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BRINGING TIL THERAPY BEYOND MELANOMA: ADVANCING THE TREATMENT OF ADVANCED PANCREATIC AND OVARIAN CANCERS

Donald Sakellariou-Thompson

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BRINGING TIL THERAPY BEYOND MELANOMA: ADVANCING THE TREATMENT OF ADVANCED PANCREATIC AND OVARIAN CANCERS

A

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by

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Dedication

The work of this dissertation is dedicated to my parents who always showed me the way and never stopped encouraging me. Σας αγαπώ πάρα πολύ.

Acknowledgements

The work in this dissertation would not have been possible without the tremendous support, guidance, encouragement, and collaboration of the so many wonderful people around me. I owe the greatest debt of gratitude to several people without whom none of this would have been possible. Chantale, your constant patience, encouragement, and support were a calming force that allowed me to keep pushing forward. Dr. Hwu, I appreciate you always challenging me to go further and providing inspiration through your dedication. Thank you both for taking a chance on me and giving me this opportunity. Cara and Marie, thank you for taking me under your wing. Your guidance, knowledge, infinite patience, and unwavering support were invaluable. It was an honor and a privilege to learn from all of you.

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Thank you to the rest of my Advisory Committee members, past and present, for your commitment, advice, and encouragement.

Thank you to all my amazing collaborators throughout MD Anderson, without whom I could not have completed this work. There is no better collaborative environment than here.

Thank you to the administration, faculty, and staff at the GSBS for their help and understanding, and for creating a great environment in which to pursue our studies.

Finally, to my family and friends, I don't have the words to convey the depth of my gratitude. I would be nothing without your unconditional love, support, and encouragement.

Abstract

BRINGING TIL THERAPY BEYOND MELANOMA: ADVANCING THE TREATMENT OF ADVANCED PANCREATIC AND OVARIAN CANCERS

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The success of checkpoint blockade immunotherapy (CBI) has reinvigorated the cancer therapy field, particularly for advanced melanoma which has doubled the survival rates compared to 20 years ago. However, there are many solid tumor types that are yet to receive substantial benefit from this ground breaking therapy, two of which are pancreatic cancer (PDAC) and ovarian cancer (OvCa). As such, the 5-year survival rate for PDAC and OvCa stand at 9% and 28% respectively. Despite the lack of efficacy of CBI so far, there is still evidence for the role of immune control in these cancers as evidenced by presence of tumor-infiltrating lymphocytes (TIL) correlating with increased survival. Since *in vivo* manipulation of TIL through CBI does not seem to be sufficient to generate a strong clinical response, approaches involving *ex vivo* manipulation of immune cells, such as adoptive cell therapy (ACT) using autologous TIL, might be able to provide therapeutic benefit. The effectiveness of TIL ACT has been demonstrated in metastatic melanoma with overall response rates around 50%. Given this success and the anti-tumor potential of the immune infiltrate in PDAC and OvCa, I sought to assess the feasibility of TIL ACT in these solid tumors. My work here has demonstrated the feasibility of TIL ACT for PDAC and OvCa by showing that they harbor an activated, anti-tumor $CD8^+$ T-cell infiltrate that can be robustly and reliably expanded using an improved 3-signal culture method consisting of a CD3 stimulation, an

agonistic 4-1BB mAb, and high-dose IL-2. Additionally, these results show that TIL ACT for PDAC and OvCa is viable regardless of primary or metastatic site and regardless of prior chemotherapy. Furthermore, given the difficulty of treating PDAC, single-cell RNA sequencing was used to interrogate the immune landscape to further our understanding of the TIL heterogeneity in PDAC. Paired transcriptomic and T-cell receptor sequencing revealed novel TIL populations with potential prognostic and therapeutic implications. A Phase II clinical trial based on this work has been initiated at MDACC to evaluate the feasibility of the adoptive transfer of autologous TIL in recurrent or refractory PDAC and OvCa (NCT03610490).

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Chapter 1: Introduction

The immune system is the body's main defense mechanism against disease and this protection is mediated by a complex system of specialized cells and molecules. Although its major mechanisms were only elucidated within the last 150 years, the concept of immunity has been known, at least implicitly, since the time of Ancient Greece when it was observed that only those people who had survived the plague could take care of the sick because they would not contract the disease again [1]. In the 18th and 19th centuries, work by Edward Jenner, Louis Pasteur, Robert Koch, Emil von Behring, Shibasaburo Kitasato, and Jules Bordet provided empirical evidence for immunity [1]. However, major mechanisms of the immune system were not revealed until observations by Elie Metchnikoff and Paul Ehrlich. Metchnikoff discovered that there were phagocytic cells, which he termed macrophages, which were always present in order to respond and clear pathogenic microorganisms. Ehrlich's research established the idea that neutralizing antibodies could be induced against a wide array of foreign substances named antigens. Fittingly, the importance of their work was recognized with the Nobel Prize in 1908. Since then, the importance of this work has only grown as improved understanding of the mechanisms of the immune system has allowed for the ability to harness its potential for treating disease. In this context, the disease that looms large for many people is cancer in all its various forms. The Word Health Organization estimates that the global cancer burden has risen to 18 million new cases with 9.6 million deaths in 2018 [2]. In this introductory chapter, I intend to lay out (1) the relationship between the immune system and cancer, (2) provide an overview of how the current understanding of this relationship has led to the immunotherapy revolution in cancer

treatment, with particular focus on (3) a type of immunotherapy involving the adoptive transfer of tumor-infiltrating T cells and (4) our efforts to improve and expand its application.

1.1: The Immune System and Cancer

Metchnikoff and Ehrlich's work laid the foundation for the two major compartments of the immune system, innate immunity and adaptive immunity, which work in concert to protect the body from foreign pathogens. All blood cells derive from stem cells in the bone marrow, called pluripotent hematopoietic stem cells (HSCs), including the components of the innate and adaptive immune system (Figure 1). The major cellular elements of both will be discussed below in order to lay a foundation for how the actions of the immune system are mediated.

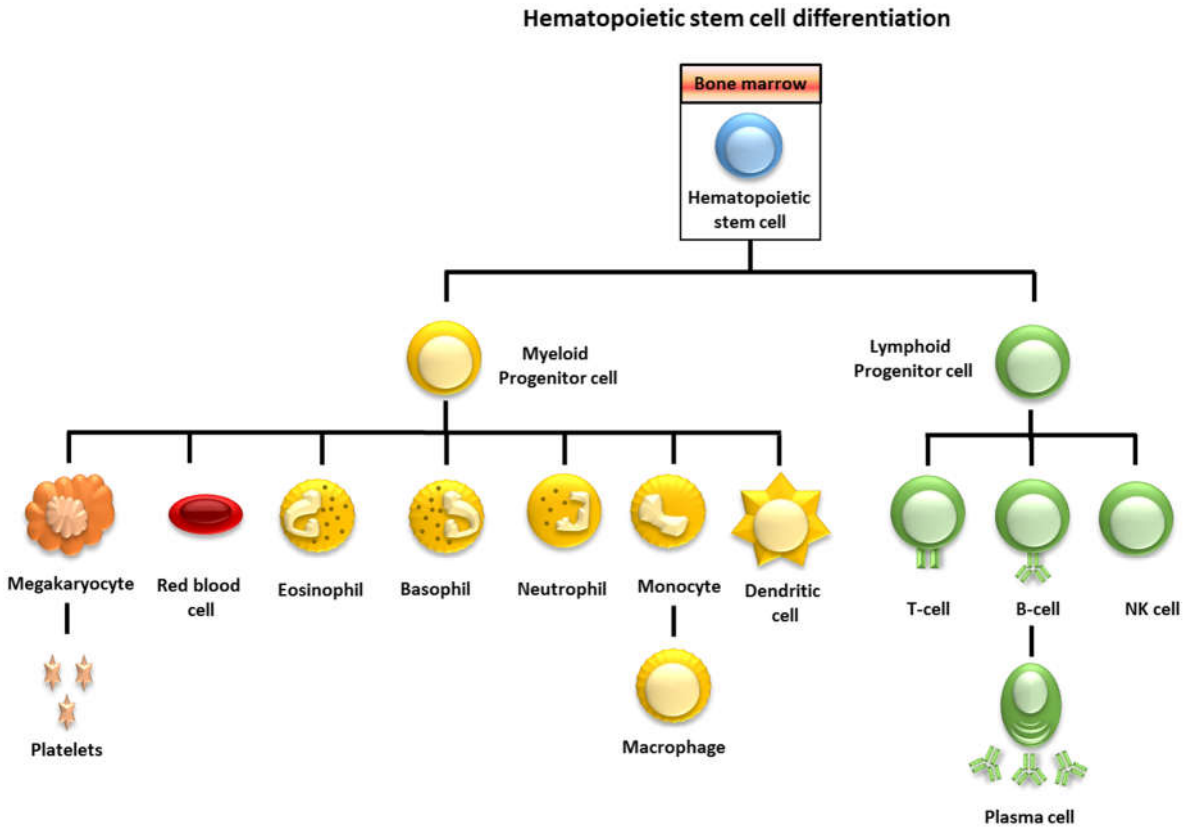

Figure 1. Hematopoietic stem cell differentiation.
Bone marrow-derived hematopoietic stem cells give rise to the common myeloid and lymphoid progenitor cell, which themselves differentiate into the major cellular components of the immune system.

1.1.1 Innate Immunity

Innate immunity is the first line of defense, and it is responsible for non-specific but immediate responses that can feed into adaptive immunity. This consists of several components, but the main and largest one that I will highlight is the myeloid cellular component. Myeloid cells arise from the common myeloid progenitor cell (CMP), which itself originates from bone marrow HSCs [1]. Along with the macrophages first described by Metchnikoff, the myeloid component is comprised of other phagocytic cells like dendritic cells and several kinds of granulocytes. While having the similar property of engulfing pathogens, they also have other important functions that are briefly described below:

Macrophages. One of the blood cell types that arises from CMPs following differentiation from HSCs are monocytes, which circulate in the blood before entering various tissues and maturing into macrophages. These cells are the main phagocytic cell (i.e. they engulf pathogens) in the body and are found in most tissues trawling for foreign microorganisms to clear. Macrophages also participate in initiating larger immune responses by producing inflammatory mediators and by functioning as antigen presenting cells (APCs). APCs present antigens to the effector cells of the immune system using a class of surface receptor known as the major histocompatibility complex (MHC; also known as Human Leukocyte Antigen [HLA]). Antigen presentation is a highly integral part of bridging the innate and adaptive immune responses which will be discussed further in section 1.1.5.

Dendritic cells. Also deriving from the CMP, immature dendritic cells (DCs) exit the bone marrow into circulation and enter various tissues, such as the skin or gastrointestinal tract, constantly checking for pathogens. While DCs have phagocytic capacity as well, they also constantly sample material from the extracellular space, process it internally, and present small pieces of this material, known as antigens, on their surface. If a DC becomes activated during this process, it will migrate from the tissue to the nearest lymph node to interface with adaptive immune cells. This process allows them to perform their main and most important function as an APC.

Granulocytes. The third major cell population that arises from the CMP is the granulocytes, which consists of neutrophils, eosinophils, basophils, and mast cells. They are named granulocytes due to the many granules of degradative enzymes they secrete or use to breakdown phagocytosed pathogens. Neutrophils are the most abundant granulocyte (60-70% of white blood cells) and are a major responder to infection. Eosinophils, basophils, and mast cells are less abundant and mainly respond to parasites as well as allergic reactions. All provide a very fast response (minutes) and are short-lived.

The components of the innate immune system are often called sensor cells because they express receptors that allow them to detect various inflammatory mediators. These innate receptors are called pattern recognition receptors (PRRs) because the recognize molecules common to various pathogens or indicators of cell stress. Unlike the lymphocyte receptors that will be described in the following section, these innate receptors do not have a highly variable repertoire of specificities.

1.1.2 In between innate and adaptive immunity

Two other cell types worth mentioning with regard to innate and adaptive immunity are the natural killer (NK) cell and the gamma-delta (γδ) T cell. While these cells derive from the CLP and are considered lymphocytes, they do not possess the hypervariable antigen receptors that define B cells and T cells [1]. These cells can be thought of as occupying a transitional space between innate and adaptive immunity due to sharing characteristics of both systems. They are briefly described below:

NK cells. Although it is a lymphoid cell, NK cells also display several characteristics of innate immune cells. They lack hypervariable antigen-specific receptors and instead express several types of innate receptors that allow them to quickly respond to cellular stress or infection. They are also able to recognize and destroy antibody-coated pathogens via Fc receptors which recognize the "base" of antibodies (Figure 2A). As such, NK cells bypass the need for antigen recognition in the context of MHC, and in fact will also target cells that do not express MHC. Additionally, recent findings suggest that they have most features of adaptive immunity except for the antigen-specific receptors. As such it has been proposed that they occupy the evolutionary space between innate and adaptive immunity [3].

γδ T cell. This type of T cell is defined by expression of distinctive, invariant TCRs that is composed of a TCRγ and TCRδ chain, hence the name. While γδ T cells have many phenotypic and functional similarities to αβ T cells, the difference in TCR allows them recognize antigens directly on the surface of target cells, outside of the context of MHC. They also have a much more limited repertoire of antigen specificity and may even function similarly to an innate immune cell in terms of pattern recognition. The complete role of γδ T cells and their antigen targets are still being elucidated.

