. For example, high predicted neoantigen load associates with longer PFS and response to anti-PD-1 therapy in NSCLC and with clinical benefit in melanoma (response or SD for greater than 1 year)[637, 639]. On the contrary, neoantigen loss has been observed in tumors of ICI resistant NSCLC patients[647]. Neoantigen heterogeneity has also been shown to be predictive of benefit to ICI therapy, with low heterogeneity (<1% associated) associated with greater OS in NSCLC patients treated with pembrolizumab[648]. Notably, the relationship of the neoantigen load and greater tumor immunogenicity is not always correlative; to invoke a T-cell response, the antigen must be presented via the MHC molecule[649]. Therefore, there is often a gap between the predicted neoantigen load and what actually occurs *in vivo*.

The role of mutations in specific genes may also play a role in therapeutic response to ICIs. Previous studies have demonstrated that loss or mutation of DNA mismatch repair (MMR) genes can lead to enhanced TMB and generation of neoantigens[650, 651]. Thus, there is also often a relationship between DNA MMR genetic loss or mutation and response to therapy; this was shown in a study whereby response rates to pembrolizumab in patients with cancers containing MMR-deficient genes was greater than the patients that contained functional MMR cancer types[651, 652]. Also, patients

with mutations in MMR genes *POLE* and *POLD1* have higher mutation burdens and in patients with NSCLC, have greater response to anti-PD-1[637]. Other specific genes and pathways that have been identified in association with response to ICI will be discussed in the transcriptomic profiling subsection.

1.4.1.3. Soluble Factors

Soluble factors are a broad category of molecules that are produced by cells, which can include but are not limited to cytokines/chemokines, serum proteins, circulating DNA/RNA, and soluble receptors[653]. In this section, I will present both soluble factors found in the peripheral blood and those found in the TME.

1.4.1.3.1. Soluble Factors in Peripheral Blood

Measuring the circulating soluble factors in the peripheral blood is an attractive method to identify biomarkers due to its clinical feasibility and minimally invasive nature for the patient[654]. Through this method, VEGF and C-reactive protein (CRP, important in opsonization) levels in the serum of patients initially were found to associate with response to the first immunotherapy, IL-2[655, 656]. When investigated in melanoma patients treated with ipilimumab, the diminished expression of CRP associated with response and improved survival, while high VEGF levels pre-treatment were correlated with poor OS in melanoma[657, 658]. In contrast, the pre-treatment VEGF concentration in the serum of melanoma patients treated with PD-1 blockade is not associated with poor response[659]. Measurement of lactate dehydrogenase (LDH) revealed that low basal serum level was associated with response and survival in metastatic melanoma patients treated with anti-CTLA-4 therapy; notably, LDH has been shown to be important in glycolysis, can be indicative of tissue damage, and can testify of tumor metastases[657, 660]. Further investigations interrogating soluble receptors present in the serum led to the discovery that the soluble IL2Ra (also known as sCD25), which is shed by activated T cells and sequesters IL-2 compromising further T-cell activation, was shown to be greater in baseline serum of melanoma patients that had shorter OS after ipilimumab treatment independently of

response[661]. This observation was also true in the patient's serum 3 weeks post-therapy. Other soluble receptors have been correlative with response to ICIs, including high expression soluble CTLA-4 in baseline serum (sCTLA-4) which has been associated with clinical benefit to ipilimumab and improved survival following ipilimumab treatment in melanoma[662]. In anti-PD-1 therapy, higher concentration of soluble PD-1 (sPD-1) and PD-L1 (sPD-L1) in the sera of patients prior to treatment are associated with worse response, OS, and PFS; similarly, lower concentration of sPD-L1 before treatment has been correlated with clinical benefit in NSCLC (defined as CR, PR, or SD) [663, 664]. A more extensive list of soluble factors in response to anti-PD-1 or anti-CTLA-4 in melanoma can be found in Table 3.

Table 3. Soluble Factors in Serum associated with Response to ICI Therapy in Melanoma

<i>ICI</i>	<i>Soluble Factors</i>	<i>Time of Assessment</i>	<i>Correlation with Outcome</i>	<i>Reference</i>
<i>Anti-CTLA-4</i>	IL-6	During Treatment	High levels associated with lack of response	Bjoern et al., 2016[665]
<i>Anti-CTLA-4</i>	CXCL11 and sMICA	Pre-treatment	High levels associated with poor OS	Koguchi et al., 2015[666]
<i>Anti-CTLA-4</i>	IL-8	Post-treatment	Decrease associated with response	Sanmamed et al., 2014[406]
<i>Anti-PD-1</i>	TNF α	Post-treatment	Decrease associated with response	Tanaka et al., 2017[667]
<i>Anti-PD-1</i>	IL-8	Post-treatment	Decrease associated with response	Sanmamed et al., 2017[668]
<i>Anti-PD-1</i>	sCD163	Post-treatment	Increase associated with response	Fujimura et al., 2018[669]
<i>Anti-PD-1</i>	IFN γ , IL-6, and IL-10	Pre-treatment	Higher in responders	Yamazaki et al., 2017[670]
<i>Anti-PD-1</i>	TGF β	Pre-treatment	High levels in responders	Nonmura et al., 2016[671]

1.4.1.3.2. **Soluble Factors in the Tumor Microenvironment**

Utilizing methods of immunohistochemistry and most recently genetic sequencing to identify soluble factors measured in tumor tissue, the association between a patients' response to ICIs and

these molecules can be directly uncovered in the TME. Some of the most studied include, IDO and IFN γ [654]. As previously discussed, IDO is immunosuppressive to T cells through inhibiting cellular activation and proliferation and by promoting CD4 polarization to T_{regs}[25, 672]. Surprisingly, IDO is found to be more highly expressed in the pre-treatment tumors of melanoma patients treated with CTLA-4 blockade that experienced clinical benefit (defined as responders or greater than 24 week SD) and responders to anti-PD-L1 therapy[628, 673]. Further, in RCC patients treated with anti-PD-1 therapy, greater expression of IDO in tumor endothelial cells was associated with greater response and PFS; the expression of IDO was also observed to be correlative with the presence of CD8 TIL[674]. Thus, although an immunosuppressive cellular factor, IDO may be indicative of a more inflamed TME, potentially explaining its correlation with greater response to ICIs[628, 674].

As aforementioned, IFN γ is typically associated with an active immune response, as it can be secreted during a T-cell mediated immune response and can cause MHCI upregulation[387, 675]. When measured in the TME, there is also a correlation of response and survival to anti-PD-1 therapy in melanoma patients, as identified using transcriptomic profiling of baseline samples (defined in the next section)[676, 677]. Utilizing a similar method in pre-treatment tumor tissues of NSCLC, head and neck squamous cell carcinoma (HNSCC), and melanoma patients treated with PD-1 blockade, an IFN γ signature was correlated with greater response and survival[678, 679]. Similarly, there was dysfunctional IFN γ signaling in the tumors of melanoma patients that did not respond to anti-CTLA-4 therapy[680]. Thus, the presence of IFN γ in the TME can also be indicative of an active immune response[681].

1.4.1.4. Transcriptomic Profiling

The transcriptome encompasses all non-coding and coding RNA within a cell or organism and is often analyzed by two methods: RNA sequencing or DNA microarray[682, 683]. Utilizing all expressed messenger RNA (mRNA) and long non-coding RNA (lncRNA), transcriptomic profiling by RNA sequencing can identify different genes expressed or enriched in a particular organism (e.g., tumor). These genes

then undergo bioinformatic analysis using software systems that identify patterns of expression (including GAGE, GSEA, etc.), thereby determining which pathways may be implicated[683, 684]. This robust methodology is therefore useful in identifying potential biomarkers to ICI therapy, by indicating which biological pathways are important in responding or non-responding patients[685]. Due to the nature of this thesis, I will focus on introducing studies of transcriptomic profiling of the whole tumor tissue (not single cell or blood transcriptomic profiling).

Initial studies investigating transcriptomic differences between patients receiving benefit to ICI therapy revealed that the baseline tumors of melanoma patients treated with ipilimumab with longer survival and/or clinical benefit (defined as response and SD greater than 1 year) had greater expression of cytolytic molecules granzyme A and perforin along with increased expression of CTLA-4 itself[639]. Further investigation into transcriptomes of ipilimumab treated melanoma patients' demonstrated enrichment in the Type I IFN response and tissue resident cells in responding patient tumors[686]. Overall, corroborating what has previously been shown in the above sections, responding patient tumors seem to have a more active immune response with increased cytolytic cell presence and IFN expression[639, 686].

Similarly, there have been studies investigating tumors of melanoma patients treated with anti-PD-1 therapy[638, 687]. In one such study, there were over 693 genes discovered to be differentially expressed between pre-treatment tumors responders and non-responders to therapy, with most genes enriched in non-responding patient tumors[638]. Pathway analysis determined that genes enriched in these non-responders were associated with the epithelial to mesenchymal transition (transition to a more aggressive phenotype), immunosuppression, monocytic cells chemotaxis, and angiogenesis; together, Hugo et al. defined this signature as the Innate anti-PD-1 Resistance Signature (IPRES)[638]. Unfortunately, this IPRES signature was not significantly different between pre-treatment tumor tissues of melanoma patients treated with nivolumab in a separate study, but an enrichment of a T-cell

activation or “hot tumor” microenvironment (high T-cell infiltration and pro-inflammatory cytokines) in the responders was still observed[687]. This underlies the variability caused by different patient cohorts regarding signature profiling and often necessitates the ability to perform confirmatory sequencing with a larger or secondary cohort or using another assay for validation (e.g., polymerase chain reaction, PCR)[688]. Thus, validation can be used to answer two distinct questions, as it confirms the methodologies and discoveries from the assay and the approach used for analysis.

With the more prevalent use of combination ICI treatment, transcriptomic profiling of melanoma patient samples have identified notable differences in the immune response and TME between the two therapies; for example, circulating T cells from CTLA-4 treated patients demonstrated an enriched proliferation signature compared to anti-PD-1 treated patient T cells that had greater expression of a cytolytic signature[685]. More recent data has also shown that prior exposure to anti-CTLA-4 therapy can impact response and alter the transcriptomic profile of anti-PD-1 treated melanoma patients; for example, the IFN γ and IFN α pathways were enriched in responders to PD-1 blockade, but this was no longer the case after prior exposure to anti-CTLA-4[689]. Thus, this demonstrates that not all immunotherapies impact the immune microenvironment or TME in tumors in the same fashion and underscores the importance of understanding different biomarkers tailored for different therapies and the order in which they are administered can also interfere with the biomarker reading. In the next section, I will introduce some of these biomarkers associated with response to ACT.

1.4.2 Biomarkers for cellular based immunotherapy

1.4.2.1. T-cell Infiltrate

The ability to grow TIL acts as the first filter for patients to be eligible to receive TIL therapy. Multiple institutions have adopted a cut-off of 50 million cells within the pre-REP stage as a determinant of “successful growth” and have demonstrated a 60-70% successful culture rate[583, 585, 690]. [At MD Anderson, with the incorporation of the TIL 3.0 method of culture, there is almost a

removal of this filter for patients in terms of pre-REP growth, with dramatically improved culture rates (nearly 100%) in both cutaneous and uveal melanoma within 3 weeks][499]. A threshold of specific TIL phenotypes have been observed to correlate with growth, as patients with successfully expanded cultures had greater presence of CD3, CD4, and CD8 as measured by IHC[691]. In terms of threshold of T-cell infiltration to enable TIL growth, when utilizing IL-2 for the pre-REP phase of culture in ovarian cancer, TIL growth correlated with CD3 presence and tumors with less than 2% of CD3 TIL infiltration (as measured by flow cytometry in freshly disaggregated tumor tissue) often did not grow TIL[613]. To promote T-cell infiltration in the tumor tissue before harvest for TIL expansion as well as potentially augment the anti-tumor activity of the TIL product, treatment with ipilimumab prior to tumor resection for TIL therapy was evaluated, as this strategy had previously led to improved TIL infiltration in preclinical models [665, 692]. Ipilimumab given prior to TIL harvest in a small cohort of 13 patients led to successful TIL outgrowth in all 13 patients but no significant improvement in anti-tumor reactivity of the TIL cultures was noted, and it did not change the TIL fold expansion in the REP or number of TIL in the infusion product[693]. Also, not all immune cell infiltration into the tumor promotes TIL growth, as the presence of NK cells and regulatory innate lymphoid cells have been observed to be inhibitory[592, 694]. Thus, using a mechanism like TIL 3.0 to allow for particularly CD3 expansion, without expanding the inhibitory NK cell populations, is significant for improving therapeutic outcome.

Although the newer methodologies to grow TIL render the success of expansion independent of the level of infiltration, little is known regarding the importance of TIL infiltration and the nature of the infiltrate in pre-treatment lesions and response to therapy. Data from our lab suggests that increased infiltration of both CD8⁺ and FoxP3⁺ cells are associated with longer OS after TIL ACT, potentially hinting at the need for a “T-cell permissive” environment for the success of T-cell therapy[691]. The loss of expression of a repressor of PI3K signaling, PTEN in the tumor, has been shown to promote resistance to T-cell mediated killing by creating “immune deserts” or areas of T-cell exclusion, and is associated with lack of response to anti-PD-1 immunotherapy[695]. In our cohort, 44 of 48 TIL treated patients

expressed PTEN; thus there was no difference in PTEN expression based on response to TIL therapy[695]. This result is consistent with the importance of PTEN in T-cell infiltration. It is conceivable that tumors with PTEN loss did not have T cells to expand and thus, did not allow patients to be treated with TIL therapy. Consequently, PTEN expression did associate with TIL growth, as patient tumors that did not grow expressed more PTEN loss[695]. Further, CD74 expression on the baseline tumor used for generation of TIL was correlated with response to therapy in metastatic melanoma tumors[696]. CD74 is an important molecule in antigen presentation via MHCII and acts as a cell surface receptor for the pro-inflammatory cytokine macrophage migration inhibitory factor (MIF)[697, 698]. Interestingly, expression of MIF by melanoma tumor cells was associated with poor OS and PFS of patients receiving TIL therapy[696]. Further characterization of TIL infiltration in pre-treatment tumors and association with response or survival to therapy is still ongoing and needs further exploration.

The cellular infiltration of the tumor shapes the ultimate TIL infusion product used in treatment, and thus the identification of TIL ACT biomarkers has mostly been based upon characterization of the pre-REP TIL/cellular infusion product and its correlation with therapeutic outcome. To define which patients may respond to TIL therapy, initial studies first investigated the reactivity of TIL during expansion, as naturally it was hypothesized greater TIL-tumor reactivity would yield greater therapeutic responses; however, it has been shown that there is no difference between the ability of responding or not responding patient pre-REP TIL to recognize their autologous tumor cell line[591, 601, 690, 699]. Similarly, TIL TCR antigen specificity for melanoma markers like MART-1 and melan-A have also not been associated with response to therapy[700]. In the same manner for the pre-REP TIL, characterization efforts have also been performed for the infusion (REP) TIL product. For example, TIL TCR specificity for melanoma-associated proteins like MART-1/melan-A have also not been associated with response to therapy[700]. Also, as mentioned in the cellular based immunology section, the presence of CD3 TIL, particularly CD8 TIL, in the infusion product correlates with response to therapy[592-595]. Further, patients with TIL having longer telomere length (less proliferation cycles)

responded to TIL therapy[586]. Other TIL phenotypes associated with differentiation status have correlated with response, but with somewhat conflicting reports. Initially, the NCI published data suggesting that less differentiated CD8⁺CD27⁺ T_{em} cells were associated with response to therapy, but cells that expressed CD28 rather than CD27 had greater persistence[571, 601, 701, 702]. Further, CD8⁺CD27⁺ T_{em} cells was not found to be correlative with therapeutic response in other cohorts[595, 603]. Our group also observed there is greater expression of B and T lymphocyte attenuator (BTLA) on CD8 TIL in the infusion product of responding patients, which has been shown to be a newly defined marker for less differentiated T_{em} cells[595, 703]. Thus, overall, less differentiated TIL in the infusion product correlate with response to therapy; however, research is still ongoing to further elucidate the ideal TIL differentiation status and phenotype that can be used to generate greatest response rates.

1.4.2.2 Tumor Genomic Alterations

The TMB, as defined in the previous section, is comprised of the non-synonymous mutations found within the tumor and is particularly influential in the generation of tumor neoantigens[16, 645]. Neoantigens are antigens absent from the genome arising from somatic mutations in the tumor and are often considered to be ideal therapeutic targets, due to their ability to elicit T-cell mediated responses without off-target or autoimmune adverse events[704]. The ability of neoantigens to improve the response of TIL has thus become a major focus to harness and augment existing immune responses to allow for enhanced tumor clearance.

The field of cellular therapy became increasingly interested in the TMB and thus neoantigens after several studies of metastatic melanoma patients who experienced tumor regression reported that the patient was treated with a high proportion of mutated antigen specific T cells[614, 705-708]. Further, it was also demonstrated that TIL have low reactivity to shared melanoma associated TAAs such as MART-1, gp100, and tyrosinase, and that the summation of the shared antigen reactivity represents only a small fraction of the overall TIL reactivity to autologous tumor for each patient, thus

failing to identify the bulk of tumor antigens recognized by TIL[709, 710]. Most of these studies demonstrated persistence of the neoantigen-specific TIL in the blood for years post infusion in responder patients; ultimately, this suggesting that reactivity of TIL to specific neoantigens can yield complete and durable tumor regression[614, 706-708].

The relationship between TMB, neoantigen load of melanoma tumors, and the outcome to TIL therapy was examined in a cohort of 25 patients. Using pre-treatment TIL therapy frozen patient samples, Lauss et al. showed that neither TMB nor predicted neoantigen load correlated with RECIST response to TIL ACT, however both TMB and predicted neoantigen load were predictive biomarkers with survival and benefit to therapy, as both correlated with longer PFS, longer OS, and clinical benefit (defined as either CR, PR, or OS of greater than 2 years) to therapy[711]. Similarly, in our cohort of TIL treated patients at MDACC, we also observed that there was no association between response to therapy and TMB[604]. For further analysis within our cohort, we thus deemed it also appropriate to interrogate deeper the association of TMB with PFS and OS and possible correlations with neoantigen load. In depth rationale and analysis of this study will be addressed in detail in Chapter 4 of this thesis.

1.4.2.3. Soluble Factors

Similarly to the *Biomarkers for antibody based immunotherapy* section, I will present soluble factors (molecules produced by cells, including cytokines, chemokines, etc.) that correlate with clinical outcome to TIL therapy found in the peripheral blood and those found in the TME.

1.4.2.3.1 Soluble Factors in Peripheral Blood

The serum of patients receiving TIL ACT can serially be investigated prior and post receipt of the TIL product, allowing for monitoring of changes in circulating cellular composition and identification of potential soluble factors that may correlate with therapeutic response. Most of the current knowledge regarding soluble factors in serum of TIL patients has been generated at MDACC[604]. By interrogating the serum prior to TIL therapy of 50 patients, greater IL-9 levels were associated with clinical

response[604]. As previously described in the T-cell subsection, IL-9 is a pro inflammatory cytokine that can be secreted by Th9 T cells, which are able to control melanoma tumor growth in mouse models[216, 217]. High lactate dehydrogenase (LDH), which is indicative often of tumor burden and metastases, measured in the serum prior to TIL therapy was associated with shorter OS in 73 metastatic melanoma patients at MDACC[604]. This finding was not surprising as elevated serum LDH is one of the strongest independent prognostic factors in metastatic melanoma[660]. A similar observation is found in ICI treatment, with the association of LDH with response and survival to both anti-PD-1 and anti-CTLA-4 therapy [650, 657, 712-714]. The differences observed between both therapies will be revisited in the following chapters of this thesis.

Investigation of circulating serum factors from patients after receiving TIL therapy revealed interesting trends. For example, soluble factors measured at week 3 post-TIL infusion (prior to second dose of IL-2) identified soluble tumor necrosis factor receptor 1 (sTNFR1), a molecule that sequesters TNF α , preventing inflammation, was significantly enriched in the serum of patients that did not respond to therapy, with a trend of enrichment also in non-responding patients serum at both baseline and 3 months post-therapy time points (cohort n=48)[604]. sTNFR1 has also been found to correlate with worse outcome in patients treated with checkpoint blockade, where patients that did not reach 2 year survival post-ipilimumab treatment had enriched sTNFR1 in their serum at 3 and 7 weeks post therapy[715]. Finally, soluble UL16 binding protein 1 (ULBP-1) and soluble MICB were both found to be greater in the serum of responding patients 3 months after TIL infusion[604]. Both ULBP-1 and MICB are expressed by melanoma tumors and bind to NKG2D found on NK, CD8, and $\gamma\delta$ T cells, which stimulates immune cell activation; tumor cells are known to shed the NKG2d ligands ULBP-1 and MICB to avoid immune detection[716-718]. Interestingly, ULBP-1 and sMICB in baseline serum levels correlate with worse survival and advanced disease stage in melanoma and have been correlated with shorter OS to ICI treatment, with ULBP-1 also associated with worse response[719, 720]. It is unclear

why soluble ULBP-1 and MICB correlate with better outcome to TIL therapy, but this again highlights the potential differences between ICI and TIL therapies.

1.4.2.3.2 *Soluble Factors in the Tumor Microenvironment*

Unfortunately, there has been relatively limited study of soluble factors in the TME of patients that receive or have received TIL therapy. At MD Anderson, nitrotyrosine (NT) in tumors used to grow TIL was associated with both poor TIL growth and lack of response to therapy[696]. Further, MIF and inducible nitric oxide synthase (iNOS) produced by melanoma cells in baseline tumor samples also were associated with poor OS and PFS after TIL therapy[696]. All three of these molecules, MIF, NT, and iNOS, can promote tumor progression and immunosuppression[721, 722].

There is a current need to further investigate potential other soluble factors for use as either biomarkers for outcome to TIL ACT, patient selection prior to treatment, or potentially novel targets to improve therapeutic efficacy. With this current deficiency in knowledge, it is important to expand and explore other methods of identifying biomarkers, including quantifying RNA and protein expression.