1.1.3 Adaptive immunity

Ehrlich's work, among others, hinted at the existence and function of adaptive immunity. This system is composed of highly specialized cells, called lymphocytes, which arise from the common lymphoid progenitor (CLP) in the bone marrow [1]. Each of these lymphocytes express antigen receptors with a specificity unique to each cell (Figure 2). When these cells divide, the unique antigen receptor will be passed on to the daughter cells which are often referred to as clones due to sharing the unique receptor. This diverse repertoire of antigen specificity is what allows the immune system to ultimately respond to almost any challenge. The hypervariability of these antigen receptors is accomplished through random genetic recombination of several hundred gene segments to generate a seemingly infinite range of specificities. The two main lymphocyte cell types are described below:

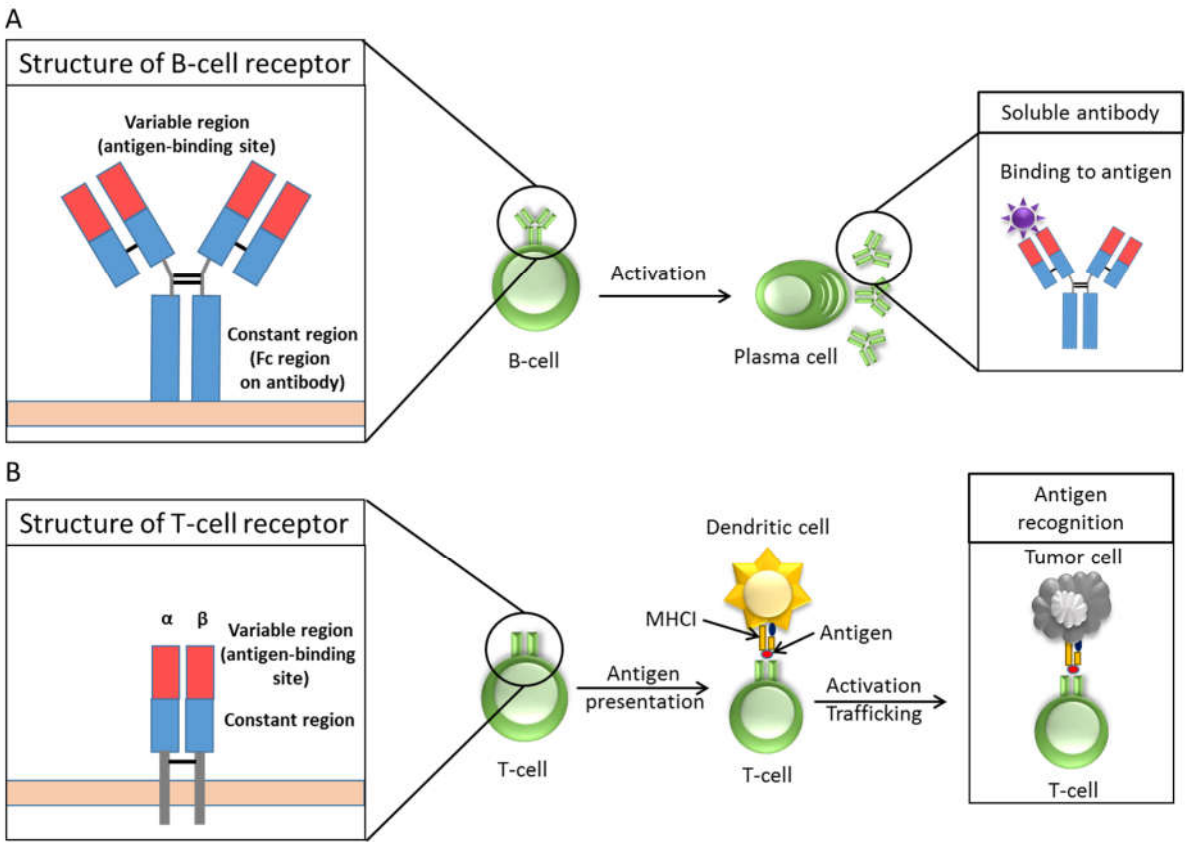

Figure 2. Structure and function of B- and T-cell antigen receptors.
(A) The B-cell receptor is a Y-shaped protein consisting of two chains. At the forked-end of the Y is the variable region where the receptor binds its cognate antigen. After a B cell is activated, it beings to secrete its receptor in the form antibodies. These bind to their targets in order to neutralize or mark them for clearance. **(B)** The T-cell receptor consists of two chains, the most common pairing being the alpha- and beta-chains. The T-cell becomes activated after recognizing its antigen in the context of MHCI, which is generally on the surface of a dendritic cell. After activation, the T cell will traffic through the body until it again recognizes its target on the surface of a tumor cell and destroy the target cell.

B lymphocytes. Also known as B cells, these lymphocytes are white blood cells that differentiate from the CLP in the bone marrow and then circulate in the peripheral blood as well as reside in lymph nodes. As mentioned, each express a unique antigen receptor (the B cell receptor or BCR) that is essentially a membrane-bound antibody. Upon recognition of an antigen via their BCR, B cells become activated and secrete Y-shaped proteins called antibodies with the same specificity as the BCR (Figure 2A). These antibodies can bind to

targets to either neutralize them directly or mark them for clearance by innate immune cells like macrophages. Therefore, B cells are the cellular mediators of the humoral immune response that Ehrlich described. Additionally, B cells can also act as APCs for T lymphocytes to direct their response.

T lymphocytes. Also known as T cells, this class of lymphocyte is the main effector cell of the adaptive immune system. T cells express their own antigen-specific receptor known as the T-cell receptor (TCR) that is composed of a TCRα chain and TCRβ chain (Figure 2B). Thus these T cells are often called αβ T cells. Upon recognition of a unique antigen through their TCR, T cells become activated and differentiate into either cytotoxic, helper, or regulatory T cells. Respectively, these cells can clear cells that express the antigen target ($CD8^+$ cytotoxic T cells), help support the immune response ($CD4^+$ helper T cells), or even suppress the response of other immune cells ($CD4^+$ regulatory T cells). Importantly, T cells can only recognize their antigen when it presented in the context MHC Class I molecules, which are present on the surface of all cells in the body (Figure 2B). T cells do not secrete their antigen receptor like B cells. Instead, they engage the target cell directly through the TCR:MHCI interface.

The adaptive immunity arm of the immune system is an amazing and powerful weapon for not only its vast array of antigen specificity, but also its capacity for immunological memory. The name adaptive refers to this extremely important function. After being exposed to a pathogen, the body "adapts" by maintaining a small population of lymphocytes that were specific for the pathogen so that it can more readily respond in the event that pathogen is encountered again. This was the principle behind the success of early vaccine studies done by Jenner and others.

1.1.4 T-cell differentiation and plasticity

T-cell development, occurs primarily in the thymus, a specialized lymphoid organ located behind the sternum. After development, T cells enter the blood as so-called naïve T cells (T_N) and migrate to peripheral lymphoid organs, such as lymph nodes, where they can be activated by antigens. Following activation, both $CD8^+$ and $CD4^+$ T cells can differentiate into several classes of T cells that are discernable by expression of certain surface markers and different, specialized functions [4]. Beyond T_N, the generally accepted classes of T cells are central memory (T_{CM}), effector memory (T_{EM}), and terminally-differentiated effector memory (T_{EMRA}) (Figure 3). In addition to these, recent data has suggested the existence of T-cell subset between T_N and T_{CM} called T stem-cell memory (T_{SCM}) with putative stem-cell like attributes like self-renewal and multipotency [5]. While the exact model for T-cell differentiation is still debated, the general consensus is that these main T-cell states fall along a spectrum of progressive differentiation where T_N leads to T_{SCM}, then T_{CM}, then $T_{EM,}$ and then finally ending with T_{EMRA} [5] (Figure 3A). As T cells progress along this continuum, they become more differentiated and more short-lived. For example, T_{CM} can persist long term in the body until they encounter their antigen, upon which a subset can differentiate into T_{EMRA} and die off shortly after executing their effector function [4].

The contribution of $CD4^+$ effector T cells is key to an efficient $CD8^+$ T-cell immune response, as well as programming of optimal memory T-cell subset formation. As such, they are often referred to as $CD4^+$ helper T cells (T_H). Within the $CD4^+$ T_{EM} subset, there are several well-defined T_H subsets: T_H1, T_H2, T_H17, and follicular helper T cells (T_{FH}) (Figure 3B) [1, 6]. The differentiation of naïve $CD4^+$ T cells into each of these subsets is mainly governed by specific cytokines (immune-stimulating proteins secreted by immune cells) in

the local environment after activation. T_H1 are mainly involved in augmenting the $CD8^+$ T-cell immune response against intracellular pathogens, and have been known to possess cytotoxic effector function as well [7, 8]. T_H2 coordinate the immune response against parasites while T_H17 participate in clearance of extracellular bacteria and fungi. T_{FH} a unique subset of helper T-cell that helps recruit B cells to peripheral lymphoid tissues (e.g. lymph nodes) as well as help regulate their response. In addition to these $CD4^+$ effector T cell subsets, there is another important subset called regulatory T cells (Tregs) that modulate and suppress the response of $CD8^+$ and $CD4^+$ effector T cells [9]. While Tregs can develop naturally in the thymus, they can also be induced in the periphery (iTreg) by exposure to certain cytokines, similar to the other CD4 subsets.

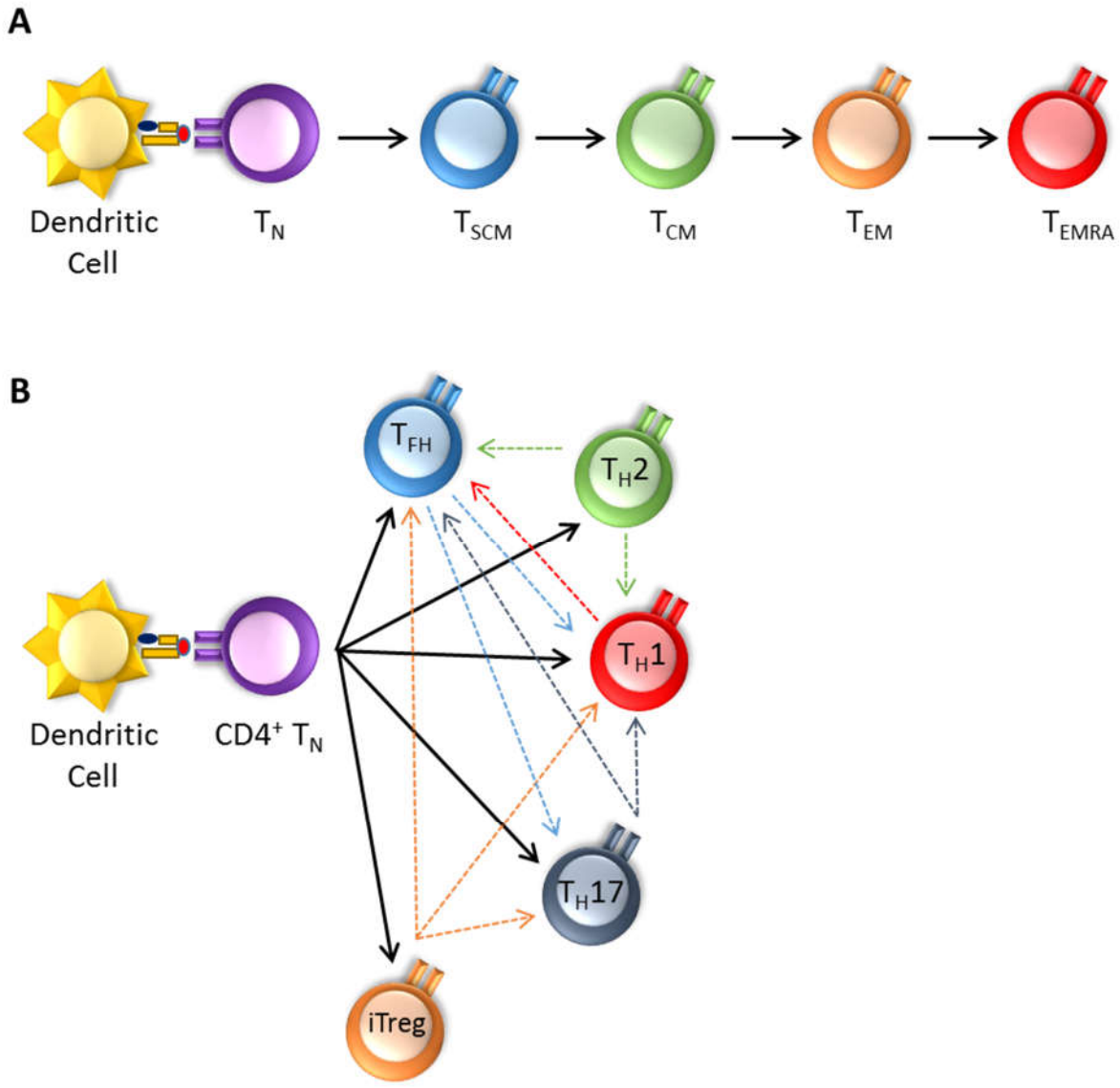

Figure 3. Differentiation and plasticity of T cells.
(A) After a naïve T-cell gets activated by an antigen presenting cell like a dendritic cell, it starts to proliferate and follow a progressive differentiation trajectory involving the different T-cell states. This trajectory can pause at the T_{SCM} or T_{CM} stage, but it eventually ends with the short-lived terminally differentiated effector cell (T_{EMRA}). **(B)** $CD4^+$ Helper T cells can differentiate (solid arrows) into several different classes initially depending on the signals received but also maintain the flexibility to repolarize (dashed arrows) to another state if the signals in the local environment change.

It is important to note that T_{EM} are not necessarily locked-in to the effector lineage they initially occupy. There is flexibility in the programming of their phenotype, which is a concept known as T-cell plasticity [4, 10]. Depending on changes in the T cells' local environment, T_{EM} can transition to a different phenotypic state. Evidence has been presented for this regarding the ability of the various $CD4^+$ T_H subsets to be repolarized to a different state based on the cytokine signals they receive [10]. For example, it is well-known that T_H17 cells can be reprogrammed into T_H1 cells with the right cytokine signals [11]. The idea of T-cell states being affected by their environment has taken on particular importance in regard to understanding $CD8^+$ T_{EM} function in the context of tumors. The data strongly suggest that functional $CD8^+$ T_{EM} that have infiltrated tumors follow a trajectory that eventually ends at a state of dysfunction that is driven by cues from the tumor and its microenvironment [12].

The phenotypic and functional changes that mark T-cell differentiation and state changes are mediated by a cascade of alterations in gene expression. These transcriptional changes are governed by expression of key transcription factors that act as master regulators of each T cell state, either independently or in a graded manner [5-7]. The expression of transcription factors associated with T_N cells (e.g. TCF7, LEF1, KLF2) is progressively lost as cells transition through the different differentiation states (i.e. T_{SCM} to T_{CM} and T_{EM}). In their place, transcription factors associated with effector cell function are upregulated (e.g. EOMES, TBET, BLIMP-1). In fact, for $CD8^+$ T-cell differentiation, the interplay between EOMES and TBET are key to determining the properties of T cell populations within the T_{EM} subset [13]. Similarly, the different $CD4^+$ T_{EM} helper T-cell subsets are controlled by well-defined transcription factors. The T_H1, T_H2, T_H17, T_{FH}, and iTreg phenotypes are

regulated by expression of TBET, GATA3, RORγC, BCL-6, and FOXP3 respectively [6, 7]. Expression of these transcription factors changes accordingly during conversion of one T_H subset to another. Ultimately, understanding mediators of these different T-cell states, as well as the relationship between the different states, is key to harnessing the immune system's potential.

1.1.5 Initiation of an adaptive immune response

With the major players of the immune system laid out, it will be important to delineate the general cascade of events that comprises an immune response as they will be relevant later. Furthermore, while all the cells discussed in the previous section play an important role in an immune response, the role of cytotoxic $CD8^+$ T cells is germane to the core of this dissertation, and so the events discussed in this section will focus specifically on their function (Figure 4).

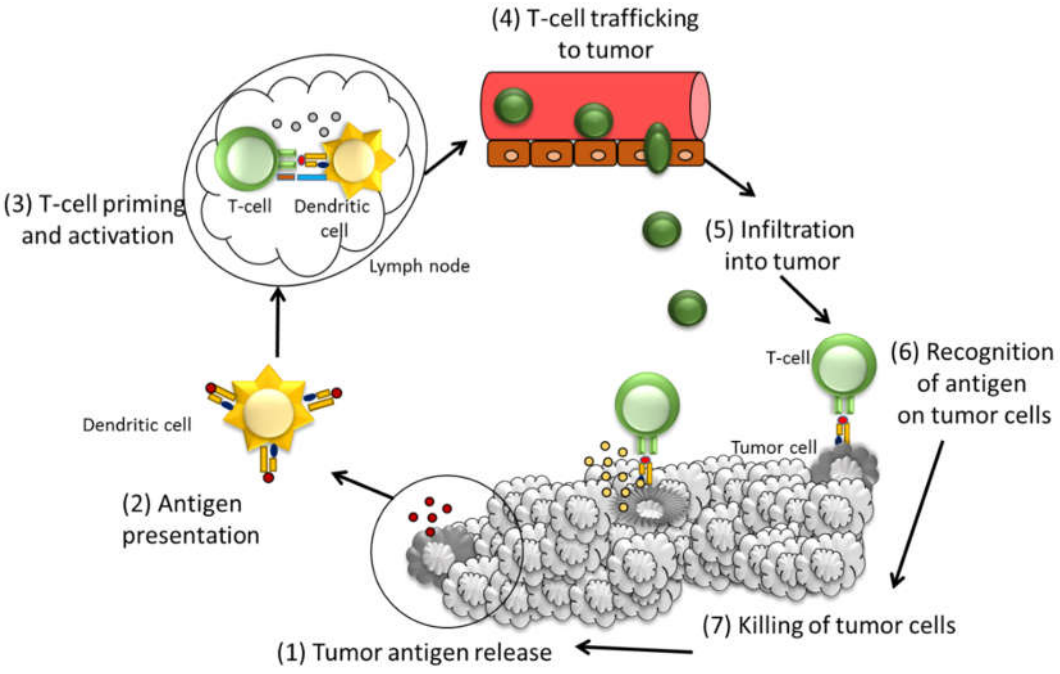

Figure 4. Initiation of an adaptive immune response against tumor.
In step (1) of the cycle, cancer antigens are released due to oncogenesis. These are eventually taken up by dendritic cells in step (2). Dendritic cells traffic to the lymph node in step (3) in order to present the antigen to T cells, which activates them. Upon activation, T cells leave the lymph node and traffic to the tumor site (4) and infiltrate it (5). Up recognition of its cognate antigen (6) that is bound to MHCI and expressed on the surface of the tumor, the T cell kills the target cell (7).

In this context, an adaptive immune response involving T cells is initiated when they encounter antigens for which their TCR is specific [1]. This encounter occurs in the context of DCs displaying the antigen on their surface while bound to an MHC Class I molecule (MHCI). DCs accomplish this through a process called cross-presentation. As mentioned in section 1.1.2, DCs regularly uptake pathogens or molecular components from the environment which are then degraded intracellularly. The proteins are broken down into small fragments called peptides, which must be 9-12 amino acids in length for $CD8^+$ T cells to recognize. These peptides are then loaded onto MHCI receptors inside intracellular vesicles in DCs. The peptide:MHCI complexes then traffic to the surface of the DC where they are displayed for T cells to interact with. The antigens that $CD8^+$ T cells recognize via DC presentation are the peptides that have gone through this antigen processing pathway.

DCs usually encounter antigens at the site of pathogenic inflammation (e.g. infection). While there, inflammatory signals in the milieu also activate the DCs which cause them to express co-stimulatory surface molecules and secrete stimulatory cytokines. After activation, DCs will migrate to nearby lymph nodes, which are specialized immune organs that contain high densities of innate and adaptive immune cells in close proximity, in order to present it to a T cell with a TCR specific for that antigen. Concomitant co-stimulation of a T cell after activation of its TCR is also required in order to sustain the T cell activation and

proliferation. These co-stimulatory signals are provided by the activated DC expressing the aforementioned cytokines and cell-surface molecules. After activation, T cells will clonally expand, leave the lymph node as effector cells and travel to the tissue site where they will act to clear the threat. $CD8^+$ T cells are able to recognize their target cells because they will also be displaying the antigen, bound to MHCI, on their surface. This migration is helped by inflammatory and chemotactic signals produced at the site of inflammation and that diffuse into gradients that direct immune cells. The antigen-specific population of T cells will contract once the target is cleared, leaving behind a small percentage of these cells that have differentiated into long-lived memory cells. These memory cells persist, either in the peripheral blood or at the tissue site, and serve as a line of rapid response in the event the antigen is encountered again.

Following this general set of events, T cells are involved in the immunological clearance of many foreign pathogens that replicate inside the body's cells like bacteria, viruses, and some parasites. After inflammatory signals produced by the pathogenic invasion draw T cells to the area, the T cells are able to recognize the infected cells because they display antigens derived from the pathogens on their surface in association with MHCI. These antigens are recognized as non-self and trigger the T cells' cytotoxic function. The recognition of non-self by T cells goes beyond pathogens or microorganisms to include a multifaceted disease that is derived from normal cells in the body: Cancer. Indeed, it has been well established that abnormally expressed proteins in cancer are a major source of immunogenic antigens [14, 15]. With cancer being major health problem worldwide and the second leading cause of death in the United States, cancer therapy has also been a major focus of research for decades [16]. In recent years, particularly attention has been given to

understanding the immune system's role in controlling cancer and developing methods to harness its potential.

1.1.6 The immune surveillance of cancer

Cancer is characterized by the accumulation of several genetic mutations that confer aberrant regulation of cell proliferation and developmental pathways, leading to unchecked tumor growth and spread. The steps needed for malignant transformation of normal cells to cancer cells were famously laid out by Hanahan and Weinberg in their "Hallmarks of Cancer" report [17]. These were as follows:

1. Evasion of cell death (apoptosis)
2. Self-sufficiency in growth signals
3. Insensitivity to anti-growth signals
4. Aberrant vascular growth (angiogenesis)
5. Unlimited replicative potential
6. Tissue invasion and metastasis

In addition to the original six hallmarks identified, one of the new hallmarks posited by a follow up to this report in 2011 is the ability for cancer to evade the immune system [18]. While not a new idea, the inclusion of this hallmark highlights the relatively recent widespread acceptance of the role of the immune system in cancer control.

In 1909, it was again Paul Ehrlich who is credited with first proposal for the role of the immune system in controlling cancer, even though he was unable to confirm this experimentally [19]. Perhaps the first experimental report of stimulating an immune response against cancer was reported by Ludwik Gros in 1943. Gros showed that intradermal immunization of mice with a sarcoma tumor line was able to induce protection against

rechallenge in some mice [20]. In the late 1950's, the theory of immune surveillance of cancer was proposed separately by Frank MacFarlane Burnet and Lewis Thomas [21, 22]. They hypothesized that the immune system could recognize cancer cell neoantigens and respond to eliminate the nascent tumors. However, it was not until the 1990's that the tools were available to truly evaluate the role of immunity in cancer. Using mice that lacked fully functional immune systems, Robert Schreiber and colleagues showed that these mice were more susceptible to tumor formation and growth [23-25]. In the years since, this finding has been recapitulated by several others [26].

As alluded to, mutations acquired by cancer cells result in the expression of antigens that are not natively expressed and thus elicit an immune reaction. This then raises the question: if the immune system is constantly surveilling the body for signs of non-self, such as those displayed by cancer, then how does a tumor manage to form? This question led to a revision of the cancer surveillance hypothesis that is now referred to as cancer immunoediting and encompasses three phases of tumor growth: (1) elimination, (2) equilibrium, and (3) escape [1].

1. The elimination phase is simply the previous working theory of immunosurveillance where the immune system recognizes and eliminates cancer cells.
2. The equilibrium phase involves tumor dormancy where residual tumor is kept in check by the immune system. It is likely during this phase that the immune system effectively selects populations of tumors cells that have acquired immune evasion mechanisms.

3. The final escape phase consists of these resistant tumor cells breaking through the control. Mechanisms for immune evasion can be due to either changes in the tumor cells or the tumor microenvironment (TME).

The phases of cancer immunoediting illustrate the potential of the immune system to not only affect tumor control but shape its immunogenicity. Understanding this has launched the immunotherapy revolution over the past two decades by helping to inform development of immune-oncology strategies that can harness the power of the immune system to prevent and treat cancer.

1.2: Cancer Immunotherapy

With the role of the immune system in tumor control having been well established, there has been a large effort to develop immunotherapies that can exploit and augment this control. Targeting of $CD8^+$ T cells has been of particular focus due their role as main cytotoxic effector cells of the adaptive immune system and because the presence of $CD8^+$ tumor-infiltrating lymphocytes (TIL) has been correlated with better prognosis in several cancer types [27]. However, factors either intrinsic to the tumor or its local environment often develop that lead to dysfunctional tumor-immune cell interactions [18]. Tumor escape can be due to a single factor such as impaired antigen recognition or due to a more complicated web of immunosuppressive factors in the TME. An immunosuppressive TME can be mediated by recruitment of regulatory immune cells, lack of co-stimulatory signals due to expression of inhibitory receptors, or a milieu inhospitable to immune cell function [26]. As a result, immunotherapy development has been focused on overcoming these

barriers and several important modalities tested in clinical trials are summarized in the following subsections (Figure 5).