1.4.2.4. *Transcriptomic Profiling*

Transcriptomic signatures that may correlate with patient response and survival to TIL therapy are not known. Investigating transcriptomic data utilizes predominantly RNA sequencing to identify not only specific genes, but also pathways that may be implicated in better or worse clinical outcome[683]. Whole tumor tissue profiling (not blood or single cell) will be the focus of this subsection.

Thus far, transcriptomic profiling of tumors from patients treated with TIL ACT has been studied by Lauss et al., who performed RNA sequencing on 24 frozen baseline tumor samples of metastatic melanoma patients along with TMB evaluation as previously discussed in the biomarker section[711]. From this data, TIL treated patients that received clinical benefit (response or SD greater than 2 years) or longer OS did not have a similar genetic signature as anti-PD-1 responding patients (as compared to

IPRES signature, described in detail in the previous section)[638, 711]. This further supports the idea that the response to ICI and TIL therapy is divergent. Interestingly, gene set enrichment analysis (GSEA) showed patients with clinical benefit to therapy were found to have a greater enrichment of genes associated with an IFN γ signature and MHC-I antigen presentation[711]. Also, in patients where no clinical benefit was observed, there was an enriched cell cycle genetic signature, which could correspond to the proliferation of the melanoma tumor cells[711]. Ultimately, these results would support a hypothesis that TIL in tumors of patients receiving no clinical benefit from therapy were less able to recognize their cognate antigens, causing diminished IFN γ secretion, and thereby leading to enhanced tumor proliferation[711]. These findings will be revisited in the fourth chapter and discussion of this thesis.

Transcriptomic data can be a powerful tool to identify biomarkers of cellular dysfunction that distinguish clinical outcome. Harnessing transcriptomic signatures associated with response to TIL therapy will allow us to uncover mechanisms of resistance that can be targeted with drugs or TIL genetic manipulation to create an ideal functional infusion product for the patient and/or even create a basis for patient selection.

Though we have insight regarding transcriptomic features that correlate with clinical benefit (response and greater than 2 year OS), little is known regarding what features in the TME may correlate specifically with response to therapy, PFS, or OS. Further, it is unknown what genes or pathways may be enriched in patients that have considerably “good outcome” to TIL ACT, including long OS, long PFS, and response to therapy. Using our cohort of TIL patients treated at MD Anderson, we thereby investigated potential transcriptomic biomarkers for “good outcome” to therapy; this will be addressed in detail in Chapter 4 of this thesis.

Chapter 2: Materials and Methods

A portion of this chapter is based on the original research article “Exposure to anti-PD-1 causes functional differences in tumor-infiltrating lymphocytes in rare solid tumors” published by Creasy et al. in the *European Journal of Immunology* on September 18, 2019 (DOI:10.1002/eji.201948217). It is presented with the permission of Wiley Publishing.

2.1 Clinical cohort development and study design

2.1.1. Rare Tumor Project

Following informed consent, patients with rare tumor types divided into ten separate cohorts were enrolled to this study as shown in Table 5. Mandatory core needle biopsies were obtained at baseline and day 15-21 after the first cycle of anti-PD-1 (pembrolizumab, 200 mg) from an ongoing Phase II Clinical Trial (NCT02721732) approved by the Institutional Review Board of The University of Texas MD Anderson Cancer Center. Pre and on-treatment biopsies were taken from the same tumor site. Patients marked as experiencing tumor regression have experienced at least a 30% decrease in tumor burden by ir-RECIST criteria.

2.1.2. Melanoma Biomarker Project

All tumor tissues utilized for this study were obtained from stage III or IV metastatic melanoma patients enrolled on the TIL ACT clinical trial MDACC (Institutional Review Board approved protocol #2004-0069, NCT00338377) and Moffitt (Institutional Review Board protocol, #18309, NCT01005745, NCT01701674, and NCT01659151). All studies were approved by the United States Food and Drug Administration and the Institutional Review Board of each independent center. The studies were written and conducted in accordance with the principles from the Declaration of Helsinki and all the patients signed written informed consent prior to treatment initiation. Tumor samples were obtained from 73 patients treated at MDACC and 10 patients treated at Moffitt. Patients from Moffitt were recruited in three different trials: TIL ACT (n=5), TIL + ipilimumab (n=2), and TIL + BRAF inhibitor (n=3). At MDACC, patients were enrolled in 3 cohorts of this trial: TIL ACT (n=65), TIL + BRAF inhibitor (n=3), and TIL with or without dendritic cell (DC) infusion (n=5).

For all trials, patients were lymphodepleted 7 days prior to intravenous infusion of autologous TIL. TIL were expanded from 1-3 mm³ tumor fragments in RPMI based media with the addition of 6,000 IU/mL of IL-2 every 2-3 days for 3-5 weeks as previously described[594, 595]. Additionally, patients received high-dose IL-2 (720,000 IU/kg) every 8 hours beginning one day after infusion for a maximum of 15 doses at MDACC or over 5 days at Moffitt. Patients treated at MD Anderson received a second course of high dose IL-2 three weeks later. Further information regarding trial design are described in Forget, Haymaker et al. 2018 and Pilon-Thomas, et al. 2012, respectively[594, 604].

At time of tumor harvest for TIL generation, a portion of this tumor was formalin-fixed, paraffin-embedded (FFPE) for histopathological diagnosis. For the MDACC treated patients, blood samples collected at time of surgery for TIL harvest or, prior to lymphodepletion before TIL infusion (baseline) were processed as peripheral blood mononuclear cells (PBMCs). PBMCs were preserved by the MDACC Immunomonitoring Core Lab and MDACC Melcore Lab. Response to TIL therapy was measured using Immune-Related Response Criteria (irRC) at MDACC and RECIST v1.1 at Moffitt. Responders are defined as patients with complete response (CR) or partial response (PR), while patients with stable disease (SD) or progressive disease (PD) are considered non-responders. Metadata for patients for all trials are presented in Tables 6 and 8.

2.2 Expansion of TIL from rare tumors

Human recombinant IL-2 (ProleukinTM) was provided by Prometheus Therapeutics and Diagnostics. A fully human and purified IgG4 monoclonal antibody against human CD137, Urelumab (663513; Lot 6A20377) was kindly provided by Bristol Myers Squibb (BMS) through a Material Transfer Agreement. Anti-CD3 antibody (clone OKT3) was obtained from Miltenyi Biotec.

One fresh core biopsy was obtained and cut into 3 mm² fragments and placed in TIL culture media [TIL-CM, RPMI 1640 with Glutamax supplemented with 2 mM L-Glutamine, 1 mM Pyruvate, 1x of HEPES, 50 µM 2-mercaptoethanol, 1X Pen-Strep (Invitrogen) and 10% heat-inactivated human AB Serum (Sigma-

Aldrich)]. A single fragment was placed per well of a 24-well plates or 5 fragments put in a G-Rex 10 (Wilson Wolf) for expansion of 2-4 weeks as previously described[499]. Briefly, IL-2 (6000 IU/mL), anti-4-1BB agonistic antibody (10 µg/mL), and anti-CD3 (30 ng/mL, clone OKT3) were added on day 0 of the culture[499]. Every 3-4 days, half of the media of the culture was removed and was replaced with new culture media with 3000 IU/mL IL-2. Once cells reached confluency, one well of the 24 well plate was divided into multiple wells for continued growth until week 4 at maximum. TIL were frozen in 90% FBS with 10% DMSO following this expansion phase for use in functional assays.

2.3 Flow Cytometric Analysis of Expanded Rare Tumor TIL

All flow cytometric processing and analysis adhered to the guidelines outlined by Cossarizza et al[723]. TIL were incubated for 10 minutes at room temperature with 5% goat serum in FACS Wash Buffer (Dulbecco's Phosphate Buffered Saline 1x with 1% Bovine Serum Albumin) prior to staining. Samples were acquired using a BD LSRFortessa™ X-20. Surface staining included: CD3 (Clone UCHT1, Biolegend), Tim3 (Clone F38-2E2, Biolegend), Lag3 (Clone 3DS223H, eBioscience), GITR (Clone eBioAITR, eBioscience), CD25 (Clone BC96, eBioscience), PD-1 (Clone EH12), CTLA-4 (Clone BN13), CD4 (Clone SK3), CD56 (Clone B159), CD8 (Clone RPA-T8), and TCRγδ (Clone B1, all BD Biosciences), which was immediately followed by intracellular staining using Perm Buffer 1 for 20 minutes at room temp (BD Biosciences) with subsequent staining for Granzyme B (Clone GB11, BD Biosciences). Dead cells were excluded using YELLOW live/dead staining (Invitrogen). The gating strategy is found in Figure 10. Analysis was performed using FlowJo V10 (Tree star).

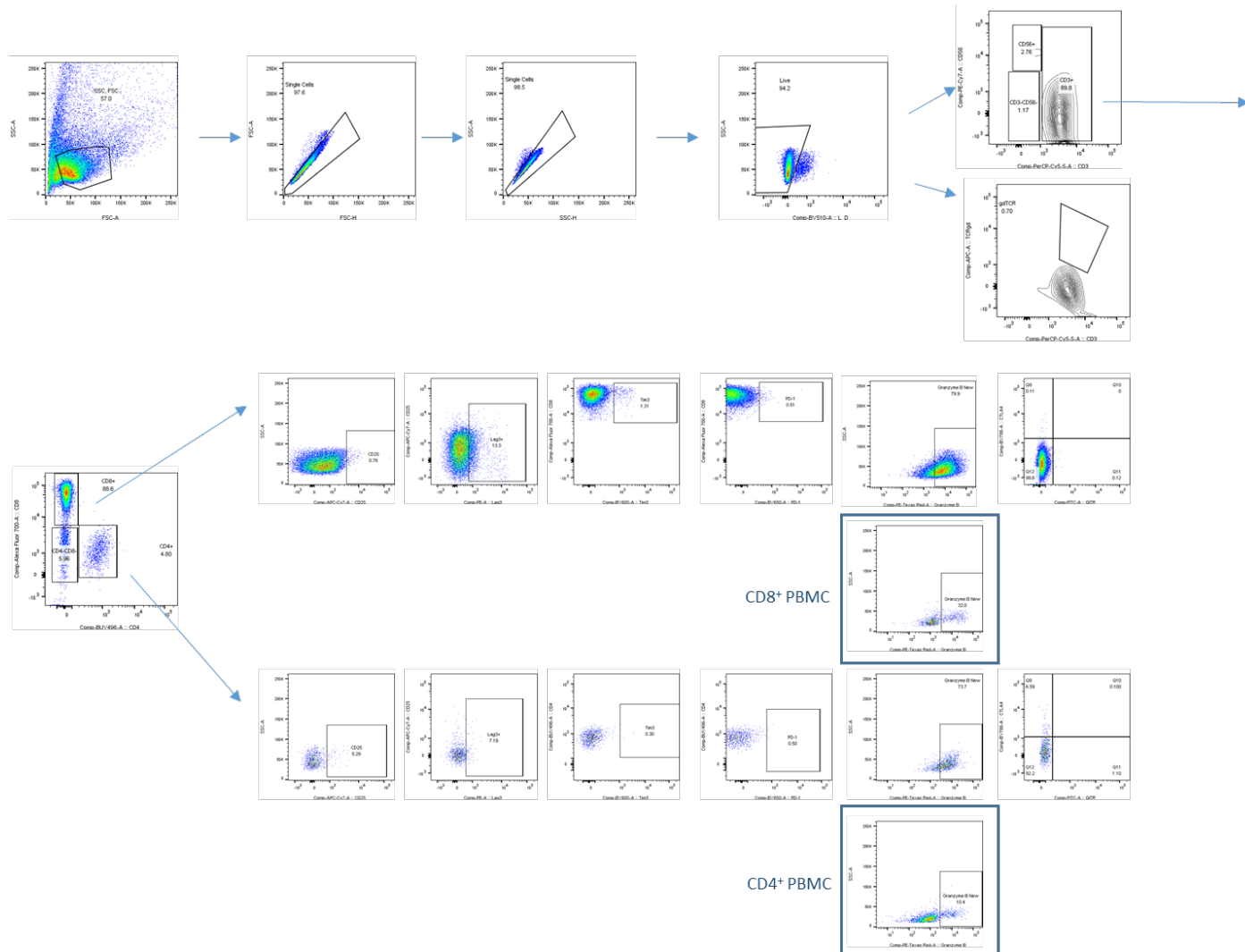


Figure 10. Gating Strategy for flow cytometry analysis of 15 color panel. Initially, samples were gated based on size and doublet exclusion, followed by Live/Dead Yellow stain exclusion for viability. Samples were gated for CD3⁺, CD56⁺, or CD3⁺γδTCR⁺. The CD3⁺ gate was subset into the CD4⁺ and CD8⁺ populations which were then stained for the following surface markers: CD25, Lag3, Tim 3, PD-1, GITR, and CTLA-4. Granzyme B was stained intracellularly and analyzed in the CD4⁺ and CD8⁺ TIL.

2.4 Detection of soluble factors following activation

Expanded TIL were thawed and immediately stained for sorting. Live/dead discrimination was based on Sytox Blue (Invitrogen) and TIL subsets were sorted into CD3⁺CD4⁺ and CD3⁺CD8⁺ populations via CD3 (Clone SK7), CD4 (Clone RPA-T4), and CD8 (Clone RPA-T8) surface staining using a FACS ARIA (BD Biosciences). Individual subsets were plated on a 96 well Co-Star Assay High-bind plate (Corning) pre-

coated with anti-CD3 (300 ng/mL, clone OKT3, Miltenyi Biotec) at 100,000 cells/well. These cells were incubated for 24 hours at 37°C in a 5% CO₂ incubator in TIL culture media with or without IL-2 (100 IU/mL, Proleukin). After incubation, cell supernatants were collected and frozen at -20°C. Cellular supernatants were plated in duplicate or triplicate and run using the Procarta Plex Multiplex Assay- Immune Monitoring Kit (Invitrogen, EPX650-10065-091). TIL culture media was used as the negative control. The plates were run on the Luminex 200 (ThermoFisher) on the same day that the assay was performed.

2.5 DNA and RNA extractions

FFPE specimens were sequentially cut in 10 µm sections for analyte extractions. The initial 2 shavings were discarded and the following 7-12 shavings were placed in an RNase free microcentrifuge tube. Samples were immediately placed at -80 °C until extraction was performed. DNA and RNA were extracted utilizing the Qiagen AllPrep DNA/RNA FFPE Kit (#80234) with the Qiagen deparaffinization solution utilized (#19093). After extraction, DNA and RNA underwent melanin removal utilizing the Zymo Research OneStep PCR Inhibitor Removal Kit (#D6030). PBMCs acquired prior to treatment were used as germline controls and extracted for DNA using the Qiagen AllPrep DNA/RNA Mini Kit (#80204). Samples were quantified for the 260/280 ratio, RIN, and DV200 with a Nanodrop 1000.

2.6 Whole Exome Sequencing (WES)

Whole exome sequencing was performed on DNA from FFPE tissue of 74 samples. Matched PBMC DNA was sequenced as germline controls and fingerprinting confirmed patient sample concordance. The library construction and hybrid capture, preparation of libraries for cluster amplification and sequencing, and cluster amplification and sequencing were performed as outlined in Forget and Haymaker et al [604]. Sequencing reads were aligned to hg19 using BWA[724]. Five samples were removed due to less than 5x mean exon coverage. Cross-contamination of samples was estimated using ContEst[725]. Four samples were removed due to possible contamination. After quality control, 64 samples were used for analysis.

2.7 WES analysis of genetic alterations

2.7.1. Tumor Purity, SNVs, and Indels

ABSOLUTE was used to call tumor purity and the cancer cell fraction (CCF) of mutations[726]. 64 pre-treatment samples met purity analysis criteria. Single-nucleotide variants (SNVs) calling and FFPE artifact filtering was performed as outlined previously[604]. Short insertions and deletions (indels) were called using Strelka[727]. The SNVs and indels were filtered to remove artifacts, based on allelic fractions observed in panels of normal samples.

2.7.2. Mutations and Neoantigen prediction

Annotating mutations, calculating the nonsynonymous mutations, and calling mutated genes were performed as outlined in Forget and Haymaker et al[604]. The HLA class I types of each patient were called with OptiType[728]. Selected mutations were confirmed using remaining DNA after WES for Sanger sequencing[729]. The region of interest was amplified using custom PCR primers. Sanger sequencing was performed on a 3730xl DNA Analyzer (Thermo Fisher/Applied Biosystems) using BigDye™ Terminator v3 chemistry (Thermo Fisher/Applied Biosystems). Mutation analysis was performed using SeqScape® Software v2.5 (Thermo Fisher/Applied Biosystems). Neoantigens were predicted using NetMHCpan 4.0[730]. The predicted neoantigen load was determined by an eluted ligand likelihood (ELL) in the top 2%.

2.8 RNA profiling and analysis

550 ng of mRNA regardless of dV200 was used for sample preparation and strand-specific cDNA synthesis at the Broad Institute (Illumina TruSeq RNA Access Kit) for 53 pre-treatment samples. A transcriptome capture approach that targets 21,415 genes, representing 98.3% RefSeq exome, was used to enrich for the mRNA. RNA Sequencing of 76 bp reads was performed on Illumina machines in two batches. Fastq files were aligned using STAR, which also provided raw read counts for each gene[731].

2.9 Gene expression profiling using droplet digital PCR (ddPCR) assay

1 µg of RNA was used from 16 FFPE tissue samples (same as for RNA-seq, when available) and from 2 positive and 2 negative control cell line samples presenting either high (U2OS ATCC #HTB-96, MCF7 ATCC #HTB-22) or low expression of *ELFN1* (Jurkat ATCC TIB-153, HEK293 ATCC #CRL-1573), was used for cDNA synthesis using the High-capacity cDNA Reverse Transcription kit (without RNase inhibitor; cat. #4368814, Thermo Fisher Scientific). The selection of control samples was performed based on The Protein Atlas gene expression, public available datasets (<http://www.proteinatlas.org>, Nov 04 2020)[732]. Next, the obtained cDNA was used to set up the ddPCR reaction following the manufacturer's instructions of the QX200 Droplet Digital PCR System using supermix for probes (no dUTP) (cat. #1863023; Bio-Rad). Each sample was run in duplicates and was run with non-template control. Briefly, the ddPCR assay was performed in 20 µL reactions containing 2x ddPCR supermix (no dUTP, Bio-Rad), two set of IDT 20x PrimeTime qPCR Assay with primers and Taqman probes (cat. #Hs.PT.58.14371910, cat. #Hs.PT.58v.45621572; IDT) at a 3.6:1 ratio (final concentrations of 900 nM and 250 nM, respectively), targeting *ELFN1* gene (labeled with FAM) and *HPRT1* (labeled with HEX) - the house keeping gene, and cDNA (50 ng of control cell line samples and 250 ng of FFPE tissue samples) or PCR grade water. The PCR reaction mixture with the addition of droplet generation oil for probes (Bio-Rad) was portioned into droplets using the QX200 Droplet Generator (Bio-Rad) according to the manufacturer's protocol. The droplets were carefully collected and next PCR amplified in C1000 Thermal Cycler (Bio-Rad) using following optimized conditions: 1 cycle at 95°C for 10 min, 40 cycles of 94°C for 1 min and 57.1°C for 1 min, 1 cycle of 98°C for 10 min, and a 12°C hold. Fluorescence signal was read in the FAM and HEX channels in QX200 droplet reader (Bio-Rad). The ddPCR data analysis was performed using QuantaSoft™ Analysis Pro Software (version 1.0.596; Bio-Rad), with positive and negative droplet populations detected using two-dimensional graphs. The gene expression was calculated from generated Poisson concentrations (copies/µL) and the data is presented as a ratio of *ELFN1* gene to housekeeping gene and graphically displayed using GraphPad Prism Software version 8.0.0. Examples of this analysis can be found in Figure 11. Primer and probe sequences for target and reference genes are provided in Table 4.