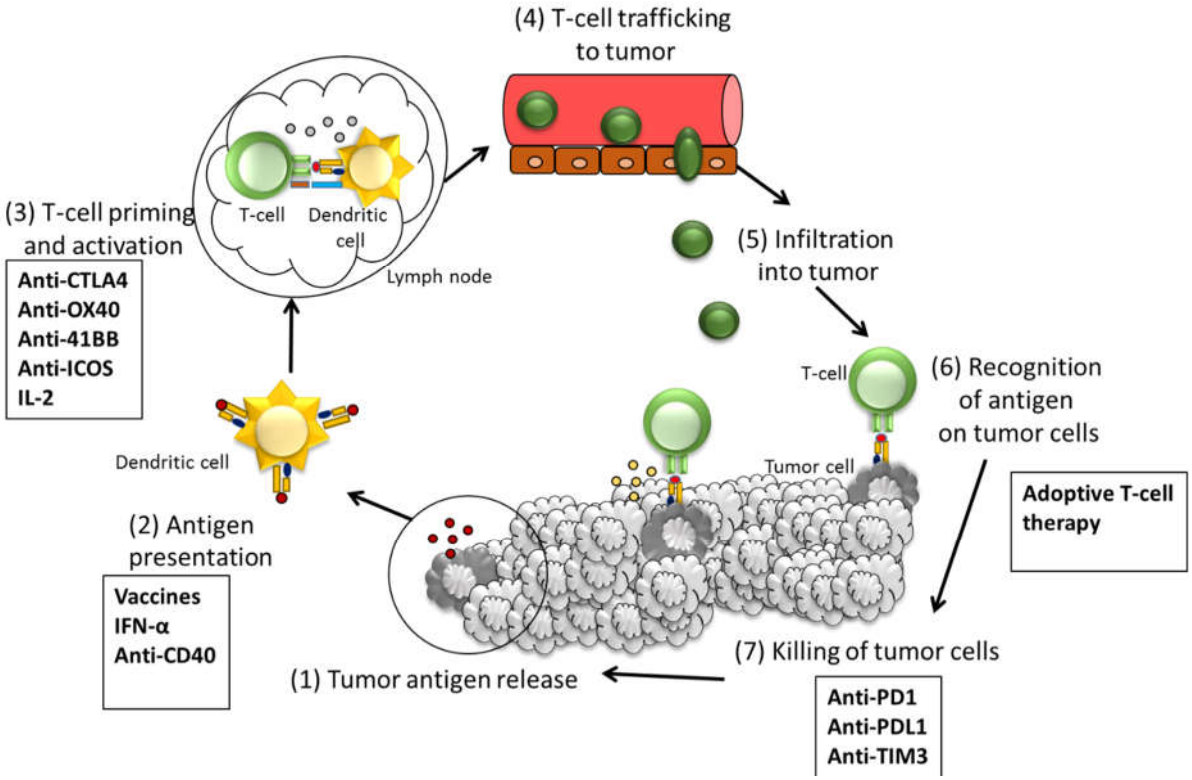

Figure 5. Immunotherapies that have been developed to boost cancer immunity. There are several immunotherapeutic options that can affect the T-cell anti-tumor response and which mediate their effect at various points throughout the cycle. Immunotherapies mentioned in this section are highlighted in the figure.

1.2.1 Cytokine Therapy

Perhaps the first example of modern immunotherapy was the use of recombinant cytokines, which are small, often immunostimulatory molecules that are secreted by all immune cells. In particular, the identification of the T cell growth factor interleukin-2 (IL-2) in 1976 was a major breakthrough for the field of immunotherapy [28]. In 1984, Steven Rosenberg demonstrated its therapeutic potential after treatment of a metastatic melanoma patient with systemically administered IL-2 resulted in a complete response (CR) that has continued for 30 years [29]. Not only was this the first example of an effective

immunotherapy in humans but it also hinted at the possibility of durable responses. Several follow up studies resulted in high-dose (HD) IL-2 being approved by the Food and Drug Administration (FDA) for treatment of metastatic renal cancer in 1992 (20% overall response rate [ORR]) and metastatic melanoma in 1998 (16% ORR) [29].

Interferon-α (IFN-α) was another notable entry in the immunotherapy treatment of metastatic melanoma. IFN-α is known to induced expression of MHCI on tumor cells as well as to activate immune cells [1]. It was approved in 1995 by the FDA after clinical trials indicated IFN-α provided a significant benefit in relapse-free survival (1 year vs. 1.7 years) of cutaneous melanoma in the adjuvant setting [30]. Since then, a modified version with an improved half-life was also approved in 2011 for resected melanoma [31]. However, using IFN-α is highly toxic is no longer used as the standard of care due to the advent of a class of immunotherapy discussed in the next section.

While IL-2 and IFN-α are the only two approved cytokine immunotherapies for cancer, others have been investigated such as IL-7, IL-15, and IL-21. IL-7 and IL-15 help promote T cell homeostasis and expansion of memory T cells while IL-21 promotes $CD8^+$ T cell activation as well as proliferation [32]. Early clinical trials for agonists of all three show signs of activity [33-35]. Additionally, a pegylated version of IL-2 that showed improved safety and pharmacodynamics in patients and is being studied in combination with other immunotherapies [36].

1.2.2 Immune Checkpoint Inhibitors

Immune checkpoint inhibitor therapy, also known as checkpoint blockade immunotherapy (CBI), is a revolutionary class of immune-oncology drugs. When T cells

become activated, they not only upregulate several costimulatory receptors to help sustain and propagate their response, but they also begin to express inhibitory receptors to counterbalance the activation [37]. These inhibitory receptors are referred to as immune checkpoints and their natural function is to help moderate the strength and duration of a T cells' activation to limit indiscriminate cytotoxicity. Tumors are able to take advantage of these immune checkpoints by recruiting cells to the TME that express the ligands for these inhibitory receptors or by upregulating these ligands themselves. Key immune checkpoint molecules that are targets for therapeutic inhibition are summarized below.

CTLA-4. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) was the first immune checkpoint receptors targeted in the treatment of cancer patients, following a body of work supporting its role in negative regulation of T cells. CTLA-4 is known to be expressed exclusively on activated effector T cells and can mediate immune suppression [37]. It was first discovered in 1987, but it wasn't until 1995 that its function as a negative regulator of T cell activation was discovered [38-40]. Shortly thereafter, its mechanism of action was first described by Jim Allison and colleagues when they showed that it works in direct opposition to CD28 costimulation, a major positive signal for T cells after TCR activation, by preferentially binding to the same ligands [41]. Allison and colleagues followed up with a pivotal experiment that showed an enhancement of anti-tumor immunity via CTLA-4 blockade with an antagonistic antibody [42]. This work led to the development and clinical testing of Ipilimumab, a fully humanized anti-CTLA4 antibody. After a key phase III trial showed Ipilimumab to be the first drug to demonstrate survival benefit in advanced melanoma patients, the FDA approved it for this indication in 2011 [43]. For his work on developing CBI, Jim Allison shared a Nobel Prize in 2018.

PD-1. Programmed cell death protein 1 (PD-1) is another key checkpoint molecule and it was the second one targeted clinically. PD-1 is known to be upregulated on activated effector T cells as a result of chronic antigen stimulation, particularly on T cells that have infiltrated the tumor tissue [44]. Tasuku Honjo and colleagues first identified PD-1 in 1992 and demonstrated its immune regulatory function a few years later [45, 46]. Further evidence by several other groups demonstrated that PD-L1, the ligand for PD-1, could be upregulated on tumor cells and mediate immune resistance [47, 48]. Evidence of upregulation of the PD-1/PD-L1 axis on T cells and tumor cells prompted development and testing of several anti-PD1 and anti-PDL1 antibodies. Two anti-PD1 antibodies, Pembrolizumab and Nivolumab, were approved by the FDA in 2014 for the treatment of melanoma after they showed durable responses in patients [49, 50]. Since then anti-PD1/PD-L1 drugs have been approved for a variety of other cancers, including the use of Pembrolizumab for cancers with specific genetic mutations (Microsatellite unstable a.k.a. MSI high) regardless of the origin. Tasuku Honjo shared the Nobel Prize with Jim Allison in recognition for his contribution to the area of CBI.

Several other immune checkpoint molecules have emerged a potential targets, such as TIM3, LAG3, TIGIT, VISTA. All of these receptors, except for VISTA which is expressed mainly on myeloid cells, are predominantly expressed on antigen-activated T cells and thus are prime axes for mediating immune suppression at the tumor site. As such, there are several clinical studies exploring their inhibition as either monotherapy or in combination with already approved checkpoint inhibitors [32, 37]. The successes seen with checkpoint inhibitors have made them the frontline option for several indications and have energized the immunotherapy field.

1.2.3 Costimulatory activation of T cells

On the opposite side of the coin from reinvigorating T cells through checkpoint inhibition is doing it by activation through costimulatory signaling pathways. The importance of CD28 as a costimulatory pathway to augment TCR signaling after T cell activation has been known since the early 1990s [51]. Other T cell costimulators have been identified as well that can potentially be targeted to augment antitumor immunity. Several of the key costimulatory receptor targets on T cells are discussed below.

4-1BB. 4-1BB/CD137/TNFRSF9 belongs to the tumor necrosis factor receptor superfamily (TNFRSF), which consists of several receptors that promote T cell survival and proliferation. This receptor can be expressed on several types of immune cells such as T cells, B cells, NK Cells, and DCs [52]. Ligation of 4-1BB with its ligand, 4-1BBL, provides pro-survival, activation, proliferation, and effector function signals through the NFκB and MAPK kinase pathways [53, 54]. With regard to T cells, 4-1BB typically becomes upregulated on recently antigen-activated $CD8^+$ T cells and reaches peak expression around 24 hrs [55]. 4-1BB agonists demonstrated improvement of anti-tumor activity in mouse models, however use of one 4-1BB agonist in the clinic resulted in severe liver toxicities [52, 56, 57]. It was found that this hepatotoxicity was mediated by a4-1BB activating IL-27-producing liver myeloid cells [58]. Anti-41BB in combination with other therapeutics is being explored in several trials [59].

OX40. OX40/CD134/TNFRSF4 is another member of the TNFRSF family that is expressed at much higher levels on $CD4^+$ T cells than $CD8^+$ T cells after peptide stimulation in humans [54]. OX40 functions similarly on $CD4^+$ T cells as 4-1BB does for $CD8^+$ T cells in that it also provides survival and proliferation signals through the same pathways [54]. Much

work on agonistic OX40 antibodies has been done in mouse models where both $CD8^+$ and $CD4^+$ T cells can express OX40 [60]. Targeting OX40 has induced T-cell mediated tumor regression in mouse models as well as promote generation of effector and memory T cells [60]. There is also evidence to suggest that OX40 costimulation can abrogate $CD4^+$ Treg activity [61, 62]. An early clinical trial showed that treatment with an anti-OX40 was able to induce tumor regression in 40% of patients [63]. Patients also showed increased $CD4^+$ as well as $CD8^+$ T cells in the blood, which was accompanied by increased *in vitro* anti-tumor response [63]. Anti-OX40 in combination with other therapeutics is being explored in several trials [59].

ICOS. Inducible T-cell costimulatory (ICOS)/CD278 is a member of the same family of costimulatory receptors as CD28 and is expressed mainly on activated $CD4^+$ T cells. Data suggests that ICOS stimulation may have indirect effects on anti-tumor response as it did not induce a strong response by itself in mouse models [59]. However, it does appear to provide benefit in the context of CBI. ICOS was found to be upregulated on $CD4^+$ T cells after anti-CTLA4 treatment, and subsequent ICOS simulation increased the cytotoxic T cell response [64]. Upregulation of ICOS after CTLA-4 blockade was also found in patients, suggesting stimulation of this pathway may enhance CBI efficacy in future trials [65]. Agonistic ICOS antibodies have only recently become available for clinical testing.

GITR. Glucocorticoid-induced TNFR-related protein (GITR)/CD357/TNFRSF18 is a third member of the TNFR family. It is found on low levels of CD4 and CD8 T cells and is upregulated after TCR activation [66]. However, it is constitutively expressed a high levels on regulatory $CD4^+$ T cells [67]. Stimulation of GITR on $CD8^+$ T cells can enhance antigen-specific $CD8^+$ T-cell response, which is probably mediated in part by inducing resistance to

Treg suppression [68, 69]. Data from mouse models showed that GITR agonism either alone or in combination with anti-PD1 or anti-CTLA4 could induce tumor regression that was T cell dependent [70, 71]. A recent clinical trial in patients with advanced, refractory cancers reported that GITR agonism combined with PD-1 blockade resulted in reduced Tregs levels systemically and in the tumor as well as enhanced $CD8^+$ T cell activity [72].

1.2.4 Cancer Vaccines

Cancer vaccines take advantage of the first step in initiating an immune response, which is antigen presentation by DCs to prime T cell responses The goal of vaccination is to use DCs loaded with either tumor cells or tumor-associated antigens (TAA), to augment the immune response against cancer. The use of an adjuvant is required to help stimulate the immune system to respond to the tumor antigens, as is the case with traditional vaccines against microbial agents.

There are currently two FDA approved cancer vaccines that use tumor antigens. The first is Sipuleucel-T, which is an autologous DC vaccine used to treat castrate-resistant prostate cancer [73]. The vaccine works by incubating the patients' DCs with a nearly universally expressed prostate cancer TAA as well as the DC stimulating cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF), and then reinfusing them into the patients. This treatment only improved survival by 4 months [73]. The second is T-VEC, which takes advantage of stimulating the immune system with tumor antigens in round-about way. T-VEC uses an oncolytic herpes virus that is modified to be less virulent as well as to express GM-CSF. The vaccine is designed to elicit a systemic anti-tumor response by infecting cancer cells and causing them to burst, spreading antigens. It was approved in 2015

for late stage melanoma and produced a 26.4% ORR vs. 5.7% ORR for patients who only received subcutaneous GM-CSF [74].

Beyond these, there are several cancer vaccines in development that use either modified tumor cells and/or TAAs. In particular, many TAAs have now been identified and cancer vaccines could be a prime way to take advantage of directing the immune system against targets specific to tumor cells. A phase III trial in stage III and IV melanoma using a gp100 peptide vaccine plus IL-2 showed only a small improvement in progression free survival compared to IL-2 alone. (2.2 months vs. 1.2 months) [75]. A TAA-based vaccine in $Her2^{+}$ breast cancer, where patients were immunized with a Her2 peptide plus GM-CSF, showed modest clinical activity in an early phase clinical trial (89.7% vs. 80.2% disease-free survival) [76]. Another clinical trial by Elizabeth Jaffee and colleagues utilized GM-CSF secreting allogeneic pancreatic tumor cells in combination with live-attenuated Lysteria expressing mesothelin, which is a pancreatic cancer TAA [77]. Unfortunately, the combination did not improve survival over chemotherapy.

In addition to targeting shared TAAs, there are also efforts to treat patients with so called personalized cancer vaccines. These studies identify neoantigens (i.e. new antigens expressed only by cancer cells typically due to a mutation) that are not only highly specific to the cancer but also to each individual patient, too. Several studies were done in melanoma patients, demonstrating induction of neoantigen specific T cells as well as clinical benefit [78-80]. Furthermore, a more recent neoantigen vaccine trial showed similar T cell responses in glioblastoma [81]. Among others, there have also been efforts at MD Anderson Cancer Center to treat metastatic colorectal cancer and pancreatic cancer patients with personalized peptide cancer vaccines. Indeed, within the past few years, several other trials involving

peptide-based anticancer vaccines were terminated early due to lack of efficacy or lack of scientific rationale to continue [82]. While vaccination can induce antigen-specific T cells, evidence suggests that combination with other immunomodulatory agents (like the ones mentioned in the previous subsections) will be needed to induce tumor clearance. As such, there are several current studies that are addressing this need by combining cancer vaccines with blockade of PD-1, CTLA4, TIM3, LAG3 or costimulation of 4-1BB, OX40, GITR, CD27 [83, 84].

1.3: Cellular Immunotherapy for Cancer

The presence of T cells in the tumor, particularly $CD8^+$ T cells, has been correlated with improved survival in a myriad of cancer types which is suggestive of their anti-tumor potential [85-92]. From current general immunology knowledge of T cell function, it is likely that at least some of the T cells in the tumor have migrated and arrested there in response to the tumor [1, 93]. As already stated, the idea that tumors can persist despite the presence tumor-infiltrating lymphocytes (TIL) led to the identification of several mechanism of tumor immune evasion which have been discussed. Futhermore, the identification of IL-2 as a soluble T-cell growth factor allowed *ex vivo* propagation of T cells in order to study their function. Taken all together, this knowledge provided the rationale for exploring cellular immunotherapies with adoptively transferred T cells. In general, adoptive cell therapy (ACT) uses the idea that by removing TIL from a restrictive and immunosuppressive environment, they can be reinvigorated and expanded to prognostically favorable numbers. Transfer of these cells back to the patient as a large boost of anti-tumor immunity can result in eradication of established tumors and long-term progression-free survival [94, 95]. T-cell

therapy was pioneered by Steven Rosenberg using TIL based ACT for the treatment of melanoma in the 1980s. Since then, attempts to build on the success of TIL ACT in melanoma have included genetically engineering lymphocytes (including NK cells) to express chimeric-antigen receptors (CAR-T), antigen-specific TCRs, or receptors to improve their functionality at the tumor site. Since ACT is central to the work in this dissertation, these strategies will be discussed at length in this section (Figure 6).

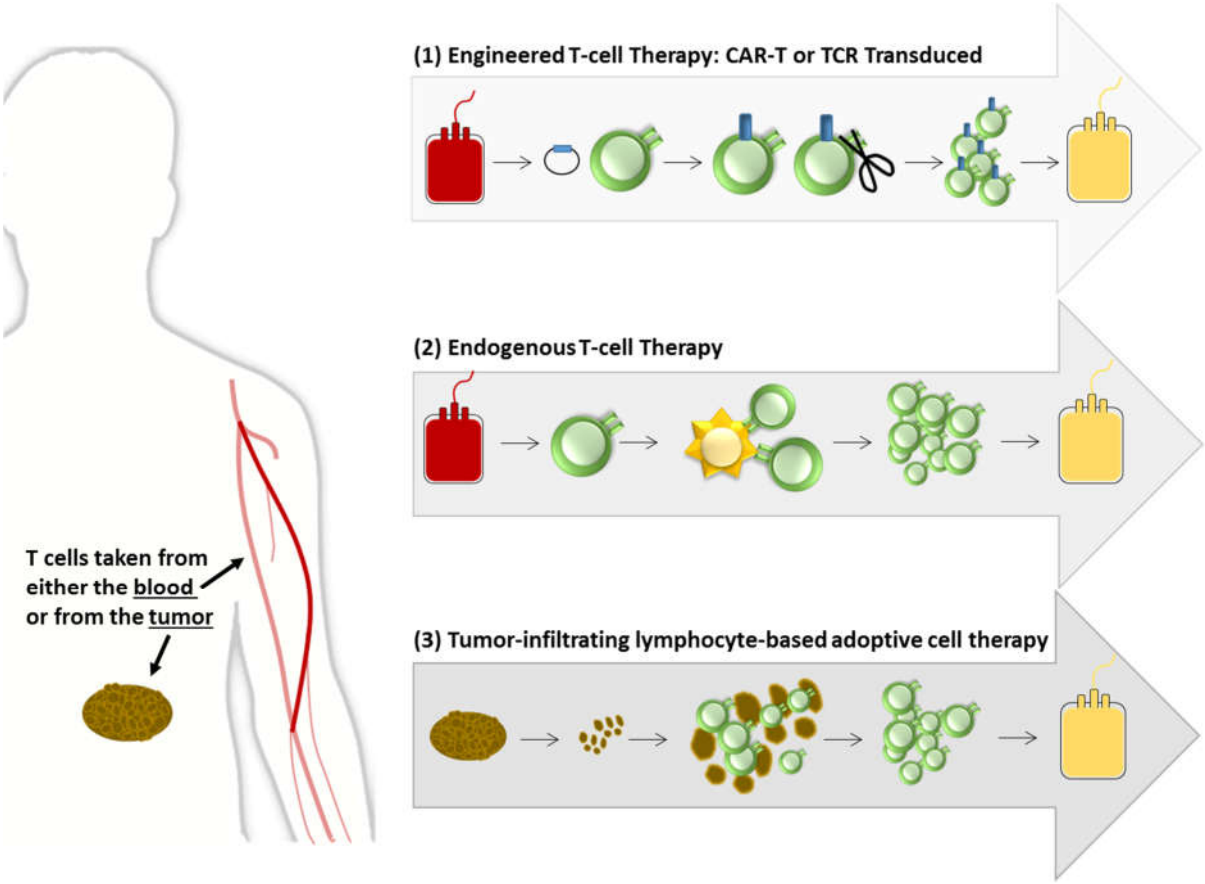

Figure 6. The three main forms of adoptive cell therapy.
Adoptive cell therapy is a form of immunotherapy that augments the patient's anti-tumor response by transferring large numbers of *ex vivo* expanded T cells. The first **(1)** approach is engineering T cells, typically taken from the blood, using viral vectors to express either a chimeric antigen receptor (CAR) or antigen-specific T-cell receptor. The second **(2)** method relies on the isolation and expansion of low-frequency antigen-reactive T cells from the blood. The third **(3)** therapy grows tumor-infiltrating lymphocytes *ex vivo* from patients' biopsies or resected tumors.

1.3.1 Adoptive cell therapy with tumor-infiltrating lymphocytes

The basic principle of TIL ACT involves the patient's tumor being resected, cut into small fragments, and cultured with IL-2 to stimulate expansive TIL growth *ex vivo* [96]. Once the TIL reach sufficient number, they undergo a second large expansion phase to be able to reinfuse billions of cells into the patient to effect tumor clearance [96]. The first example of this was demonstrated by Rosenberg and colleagues in a murine model of lymphoma, and its effect was further enhanced with systemic IL-2 administration following TIL infusion [97, 98]. Observation that human anti-tumor TIL could be consistently isolated from melanoma led to the first demonstration that ACT with autologous TIL could mediate tumor regression in melanoma patients [99-101]. Despite these early promising results, the duration of responses (DOR) was often short-lived (2-13 months) and infused TIL rarely persisted for more than a few days in the patient [102].

As such, Rosenberg and colleagues at the NCI worked to improve the early TIL generation and administration process. A major development was the observation that lymphodepletion of patients prior to adoptive TIL transfer improved response as well as engraftment of the transferred cells. Mechanistically, the superior TIL persistence afforded by lymphodepletion is believed to be mediated by the removal of endogenous homeostatic cytokine sinks by the lymphodepleting regimen [103, 104]. Furthermore, a "young TIL" culture protocol was established that reduced culture time, and increased patient eligibility was achieved by making the assumption that all TIL cultures contain anti-tumor activity and removing the TIL reactivity testing step, thus making TIL ACT more accessible. While the young TIL method did decrease the time of culture and had attributes associated with better

persistence, it resulted in response rates below what was reported for the traditional method (35% vs. 49% ORR) [94, 105, 106].

With the success of TIL ACT in melanoma established at by Rosenberg and colleagues, other medical centers have sponsored clinical TIL ACT trials for melanoma and there have been 13 studies to date that followed the general protocol laid out by the National Cancer Institute [107]. Since most (8/13) trials use HD IL-2 after TIL infusion, which is the model followed at MD Anderson Cancer Center in particular, only the results of these trials will be discussed as they are the most relevant. A meta-analysis of these trials found that the ORR at the different centers were consistent with each other, indicating the reproducibility of TIL ACT [107]. The overall ORR was 43%, which included 15% CR and 28% partial responses (PR). From the available data, the overall median DOR was not reached for CRs after 48 months and 11.5 months for PRs. Furthermore, a TIL dose consisting of $\geq 50 \times 10^9$ cells and a more $CD8^+$ TIL rich product were both correlated with better response [107-109].

Given the potential, there have also been attempts to push TIL ACT beyond melanoma, particularly for cancer types in need of improved therapies and that have not yet responded well to CBI. Several recent preclinical and clinical studies have been published in ovarian cancer, various gastrointestinal (GI) epithelial cancers, colorectal cancer, HPV^+ cervical cancer, breast cancer, pancreatic cancer, and non-small cell lung cancer [110-120]. Efforts to further improve the feasibility of TIL ACT for solid tumors other than melanoma will be delineated in subsequent chapters.

1.3.2 Antigen-specific T cell therapy

The success of TIL ACT in melanoma has provided the impetus to improve upon the therapy by improving the antigen-specificity, either through selection of antigen-specific T cells or genetic engineering of T cells to express certain antigen-specific TCRs. The natural starting place is to identify the specific T cells and their cognate antigens which mediate the immune responses seen. TAAs typically fall under the category of either shared non-mutated antigens, such as cancer testis antigens or differentiation antigens that are overexpressed in cancers, or private neoantigens deriving from tumor mutations that are usually specific to the individual's cancer [121]. Early studies in melanoma identified two differentiation antigens, MART-1 and gp100, that were well recognized by melanoma TIL [122, 123]. A clinical study using T cells that were virally transduced with high-affinity TCRs for these antigens showed cancer regression in several patients [124]. TCR-engineered T cell therapy has also shown some success in a clinical trial for patients with melanoma (45% ORR; 5/11 patients) and synovial sarcoma (66% ORR; 4/6 patients) with T cells targeting the cancer testes antigen NY-ESO-1 [125]. However, targeting shared antigens can have sometimes serious on-target off-tumor effects as they can be expressed in normal tissues also [125-127].

These potential issues have raised interest in targeting tumor-specific mutated antigens and the emergence of next-generation sequencing has made this possible [128, 129]. Several retrospective studies of ACT TIL treated patients have identified neoantigen-reactive TIL in the infusion products for melanoma, various epithelial gastrointestinal tumors, and breast cancer [112, 114, 130]. It is unclear how much these TIL contributed to tumor regression since they were not pure populations of antigen reactive cells, but it is likely they played some part given the evidence that increased mutation load correlates with increased

survival in general as well as better response to CBI [131-134]. Further studies have also identified TIL from patients that recognize antigens from driver mutations like TP53 and KRAS in colorectal cancer, ovarian cancer, and pancreatic cancer [115, 135-137]. Interestingly, Eric Tran and colleagues at the NCI identified TIL from two different colorectal cancer patients that recognized KRAS neoeptiopes restricted to the same MHCI allele, hinting at the possibility of developing TCR-engineered T-cells targeting common driver mutations across multiple tumor types [115].

In addition to finding antigen-specific T cells or TCRs at the tumor site, antigen-reactive endogenous T cells (ETCs) can also be found circulating in the blood. These ETCs can be isolated from peripheral blood and expanded *ex vivo* for ACT in a method called ETC Therapy [138]. T cells specific for the melanoma associated antigens MART1, tyrosinase and gp100 were observed in the peripheral blood of melanoma patients [139-141]. Technical hurdles face ETC given that circulating antigen-specific T cells are often <0.1% of the peripheral population, but techniques such as cell sorting with tetramers have allowed this therapy to be translated into the clinic. To date, several ETC Therapy trials have been conducted in melanoma patients [138]. While many of the studies demonstrated clinical benefit (i.e. stable disease) and some patients experienced durable complete responses, the ORR generally remained less than 25%. The only exception is a study by Khammari *et al.* that reported a 43% ORR, which is comparable to the response rates reported for TIL ACT [142]. While challenges still remain to improve ETC Therapy, it could eventually be transposed to other cancer types as evidenced by a recent report that identified circulating neoantigen reactive T cells in gastrointestinal cancer patients [143].