Table 4. Primers and Probes for ddPCR assay

Gene Query	Species	Detects all Variants	Exon Location	Primers: Probe Ratio	Concentration	Primers 5' to 3'	Probe 5' to 3'
<i>HPRT1</i>	Human	Yes	8-9	3.6:1	20x	Primer 1: GCGATGTCAATAGGACTCCAG	AGCCTAAGATGAGAGTTCAAGTTGAGTTGG
						Primer 2: TTGTTGTAGGATATGCCCTTGA	
<i>ELFN1</i>	Human	Yes	1-2	3.6:1	20x	Primer 1: TGTCCTGATGGCTCCACT	TCCGACGTTCTGTTGTGTGCAG
						Primer 2: GAATCCTCCAGCCTATTCCTC	

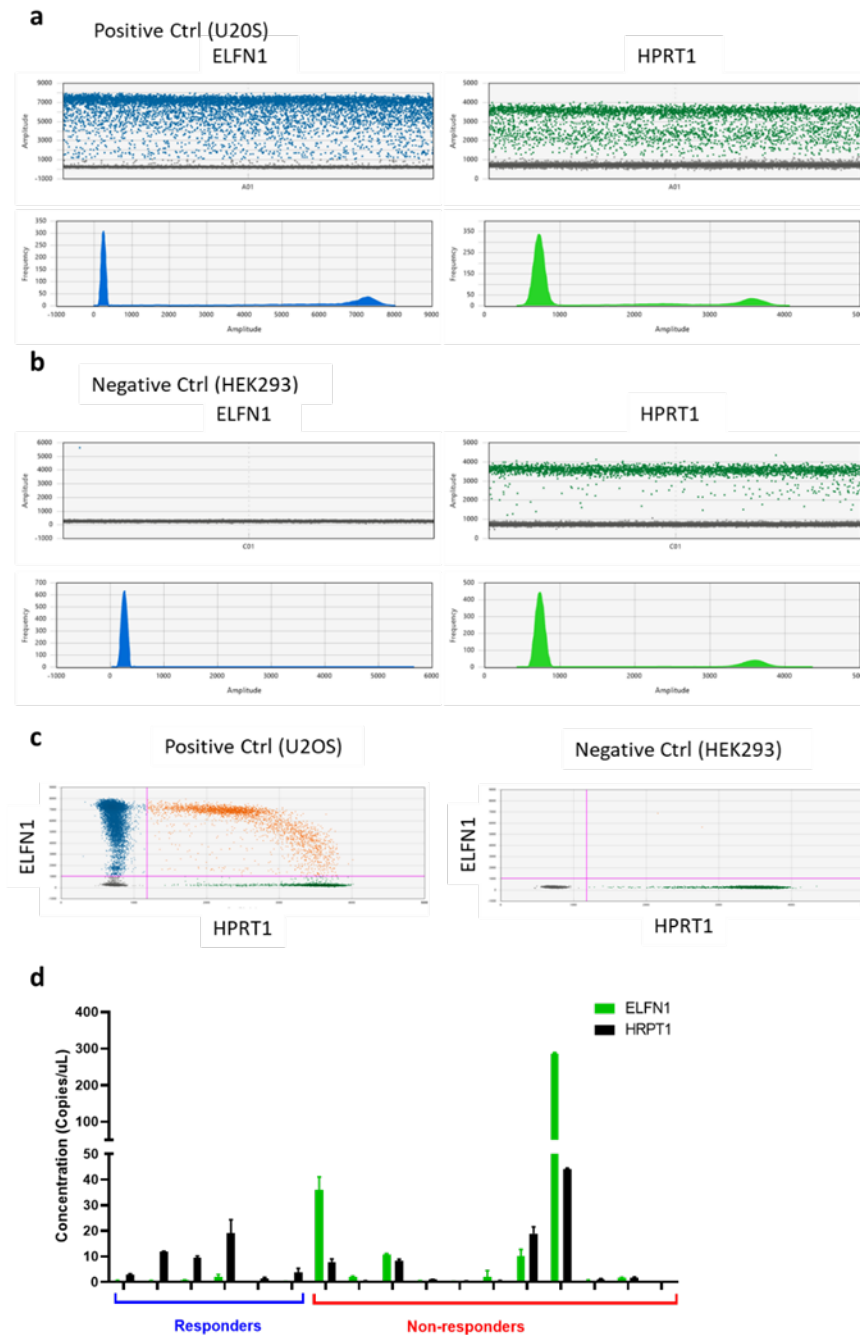


Figure 11. ddPCR amplitude, frequency, and concentration (copies/ μ L) in control cell lines and FFPE tissues. Fluorescence amplitude plots (top) and fluorescence amplitude histograms (bottom) for the (a) positive control cell line U2OS and (b) negative control cell line HEK293. Plots and histograms for ELFN1 are represented in the left side and HPRT1 found on the right side of the figures. c 2-D plots representing the positive control cell line U2OS (left) and negative control cell line HEK293 (right). Channel 1 fluorescence (ELFN1; FAM) positive droplets are represented in blue, channel 2 fluorescence (HPRT1; HEX) positive droplets are represented in green, and droplets positive for both channels are expressed in orange. d Bar graph represents the mean concentration (copies/ μ L) of either ELFN1 or HPRT1 per sample (n=6 responders; n=11 non-responders). Error bars are representative of the standard deviation, as samples were run in duplicate.

2.10 Methylation array

2500 ng of DNA from 30 FFPE blocks at MDACC was utilized for methylation analysis at the USC Epigenome Center using the method outlined in Marzese, et al[733]. The Infinium HumanMethylation450 BeadChip was used to profile the methylation level of single-CpG-sites for 30 samples. After preprocessing the data as outlined in Marzese et al., the calculated betta-values of Msites for each sample were converted to M-values to avoid heteroscedasticity for highly methylated or unmethylated CpG sites[733, 734]. The M-values of 50 Msites for 20 Responders (CR/PR) and 10 NR (PD) were then used for unsupervised hierarchical clustering of the samples in terms of their methylation profiles ordered by Msites location within the *ELFN1* genes. Before the clustering, the M-values were centered and normalized by rows and columns. Hierarchical clustering was implemented by Cluster 3.0 software using the centroid linkage algorithm and the Pearson correlation as the similarity measure[735]. Visualization was done by Java TreeView 3.0[736]. The mean levels of methylation for R and NR were calculated using normalized and centered M-values and visualized as boxplots using 'beeswarm' R library. The statistical significance of difference in the mean methylation levels was evaluated by Wilcoxon tests in R.

2.11. Statistical Analysis

2.11.1. Rare TIL Project

GraphPad prism version 7.03 (GraphPad Software) was used for graphing and statistical analysis. Statistical analysis of flow cytometry and Luminex data was performed using the Wilcoxon matched-pairs signed rank test. Bar graphs with error bars represent mean and standard deviation. Further statistical analysis using a paired, t-test and heat map creation was performed using Qlucore Omics Explorer version 3.4 (Qlucore). Normalization of the heat map data is based on the mean as equivalent to zero, with values on the heat map depicting -2 to 2 standard deviations from the mean.

2.11.2. Melanoma Biomarker Project

Statistical analyses were performed in R 3.5. For correlations of non-silent mutation load and neoantigen load with overall survival (OS) and progression-free survival (PFS), the log₁₀ (mutation or neoantigen load) was used in a Cox proportional hazards model. Maftools was used for the sample-by-gene mutation plot[737]. For the analysis of mutations in specific genes, each gene was classified as having at least one non-silent mutation or not in each patient, and the binary results were used in Mann-Whitney tests for responder vs non-responder comparisons or in Cox proportional hazards models for correlations with OS or PFS.

For the transcriptomic analysis, samples with fewer than 12,500 detected genes were excluded for quality control, leaving 34 samples for subsequent analyses. The raw read counts for each gene were fed into DESeq2 for differential expression analysis.[738] Only protein-coding genes were analyzed. Genes with zero read counts in 75% or more of the samples were excluded to prevent outliers from influencing results. This removed 2290, or 12.4%, of the protein-coding genes in the 34 pre-treatment samples. We used log(OS +1) and log(PFS +1) response as the variables of interest in DESeq2, and batch effects were controlled by introducing a batch covariate into the model[738].

The statistical test for ddPCR utilized were Pearson's correlation and Mann-Whitney t-test in GraphPad Prism version 8.0.0. The statistical significance of difference in the mean methylation levels was evaluated by Wilcoxon tests in R.

Chapter 3: Exposure to anti-PD-1 causes functional differences in tumor-infiltrating lymphocytes in rare solid tumors

This chapter is based on the original research article “Exposure to anti-PD-1 causes functional differences in tumor-infiltrating lymphocytes in rare solid tumors” published by Creasy et al. in the *European Journal of Immunology* on September 18, 2019 (DOI:10.1002/eji.201948217). It is presented with the permission of Wiley Publishing.

3.1 Introduction

Checkpoint blockade has become the standard of care in many solid tumor malignancies. T cells within a tumor can now be protected against multiple inhibitory mechanism deployed by the tumor and its environment through the use of targeting antibodies. This approach was originally developed in melanoma, which showed initial promise with anti-CTLA-4 antibodies. Inhibiting the checkpoint molecule CTLA-4 expressed by activated T cells demonstrated the efficacy and ability of immunotherapy to accomplish long term response rates, but had notable immune related toxicities[309]. Another inhibitor targeting a second checkpoint molecule expressed at the surface of activated T cells, PD-1, was discovered and utilized in humans for the first time in 2006[739]. The anti-PD-1 antibody demonstrated lower toxicity and prolonged progression-free survival and overall survival[740].

Pembrolizumab demonstrated a 47.3% six month progression-free survival rate in advanced melanoma[741]. This antibody as compared to standard chemotherapeutics significantly enhanced overall survival in patients with non-small cell lung cancer[348, 742]. Initial success using anti-PD-1 for melanoma and non-small cell lung cancer led to the eventual expansion for use in other cancer types. Most success has been reached in tumor malignancies caused by known carcinogens such as infectious agents and ultraviolet light which can result in a high mutation burden, with highest response rates in Hodgkins disease, desmoplastic melanoma, merkel cell carcinoma, and MSI-high cancers[739].

Rare tumor types are plagued with lack of investment in treatment options. Little is currently known regarding the immune landscape of many of these tumors, including the potential benefit that

could be attributed to checkpoint inhibitors. The far-reaching success of pembrolizumab and its approval in other cancer types warrants its testing in rare tumors. Therefore, we designed a Phase II clinical trial using pembrolizumab for patients with rare cancer types from various origins.

Although much has been studied regarding the theoretical mechanism(s) of action of anti-PD-1 therapy in metastatic melanoma, little is known about the actual alterations that occur within the tumor microenvironment caused by initial dosing. Our trial was designed to collect a core needle biopsy from the tumor at baseline and on-treatment from the same tumor lesion. This allowed for the opportunity to investigate these changes, particularly on the tumor-infiltrating lymphocytes (TIL), which predominantly express PD-1, making them the primary target in this therapy. We hypothesized that there would be distinct alterations within the TIL, as it has been shown that TCR repertoire and T-cell infiltration is impacted by checkpoint inhibitors[687]. In this study we aim to determine an impact on the TIL, in both phenotype and function early on-treatment, immediately following the initial dose of pembrolizumab.

3.2 Results

3.2.1 Exposure to one dose of anti-PD-1 does not impair TIL expansion from a core needle biopsy

To assess potential functional changes resulting from one exposure to anti-PD-1, TIL were expanded from baseline and matched on-treatment biopsies (baseline vs C1D15-21). Given that this collection was from a Phase II study in rare tumors and that only one core needle biopsy was available for assessment, we chose to assess functional changes on expanded TIL. Only those samples with positive TIL growth from baseline and on treatment were used for subsequent analysis. As shown in Figure 12A, using the TIL 3.0 pre-REP (rapid expansion protocol) method, we were able to expand TIL from various rare solid tumor types (n=33)[499]. Table 5 shows stratification by rare tumor type as well as range of successful pre-REP TIL expansion. We further demonstrated that there was no difference in our ability to grow TIL based on exposure to anti-PD-1 by collecting longitudinal samples derived from the same biopsy

site (Figure 12B). Overall, our results confirm our ability to grow TIL from multiple rare solid tumor types, regardless of one anti-PD-1 dose.

Table 5. Patient metadata for rare tumors that grew TIL before and after exposure to anti-PD-1

Tumor Type	Baseline			On-Treatment			Response to Pembrolizumab R= tumor regression, NR= no tumor regression
	Number of Samples at Baseline	Number of Samples (over 2 million cells per fragment)	Range of Growth per Fragment (in millions of cells)	Number of Samples after One Cycle of anti-PD-1 (C1D15-21)	Number of Samples (over 2 million cells per fragment)	Range of Growth per Fragment (in millions of cells)	
Squamous Cell Carcinoma of the Skin	5	4	0.27-38.46	5	5	14.31-59	2 R 3 NR
Small Cell Malignancies of Non-pulmonary Origin	4	4	19.92-57.43	4	4	17.78-43.20	4 NR
Adrenocortical Carcinoma	3	3	8.58-53.40	3	3	6.28-43.16	3 NR
Medullary Renal Cell Carcinoma	2	2	36.12-46.75	2	2	8.90-81.51	2 NR
Carcinoma of Unknown Primary	4	4	3-43.8	4	4	11.31-60.17	4 NR
Penile Carcinoma	0	0	n/a	0	0	n/a	n/a
Vascular Sarcoma	2	2	8.33-46.31	2	2	2.90-29.40	2 NR
Testicular Carcinoma	1	0	1.78	1	0	1.76	1 NR
Paraganglioma-pheochromocytoma	2	2	2.81-21.53	2	2	15.39-29.40	2 NR
Other Rare Tumor Histologies	10	10	4.55-50.24	10	10	3.17-50.48	2 R 8 NR

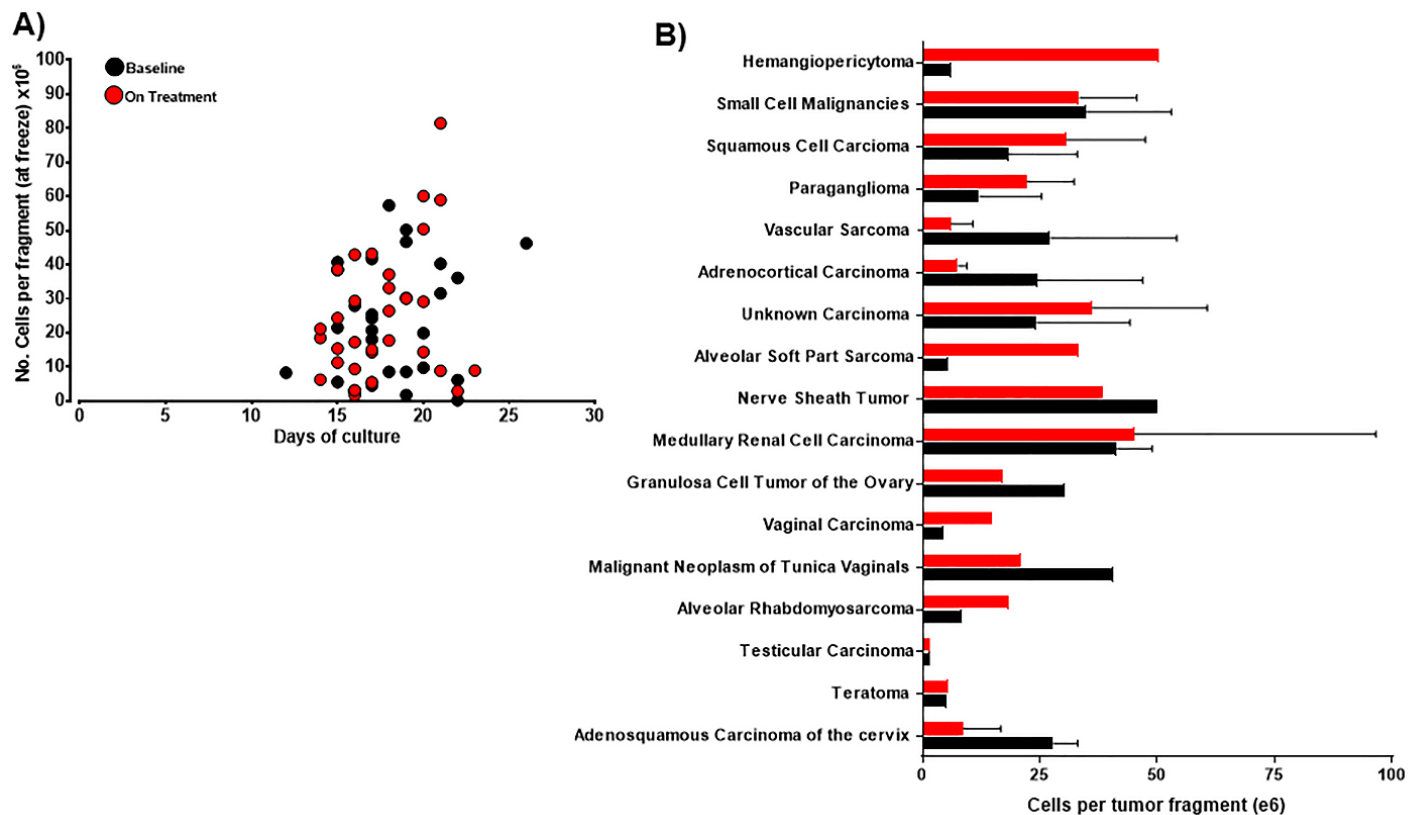


Figure 12. Exposure to one dose of anti-PD-1 does not impair TIL expansion from a core needle biopsy. A single core biopsy was obtained prior to anti-PD-1 therapy and 15-21 days after the first treatment with the antibody across multiple rare tumor types. (A) The number of expanded cells and culture duration are shown from baseline (black circles) and on-treatment (red circles) tumor tissue (n=33 paired samples from 33 individual patients). (B) The TIL growth per rare tumor type before and after exposure to anti-PD-1 is shown. All graphs are representative of the growth normalized by fragment and depict samples that were confirmed to have tumor presence (via hematoxylin and eosin staining) at baseline (BL) and on-treatment (OT), n=33 patients. Bar graphs with error bars represent mean and standard deviation.

3.2.2. CTLA-4 surface expression on expanded TIL differs between baseline and post single dose anti-PD-1

We initially hypothesized that cells recruited into the tumor microenvironment after antibody treatment would have a superior functional phenotype based on previous studies done in metastatic melanoma[743, 744]. When looking at the distribution of the T-cell subsets using flow cytometry, we found that cultures were uniformly rich in CD8⁺ TIL (n= 27, $p=0.1226$, Figure 13A, left panel) which was

expected as our culture technique includes anti-4-1BB which is known to preferentially expand CD8⁺ TIL [501]. While a decreased percentage of CD4⁺ TIL was also expected in all cultures for the above reason, there was a further decrease observed after one treatment of anti-PD-1 (n= 27, $p=0.0182$, Figure 13A, right panel). This was also reflected by the ratio of CD8⁺ to CD4⁺ TIL, which increased after anti-PD-1 exposure (n=27, $p=0.0317$, Figure 13B). It is worth noting that the decrease in CD4⁺ TIL post anti-PD-1 was also observed in the 4 patients who experienced tumor regression represented in green circles in Figure 13 (A and B). There was no appreciable difference in the level of gamma delta T cells or CD3⁺CD4⁺ CD8⁻ T cells found in the expanded TIL cultures between baseline and on-treatment samples (Figure 14).

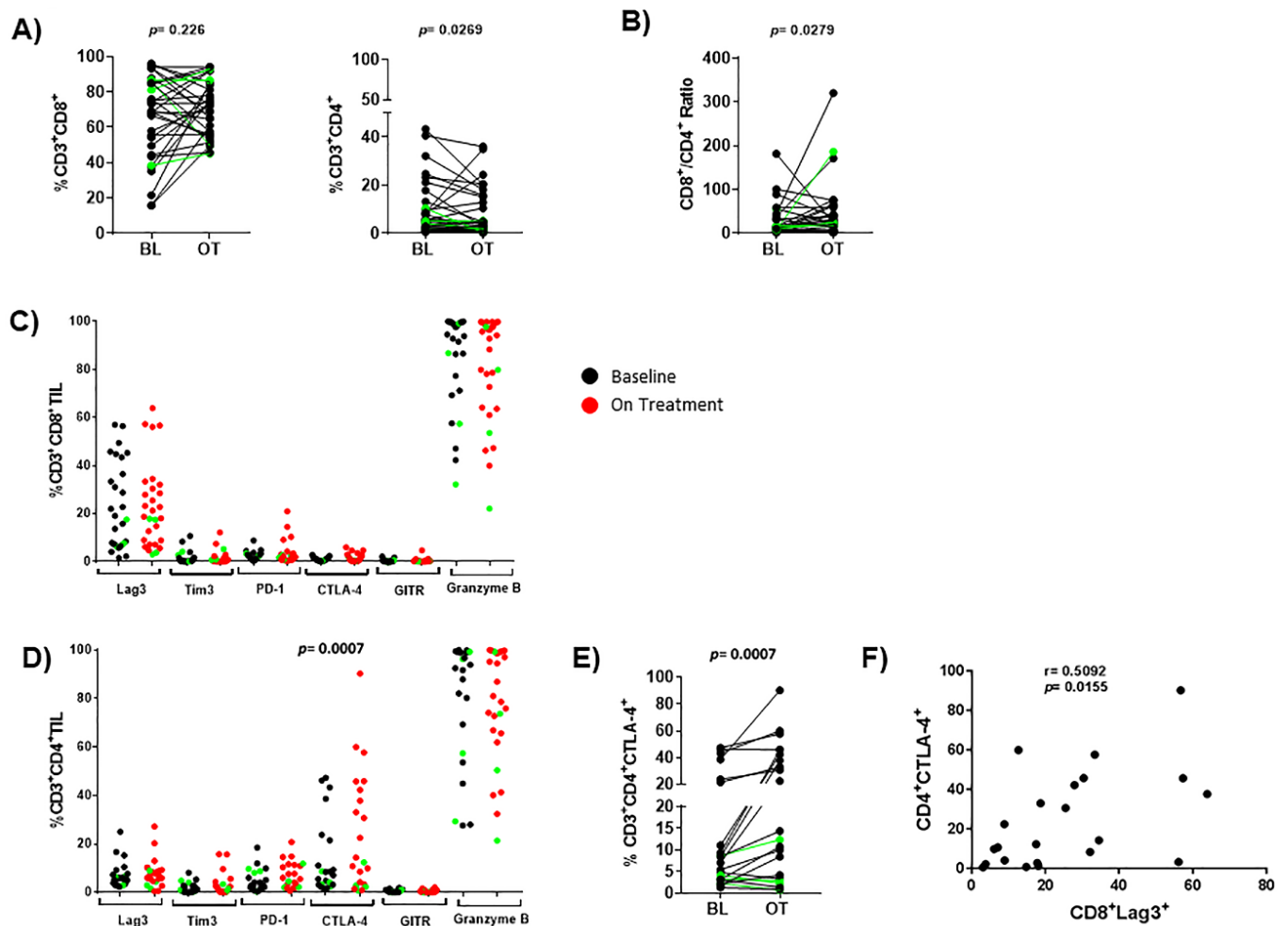


Figure 13. Phenotypic analysis of expanded TIL reveals differences between paired baseline and post single dose PD-1 in CTLA-4 surface expression. TIL were expanded from tumor tissue harvested at baseline and after treatment and analyzed by flow cytometry. (A) Stable CD8⁺ TIL population ($p=0.226$) and a diminished CD4⁺ TIL after one treatment of anti-PD-1 ($p=0.0362$) from analysis of pre and post treatment samples (pairs) from 33 patients ($n=33$ pairs). **(B)** Ratio of CD8⁺ to CD4⁺ TIL, showing CD8⁺ TIL increase after anti-PD-1 exposure ($p=0.0279$), from analysis of pre and post treatment samples from 33 patients ($n=33$ pairs). **(C&D)** These graphs represent surface expression of different phenotypic markers on CD8⁺ **(C)** or CD4⁺ **(D)** T-cell subsets before (black circles) and after anti-PD-1 therapy (red circles) ($n=27$ pairs, $n=22$ pairs respectively). **(E)** Surface CTLA-4 expression in the CD4⁺ TIL is shown, with an increase in patients who did not experience tumor regression (black circles) after therapy ($p=0.0003$, $n=18$ pairs). Green samples represent patients who experienced tumor regression to treatment ($n=4$, $n=22$ pairs total assessed). **(F)** Correlation between CD4⁺ CTLA-4⁺ and CD8⁺ Lag3⁺ subsets in the post-PD-1 exposure samples ($n=22$, $r=0.5092$, $p=0.0155$). Statistical analysis for all panels was done using paired t-tests.