1.3.3 Chimeric-antigen receptor T cell therapy

In the effort to improve the antigen-specificity and function of adoptive transferred T cells, genetically engineering them to express chimeric-antigen receptors (CARs) is the next logical step. Endogenous $CD8^+$ T cell activation by its cognate antigen relies on recognizing it while bound to MHCI and also requires costimulation to help sustain the activation [1]. However, MHCI expression loss and lack of proper costimulation are two well-known mechanism of immune evasion [1]. Additionally, TCR-affinity for shared TAAs can sometimes be weak. As such, CAR-T cells, have been proposed as a way to overcome these potential issues. CARs were pioneered in the late 1980s and essentially use a receptor that is composed of an extracellular antigen-binding domain of an antibody that is linked to intracellular signaling chains. These are typically the CD3-ζ chain from endongenous TCRs stacked on top of costimulatory domains (e.g. CD28, 4-1BB, OX40) to provide complete activation [144-146]. CARs can be easily transduced into patient T cells using viral vectors. In this way, CAR-T can easily provide non-MHC–restricted recognition of cell-surface TAAs.

Unfortunately, the same pitfalls with using endogenous TCRs specific for non-mutated public antigens can also apply to CAR-T [147]. This makes targeting these kinds of cancer antigens that are also normally expressed on non-essential tissues an important consideration to avoid serious toxicity. In fact, it is in this realm that CAR-T has seen overwhelming success. Targeting B cell malignancies like acute lymphoblastic leukemia with CD19-targeted CAR-T has produced complete response rates ranging from 70% to 91% in children and adults [148]. In 2017, this led to a CD19-directed CAR-T (Kymriah) to being not only the first cellular therapy approved by the FDA, but also the first treatment to include

gene therapy. There are currently several more trials ongoing for other hematological malignances using CAR-T against new antigen targets [148]. While blood cancers have enjoyed remarkable response rates with CAR-T, the efficacy in solid tumors is still unclear. So far, the only example of strong regression in solid tumors is in glioblastoma using CAR-T targeting Interleukin-13 receptor alpha 2 [149]. Unfortunately, this patient recurred after about 8 months. Several CAR-T trials in solid tumors are currently ongoing for prostate cancer, mesothelioma, pancreatic cancer, glioma, glioblastoma, and sarcoma [148]. Several CAR-T trials in solid tumors are currently ongoing for prostate cancer, mesothelioma, pancreatic cancer, glioma, glioblastoma, and sarcoma [148].

CAR-T also suffers toxicities associated with cytokine release syndrome and neurotoxicites, which are probably a result of patients being infused with a large amount of activated T cells [147]. Finally, while there is also the issue of antigen loss being a mechanism of immune escape since CAR-T only target one antigen, there are some preclinical studies addressing this issue by using CAR-T multiple specificities [150].

1.4: Improvement of TIL-based Adoptive Cell Therapy

While TIL ACT has provided the best results for solid tumors in terms of cellular therapy, there are still opportunities for improvement. The numerous preclinical and clinical studies over the past 30 years have revealed several factors important the efficacy of TIL ACT: (1) Generation of a clinically relevant number of TIL that are (2) enriched for antigen-specific TIL and (3) that display phenotypic characteristics that confer improved

functionality and durable persistence. Previous work from our group and others has focused on addressing these points so as to increase the scope and efficacy of TIL ACT.

1.4.1 Adding 4-1BB costimulation

Several TIL ACT trials for melanoma patients demonstrated that a high number of total TIL infused, and in particular a higher amount of $CD8^+$ TIL, is correlated with objective response [108, 151, 152]. However, several studies have found inconsistent generation of a large amount of $CD8^+$ TIL from tumor fragments with high-dose IL-2 (6000 U/mL). Reported success rates for general TIL growth range from 31-94%, although no consistent cut-off number was used between studies [109, 153-155]. With regard to the TIL ACT experience and MD Anderson Cancer Center, it was found that $40x10^6$ cells were needed in the initial expansion to confidently move forward to the expansion phase [156]. The reported success rate with this cut-off was 62%. Likewise, the reported fraction of $CD8^+$ TIL in the initial expansion product or infusion product varied widely, although the overall average CD8% was >50% [108, 152, 157, 158]. Finally, the time of initial TIL expansion phase reported in these studies showed that it can last from 3-5 weeks. Tran *et al*. demonstrated that the longer TIL remain in culture, the more differentiated they become (based on CD27 and CD28 expression) and the shorter their telomeres [158]. Both of these characteristics have been correlated with worse clinical response, TIL persistence, and proliferation [159-161].

Taken together, these identified shortcomings provided points of attack to improve the quality of the TIL product generated. As such, 4-1BB costimulation provides a way to address these points given the signaling pathway's ability to improve the proliferation, survival, and effector function of $CD8^+$ TIL [53, 162-164]. 4-1BB signaling accomplishes these results by activating the NF-κB and MAPK pathways, which are well-known regulators

of cell survival, maturation, and proliferation (Figure 7) [165-168]. Ligation of 4-1BB, either through interaction with trimers of its natural ligand 4-1BBL or through crosslinking with an agonistic antibody, recruits TNFR-associated factor (TRAF) 1, TRAF2, and TRAF3 to its cytoplasmic tail, which form heterotrimers themselves [169]. TRAF1 functions to sustain TRAF2 expression and signaling while 4-1BB signaling seems to be dependent on TRAF2 participation [170]. TRAF2 has E3-ubiquitan ligase activity, acting as a scaffold for the recruitment of factors that ultimately lead to the activation of NF-κB and MAPK signaling [169]. Induction of 4-1BB signaling results in the secretion of IL-2 and IFN-γ, and the expression of the anti-apoptotic proteins Bcl-xL and Bfl-1, which protect against activation-induced T-cell death [162, 163].

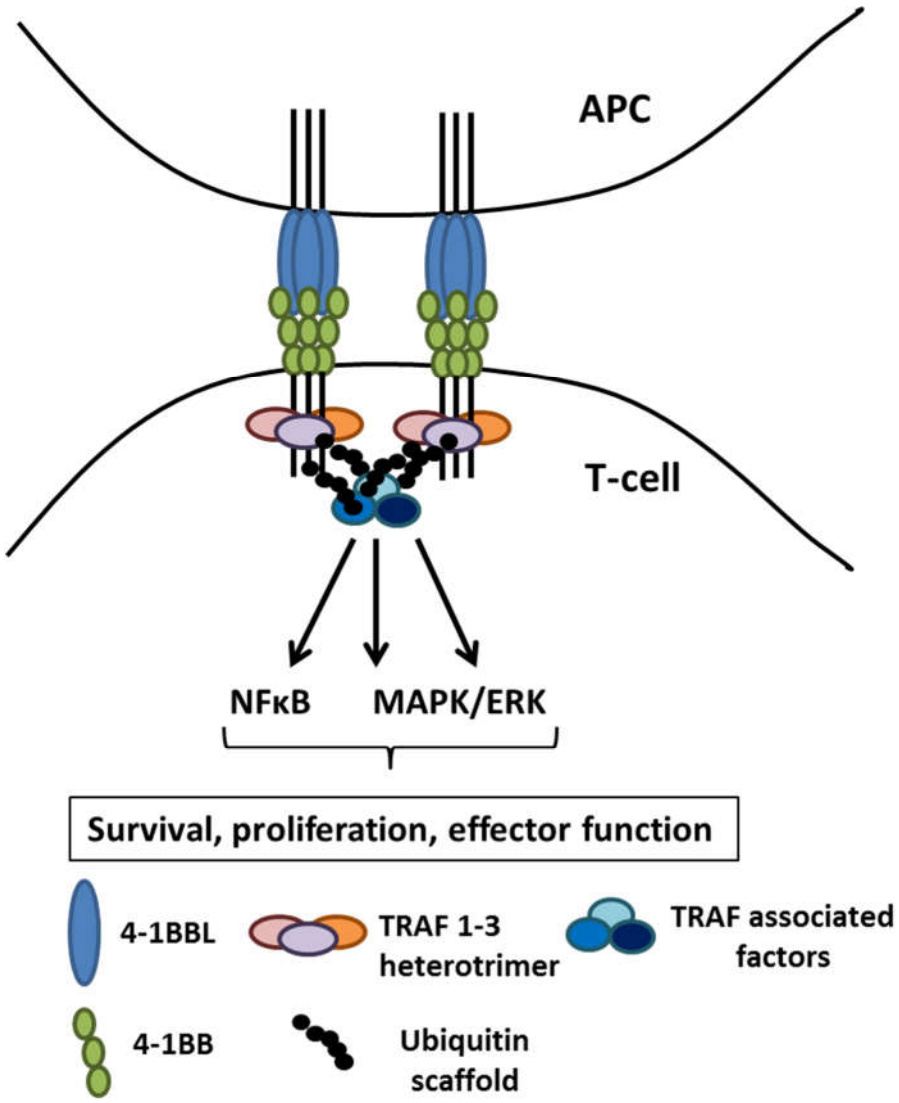

Figure 7. 4-1BB signaling pathway.
When 4-1BB signaling is triggered in the T-cell, TRAFs are recruited to the cytoplasmic tail in order to propogate signaling via the NFκB and MAPK/ERK pathways that ultimately result in changes in gene expression geared towards pro-survival, proliferation, and effector functions.

Previous work in the lab by Chacon *et al.* focused on the effects of integrating a fully-human agonistic 4-1BB monoclonal antibody (a4-1BB mAb) from Bristol-Myers Squibb (Urelumab; BMS 663513) into the TIL expansion process. Addition of a4-1BB to the tumor fragment culture (initial expansion phase) augmented and accelerated $CD8^+$ TIL growth

compared to using IL-2 only, which seemed to be helped by activation of DCs within the tumor tissue [171]. This also resulted in increased tumor specificity, which is likely due to the fact that 4-1BB costimulation targets and enriches for recently antigen-stimulated $CD8^+$ TIL [53, 55, 172]. Furthermore, use of a4-1BB also protected TIL from activation-induced cell death (AICD), to which they were previously found to be susceptible, after the second large expansion phase [173]. Additionally, its use during the large expansion phase maintained their CD28 expression which is correlated with longer telomeres [159, 171, 174].

The importance of 4-1BB costimulatory signaling has been corroborated by other groups as well. CD28 is probably the most widely recognized costimulatory signal following TCR activation. As such, most classical methods of expanding T cells *in vitro* involve anti-CD3/anti-CD28 microbeads. However, these beads typically preferentially expand $CD4^+$ T cells instead of $CD8^+$ T cells [175-177]. Furthermore, it has been reported that the 4-1BB costimulation better expands memory, antigen-specific, CD8+ T cells than compared to CD28 costimulation, which were mainly naïve cells [178]. Beyond improving proliferation and cytotoxicity, 4-1BB costimulation also has more beneficial effects on T cell metabolism as compared to CD28 signaling. Several studies have revealed that 4-1BB costimulation results in metabolic reprogramming that involves mitochondrial enlargement, increased mitochondrial respiratory capacity, and increased mitochondrial biogenesis [179-181]. These alterations may ultimately improve T cell persistence and anti-tumor activity. Finally, 4-1BB costimulation appears to impart lasting benefits that are mediated by epigenetic changes [182].

1.4.2 Improving TIL product with gas-permeable culture flasks

Ways to improve TIL growth are not just limited to growth factors provided in culture medium. Treating patients with ACT TIL can also be improved by the materials used and the simplification of manipulating the cultures. The traditional TIL growth protocols use 24-well tissue culture plates, various sizes of tissue-culture flasks, and gas-permeable bags [157]. Use of novel gas-permeable flasks (GREX), which have a membrane on the bottom upon which cells sit, have improved TIL expansion and simplified the logistics, thus making the process more accessible [183, 184]. In addition to this important but oft over looked component, the GREX flasks were also found to be beneficial for the TIL function and phenotype [185]. Due to the position of the TIL on top of the gas-permeable membrane, the cells can take advantage of the increased oxygen exchange. This resulted in rescue of TIL growth for cultures that did not expand in the traditional flasks, maintenance of clonal diversity, and improved mitochondrial function [185]. Furthermore, the GREX flasks also affected a beneficial phenotypic change on the TIL as marked by increased fraction of $CD8^+BTLA^+$ TIL. BTLA, or B-and-T lymphocyte attenuator, is traditionally thought of as a negative regulator on T cell function [186, 187]. However, a higher percentage of BTLA expressing $CD8^+$ TIL in the infusion product was correlated with persistence and response in TIL treated patients [108]. Further work by Haymaker *et al.* and Ritthipichai *et al.* laid out respectively a possible explanation for this by showing that, compared to $CD8^+$ TIL lacking BTLA expression, $BTLA^+CD8^+$ TIL are less differentiated ("younger") and able to serially kill cancer cells [188, 189].

1.4.3 Genetic engineering of TIL to improve function

As our understanding of biological processes continues to improve, it's likely that the next generation of cellular therapeutics will involve genetic engineering. So far this is best exemplified by the ground-breaking FDA approval of a CD19 CAR-T, not only the first cellular cancer immunotherapy approved, but the first to involve genetic engineering. Biological manipulation like this is not restricted to expression of chimeric-antigen receptors or antigen-specific TCRs, but can also produce so called "armored" T cells that will be able to resist or even exploit immunosuppressive signals as well as provide their own cytokine stimulation.

Receiving supportive stimulation from cytokines is an important aspect of a sustained immune response, with IL-2 being one of the most well-known T cell growth factors. Early preclinical studies by Rosenberg and colleagues established that transduction of human T cells with the IL-2 gene allowed prolonged persistence *in vitro* without exogenous IL-2 and could produce their own IL-2 upon TCR activation [190, 191]. Based on this, TIL ACT with IL-2 transduced TIL could be beneficial due to improved persistence *in vivo* [192]. Other promising cytokines have been explored as well, like IL-12 and IL-15, due to their effector T cell proliferative and protective abilities [193-196]. Being able to provide local administration of lymphoproliferative cytokines could help overcome suppression in the TME as well as avoid toxicities associated with systemic cytokine administration [197, 198].

There have also been efforts to improve T cell anti-tumor activity via expression of non-endogenous receptors. In melanoma, ACT with TIL expressing the CXCR2 chemokine receptor could allow for better trafficking to tumors due to their expression of the CXCR2-ligands CXCL1 and CXCL8 [199, 200]. Once there, improved activity could be endowed by

incorporation of dominant-negative receptors (lack intracellular signaling domains) for TGF-β or Fas, thus resisting immunosuppression of apoptosis respectively [201-203]. A method to expand genetically engineered TIL for ACT has been established and will facilitate future studies of this type [204].

1.5: Main Theoretical Questions

The success of CBI has reinvigorated the cancer therapy field, particularly for advanced melanoma which has double the survival rates compared to 20 years ago [16, 205]. Prompted by this, the race to expand treatment to other cancer types is in full swing. Several tumor types have received great benefit (e.g. NSCLC, RCC, MSI-Hi), but they often belong to the same category of being highly immunogenic and/or being immune-inflamed [206-208]. Unfortunately, there are many more solid tumor types (e.g. pancreatic, ovarian, breast, MSS colorectal, prostate cancer) that fall on the other end of the spectrum and in which CBI has been ineffective so far. Given the heterogeneity of cancers, the immune contexture of some cancer may not be conducive to CBI and instead that might benefit from immunotherapies that approach the issue from another angle. Despite the lack of efficacy of immunotherapy so far, there is still evidence for the role of TIL in control of these tumors [27]. Given this fact coupled with the success of TIL ACT, which can take advantage of antigen-specific TIL by expanding them *ex vivo*, I sought to assess the feasibility of this therapy in other solid tumors that still have an unmet need.

One such tumor type is pancreatic cancer. Several immunotherapy trials, CBI or otherwise, have failed to provide improved clinical benefit compared to

surgery/chemotherapy, which remain the standard of care [77, 209-213]. As such, the 5-year survival rate remains the lowest of any cancer type at <10% [16]. In contrast to melanoma, pancreatic cancer TME is characterized by an extensive stromal compartment composed of immunosuppressive immune cells and a fibrotic extracellular matrix [214, 215]. In spite of this seemingly restrictive immune TME, the presence of TIL is correlated with increased survival [85, 216]. Therefore, I investigated transposing TIL ACT to pancreatic cancer with the following hypothesis and specific aims to guide me:

Hypothesis: Tumor-specific TIL exist in solid tumors beyond melanoma and their potential for tumor clearance can be harnessed through TIL-based Adoptive Cell Therapy.

I. **Specific Aim 1:** Determine the immune and molecular landscape of TIL in pancreatic cancer (PDAC) and ovarian cancer (OvCA)
II. **Specific Aim 2:** Elucidate requirements for TIL expansion in PDAC and OvCa, and determine phenotypic attributes of the expanded TIL
III. **Specific Aim 3:** Interrogate the presence of tumor-reactive TIL in TIL generated from tumors generally unresponsive to current immune checkpoint blockade therapy

Chapter 2: Materials and Methods

Patient selection

Pancreatic Cancer Samples. After obtaining written informed consent, 61 patients with primary or metastatic pancreatic ductal adenocarcinoma underwent surgical resection. Patients are referred to by their de-identified "MP" number. Tissue from surgical resections was used to expand TIL under protocols (PA15-0176, LAB00-396, PA15-0014 for PDAC samples and LAB06-0755 for melanoma samples) approved by the Institutional Review Board of The University of Texas MD Anderson Cancer Center.

Ovarian Cancer Samples. Patients with primary or metastatic high-grade serous ovarian carcinoma underwent surgical resection (n=98, 84 evaluable for flow cytometry). In 47 patients, platinum-based chemotherapy and/or chemoradiation was administered. Patients are referred to by their de-identified number. Informed consent was obtained from all individual participants included in the study for their specimens and data to be used in research and for publication.

Ethical approval and tissue from surgical resections used to expand TIL were both obtained under protocol (PA16-0912 and LAB02-188) approved by the Institutional Review Board of The University of Texas MD Anderson Cancer Center.

Reagents and cell lines

A fully human and purified IgG4 monoclonal antibody (mAb) against human CD137/4-1BB, Urelumab (663513), was kindly provided by Bristol-Myers Squib (BMS). Human recombinant interleukin-2 (IL-2) (Proleukin^{TM}) was generously provided by Prometheus Therapeutics & Diagnostics. MHC class I blocking antibody (clone W6/32) and

isotype control (mouse IgG2a, clone eBM2a) were purchased from Invitrogen and eBioscience, respectively. Autologous pancreatic tumor targets were found to match the patients using STR DNA fingerprinting performed at MDACC and the tumoroid was confirmed mycoplasma-free. CAPAN-1 and SKOV3 cell lines were purchased from ATCC. COV318 and COV362 were purchased from Sigma-Aldrich (now Millipore-Sigma, St. Louis, MO). All cell lines were HLA-typed, STR fingerprinted, and confirmed mycoplasma-free at MDACC.

Isolation and expansion of TIL from human solid tumors

The tumor samples were cut into 1-3 mm^2 fragments and placed in TIL culture media [TIL-CM: RPMI-1640 with GlutaMax (Gibco/Invitrogen), 1× Pen–Strep (Gibco/Invitrogen), 50 μmol/L 2-mercaptoethanol (Gibco/Invitrogen), 20 μg/mL Gentamicin (Gibco/Invitrogen), and 1 mmol/L sodium pyruvate (Gibco/Invitrogen] with 6000 IU/ml IL-2 in 24-well plates for up to 5 weeks, as previously described [108, 217]. The same method was applied for the metastatic melanoma samples. For the 4-1BB mAb (a41BB) condition, both 6000 IU/ml IL-2 and 10 ug/ml 4-1BB mAb were added in the culture plates on day 0 and day 4 or 5. TIL were expanded for up to 35 days prior to performing the described assays or the rapid expansion protocol (REP). For the IL-2+OKT3+a41BB condition, five tumor fragments were put in a G-Rex 10 flask (Wilson Wolf Manufacturing, New Brighton, MN) in 20 mL TIL-CM with 6000 IU/mL IL-2, 10 ug/mL 4-1BB mAb, and 30 ng/mL anti-CD3 (OKT3) as previous described [218]. Four to five days after culture initiation, 20 mL of additional TIL-CM with 6000 IU/mL IL-2 was added. Half-media changes were done every 3-4 days with fresh TIL-CM containing 6000 IU/mL IL-2 for up to 35 days or until the cells formed a thick layer

completely covering the bottom of the flask. The cell suspensions were collected and cryopreserved for later testing.

Cell Sorting and Rapid Expansion of $CD8^+$ OvCa TIL

To control for reactivity of CD8+ TIL, bulk TIL products were stained with CD3 FITC, CD8 APC-H7, and SYTOX Blue Dead Cell Stain in order to isolate the $CD8^+$ T cells using a BD FACSAria IIIu in the MD Anderson Cancer Center (MDACC) Flow Cytometry and Cellular Imaging Core Facility. Then, to provide greater cell numbers for functional assays, the sorted $CD8^+$ TIL underwent the rapid expansion protocol in G-Rex10 flasks, which was previously described by Forget et al. and then viably-frozen [217]. Briefly, the REP was performed in the G-Rex 10 device (Wilson Wolf Manufacturing) following a scaled-down version of the previously described protocol [217]. Briefly, TIL were put in culture with pooled allogeneic irradiated PBMC feeder cells at a ratio of 1 TIL to 200 feeders in combination with 6000 IU/ml IL-2 and 30 ng/mL of anti-CD3 (OKT3 clone) on day 0 of the REP. The REP process lasted for 14 days, with REP-CM (half TIL-CM and half AIM-V (Invitrogen)) used for the first 7 days and only AIM-V for the last 7 days of expansion. The cells were collected and cryopreserved for later assays.

Immunohistochemistry (IHC)

Four-micrometer-thick serial sections were obtained from representative formalin-fixed, paraffin-embedded (FFPE) blocks for IHC, as well as hematoxylin and eosin (H&E) staining. H&E slides were examined by a pathologist to confirm the presence of tumor. IHC was performed using a Leica Bond Max automated staining system (Leica Microsystems) with antibodies against CD3 (dilution 1:100; Dako). The expression of the marker was

detected using a Leica Bond Polymer Refine Detection kit (Leica Microsystems) with diaminobenzidine reaction to detect antibody labeling. Counterstaining was done using hematoxylin. Human tonsil FFPE tissues with and without CD3 primary antibody were used as positive and negative controls, respectively. For quantification of CD3 expression, the slides were digitally scanned at 200 magnification using the Aperio AT2 scanner (Leica Microsystems). The images were visualized using the ImageScope software (Leica Microsystems) and analyzed using the Aperio Image Toolbox (Leica Microsystems). Five regions of interests were randomly selected within the tumor area of each slide. The number of CD3 positive cells per mm^2 (cell density) was evaluated, and the final score was expressed as the average density of the five areas.

Flow Cytometric Analysis of TIL

Fresh tumor samples were manually disaggregated between frosted-glass slides to obtain a single-cell suspension for analysis. Both the disaggregated tissue samples and expanded TIL were blocked in FACS Wash Buffer (Dulbecco's Phosphate Buffered Saline 1X with 1% Bovine Serum Albumin) supplemented with 5% goat serum (Sigma-Aldrich) for 10 min at room temperature. Cell suspensions were then stained in FACS Wash Buffer for 30 min using fluorochrome-conjugated monoclonal antibodies for CD3, CD4, CD8, CD16, CD56, CD57, Granzyme B, TBET, γδ TCR, CD27, CD28, CD45, CD45RA, CD45RO, CCR7, CD103, CD152/CTLA4, BTLA (clone J168) (BD Bioscience), CD25, CD279/PD-1, KLRG1, CD278/ICOS, CD366/TIM3 (Biolegend), and CD223/LAG3, FoxP3, Ki67, CD357/GITR, Eomes (eBioscience/ThermoFisher). Stained cells were fixed in 1% paraformaldehyde solution for 20 min. Intracellular staining was performed using eBioscience transcription factor staining kit according to the manufacturer's instructions.

Samples were acquired using the BD FACSCanto™ II or BD LSRFortessa X-20 and analyzed using FlowJo Software v10.2 (Tree Star). Dead cells were excluded using an AQUA or Yellow live/dead staining (Invitrogen).

T-cell Receptor Beta Sequencing

Genomic DNA was extracted from samples using DNeasy Blood & Tissue Kit (Qiagen) as per manufacturer's instructions. TCRβ CDR3 regions were amplified from between 0.2–3 μg of DNA. All samples had the ImmunoSEQ^{TM} assay performed at Adaptive Biotechnologies, with deep sequencing for PBMC DNA and survey-level sequencing for all others. Data analysis was performed at MDACC.

Preparation of Single-Cell RNA Sequencing Libraries for PDAC TIL

Fresh tumor samples were disaggregated using a MediMachine (BD Biosciences) to create a single-cell suspension. After disaggregation, $CD3^+$ T cells were isolated for sequencing via magnetic bead separation using the EasySep^{TM} Release Human CD3 Positive Selection Kit (StemCell Technologies) according to the manufacturer's instructions. For cultured TIL, previously cryopreserved samples were thawed, washed with PBS, and resuspended at $1x10^6$ cells/mL. The single-cell RNA sequencing libraries were prepared according to the protocol for the 10x Genomics Chromium^{TM} Single-cell V(D)J Regent Kits using the v1 chemistry. T cells were loaded on a Chromium single-cell A chip and placed in a Chromium controller where they were partitioned into single-cell droplets to undergo cell barcoding and cDNA synthesis. After isolation of the cDNA by DynaBeads MyOne Silane beads, it was then PCR amplified, again purified by size-selection using SPRIselect (Beckman), all according to the manufacturer's instructions. For construction of the gene

expression library, 50 ng of amplified cDNA was fragmented, end repaired, sized-selected using SPRIselect and PCR amplified again according to the manufacturer's instructions. For the TCR library, 2 ul of amplified cDNA was used for enrichment of TCR transcripts via the Chromium Human T cell V(D)J Enrichment Kit and purified using SPRIselect, both following the manufacturer's instructions.

Sequencing of the TCR and gene expression libraries

The transcriptome libraries were pooled and sequenced paired-end (26 cycles read 1, 91 cycles read 2, 8 cycles i7) on the Illumina Novaseq to a targeted depth of 600M paired-end reads per library. TCR libraries were pooled and sequenced on the Illumina Miseq 150bp paired-end to a targeted depth of 1000 reads per T-cell.