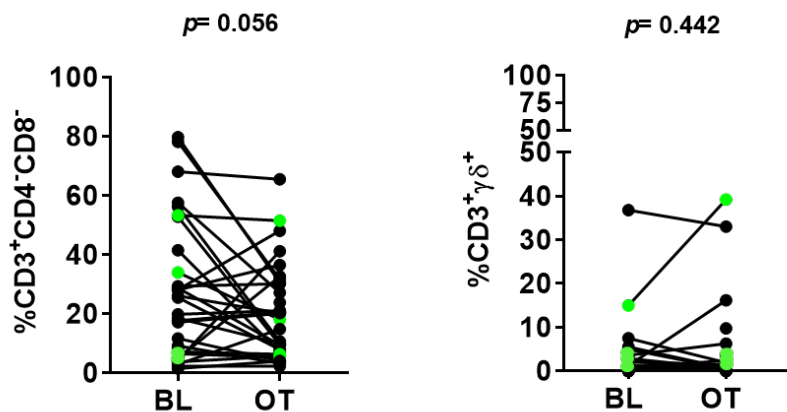


Figure 14. Further phenotypic analysis of expanded TIL pairs.

Flow cytometry analysis of expanded TIL ($n=33$ pairs) demonstrates no difference between time points in CD3⁺CD4⁺CD8⁻ (left panel) or CD3⁺γδ⁺TCR⁺ (right panel). Green dots represent patients who experienced tumor regression.

Assessment of the state of activation/inhibition as well as function was performed using high order flow cytometry in both CD8⁺ and CD4⁺ propagated TIL pre and post-pembrolizumab. There was no difference associated with anti-PD-1 exposure in the CD8⁺ TIL for expression of Lag3, Tim3, PD-1, CTLA-4, GITR, or granzyme B (Figure 13C, $n=27$). Unexpectedly, there was enhanced surface expression of CTLA-4 in the CD4⁺ TIL after anti-PD-1 exposure in TIL grown from paired patient samples ($n=22$, $p=0.0007$, Figure 13D and E). Conversely, in the patients who experienced tumor regression (green circles), CTLA-4 expression is not altered or increases slightly in the CD4⁺ TIL subset independently of anti-PD-1 exposure (Figure 13D and E). Given the high expression pattern of Lag3 on CD8⁺ TIL and membranous CTLA-4 on CD4⁺ TIL expanded post anti-PD-1 exposure, we explored the co-existence of these two subsets in matched patient samples. We observed that these subsets do correlate with each other ($r=0.5092$, $p=0.0155$, $n=22$, Figure 13F). Increased CTLA-4 expression in TIL following anti-PD-1 treatment was reported

in a mouse model of melanoma however this phenotypic change was seen in freshly isolated TIL, and occurred in CD4⁺ and CD8⁺ T cells [745]. Our findings suggest that the increased surface expression of CTLA-4 is uniquely maintained in CD4⁺ TIL after activation followed by 14-21 day culture. Similar findings were reported in melanoma comparing TIL phenotypes *ex vivo* following exposure to a single dose of anti-PD-1; however both studies reported a positive correlation with the presence of CD8⁺PD-1^{hi}CTLA-4^{hi}, not with the CD4 population [294, 635]. While the observation that patients who experienced tumor regression to anti-PD-1 therapy do not experience a sustained elevation of CTLA-4 on CD4⁺ TIL is interesting, additional patients who experienced tumor regression would be needed to see if the pattern holds. Nevertheless, it does suggest that inhibition through CTLA-4 may limit the efficacy of the therapy and argues for testing the combination of anti-PD-1 and CTLA-4 blockade in rare solid tumors.

3.2.3. A single exposure to anti-PD-1 unveils an altered cytokine expression profile in expanded CD8⁺ TIL

Although the expression of granzyme B was not impacted, we decided to further investigate possible changes in functionality that may occur post PD-1 exposure such as secretion of cytokines and chemokines upon activation. Cytokine profiling of sorted CD4⁺ and CD8⁺ TIL subsets was performed 24 hours post TCR engagement. No consistent difference in the secretion profile of CD4⁺ TIL was observed when comparing pre and post anti-PD-1 treatment (Figure 15). Only 6 pairs could be tested in the secretion assay due to the modest CD4⁺ product expanded. Interestingly, distinct differences in analyte secretion were observed between the CD8⁺ TIL pairs (n=26 pairs, $p = \leq 0.05$, Figure 16). As shown in the heatmap of Figure 15, there was an overall diminished cytokine secretion in samples post PD-1 exposure independently of the rare tumor type from which the TIL were expanded. The cytokines and soluble factors secreted that were the most significantly impacted after checkpoint blockade are: IL-23, IL-22, VEGF-A, SDF-1 α , and HGF (Figure 15B). Soluble IL-2R (sIL-2R, CD25) was included because of its association with anti-CTLA-4 treatment and response failure. It has been previously shown that high

levels of sIL-2R within the serum is associated with resistance to anti-CTLA-4 therapy[661]. Interestingly, the secretion of IL-2R is reduced in our CD8⁺ TIL population post PD-1 exposure for most of the patients which are also a majority of patients who did not experience tumor regression (black circles, Figure 16B); there was no clear pattern in the 4 patients who experienced tumor regression (green circles, Figure 16B).

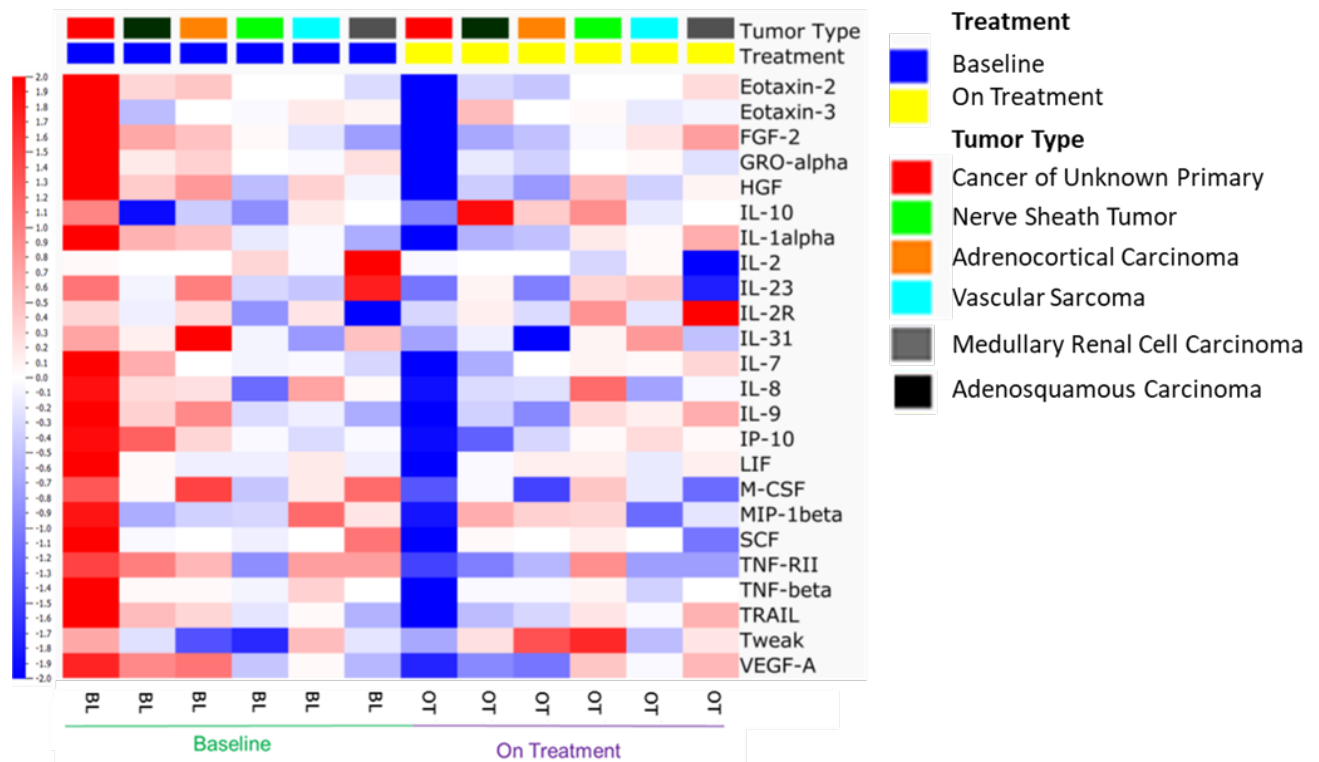


Figure 15. Expanded CD4⁺ do not have an altered secretion profile after exposure to anti-PD-1. Cytokine analysis of supernatants from sorted, activated CD4⁺ TIL expanded from baseline and on-treatment biopsies. Treatment time point and tumor type are indicated in the heading of heat map. Shown are soluble factors that are not statistically different before and after one treatment with anti-PD-1 ($p < 0.5$, $n=6$ pairs).

IL-23 was one of the most significantly diminished cytokines secreted post-pembrolizumab exposure across all patients, (Fig. 3B, $p=0.0079$), along with IL-22, whose expression is usually dependent on IL-23 (Figure 15B, $p=0.0072$)[746]. IL-23 leads to the differentiation of CD8⁺ T cells into an enhanced pro-inflammatory Tc17 subset[746]. The reduction of this subset after treatment could promote a tumor permissive environment[747]. High levels of IL-22 secreting Tc-22 cells are associated with poor prognosis in squamous cell carcinoma[748]. This was not witnessed in our data, with no association of higher IL-22 production in patients who did not experience tumor regression (black circles, Figure 15B). It has also been previously shown that VEGF-A induces enhanced expression of checkpoint molecules on TIL [749]. A drop in expression of VEGF-A was observed in our post therapy samples regardless of response (Figure

15B ($p=0.0004$). We did not observe a drop in surface expression of CTLA-4 or PD-1 by flow cytometry in post therapy samples (Figure 12). Further, we observed a decrease in production of SDF-1 α and HGF post exposure to pembrolizumab (Figure 15B, $p=0.0051$ and 0.002 , respectively). Previous data demonstrates that SDF-1 α induces iNOS production in T cells, leading to impaired function[750], while HGF has been shown to be ineffective in generation and activation of cytotoxic T cells from naïve CD8⁺ cells (Fig. 3B)[751]. High serum concentrations of HGF have been correlated with the absence of response to anti-PD-1 in melanoma and HGF has been found to downregulate the expression of perforin[752]. The reduction in secretion of SDF-1 α and HGF would suggest an enhanced on-treatment CD8⁺ TIL functionality. In summation, however, the cytokine profile does not fully demonstrate a “good” or “bad” functional phenotype that is induced by exposure to one cycle of anti-PD-1, but must be taken in the totality of how these cytokines interplay with both the TIL and overall TME for enhancing TIL function or suppressing tumor growth.

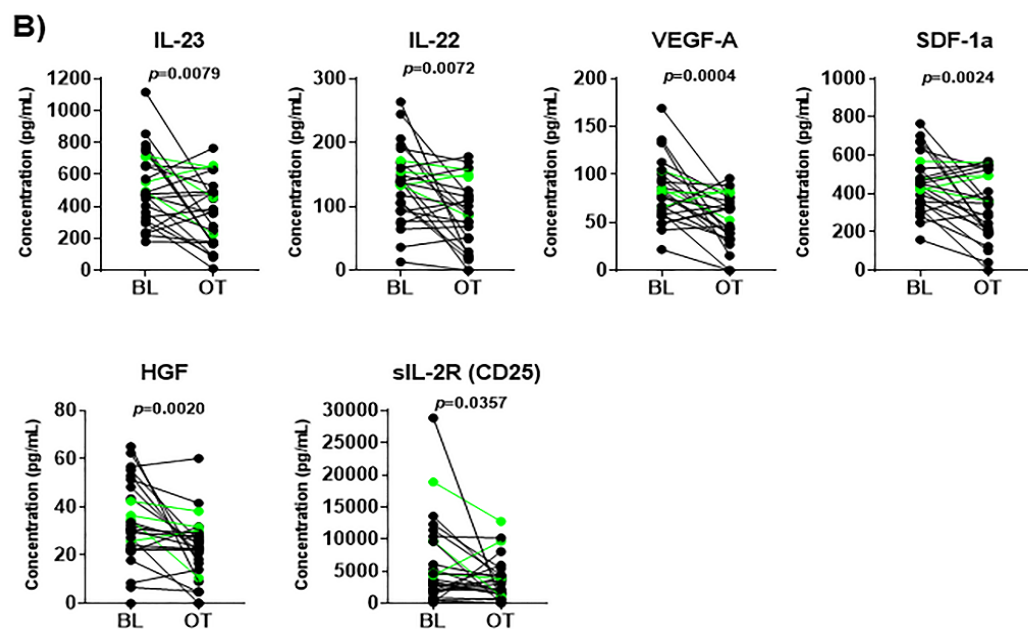
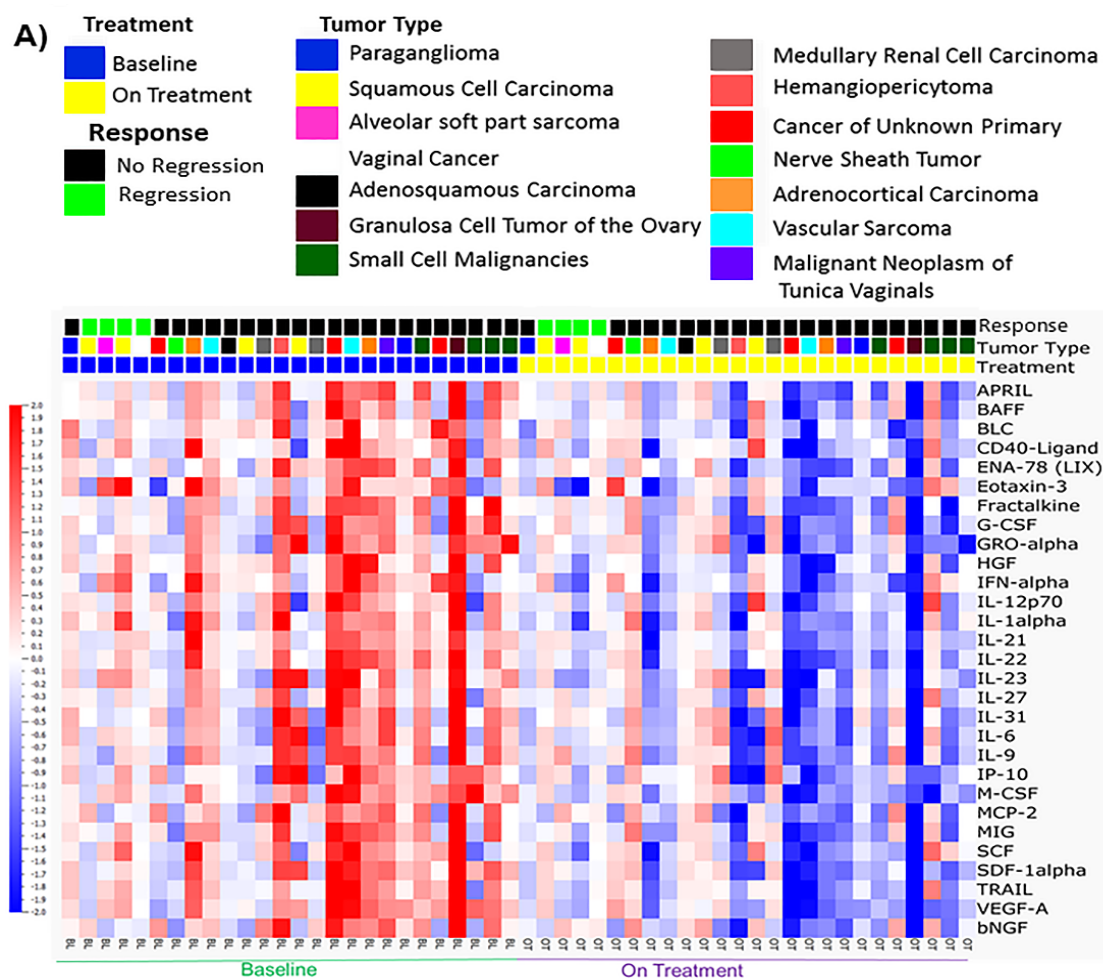


Figure 16. A single exposure to anti-PD-1 unveils an altered cytokine expression profile in expanded CD8⁺ TIL were expanded from tumor tissue harvested at baseline and after treatment, sorted for CD8⁺ T cell subset, activated with plate-bound anti-CD3 and assessed for soluble factor secretion in the supernatant after 24 hours. **(A)** Heat map depicting CD8⁺ TIL secretion profile with treatment time point and tumor type indicated in the heading. Shown are normalized, soluble factors significantly different before and after one treatment with anti-PD-1 in 26 patients ($p < 0.05$). Statistical analysis using a paired, t-test and heat map creation was performed using Qlucore Omics Explorer version 3.4 (Qlucore). Normalization of the heat map data is based on the mean as equivalent to zero, with values on the heat map depicting -2 to 2 standard deviations from the mean. **(B)** Selected soluble factors showing linear concentrations detected in 26 patients from (A) Paired t-tests on individual factors and associated p values are presented. Green dots represent patients who experienced tumor regression to treatment (n=4 pairs).

3.3 Summary

Overall, this chapter presents the first study to our knowledge that investigates the impact of one dose of pembrolizumab on expanded TIL phenotype and function in patients with rare tumors. Through this work, we also are able to demonstrate the ability to grow TIL from a single core needle biopsy. We observe a CD8⁺ phenotype that is relatively unchanged with a CD4⁺ phenotype characterized by enhanced surface CTLA-4 expression in comparing TIL before and after exposure to anti-PD-1. Interestingly, membranous CTLA-4 expression remained unchanged or was reduced in the four patients that experienced tumor regression; conclusions cannot be made, however, due to this small number and additional samples would be needed to confirm this divergent trend. Finally, we determine a diminished cytokine secretion profile within CD8⁺ TIL after pembrolizumab. Together, this provides evidence of phenotypic and functional changes occurring within TIL after a single dose of anti-PD-1, which can help understand mechanisms of resistance and design new therapeutic approaches in rare solid tumors (Figure 17). Ultimately, this chapter suggests expression of phenotypic markers on TIL after one dose of anti-PD-1 could potentially be utilized as biomarkers for therapeutic response.

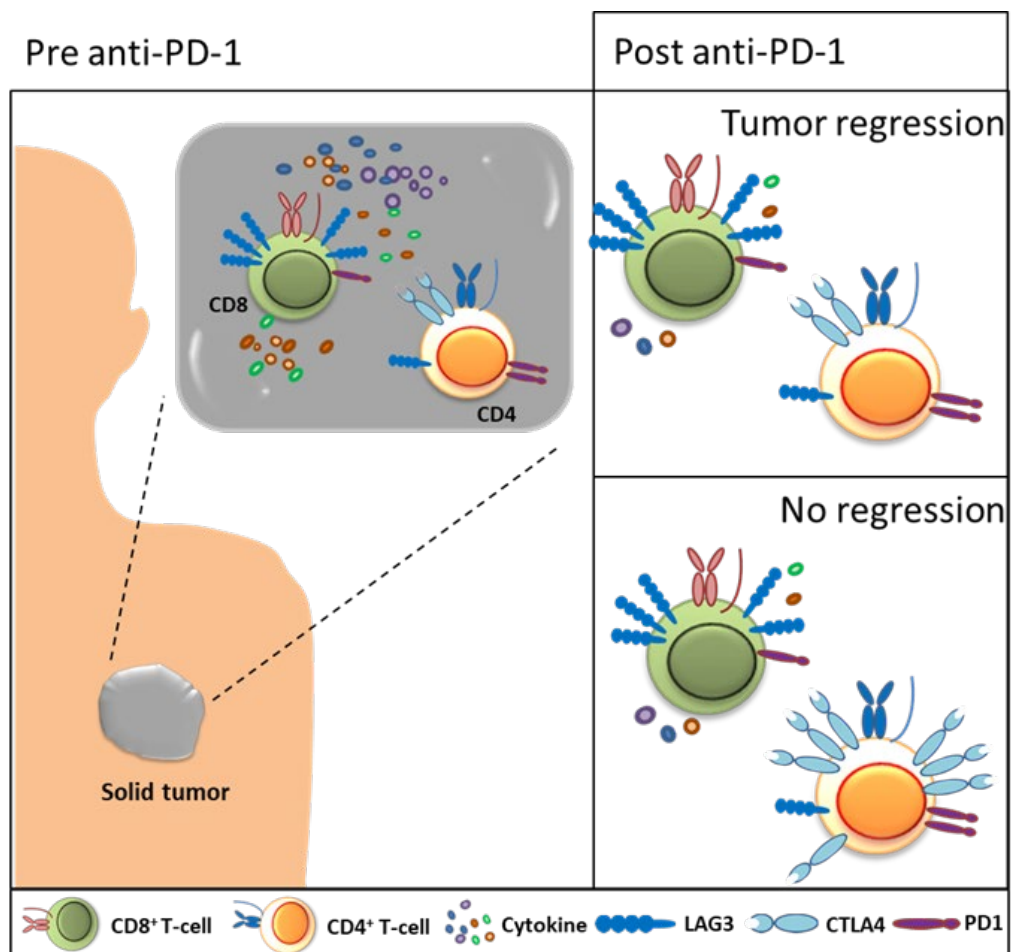


Figure 17. Schematic representing the inhibitory side-effects of anti-PD-1 therapy on TIL function.

Post anti-PD-1 exposure, in tumors that experience regression, demonstrate roughly equivalent expression of CTLA-4 on CD4 T cells, while patient tumors that did not experience regression had higher expression of CTLA-4 on CD4 T cells. Further, there was an observed decrease in soluble factor expression by particularly CD8 TILs after one dose of anti-PD-1.

Chapter 4: The Roadmap to Tumor-Infiltrating Lymphocyte (TIL) Therapy: Understanding genomic alterations for improved patient outcome

4.1 Introduction

The treatment landscape for metastatic melanoma has been revolutionized by immunotherapies. Immune checkpoint blockade has achieved objective response rates of 40-60%[350, 753]. Cell-based immunotherapy using tumor-infiltrating lymphocytes (TIL) yields similar response rates as the antibody-based immunotherapies, but has had more difficulties reaching the mainstream because of the challenges of manufacturing approaches and the toxicities related to IL-2 administration, resulting in this treatment being limited to a few centers worldwide. Nonetheless, adoptive cell therapy (ACT) of TIL, has led to durable responses (40-50%) even in patients refractory to checkpoint inhibitors[587][594, 604, 754, 755]. However, recent studies have shown promising results with a commercial TIL product in patients with metastatic melanoma and in patients with gynecological malignancies, supporting the potential for more widespread application of TIL therapy in the future. The use of TIL could be personalized and improved by developing better determinants of clinical benefit from this treatment; therefore, there is a strong rationale in doing so.

Previously our group has reported TIL attributes that correlated with clinical responses in metastatic melanoma patients treated with ACT TIL. Higher number of TIL infused, and higher proportion of CD8⁺ TIL and CD8⁺ BTLA⁺ TIL in the infusion product, both correlated with improved clinical response[595]. While these features raise hypotheses about strategies to improve the efficacy of TIL, they are not helpful for selecting patients for this treatment. We also identified clinical features, including low serum LDH levels and fewer prior systemic therapies, at the time of treatment as predictors of improved response to ACT TIL[604].

There is also a strong rationale to evaluate tumor features for associations with TIL outcomes. In addition to improving patient selection, the identification of molecules or pathways associated with resistance could suggest rational combinatorial approaches to improve outcomes. In a small cohort of

patients treated with TIL therapy, mutation load was associated with favorable outcome[756]. However, our group was unable to confirm these findings[604].

In this study, we expanded our investigation of genomic factors in relation to outcomes of TIL ACT. We analyzed whole exome sequencing (WES) and RNA sequencing (RNAseq) of tumors used to generate the TIL product for 55 metastatic melanoma patients treated with TIL therapy at the MD Anderson Cancer Center (MDACC), as well as 9 patients treated with TIL therapy at the H. Lee Moffitt Cancer Center and Research Institute (Moffitt). Our integrated analysis of the molecular features of the tumors with patient response, progression-free survival (PFS), and overall survival (OS) has identified new candidate predictors of clinical outcomes with ACT TIL.

4.2 Results

4.2.1. Study Design

To investigate the association between the genetic makeup of the tumor and clinical outcome in TIL ACT, we evaluated the baseline melanoma tumor tissue samples from patients treated at MDACC and Moffitt (Table 6). Following surgical resection, this tumor sample was first utilized to grow TIL for clinical use and leftover tissue was used to generate an array of different biological reagents such as DNA and RNA (Figure 18). To identify biomarkers of outcome and response, we interrogated all of these biological reagents. In-depth characterization of the TIL infusion product and detailed clinical benefit reported following TIL ACT can be found in Forget, Haymaker et al. 2018[594, 604]. Here we describe the results of our in-depth molecular characterization of the DNA and RNA collected from these tumors.

Table 6. Patient metadata for samples used for WES

<i>Number of patients</i>		<i>Total</i>	<i>Complete Responder</i>	<i>Partial Responder</i>	<i>Stable Disease</i>	<i>Progressive Disease</i>
Age						
	<30	7	0	4	2	1
	31-40	13	1	2	4	6
	41-50	18	4	5	6	3
	51-60	24	2	3	8	5
	>61	8	1	2	4	1
Gender						
	Male	37	5	6	14	12
	Female	27	3	10	10	4
Stage						
	IIIC	4	2	1	1	0
	M1a	1	0	0	0	1
	M1b	9	1	1	5	2
	M1c	50	5	14	18	13
TIL Harvest Site						
	Lymph Node	18	3	2	7	6
	Soft Tissue	27	0	9	13	5
	Visceral	9	3	2	2	2
	Metastasis					
	Soft Tissue+	1	0	1	0	0
	Visceral					
	Lymph Node+	3	1	1	1	0
	Visceral					
	Soft Tissue+	6	1	1	1	3
	Lymph Node					
Progression on TIL						
	Yes	53	2	11	24	16
	No	11	6	5	0	0

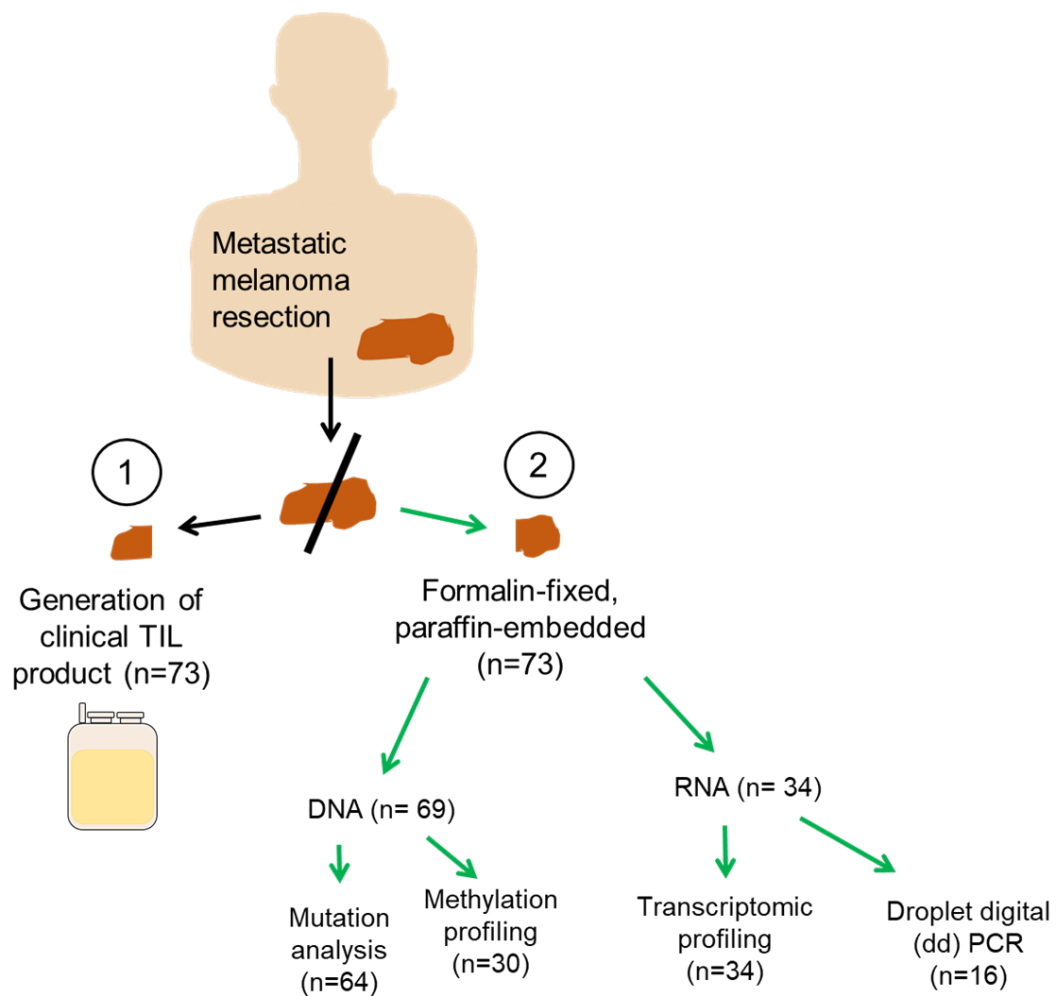


Figure 18. Resected tumor workflow on the metastatic melanoma TIL study. Schematic depicting the distribution of the metastatic melanoma tumor after surgical resection at MDACC (n=64) and Moffitt (n=9). Numbers in circles indicate initial sample prioritization and distribution for 1) generation of the clinical TIL product for patient treatment and 2) formalin-fixation, paraffin embedding (FFPE). The 73 patients with generated clinical TIL product, thus had 73 corresponding FFPE blocks. DNA was extracted from all blocks, with 64 samples passing QC for mutation profiling via WES and 30 samples were also used for DNA methylation profiling (5 samples passed DNA methylation QC that did not pass WES QC). Extracted RNA from 34 patients passed QC for transcriptomic profiling for RNAseq, with remaining RNA or subsequently extracted RNA utilized for ddPCR from 16 samples.

4.2.2. Mutation analysis of TIL treated patients identifies enriched recurrently mutated genes in this cohort

Total tumor mutation burden (TMB) and mutations in specific genes have been shown to be associated with outcomes with immunotherapy, particularly checkpoint inhibitors, in several tumors types[614, 636, 638, 639, 705]. We first compared our results to the Cutaneous Melanoma Genome Atlas (TCGA) study [757]. We found that the mutations detected in both cohorts were similar overall. The median number of mutations in our cohort, including single-nucleotide variants (SNVs) and short insertions and deletions (indels), was 455.5 (range 21–1941, Figure 19a), which is comparable to what was previously reported in the in TCGA in 333 melanoma primary and/or metastatic samples, indicating we had sufficient sensitivity to detect the vast majority of mutations in these samples[757]. Most of the SNVs detected were C>T transitions, and the overall mutational signature was highly consistent with UV-induced mutations as defined by COSMIC Signature 7 and not formalin-fixed tissues (Figure 19b; Figure 20)^[758].

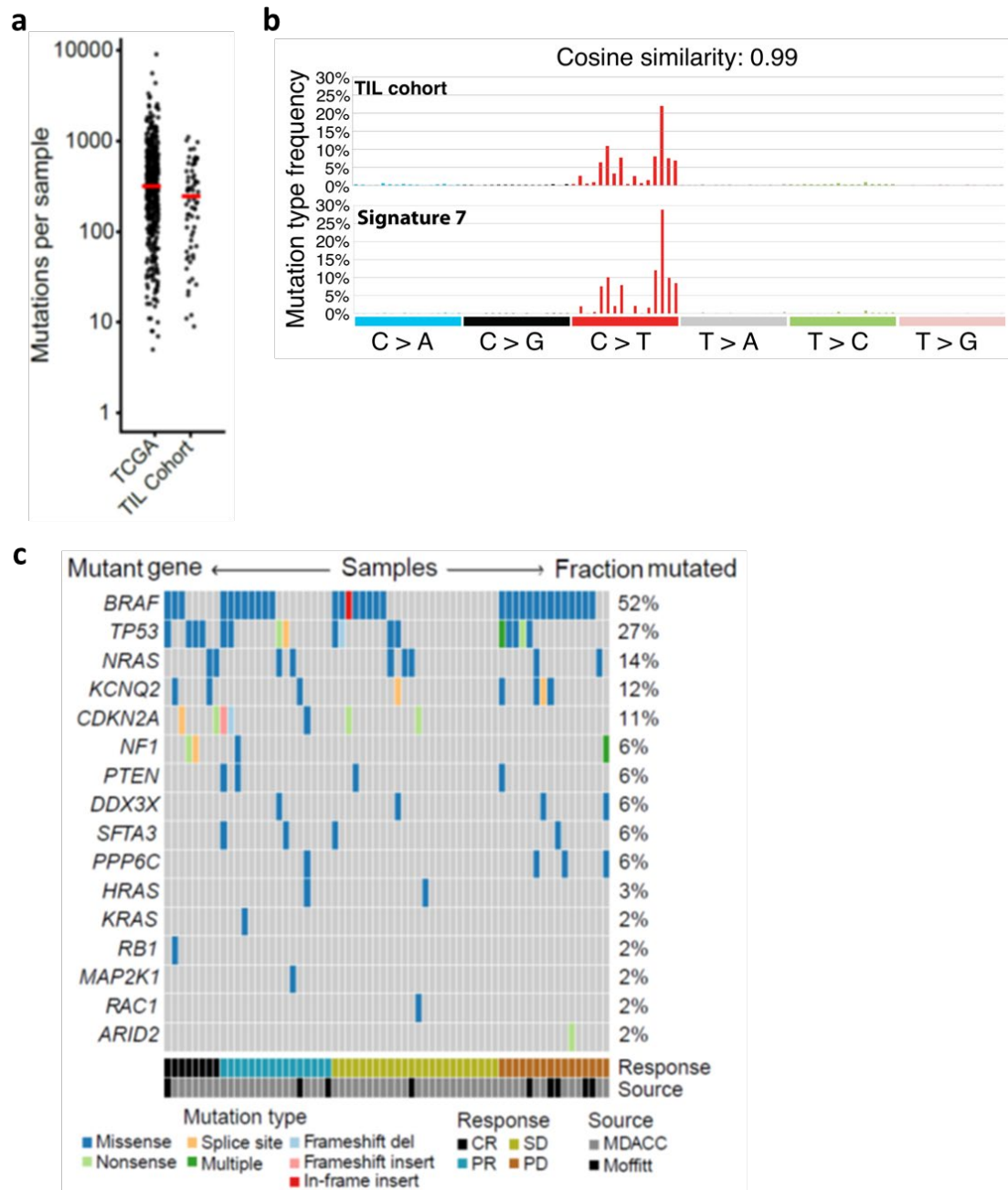


Figure 19. Mutation analysis of pre-treatment tumors from TIL treated patients identifies recurrently mutated genes in this cohort **a** Mutation load in the TCGA cutaneous melanoma cohort and our TIL cohort, consisting of the pre-treatment tumor of the 64 TIL treated patient samples. Red line identifies mean number of mutations. **b** The upper panel shows the mutation signature of SNVs in the TIL patient cohort (n=64), while the lower panel depicts the signature of UV-induced mutations as described by COSMIC Signature 7. The two signatures have a cosine similarity of 0.99. **c** The MutSig2 plot of the recurrent mutations (left side) found within the sample set ($q < 0.1$ is significantly enriched) and the frequency (in percentage) of the mutations (right side). The type of mutation, response to therapy, and cancer center of origin for each sample are denoted below the main plot.

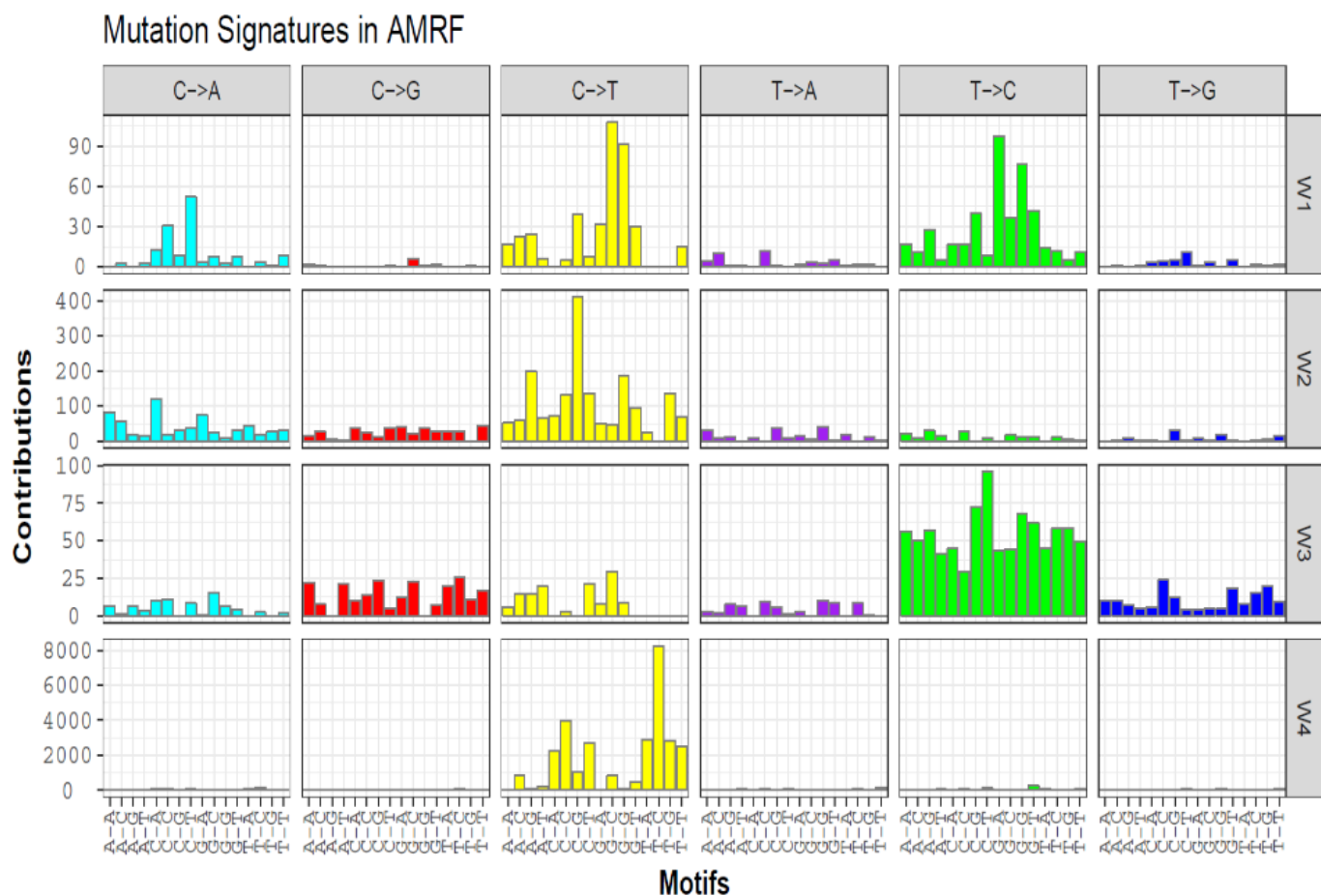


Figure 20. C-T transitions are due to UV exposure rather the tissue fixation. C-T transition in the W4 region are higher then the W3 region suggesting that mutations find in our cohort of formalin-fixed paraffin embedded tissues are due to UV exposure and not tissue fixation.

In addition to UV-induced mutations, driver mutations such as *BRAFV600E* and *NRASQ61R* have been well characterized in melanoma regarding their impact on tumor development, frequency, and therapeutic benefit. In our patient cohort, we identified six significantly recurrently mutated genes (Figure 19C), including *BRAF*, *NRAS*, *CDKN2A*, *TP53*, *KCNQ2*, and *SFTA3*. Comparing our cohort to previously published datasets, we find that *BRAF*, *TP53*, and *CDKN2A* were mutated at similar rates, within 10% of what was previously reported [52%, 27%, and 11%, respectively (Figure 19c)][759][760][761][762]. However, *NRAS* was less frequently mutated in our cohort (14% vs. 26–28%), while *HRAS* and *KRAS* were mutated at similar rates [3% and 2%, respectively (Figure 19c)][759][760][761].

We also identified significantly recurrent mutations in *KCNQ2* and *SFTA3* (MutSigCV $q = 0.03$ and 0.04 , respectively) which were more frequent than reported in TCGA (12% vs. 1.9% for *KCNQ2*, 6% vs. 0.6% for *SFTA3*, Figure 19c)[759]. *KCNQ2* had missense mutations in 6 patient samples and splice site mutations in 2 patient samples, *SFTA3* had missense mutations in 4 samples, with a hotspot (in 3 of the 4 samples) with C to T transitions at chr14:36946289, leading to Glu50Lys. Sanger sequencing confirmed the presence of these mutations (Figure 21, Table 7). Notably, TIL ACT can only be offered if TIL are expanded from the tumor sample, which was the case for 60-70% of the patients accrued on this trial; thus treated patients have undergone a selection process that may influence the mutational profile [499].

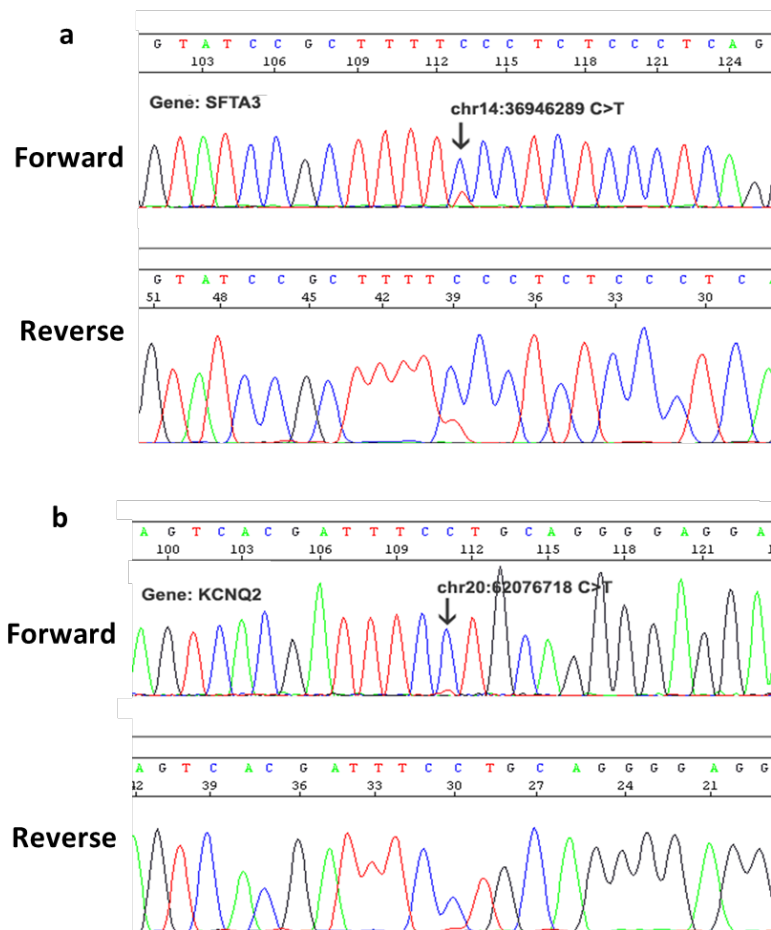


Figure 21. Validation for mutations in *SFTA3* and *KCNQ2* via Sanger Sequencing. Representative samples depict heterozygous mutations in (a) *SFTA3* (n=3) and (b) *KCNQ2* (n=6), with results plotted for forward and reverse PCR sequences.

Table 7. Primers used for Sanger sequencing assay

Gene Query	Sample Name	Chromosome Location	Exon/Intron Location	Primers
KCNQ2	A90	chr20:62038245	Exon 15	Forward: TGACTCTCCCTCCGCAATGT Reverse: GTCGCTGTCCGCCTACGG
	I40104	chr20:62038245	Exon 15	Forward: TGACTCTCCCTCCGCAATGT Reverse: GTCGCTGTCCGCCTACGG
	A63	chr20:62076718	Intron 2-3	Forward: GCCCTGATTCTAGCAATACCAC Reverse: AGAACTGCTCCTGTGGGTGT
	A99	chr20:62103638	Exon 1	Forward: CACGTAGGCGTGGTAGATGAA Reverse: AAGCTGAAGGTGGGCTTCG
	A68	chr20:62038637	Exon 15	Forward: GAGCGCACGATCTTGACAAT Reverse: AGCTGGACTTCCTGGTGAATA
	M40091	chr20:62038073	Exon 15	Forward: GCCCACTCAGTTACTGTAAGA Reverse: GAACCTGGATGCTCTCAACA
	A10	chr14:36946289	Exon 3	Forward: AAGTCAGCGTCCCTACTTTAAC Reverse: GCACAAACATATTGGGCATATCA
	A56	chr14:36946289	Exon 3	Forward: AAGTCAGCGTCCCTACTTTAAC Reverse: GCACAAACATATTGGGCATATCA
	V40108	chr14:36960444	Exon 2	Forward: GCGATCCCATATTATGCAAGTG Reverse: TGTATGTTGAGTCTACTGCCAA
SFTA3				

4.2.3. Predicted neoantigen load correlates with overall survival

High mutation burden was reported to positively correlate with response to some immunotherapeutic approaches, and previous reports suggested an association with PFS and OS in TIL treated patients[614, 763]. In our cohort, composed of patients from two independent centers, we observed a trend between longer OS and greater non-silent mutation load ($p=0.07$ for OS, Figure 22a), which was not observed with PFS or response ($p=0.14$, $p=0.77$, Figure 23a and b)[604]. Of note, sample

purity did not correlate with any outcomes, indicating there was no bias in the proportion of tumor cells within the samples between the groups (Figure 24).

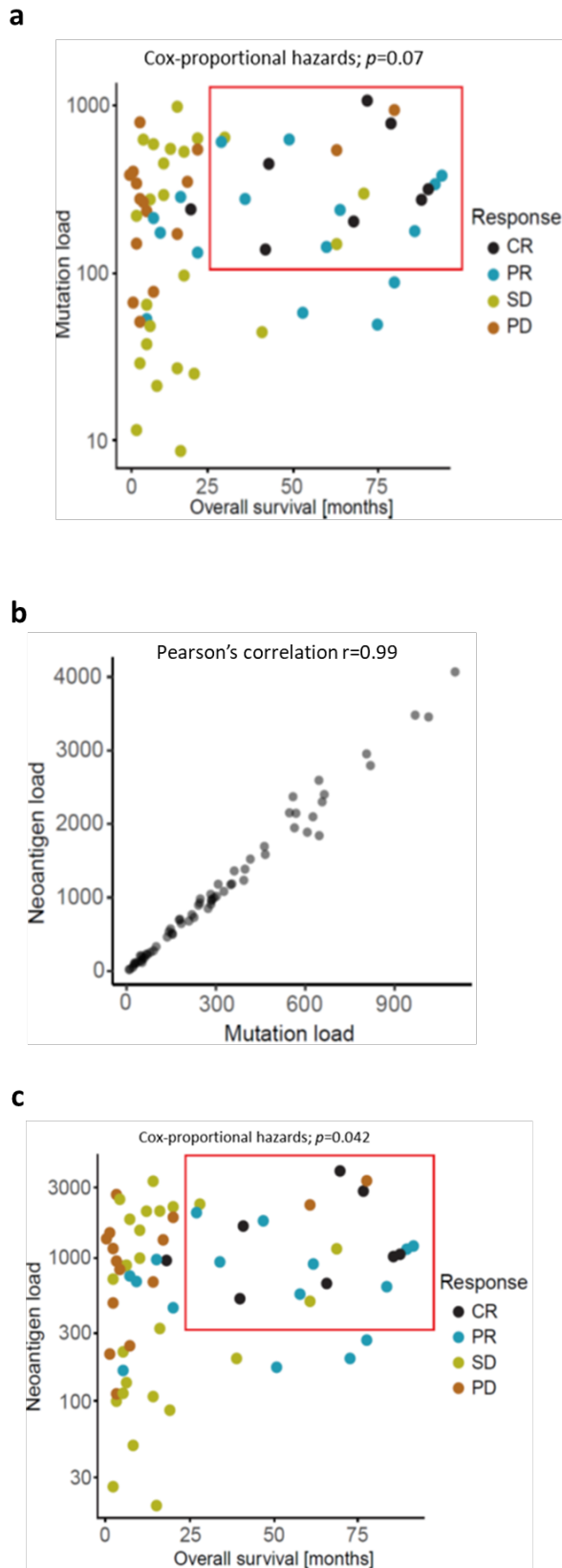


Figure 22. Non-silent mutation burden and predicted HLA class I neoantigen load associate with overall survival. The three graphs represent pre-treatment tumor samples from 64 TIL treated patients. **a** Graph showing non-silent mutation load and overall survival of patients color-coded their response to TIL ACT. The red box highlights patients with the longest overall survival and highest mutation burden. The p-value was generated using the Cox proportional hazards model. **b** Correlation between predicted neoantigen load and nonsilent mutation load. The r-value depicts the Pearson's correlation coefficient. **c** Predicted neoantigen load versus overall survival color-coded by patient response status. The red box highlights patients with the longest overall survival and highest neoantigen load. The p-value was generated using the Cox proportional hazards model.

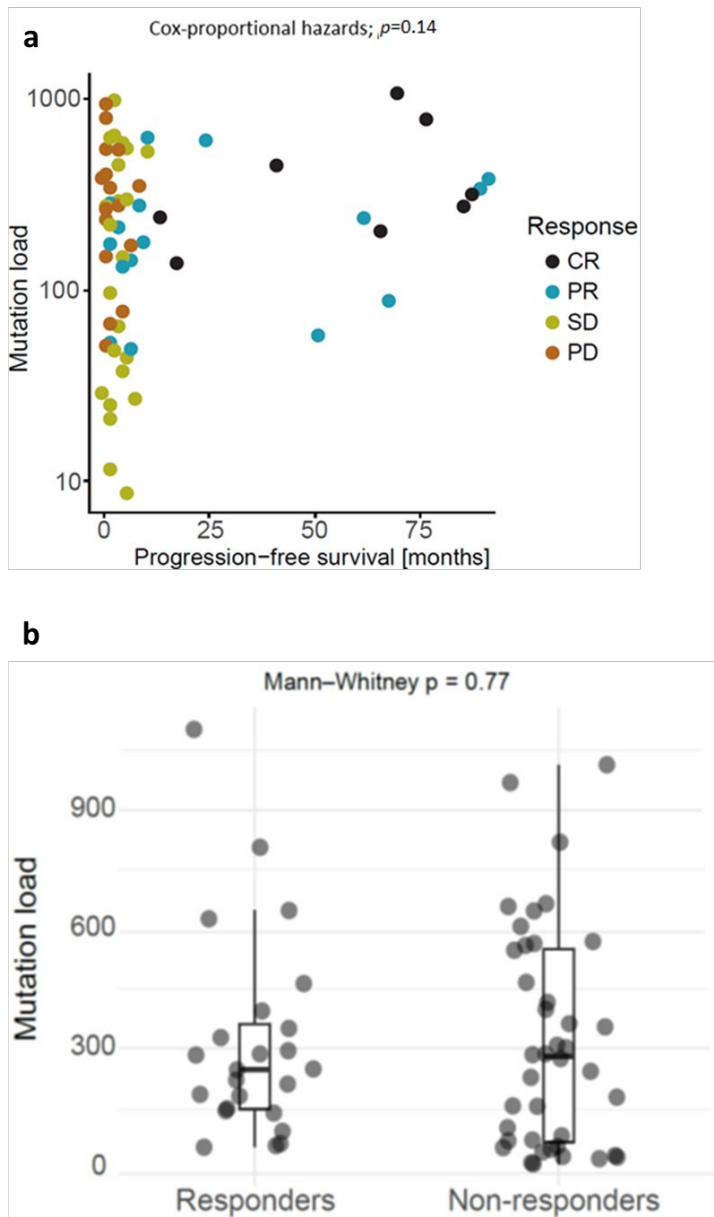


Figure 23. Mutation load versus PFS and response to therapy. **a** Scatterplot representing the association between mutation load and PFS, with patient response color coded (n=64). The p-value was generated through the Cox-proportional hazards model. **b** Box plots depicting the mutation load and association with response (responders- CR and PR or non-responders- SD and PD) (n=64). The p-value was generated using a Mann-Whitney t-test.

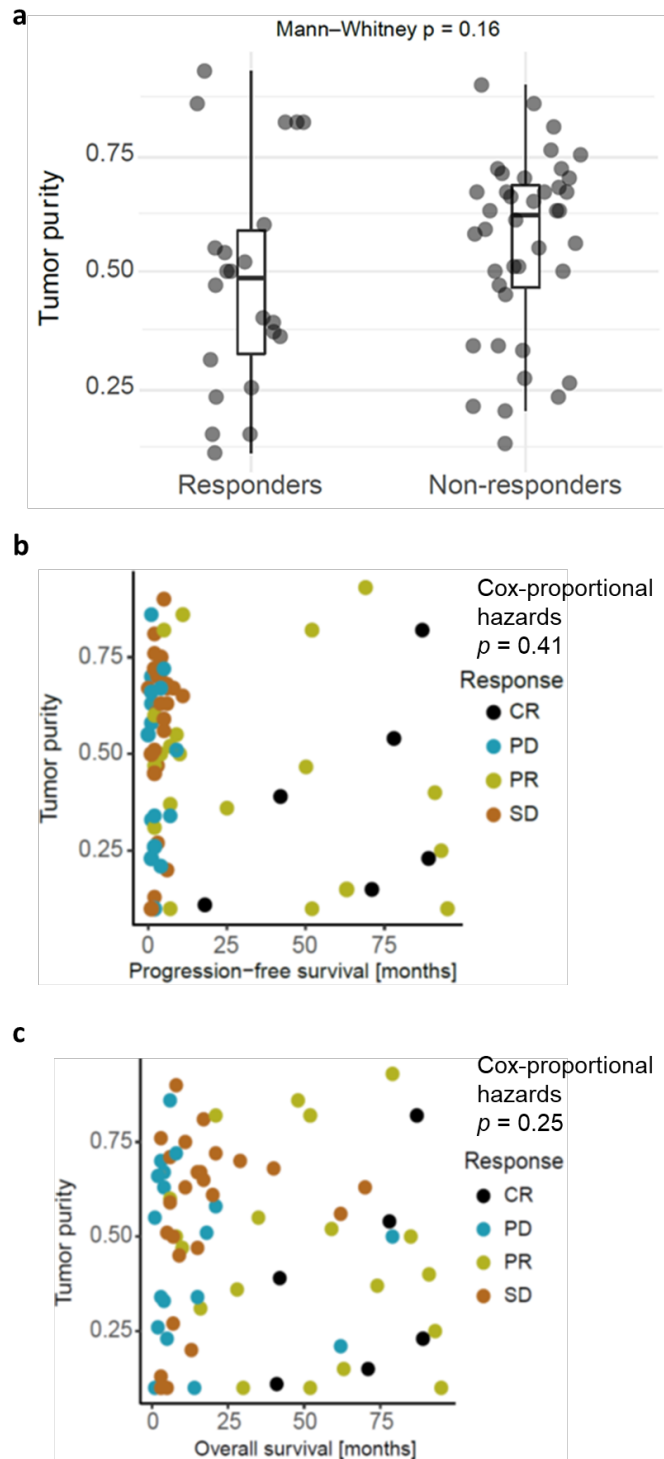


Figure 24. Tumor purity related to outcomes. The cancer cell fraction (CCF) representing sample tumor purity in baseline tumor samples of 64 patients compared to response (a), PFS (b), and OS (c). The p-value for (a) was generated using a Mann-Whitney t-test. The p-values for (b) and (c) were generated using a Cox-proportional hazards model.

The more mutations that are found in a tumor, the more potential there is for the generation of neoantigens. Neoantigens can induce a powerful T-cell mediated immune response leading to improved tumor clearance in the patient. The neoantigen load can be calculated by predicting the ability of the specific HLA molecules expressed by the patient's tumor cells to bind peptides derived from mutated

antigens. We observed a strong correlation between predicted HLA class I neoantigen load and non-silent mutation burden (Pearson's $r = 0.99$, Figure 22b). We also detected a significant association between OS and high tumor neoantigen load ($p = 0.042$, Figure 22c). However, the relationship between neoantigen load and PFS ($p = 0.12$) and clinical response ($p = 0.78$) were not significant (Figure 25a and b).

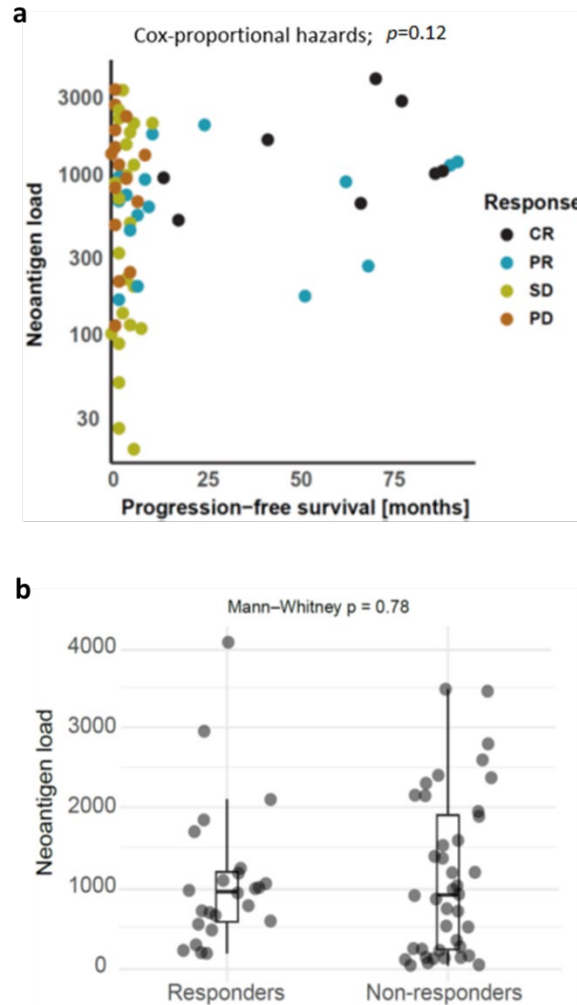


Figure 25. Association between predicted neoantigen load and PFS or response. a Scatterplot representing the association between predicted neoantigen load and PFS, with patient response status indicated. ($n=64$). The p -value was generated using the Cox-proportional hazards model. **b** Depicted is the predicted neoantigen load and association with response, either responders (CR and PR) or non-responders (SD and PD) ($n=64$). The p -value was generated using a Mann-Whitney t -test.

4.2.4. *PDE1C*, *RTKN2*, *NGFR*, and *ELFN1* correlate with survival and response

To further gain insight into potential biomarkers and novel targets to improve TIL therapy, we performed RNA sequencing on the tumor samples ($n = 34$, Figure 18, Table 8). We identified nine genes significantly enriched in responders and one gene enriched in non-responders (Figure 26a). This

observation was carried through when looking at PFS, as we identified 52 genes enriched in patients with long PFS and 31 genes enriched in patients with short PFS (Figure 26b), and 60 genes enriched in long OS and 47 genes enriched in short OS (Figure 26c) (FDR $q = 0.1$ for all).

Table 8. Patient metadata for samples used for RNA sequencing

	<i>Number of patients</i>	<i>Total</i>	<i>Complete Responder</i>	<i>Partial Responder</i>	<i>Stable Disease</i>	<i>Progressive Disease</i>
Age						
	<30	5	0	2	2	1
	31-40	4	0	0	0	2
	41-50	8	1	2	2	0
	51-60	12	1	1	1	5
	>61	5	0	2	2	0
Gender						
	Male	25	2	3	13	7
	Female	9	0	4	4	1
Stage						
	IIIC	1	0	0	1	0
	M1a	1	0	0	0	1
	M1b	4	0	2	2	0
	M1c	28	2	5	14	7
TIL Harvest Site						
	Lymph Node	11	1	1	3	6
	Soft Tissue	15	0	5	9	1
	Visceral	3	0	1	2	0
	Metastasis					
	Soft Tissue+	0	0	0	0	0
	Visceral					
	Lymph Node+	2	1	0	1	0
	Visceral					
	Soft Tissue+	3	0	0	2	1
	Lymph Node					
Progression on TIL						
	Yes	30	0	5	17	8
	No	4	2	2	0	0

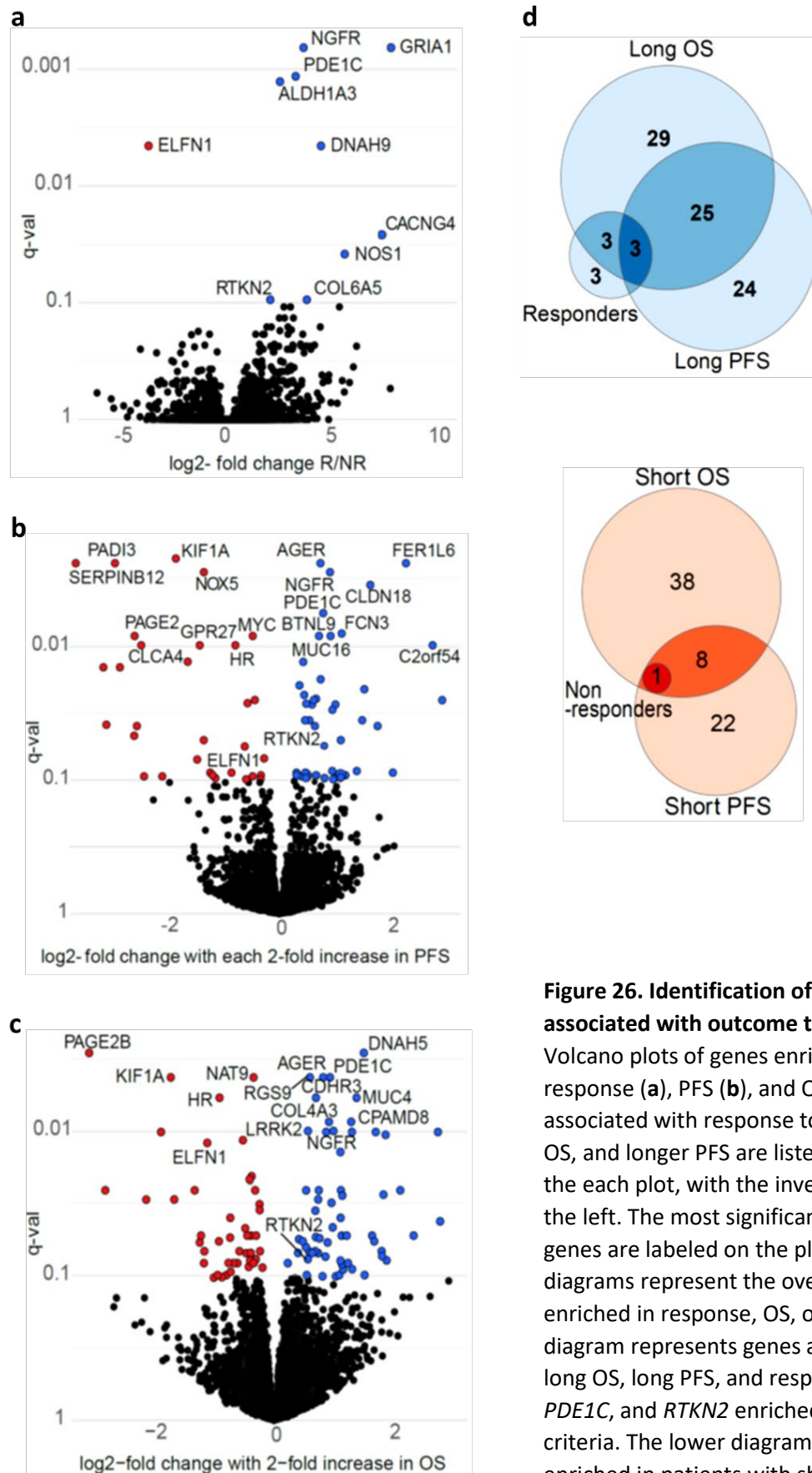


Figure 26. Identification of gene signatures associated with outcome to TIL ACT.

Volcano plots of genes enriched by response (a), PFS (b), and OS (c). Genes associated with response to therapy, longer OS, and longer PFS are listed on the right of the each plot, with the inverse indicated on the left. The most significantly enriched genes are labeled on the plots. **d** Euler diagrams represent the overlapping genes enriched in response, OS, or PFS. The upper diagram represents genes associated with long OS, long PFS, and response, with *NGFR*, *PDE1C*, and *RTKN2* enriched in these criteria. The lower diagram identifies *ELFN1* enriched in patients with short OS, short PFS, and lack of response to therapy.

To identify the most prominent genes whose expression associated with good or poor outcome, we looked for genes at the intersection of all three outcome metrics. We found *PDE1C*, *RTKN2*, and *NGFR* to be enriched in patients' samples associated with therapeutic response, long PFS, and long OS (Figure 26). In turn, we found *ELFN1* to be enriched in patient samples associated with lack of response, short PFS, and short OS (Figure 26d).

Given our high interest in finding genes associated with resistance to TIL ACT to identify potential mechanisms of resistance to target, we decided to focus on further characterizing *ELFN1*. For baseline patient samples for which RNA was still available after RNA sequencing, we further validated *ELFN1*'s expression as a "stand-alone" gene utilizing the droplet digital PCR (ddPCR) assay. Results showed high concordance between the mean ratio of *ELFN1* to the reference gene, *HPRT1*, as compared to the transcripts per million (tpm) from RNAseq ($r^2=0.94$; Pearson's correlation $p<0.001$, Figure 27a). Consistent with the RNA-seq analysis, we confirmed the significant enrichment of *ELFN1* gene expression in non-responders compared to responders to TIL-therapy (Figure 27b). Therefore, the enrichment of *ELFN1* in melanoma tumor tissues of non-responding patients was corroborated by two independent assays.

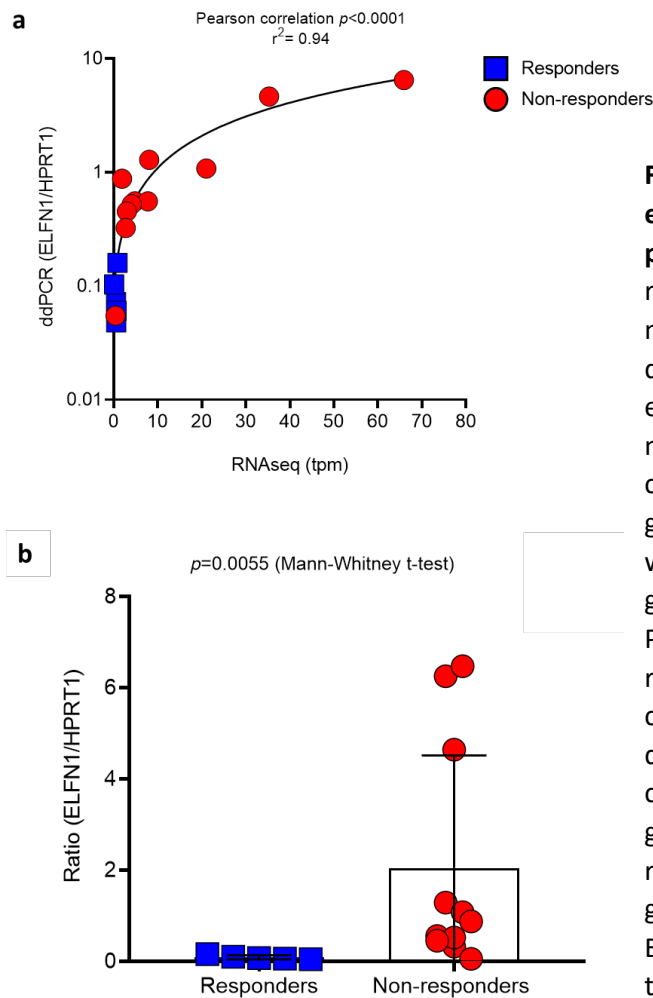


Figure 27. ddPCR confirms enriched *ELFN1* expression in tumors of non-responding patients. Graphs depict 5 samples from responding patients and 11 samples from non-responding patients. **a** Scatterplot depicts correlation between *ELFN1* expression by RNAseq (transcripts per million; tpm) and the mean ddPCR ratio of concentration of target gene to reference gene (*ELFN1/HPRT1*) per sample, plotted with the line of best fit. The p-value generated tests the significance of the Pearson's correlation and the r^2 value is represented on the graph. Samples are color coded by response to TIL therapy. **b** Graph depicts the mean ddPCR ratio of concentration of target gene to reference gene (*ELFN1/HPRT1*) per sample stratified by response to TIL therapy. The p-value was generated using a Mann-Whitney t-test. Error bars on the graph are representative of the standard deviation.

4.2.5. Distinct methylation profiles of *ELFN1* creates a signature that delineates response

These differences in expression between responders and non-responders could be due to underlying differences in genetics, differential activation of pathways, differences in epigenetic state, or a combination of these. To evaluate the role of epigenetics in determining these expression patterns, we interrogated methylation across the entire *ELFN1* gene. We determined that indeed, *ELFN1* is hypermethylated in responding patients (with either complete or partial response) and hypomethylated in non-responders (a category that includes only patients with progressive disease) ($n = 30$, $p = 0.04$, Figure 28a). When focusing on methylation at the 3' untranslated region (UTR), 5' UTR, body, and transcription start site (TSS), we observe two distinct clusters of patients, corresponding with the response to therapy ($p = 0.0068$, Figure 28b). The methylation of *ELFN1* in the CpG island of the 3' region is observed in a

majority of responding patient samples, which may yield RNA expression. In contrast, the methylation of *ELFN1* at the 5' region is observed in most of the non-responding samples, which may suggest a mechanism to regulate *ELFN1* expression. The methylation data supports the differential expression of *ELFN1* based on outcome to TIL ACT.

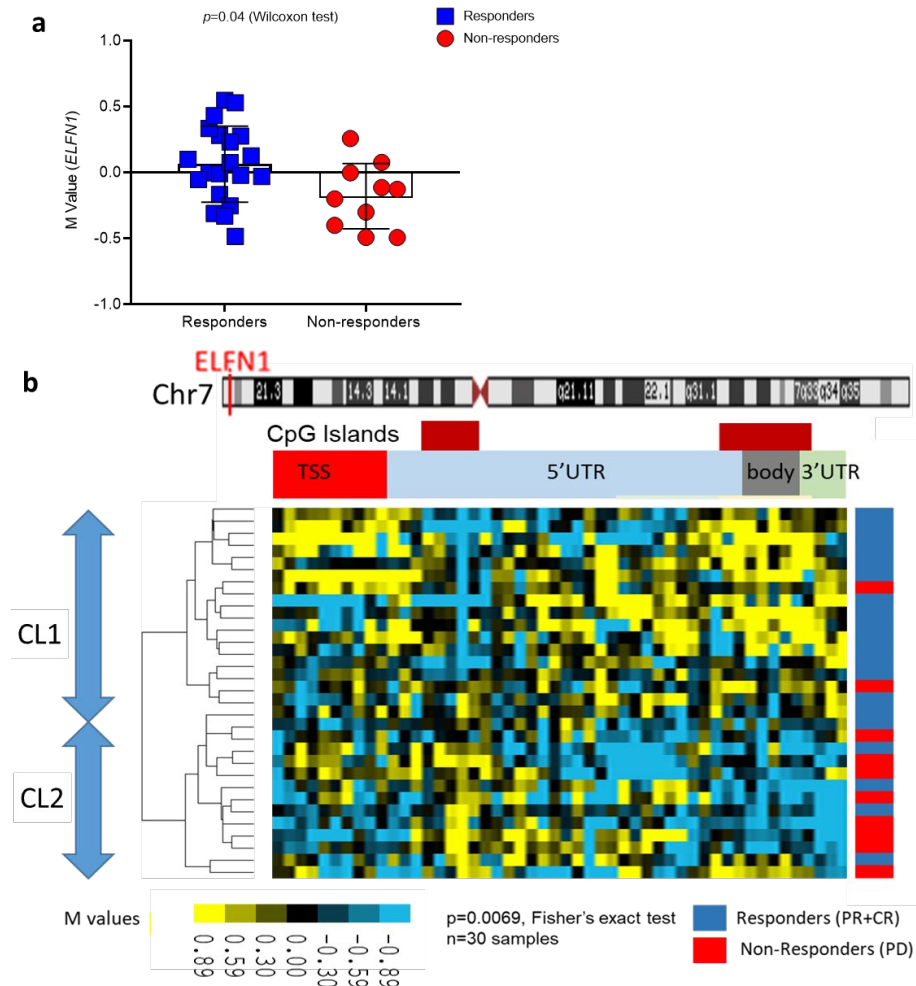


Figure 28. *ELFN1* methylation status in tissues from responders and non-responders. **a** Global mean methylation of *ELFN1* in 30 tumors harvested for TIL propagation, comparing responders (blue, n = 20, CR+PR) to non-responders (red, n = 10, PD) patients. Error bars on the graph are representative of the standard deviation. **b** Unsupervised hierarchical clustering of the 30 tumors shown in (a) by methylation profiles of the *ELFN1* gene. Location of the methylation site (M site) is presented within the gene (5 prime to 3 prime, top of the heat map) with the CpG islands in maroon. The location of *ELFN1* on chromosome 7 is also shown above. The p-value was generated with a Fisher's exact test.

4.3 Summary

This chapter summarizes our study on potential genomic and transcriptomic biomarkers for metastatic melanoma patient outcome to ACT of TIL. Through this research, we identify that there is a trend in greater mutation burden correlating with longer overall survival (OS), which was not witnessed with progression free survival (PFS) or response to therapy. Building upon this, when examining the immunogenicity of these tumors by examining the neoantigen load, we determine that higher neoantigen burden significantly correlates with greater OS, but again, does not associate with PFS or response to TIL ACT. When delving deeper into what specific genes or pathways may be implicated in determining outcome, we find that three genes are associated with favorable outcome (long PFS, long OS, and response): *NGFR*, *RTKN2*, and *PDE1C*. On the contrary, we identify one gene, *ELFN1*, associated with poor outcome (short PFS, short OS, and lack of response); we confirmed this enriched expression utilizing another transcriptomic assay, ddPCR. The significance of each of these genes will be detailed in the subsequent chapter of this thesis. Further investigation into the epigenome of these tumors through methylation profiling did support two divergent methylation patterns within responding and non-responding tumor pre-treatment FFPE tissue, which we thereby hypothesize supports our original findings. Overall, in this chapter, we identify biomarkers and novel genes that are associated with response or lack of response to TIL ACT in metastatic melanoma patients. The identification of *ELFN1* as a gene associated with poor response to therapy also provides an opportunity for modulation using genetic knock out or repression to ultimately improve the final TIL product, yielding improved therapeutic response rates.

Chapter 5: Discussion

The immunotherapy revolution has overwhelmingly impacted the current ways in which cancer patients are now treated. While it brought about new, effective, therapeutic options, including ICIs and cellular therapy, the biological underpinnings of these new therapies are not completely understood. It is worth pausing to consider the biological relevance and implications of these choices. Understanding the impact of such therapeutics on the immune system or TME itself can thus play a major role in uncovering factors associated with response to the current treatment and to decide on subsequent therapeutic options. This is where the importance of biomarkers truly comes into play, allowing for understanding of both impacts of and outcome to therapy.

As described at length in the introduction section of this thesis, ICIs, particularly anti-CTLA-4 and anti-PD-1, are prevalently and successfully used for the treatment of multiple cancer types, including melanoma and NSCLC[341]. Initially, ICIs were tested and deemed successful in cancers that had high immune infiltration and high mutation rates often caused by known carcinogens[739]. These successes have ultimately spurred the investigation of using ICIs, most commonly anti-PD-1, in other cancer types that may not necessarily fit this original mold. For example, treatment success to anti-PD-1 has been seen in cancers with low TMB, including RCC[341]. Investigating the use of an effective immunotherapeutic drug like pembrolizumab in patients with rare tumor types thus makes sense, ultimately offering a potential therapy to an under-served and under-represented patient population in clinical trials. However, there is little scientific evidence regarding the TME and immune profile of these tumors that would give insight on the use and efficacy of ICIs in these rare tumor types.

Further, ICI therapies are given in multiple cycles, for example every 2 to 3 weeks for up to 2 years in the case of anti-PD-1. The immunologic impacts of each dose of therapeutic blockade and the importance of repeated dosing for response to therapy is unclear. Understanding the early changes induced by ICI would be especially pertinent to make treatment decisions for patients failing to

complete all recommended cycles of therapy, often due to irAEs[764]. The current knowledge we have regarding the TME and immune microenvironment modulation upon checkpoint blockade exposure typically comes from studying the tumor upon recurrence to therapy, and is often associated with the methods in which the immune system becomes suppressed or dysfunctional. In collaboration with the Investigational Cancer Therapeutics department at MDA, we were given a unique opportunity to investigate early ICI-induced changes through collection of rare tumor core needle biopsies before and immediately following one dose of pembrolizumab. With the impact on the T-cell component by ICIs, we reasoned that TIL growth success as well as phenotypic, and functional profiling of TIL present before and after therapy can yield insight as to how anti-PD-1 is changing the immune landscape post exposure to ICI treatment. Overall, our study helped to identify biomarkers for the initial impacts of checkpoint blockade and the effects it has on the immune microenvironment.

As it has been previously established that the blockade of CTLA-4 or PD-1 leads to improved T-cell functionality and often improved T-cell infiltration in tumors, we initially hypothesized that the TIL of patients previously treated with ICIs would exhibit superior functionality[295, 297, 339, 745]. However, examination of the outcome of melanoma patients receiving TIL therapy at MD Anderson after progression on checkpoint blockade, mainly anti-CTLA-4, revealed a lower response rate, a shorter duration of response and a decreased ability of the TIL to expand in the final rapid expansion step, following TCR engagement for patients pretreated with checkpoints[604]. Interestingly, T-cell attributes found to correspond with better clinical outcome in checkpoint naïve patients (total number of TIL infused, percentage of CD8 and expression of BTLA on CD8) did not correlate with outcome in patients with prior progression on anti-CTLA-4 alone or in sequence with anti-PD-1. A recent study rather demonstrated a negative impact of progression on anti-PD-1 before TIL therapy but did not find that prior progression on CTLA-4 affected outcome to TIL therapy. In this study, melanoma patients previously exposed to anti-CTLA-4 had longer PFS though no difference in OS or response to TIL therapy[606]. Unlike CTLA-4, melanoma patients with prior PD-1 exposure had poorer PFS following TIL

infusion[606]. Therefore, there is variability in the emerging data regarding the impact of pre-treatment with ICIs on the outcome to TIL therapy, but thus far the data suggests a negative impact of one or more lines of ICI before TIL therapy. Further examination is needed to resolve the impact of ICI on TIL function.

In our study, we initially were able to demonstrate the ability to grow TIL from a single core needle biopsy, showing that the threshold of tissue to generate TIL can be much less than previously described[459, 538]. Further, with the use of such small tissue, less invasive surgeries would be required for patients to undergo and may open the door to more patients being enrolled on TIL trials. Also, core needle biopsies can be utilized for tumors that cannot be surgically resected, thus adding more treatment options for these patients.

We set out to interrogate functional and phenotypical changes in the TIL driven by exposure to anti-PD-1. The first challenge encountered was the very small size of tumor tissue available for our studies (1 core needle biopsy pre and post therapy). Given this limitation, we first tried to maximize the use of our tumor tissue sample. We prioritized TIL expansion and thus could not perform phenotyping of TIL in the fresh tumor tissue, but did perform phenotyping of TIL after TIL pre-REP expansion. However, the bias introduced in this assay is that we only can phenotype markers that are not lost after expansion and will only be profiling cells that successfully grew. But due to the use of the TIL 3.0 method, which yields a 100% successful culture expansion of TIL in melanoma, we were able to phenotype almost every paired baseline and on treatment sample received, from a variety of rare tumor types (n=33; Fig 1A, Chapter 3)[499]. We did not witness any TIL growth differences based on type of cancer nor exposure to anti-PD-1.

The main interest of this study was to uncover potential biomarkers and changes to TIL before and after exposure to anti-PD-1. Using our expanded TIL from pre- and post-treatment, we were thus able to look at lasting phenotypic changes that occurred post exposure to one dose of pembrolizumab.

One caveat that must be noted, however is that based on our use of the TIL 3.0 method of expansion, we know that we skewed our cellular population to a CD8 phenotype due to the addition of agonistic 4-1BB antibody[602]. To overcome this, we could utilize the 3-5 core needle biopsies obtained by pathology for FFPE for multiplexed IHC or immunofluorescence to obtain a general idea of the cellular profile in the fresh tumor, which could be compared to our expanded TIL phenotype. Besides holistically characterizing T-cell subtypes, we further looked to the expression of other ICIs expressed on the surface of TILs, as these would give hints to potentially mechanisms of tumor immunosuppression and targetable molecules to combine with anti-PD-1 blockade to improve TIL function. In doing so, we noticed both CD4 and CD8 expanded TIL surface expression did not vary in terms of expression of Lag3, Tim3, PD-1, or GITR after exposure to anti-PD-1 (Fig 2C and 2D, Chapter 3).

We noted an increase in CTLA-4 surface expression in the pre-REP grown CD4 TIL after one dose of anti-PD-1 (Fig 2D and 2E). The upregulation of CTLA-4 upon the blocking of anti-PD-1 had been previously observed in mice and was demonstrated on freshly isolated CD4 and CD8 TIL (Source 12). Interestingly, the expression of CTLA-4 in this trial was witnessed after 14-21 days of cell culture and last exposure to anti-PD-1, hinting that these are lingering effects not lost during cellular expansion over time. The modulation of CTLA-4 upon exposure to pembrolizumab supports the testing of the combination of anti-CTLA-4 and anti-PD-1, and may help to explain the success which is witnessed with this combined approach in different tumor types in these trials[350, 351, 353]. We also observed an association between higher Lag3 expression on CD8 TIL and higher surface CTLA-4 expression on CD4 TIL of the same TIL culture in post-PD-1 exposure samples (Fig 2F; $r=0.5092$; $p=.0155$). Thus, Lag3 may be the next checkpoint molecule upregulated to support TIL suppression by the TME. This observation supports the idea of testing the triple combination of CTLA-4, PD-1 and Lag3 blockade as the next evolution in therapy. There are currently clinical trials in process utilizing the combination of Lag3 blockade and one or both of these molecules (trial including blockade of all 3: NCT03459222)[765].

Further, this work provides evidence of the lasting biological consequences of exposure to one dose of anti-PD-1 on both CD8 and CD4 TILs.

A significant limitation in this trial is that most of these patients did not respond to pembrolizumab treatment. The overall cohort had a 14% objective response rate[766]. In the subset of patients studied from this cohort, for which we had paired baseline and post-treatment, successfully grown TIL (n=33), we identify 4 patients that experienced tumor regression. Overall, these patients had CTLA-4 expression on CD4 that was not or minimally elevated after the anti-PD-1 dosing, which did not follow the general trend of the other samples, but did not have anything else phenotypically distinct regarding their TIL post one dose of anti-PD-1. However, due to the small sample size and the limited number of phenotypical markers assessed, we cannot make firm conclusions based on the phenotype of grown TIL as a potential biomarker for patient treatment outcome when comparing pre- and post-exposure to one dose of anti-PD-1. Having access to a larger number of samples from patients who responded to anti-PD-1 therapy would have allowed us to gain a better understanding of the phenotypical changes occurring in the setting of response to therapy.

Taking this a step further, we wanted to investigate the changes in TIL functionality, as defined by secretion of soluble factors upon stimulation, driven by a single exposure to anti-PD-1. Prior use of circulating soluble factors as biomarkers of therapeutic response and outcome has been reported for both ICI treatment and TIL therapy (see Introduction Biomarker Sections for a more detailed description). There has also been a focus in these studies in determining the expression of IFN γ , as this is often deemed a marker of TIL antigen recognition and activation[569]. In our study, we profiled 65 soluble factors produced by *in vitro* activated TIL. Unfortunately, the numbers of CD4 TIL expanded from the cultures were limiting (n=6 sorted pairs; due to culturing conditions with 4-1BB agonism), leading to most profiling performed within the CD8 TIL population. Functionally, we observe that there

is a diminished secretion of multiple soluble factors after exposure to one dose of pembrolizumab in the CD8 TIL (Fig 3A).

One important component of T-cell function is to secrete soluble factors. Previous literature has suggested that TIL functionality can be assessed by measuring the array of soluble factor secreted by T cells, with the production of multiple soluble factors per cell contributing to the polyfunctionality of TIL being associated with highly avid cells[767-769]. Further, it has been shown that TIL producing low amounts of cytokines within the TME corresponds with upregulation of markers like CTLA-4, PD-1, and Tim3 also defining dysfunction, particularly with PD-1⁺ TIL producing less IFN γ and IL-2 upon antigen stimulation[768, 769]. Thus, our results displaying a diminished soluble factor secretion profile suggest that we are expanding more dysfunctional TIL after a single exposure to anti-PD-1, and that the profile is stable even after exogenous provision of a potent costimulatory signal (anti-41BB) during the expansion phase. These results are corroborating our original observations in TIL ACT where the transfer of TIL expanded after a patient progresses on checkpoint blockade is less effective. The specific soluble factors that declined the most after one dose of anti-PD-1, include IL-22, IL-23, VEGF-A, SDF-1 α , HGF, and sCD25 (Fig 3B). As mentioned previously, high serum levels of sCD25, VEGF-A, and HGF have all been previously correlated with either poorer survival or lack of response to therapy in melanoma[658, 659, 661, 752]. Further, SDF1- α has been shown to induce iNOS production in TIL, and iNOS in the TME of samples prior to TIL therapy has been shown to correlate with poor PFS and OS in melanoma patients[696]. (For a thorough review of these studies, please refer to the Biomarker section of this thesis). Unfortunately, perhaps due again to the low number of patients experience tumor regression in our cohort, we could not find a specific association between cytokine production and response to therapy; further study would be needed to investigate this observation in survival. Overall, based on the prior association with response to other immunotherapies, the reduction in these soluble factors witnessed in the TME post-pembrolizumab exposure would suggest that these TIL were less dysfunctional and more apt to generate a therapeutic response/prolong survival.

IL-22 and IL-23 were also significantly diminished upon exposure to one dose of anti-PD-1 therapy. IL-22 and IL-23 are associated with a pro-inflammatory Tc17 and Tc22 subsets, which elicit strong antitumor effects[770-772]. Further, a recent publication observed that within ovarian cancers, Tc22 cells encompass 30% of expanded CD8 TIL and the production of IL-22 in these TIL was associated with recurrence free survival[770]. The cytokine profiling in our study may suggest the reduced presence of the Tc22 subset post anti-PD-1 dosing, which could represent an important change in the balance of TIL cellularity. However, we did not observe any of the profiled cytokines significantly going up after anti-PD-1, supporting the concept that the reduced levels of multiple soluble cytokines detected are probably the result of a per cell diminution in the capacity to secrete cytokines rather than a change in the balance of the different TIL functional populations. Thus, this magnifies the importance of understanding the ratios and interplay of these cytokines together and highlights an important caveat of selected analyte analyses. For example, we did not see a change in the secretion of IFN γ , typically used to assess the functionality of anti-tumor T cells. However, the important shift in multiple other secreted factors point to a functional change in TIL after one dose of anti-PD-1. Our TIL functional assessment was limited by tissue availability. Ideally, the TIL functionality would be tested using the autologous tumor line generated at the same time of TIL expansion, whereby actual assays regarding killing (like caspase expression) could be utilized. In future studies, single cell sequencing of TIL pre- and post-anti-PD-1 exposure may also be useful to determine the functionality of these cells, depicting which TIL would likely secrete which cytokine profile, yielding more insight to potentially relatively easily identifiable biomarkers for ICI exposure and ideally outcome to therapy. Until these confirmatory assays are performed, concrete statements regarding the impact of anti-PD-1 on TIL cannot be made. Overall, however, we can conclude that a diminished secretion profile can be observed in expanded TIL post exposure to one dose of anti-PD-1, suggesting an enduring effect of anti-PD-1 on TIL function.

Although it is important to grasp the impact of therapy on the TIL themselves, it is also important to understand the interplay and complexity of cellular functionality within the entirety of the

TME. When considering the make-up of cellular therapies, particularly TIL ACT, there are two main components: the TIL and the tumor. Thus far, characterization of the TIL have been extensively performed, often through cellular phenotyping of the infusion product as mentioned above. However, it is also important to understand how the TME holistically may be impacting patient therapeutic outcome and its modulation upon treatment. To do so, methods of cellular profiling via IHC have been predominantly been utilized[691, 696]. However, with a higher prevalence and greater accessibility to next generation sequencing, a new renaissance has begun for interrogation tumor tissue further at the genomic, transcriptomic, and epigenomic level to determine potential biomarkers. Within the realm of immunotherapy, this was initially performed to identify biomarkers of patient outcome to ICIs, which prompted our interest in defining genomic attributes of the TME correlating with outcome to TIL therapy.

In this study, we were thus interested in determining biomarkers for TIL ACT utilizing genomic, transcriptomic, and epigenomic means, querying the entire TME. To increase our sample size, we supplemented our samples with 9 samples from patients accrued at Moffitt Cancer Center for WES analysis to investigate the recurrent mutations, overall mutation burden, and predicted HLA Class I neoantigen load. Although the use of FFPE tissues for our genomic studies allowed for most samples within the TIL trial at MDACC to have representative tissue, it did introduce issues of formalin fixation based artifact and overall DNA and RNA degradation resulting in poor quality. However, previous studies have shown concordance in calls between FFPE and fresh frozen derived melanoma tumor tissues for both WES and RNA sequencing[773, 774]. Thus we elected to proceed with the analysis, in collaboration with Dr. Rameen Beroukhi from the Broad Institute, and Jeff Meng, PhD student in his laboratory, for bioinformatics analysis support. Filtering steps were put in place to remove bias from the formalin fixation[604]. Before proceeding with analysis, our team compared the overall mutation burden to that of the TCGA cohort and the SNVs within our samples to those found in Cosmic Signature 7, which represents UV Induced mutations[757, 758]. Both of these initial checks therefore gave us

confidence within our dataset to proceed with further analysis. Importantly, when working within bioinformatic datasets, this step is critical; without this transparent step, subsequent findings can be questioned as “trash in, trash out.”

Our analysis began through the verification of common recurrent mutations observed within melanoma, including *BRAF*, *NRAS*, *TP53*, and *CDKN2A*. Interestingly, in this combined patient cohort, we observed *KCNQ2* and *SFTA3* to have enriched mutations as compared with the TCGA (12% vs. 1.9%, 6% vs. 0.6% respectively)[758]. *KCNQ2* encodes potassium channels associated with membrane polarization, but recently has been associated with oncogenic properties, with amplified copy numbers in GI cancers[775]. Mutations within *KCNQ2* are mutually exclusive with those of *CDKN2A*, *KRAS*, and *TP53*; thus, potentially suggesting that the combination of these mutations could be detrimental to cell survival[775]. Further study would be needed to see if mutations within *KCNQ2* in melanoma also would be associated with oncogenesis as well, supporting new targeted therapies for this specific gene. *SFTA3* is a surfactant protein often found in the lung and eye, with the ability to enhance the phagocytic rate of alveolar macrophages[776]. Thus, in patients harboring this mutation, there would be a hypothetical disadvantage regarding the ability of these cells to generate aspects of an innate immune response within mucosal tissues. Notably, patients with recurrent mutations, including this *SFTA3* mutation may be better suited for TCR T-cell therapy, as *SFTA3* is shown to have a hotspot mutation in 3 of our 4 patients. More studies would be needed to define if this new hotspot mutation is immunogenic and if so, to clone the TCR. TCR transduced T-cell trials targeting mutations shared amongst patients are ongoing for *KRAS* and *TP53* mutations, with patients treated for the G12D mutation in *KRAS* experiencing regression[520, 521]. This would cause a need for patients to be pre-screened for these mutations prior to TIL therapy to determine the best method of ACT. However, it also must be noted that differences in the frequency of detection of new mutations between our cohort and TCGA may be attributed to the actual update in the software used to call mutations, which has become more sensitive[777]. To determine if this was the case, reanalysis of the TCGA data should

also be performed using the same software to determine if the mutation rates within these genes is augmented accordingly.

The presence of mutation-reactive TIL has been identified in the infusion products of patients who developed complete responses after TIL ACT in melanoma and patients who derived clinical benefit from TIL ACT in other solid tumor types[565, 615, 778]. Moreover, TIL products enriched for neoantigen reactivity have shown clinical promise, with durable tumor regression demonstrated within a patient for metastatic breast cancer[570]. Overall, these observations lead to the hypothesis that mutated tumor antigens may play an important role in response and outcome to TIL ACT. In support, the Lauss et al study of melanoma patients receiving TIL therapy demonstrated the TMB to be positively correlated with clinical benefit (response and OS of greater than 2 years), OS, and PFS[711]. However, Forget and Haymaker observed that within our cohort of 54 patients at MDACC, there was no association between TMB and response to TIL ACT[604]. When we investigated this within our expanded cohort including patient tissues from Moffitt, we noted that there was a trend between greater TMB and OS ($p < 0.07$), while there was no correlation with response and PFS, suggesting the involvement of subsequent therapies in deriving a benefit from high TMB.

Given that the sheer number of mutations (TMB) is not associated with response or PFS to TIL therapy, but patients infused with neoantigen-reactive TIL tend to benefit from the therapy, we investigated the likelihood of the mutations to be presented to the immune system by examining the predicted neoantigen load. Prior studies have demonstrated a relationship between predicted neoantigen load and patient therapeutic outcome, particularly to anti-PD-1 and anti-CTLA-4 in melanoma and NSCLC[637, 639, 646, 647]. The Lauss et al. group found similar findings that clinical benefit (response and OS greater than 2 years), PFS, and OS correlated with greater predicted neoantigen load for TIL therapy in melanoma patients[711]. Within our cohort of patients, we noted that predicted HLA Class I neoantigen load was correlative with OS, but did not correlate with PFS or

response. This was somewhat expected, as we observed a tight correlation between neoantigen load and TMB. This also suggest that the mutations that are witnessed within these tumors are generating neoantigens for potential use as targets for T-cell mediated clearance. As we specifically identified the mutated neoantigen load, it is important to note we are not taking into consideration other TAAs, including post-transcriptionally modified, testes, or overexpressed TAAs that also may be influential in the immunogenicity of the tumor. It is also important to distinguish that although predicted neoantigen load is useful, other factors play into this model such as including the level of expression of the mutated gene and the availability of a functional antigen presentation machinery. In summary, it is likely that mutation-specific TIL contribute to tumor clearance and prolonged survival following TIL therapy but certainly they are not the only driving factor.

We were further interested in which genes or genetic pathways were modulated between our patient cohorts to uncover transcriptomic signatures associated with outcome to TIL. The Lauss et al. group had previously published results demonstrating that there was an enrichment in HLA Class I machinery in patients that had a favorable outcome to TIL ACT[711]. Within our sample set, we identified the genes *PDE1C*, *RTKN2*, and *NGFR* enriched in patients with favorable outcome (long survival and response) to TIL ACT, while we found *ELFN1* consistently enriched in patients with poor outcome to therapy. Unfortunately, within our cohort, we did not see any enrichment in a particular pathway or cell type associated with this analysis. Delving deeper into the functionality of these genes, we find that they are typically not associated with a positive outcome in cancer treatment; thus, we looked into the impact of these genes in the immune system to perhaps explain the correlations we observe. In doing so, we find that *PDE1C* degrades cyclic AMP (cAMP), a secondary messenger that can be inhibitory to T-cell effectors[779, 780]. Therefore, expression of *PDE1C* could prevent the inhibition of anti-tumor T cells, yielding improved patient tumor clearance and ultimately response to immunotherapy. Similarly, *RTKN2* could offer protection to T cells by preventing apoptosis, leading to improved T-cell persistence and anti-tumor activity post-transfer[781]. We were intrigued to find *NGFR* enriched in our patients that had

favorable outcome to therapy, as *NGFR* is enriched during melanoma metastasis and progression[782]. However, a recent abstract presented at the American Association of Immunologist's annual meeting reported that *NGFR* can bind to the costimulatory molecule CD80 and protect against CTLA-4 mediated suppression on T cells[783].

Future studies will need to define the expression pattern of *PDE1C*, *RTKN2*, and *NGFR* in the melanoma TME. Causing either enhanced expression in one or all of these genes with positive association could thus improve clinical outcome of TIL treated patients, but to do so, understanding which cells within the TME express these genes is critical. For example, review of the literature shows that *NGFR* can be expressed on both melanoma tumor cells and dendritic cells; in this context, we would assume enhancing expression of *NGFR* particularly in dendritic cells may be the first avenue of investigation to improve therapy[784]. In contrast, *RTKN2* is shown to be expressed within T cells[781]. To enhance expression of genes within TIL has performed most commonly using retroviral constructs to generate genetically modified TIL (examples include *CXCR2* or *TGFβ* DNR), but use of CRISPR knock in systems have recently gained attention[572, 573, 785]. Mechanistic studies on the role of these genes in defining outcome to TIL therapy will need to be performed.

Conversely, one gene was associated with poor survival and lack of response to TIL ACT, *ELFN1*. Due to its involvement in resistance to TIL therapy providing a potential therapeutic target, we narrowed our focus on this gene. As mentioned above, due to the use of FFPE tumor tissue, which includes the entirety of the TME, it was difficult to determine which cell type actually expresses the *ELFN1* gene. This is vital to understand, as this would further determine the potential target ability of this gene to allow for improved patient outcome. Therefore, we first looked to the literature to determine the role of *ELFN1*, particularly in tumor biology and immunity. First, it was noted that *ELFN1* is a member of the extracellular leucine-rich repeat (LRR) superfamily, which also includes TLRs, but unlike *ELFN1*, TLRs contain a cytoplasmic toll-IL-1R domain[786]. However *ELFN1* has thus far only been associated with the nervous

system and has not been implicated in any immune function[786]. *ELFN1* is essential in induction of the electrophysiological properties of the synaptic connection within the hippocampus[787]. Further study demonstrated *ELFN1* as an allosteric modulator of glutamate receptors type III, particularly metabotropic glutamate receptors 6 and 7 (mGluR6 and mGluR7)[788, 789]. Although glutamate receptors type I and II are both expressed in T cells and involved in immune modulation, the same has not been documented with glutamate receptors type III[790]. Thus, it is unlikely that *ELFN1* is interacting directly with TIL. However, metabotropic glutamate receptors type III have been shown to be expressed in melanoma and prevent apoptosis in neurons through activation of the MAPK/P13K pathways; therefore, *ELFN1* could potentially protect tumor cells from apoptosis through their glutamate receptors[791, 792].

Expression of *ELFN1* has recently been described in both breast and ovarian cancers, however has never been reported in melanoma[793, 794]. For example, *ELFN1* is enriched in ovarian cancer cell lines that are sensitive to ROS and HSP90 targeted chemotherapy[794]. Unfortunately, little is known regarding the functional role *ELFN1* may have in tumors, however methods to interrogate its role in melanoma will be addressed later in this discussion. Also, the anti-sense, non-coding RNA *ELFN1-AS1* has been shown to be upregulated in CRC patients with poor prognosis and its silencing inhibits the proliferation and migration, as well as enhancing the apoptosis, of CRC tumor cell lines[795]. Though anti-sense RNA can often regulate expression of other genes, it has not been proven that *ELFN1* is impacted by *ELFN1-AS1*[796].

Further study in melanoma tumor cells would thus be needed to characterize the role of *ELFN1*, and possible interaction with *ELFN1-AS1*, as we would presume that its main functional impact would be within this cell type. Previously published single cell RNA sequencing data sets could be interrogated to first confirm that *ELFN1* is expressed in tumor cells rather than TIL or other cells within the TME; we did a precursory query in a previously published dataset by Jerby-Arnon et al. of 33 melanoma tumor samples

profiled via single cell RNA sequencing and found low expression of *ELFN1* in immune cells, with values around zero for macrophages, T cells, NK cells, and B cells[797]. To further confirm this, more analysis should be performed utilizing similar single cell RNA sequencing published data sets; as an alternative, fresh melanoma tumors could be utilized to perform transcriptomic single cell sequencing for expression of *ELFN1*. From available data, we would hypothesize that *ELFN1* was expressed in tumor cells. Upon confirmation that *ELFN1* is expressed within melanoma tumor cells, we would propose utilizing targeted knock out using potentially a CRISPR-Cas9 model or knock down using siRNAs of *ELFN1* in melanoma cell lines. First, cellular proliferation of these modified tumor cells (as compared to control cells) could be monitored utilizing flow cytometry. Next, these *ELFN1* targeted KO cells could be co-cultured with corresponding autologous TIL to determine differences in T-cell mediated death, secretion of soluble factors, and cellular metabolism. We would then ultimately move to mouse model systems to study the effect of *ELFN1* knock out of tumors *in vivo*; first, we would implant an established melanoma tumor cell line (*ELFN1* KO or WT B16) in C57BL/6 mice to determine how this change may inhibit tumor growth. We could then use this model of B16 in C57BL/6 with an adoptive pmel-1 T-cell transfer to mimic a TCR specific adoptive cellular therapy; this could then be similarly applied utilizing our human melanoma patient tumor and TIL pairs to perform adoptive transfer experiments using NSG mice. If *ELFN1* expression is not observed in the tumor cells, *ELFN1* KO mice may be utilized to determine if *ELFN1* expression in non-tumor cells impacts B16 tumor establishment and growth. Infiltration of tumors by immune cells and changes within the stroma would be compared to WT mice. Ideally, these results would lead to the development of a therapeutic regimen, whereby targeted *ELFN1* inhibition could occur, accompanied by TIL ACT.

We next investigated the implication of epigenetic modifications on *ELFN1* expression within the TME. In doing so, we focused on methylation, as methylation of genes in melanoma has led to both tumor suppression and modulation of a number of differentially expressed pathways, including metastasis and immune recognition[798]. Previously methylation has also been implicated in patient response to

immunotherapy. For example, patients with low methylation of CTLA-4 correlated with response and longer OS to both anti-PD-1 and anti-CTLA-4 therapies[799]. Further, patients with high methylation of 4-1BB correlated with lack of response and poor PFS to anti-PD-1 therapy[800]. Interestingly, the site of methylation is most important, often dictating whether a gene will be expressed or suppressed. Canonically, we think that methylation at the promoter region leads to suppression of gene expression; however, this is not always the case, as methylation at this site can cause release of suppressors, release of silencers, or binding of enhancers[801, 802]. Thus, to fully understand and appreciate the role of methylation, it is often necessary to perform functional assays to draw conclusions regarding the expression of genes correlating to methylation sites. Within our patient cohort, we noted that overall there was enhanced global methylation of *ELFN1*, with two distinct clusters of methylation, particularly with hypo-methylation in non-responding patients in the body and 3' CpG island. This data ultimately suggests that *ELFN1* expression could be regulated by methylation of these regions of this gene. Due to a lack of overlapping samples utilized for both methylation and transcriptomic assays within our study, we could not fully investigate this relationship. However, we tried to interrogate this question utilizing primary cell lines derived from metastatic melanoma patients that had genomic sequencing data and methylation profiling performed. Unfortunately, there were different methylation profiles in the tumor cell lines that did not correlate with the profiles observed in the FFPE tissue, which potentially could be explained by *ex vivo* culture of the cells no longer be representative of the initial tumor. Thus, in the future, it may be more appropriate to interrogate other previously published datasets of whole tumor tissues with both methylation and transcriptomic profiling performed. Samples within these datasets could be segregated by enriched methylation at the TSS, 3'UTR, body, or 5'UTR, which could then be compared with the matched sample transcriptomic expression data to determine if these regions lead to repression or expression of *ELFN1*. Further, especially based on the results regarding the regulation of *ELFN1* expression epigenomically, targeting *ELFN1* methylation may prove to be a novel therapeutic

mechanism to improve patient outcome to TIL ACT. Prior to this study, however, the role of *ELFN1* within the tumor should be elucidated.

Biomarkers for therapy can fall into two major categories: prognostic or predictive. Prognostic biomarkers inform about patient outcome regardless of treatment type, while predictive biomarkers are typically measured prior to therapy and indicate response to a particular therapy type[803]. Based on these definitions, our study does solidify greater neoantigen load as prognostic for longer OS, as previously defined in the Lauss et al. group and its previous association with clinical benefit in immunotherapies. We determine this as a prognostic marker rather than a predictive marker, as there was no association with longer PFS or response to therapy, which would be most indicative of the impact of TIL therapy; thus, we cannot attribute the greater OS directly with TIL ACT. Further, we also begin the process of identifying novel genes as predictive biomarkers to therapy[711]. In order to claim the genes we identify via transcriptomic analysis are in fact predictive, however, we would first need to determine that what is observed is specifically correlative to ACT of TIL. For example, is *ELFN1* enriched in patients that have poor outcome specifically to TIL ACT, or is this gene also enriched in patients with metastatic melanoma that have poor outcome overall or respond poorly to other therapeutic interventions? To fully make the claim that these are prognostic or predictive biomarkers, enrichment of these genes should also be investigated in other published melanoma datasets of patients either not treated (like TCGA) or treated (like Van Allen et al. or Hugo et al.) to see if enrichment is specific to this therapy[638, 639, 759].

Notably, we have a major caveat within our study that must be acknowledged: we include a heterogeneous mix of cutaneous, acral, and mucosal melanoma patients. Due to this, mutation rates may be skewed, as patients with cutaneous melanomas have a higher rate of *BRAF* mutations and a greater overall TMB, while patients with acral and mucosal melanomas have a greater prevalence of *KIT* mutations[804-806]. Further, patients with cutaneous melanomas tend to have longer OS comparatively, particularly in association with ICI treatment; for example, within a study of 428 melanoma patients,

patients with cutaneous melanoma had an OS of 45 months after ICI therapy, while patients with acral and mucosal melanoma had OS of 17 and 18 months, respectively[807, 808]. Thus, to overcome this potential bias within our cohort, we could utilize multivariate analysis to compare mutation (recurrent and TMB), survival, and melanoma tumor type.

Thus, over the course of our study, another study investigating the genomic correlates of outcome to TIL therapy has been reported by Lauss et al., but for our analysis, we had the following major changes: 1) increased the sample size of patient tissues interrogated, 2) utilized FFPE tissues, as archival blocks are often banked in pathology for all patient samples, 3) investigated epigenetic alterations that may lead to distinctions in outcome, and 4) made correlations associated with specifically OS, PFS, and response rather than clinical benefit (response and OS of greater than 2 years). We did not use this measure of clinical benefit within our study, as patients often are enrolled in other clinical trials or receive follow up treatments, contributing to their OS. Due to this, PFS, the survival until a person progresses and receives a new modality or succumbs to illness, is often a better measure of response to specifically TIL therapy.

Overall, our studies identified novel biomarkers within the TIL and TME that are associated with immunotherapy. Within the TIL specifically, we demonstrate that exposure to one dose of anti-PD-1 can lead to both phenotypic and functional changes in pre-REP expanded TIL, with increased expression of CTLA-4 in CD4 TIL and with decreased expression of soluble factors by CD8 TIL. These findings support the use of the combination of anti-PD-1 and anti-CTLA-4, and suggest a profound and lasting impact of anti-PD-1 drugs on TIL acquired very early in the course of therapy. These results emphasize that care that must be taken when choosing therapeutic strategies. Further, the diminished secretion of both pro- and anti- tumorigenic soluble factors emphasizes the importance of a holistic view regarding conclusions on cellular functionality, suggesting the need for next steps like single cell sequencing or functional cellular assays to dig deeper into the functional impairments of these TIL. Within the entirety of the TME,

we show that there are genomic correlates to clinical outcome of TIL ACT in metastatic melanoma, as discovered by using pre-treatment FFPE archived patient samples. We uncover the association between TMB and OS after therapy, with a stronger correlation between predicted neoantigen load and OS. Further, we identify 3 genes associated with favorable outcome to TIL ACT, including *NGFR*, *RTKN2*, and *PDE1C*. Particularly through these findings, we identify the gene *ELFN1* is associated with poor survival and lack of response to TIL ACT, which is also supported by epigenetic methylation profiling. Future studies that identify the particular role of *ELFN1* in the TME are needed, but ideally, we hope that this gene becomes a novel target for cellular therapy and improving patient clinical outcome in metastatic melanoma. Thus, this thesis supports the diversity, identification, and importance of utilizing biomarkers in the TIL and TME for patient treatment benefit.

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Vita

Caitlin Alane Creasy was born in Lafayette, Louisiana to Sheri and Cliff Creasy. After graduating The Woodlands High School in The Woodlands, TX, she attended Southwestern University in Georgetown, TX, where she obtained her B.A. in Biology and Environmental Studies. During her time at Southwestern, Caitlin performed research in the laboratory of Dr. Martin Gonzalez. In pursuit of her curiosity and quest for knowledge, she continued her education and earned her Masters of Science in Biomedical Sciences (Immunology) at the University of North Texas Health Science Center: Graduate School of Biomedical Sciences in Fort Worth, TX under the mentorship of Dr. Lisa Hodge. Upon completion, she joined the laboratory of Dr. Laszlo Radvanyi, and upon his encouragement, joined the MD Anderson UTHealth Graduate School of Biomedical Sciences in Houston. Caitlin carried out this dissertation in the Department of Melanoma Medical Oncology under the mentorship of Dr. Chantale Bernatchez and Dr. Michael Davies.

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