Unsupervised clustering of scRNAseq data

The Seurat package version 2.4 workflow in R (https://github.com/satijalab/seurat) was used for transcriptome processing/clustering plots. The data was integrated for batch correction using the first 50 canonical components. To separate CD8 and CD4 cells, each cell was scored for expression of genes over expressed in clearly CD8 or CD4 cells (by CD8A, CD4 transcript count). Cells were then assigned to the group where they had the higher score and then split for further processing. The tSNE embedding and clustering was performed on the first 20 principal components of the integrated embedding. The cells were clustered using shared nearest neighbors (SNN) and the heat maps represent the top over expressed genes of each cluster selected by average fold change and p-value on a Wilcox test. Clusters were named by picking one gene indicative of their functional status. The Circo plots were

generated using a custom code where the inner tree is hierarchical clustering of the mean of the transcriptome data for each clonotype. The shared clones between the fresh and grown TIL were matched via their TCRa and TCRb nucleotide sequence and then graphed based on the relative frequency in each sample.

TIL Recognition Assays via 4-1BB Upregulation and IFN-γ secretion

For the PDAC TIL: In triplicate, T cells were put at a 10:1 ratio with their autologous tumor target, CAPAN-1 (HLA mismatch control), or media (TIL alone). For some experiments the autologous tumor targets were incubated with 80 μg/ml of anti-MHC class I antibody (clone W6/32) for 3h prior to addition of T cells, as previously described [219]. After 24 h incubation, the supernatants and T cells were collected. TIL were analyzed for 4-1BB/CD137 expression via flow cytometry. Detection of secreted cytokines in the corresponding supernatants were detected using a V-PLEX Plus Proinflammatory Panel 1 (human) kit and analyzed on a QuickPlex SQ 120, both available from Meso Scale Discovery. Reported values have CVs <20%.

For the INF-γ ELISPOT: One day prior to the assay, the TIL were thawed and rested overnight in TIL-CM with 6000 IU/mL IL-2. Six hours prior to the assay, TIL were washed and rested in TIL-CM without IL-2. The tumor lines were put at 1x106 cells/mL and incubated with 80 μg/mL of the HLA-ABC blocking antibody or 80 μg/mL of its isotype control for 3 hrs in 15 mL conical tubes at 37C. The tumor cells were then added directly to the ELISPOT plate. TIL were then put at a 2:1 or 4:1 ratio with an HLA-matched OvCa cell line (either SKOV3, COV318, or COV362) in the presence of an HLA-ABC blocking antibody, its isotype control, PMA/Ionomycin, or media (TIL alone). The conditions were performed in triplicate and the cells were co-cultured for 15 hrs before developing as

previously described [220]. Spots on ELISPOT plates were counted with the ImmunoSpot machine (Cellular Technology Ltd., Shaker Heights, OH).

Statistical Analysis

GraphPah Prism v7.0 (GraphPad Software) was used for graphing and statistical analysis. Differences between groups or experimental conditions were determined using either parametric or non-parametric, two-tailed t-tests (paired or unpaired as appropriate). Linear regression and Spearman correlation analyses were also used as indicated. Two-sided p-values <0.05 were considered statistically significant and in the figures are indicated as * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$.

Chapter 3: 4-1BB Agonist Focuses CD8$^+$ Tumor-Infiltrating T-Cell Growth In to a Distinct Repertoire Capable Of Tumor Recognition in Pancreatic Cancer

This chapter is based on the original research article "4-1BB Agonist Focuses CD8$^+$ Tumor-Infiltrating T-Cell Growth In to a Distinct Repertoire Capable of Tumor Recognition in Pancreatic Cancer" published by Sakellariou-Thompson *et al.* in *Clinical Cancer Research* on September 25th, 2017 (DOI: 10.1158/1078-0432.CCR-17-0831). It is presented with permission from the American Association for Cancer Research (AACR) as per item 4 of the "Article Reuse by Authors" section of the publisher's Copyright, Permission, and Access document which states, in part:

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3.1 Introduction

Pancreatic cancer is the third-highest cause of cancer-related death for men and women in the United States and is expected to become the second-leading cause of cancer mortality by 2030 [221, 222]. The majority (85%) of pancreatic cancer diagnoses are classified as pancreatic ductal adenocarcinoma (PDAC) [223]. Patients afflicted with PDAC often present with late-stage cancer and face the poorest prognosis of all cancer types with a 5-year survival rate of around 6% [224]. Despite efforts to improve treatment, surgery, chemotherapy, and radiation remain the only options. These treatment strategies have shown limited effectiveness as most patients will recur within a year of treatment even after successful tumor resection [224, 225]. Therefore, there is a great need to broaden treatment options.

Immunotherapy has made a tremendous mark in the treatment of cancer, especially in the past decade. Its success was first observed in the treatment of metastatic melanoma with high-dose IL-2 and then more recently with agents that block CTLA-4 and PD-1 (checkpoint

blockade) [43, 226, 227]. These treatments were later transposed to several cancer types, including MSI-high cancers regardless of indication [134, 228-235]. In PDAC, however, they have been ineffective with no objective response seen with the exception of the very rare MSI-high subtype of PDAC [209, 210, 231]. The lack of efficacy could be a result of the paucity of $CD3^+$ T-cell infiltrate [236]. An alternative approach to overcome the limitation posed by the modest immune infiltrate in PDAC is the *ex vivo* amplification of TIL for re-infusion through autologous ACT.

TIL ACT expands T cells up to several hundred-fold from surgically resected tumor and re-infuses them into the patient, providing a large influx of anti-tumor T cells. Our group and others have demonstrated its effectiveness in melanoma [94, 108, 237, 238]. With an average objective-response rate (ORR) of 50%, TIL ACT is among the best treatment options for metastatic disease. The MDACC experience also demonstrated a positive correlation between $CD8^+$ TIL infused and response [108]. These results have already spurred efforts to translate ACT to other cancer types, and a handful of preliminary studies have reported encouraging data in small cohorts of patients in indications such as cervical cancer (33% ORR), ovarian cancer (avg PFS 3.7 months; Pederson Oncoimmunol 2018) and gastrointestinal malignancies (case studies reported but no ORR yet) [239, 240]. PDAC could also potentially benefit from TIL ACT as the presence of $CD8^+$ TIL is associated with greater 5-year survival [85, 216]. This suggests that endogenous PDAC TIL can exert some degree of tumor control, supporting the potential of TIL ACT.

One of the major challenges faced in growing TIL from GI cancer types for ACT trials is the difficulty of expanding $CD8^+$ T cells from the tumor tissue [119, 241]. PDAC has a well-characterized immunosuppressive tumor microenvironment that might contribute to

the difficulty of triggering the proliferation of cytotoxic $CD8^+$ T cells from this tumor tissue and account for their decreased numbers [236, 242]. A method to resolve this barrier is by manipulating 4-1BB/CD137, a member of the tumor necrosis factor receptor family, which provides a strong co-stimulatory signal for increased activation, proliferation, and survival. This receptor is predominantly expressed on recently activated $CD8^+$ T cells with peak expression at 24 h [55]. In fact, our group demonstrated that inclusion of an agonistic 4-1BB mAb (Urelumab, BMS) in TIL cultures was able to increase melanoma and triple-negative breast cancer $CD8^+$ TIL proliferation [220, 243]. Based on this previous work, we posited that use of an agonistic 4-1BB mAb in PDAC TIL culture would provide the same benefits of increased $CD8^+$ TIL yield.

Here, we demonstrate that the addition of an agonistic 4-1BB mAb increases the ability to grow TIL from PDAC, improves the total yield, and stimulates the proliferation of more $CD8^+$ T cells without overly differentiating them. In addition, these $CD8^+$ TIL have a distinct TCR repertoire compared to IL-2 only grown TIL and display MHC class I-restricted autologous tumor recognition. These results support the use of 4-1BB-expanded TIL in ACT strategies for patients with PDAC.

3.2 Results

3.2.1: PDAC TIL infiltrate is predominantly $CD4^+$ T-cells

To determine if the immune component of PDAC was sufficient for TIL ACT, we assessed the immune infiltrate by performing flow cytometry on manually disaggregated samples (n=28). The amount of $CD3^+$ TIL observed was less than 1% of all cells in the tumor sample on average as compared to metastatic melanoma with an $CD3^+$ TIL infiltrate >2%

(Figure 8A). Quantitative IHC analysis found that the mean density of $CD3^+$ TIL was 314 cells/mm2 (Figure 8B). This is fewer than what the literature reports for an immunogenic cancer like melanoma (422 cells/mm2) [244]. Additionally, metastatic (closed circle) and primary (open circle) PDAC samples did not appear to stratify. Further IHC analysis showed that all samples displayed ≥50% MHC Class I expression that was homogenous throughout the tumor tissue, suggesting that lack of Class I was not the reason for low $CD3^+$ infiltration (Figure 8C). We also evaluated the proportion of $CD8^+$ and $CD4^+$ T cells. With an average CD8:CD4 ratio of 0.75, $CD4^+$ TIL comprised the majority of the T-cell infiltrate (Figure 8D). As a point of comparison, metastatic melanoma showed a CD8:CD4 ratio of 1.5 (Figure 8D). This ratio is similar to IHC data also from Erdag et al. that exhibited a CD8:CD4 ratio of 1.6 [244]. Metastatic and primary sites did not seem to show a difference in CD8:CD4 ratio. Phenotypic analysis on the $CD8^+$ T cells determined their activation and differentiation state by assessing their expression of CD28 (50% ± 20%), CD45RA (10% ± 6%), PD-1 (45% ± 12%), and BTLA (20% ± 12%) (Figure 8E). There were not enough primary samples that could be compared with metastatic samples to discern a difference in the phenotype of their TIL. However, the low frequency of CD45RA expression combined with expression levels of the other three markers suggests a relatively activated and not terminally-differentiated immune infiltrate.

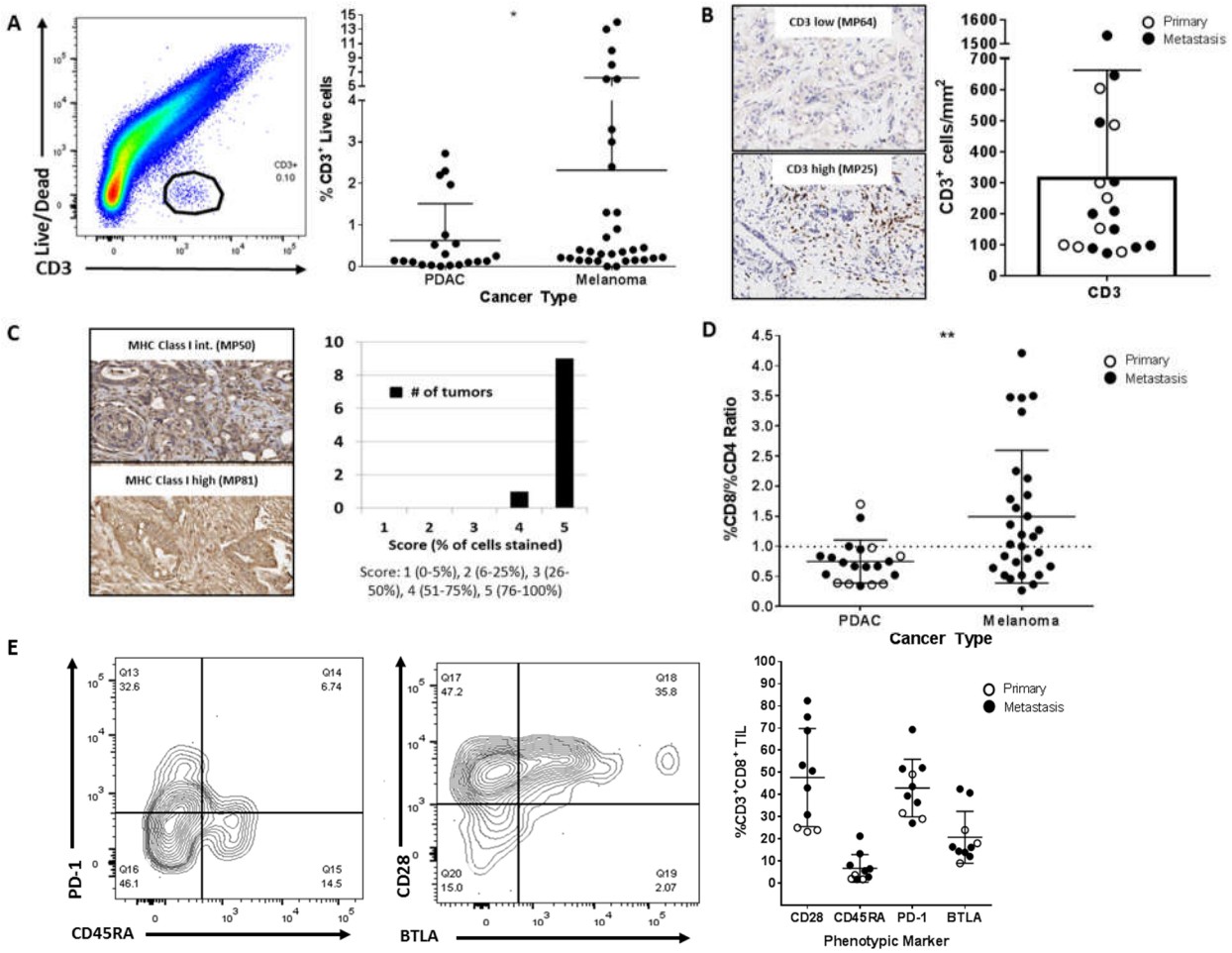

Figure 8. PDAC TIL infiltrate is largely dominated by CD4$^+$ T cells.
(**A**) Flow cytometry analysis was performed on freshly disaggregated tumor samples. A representative figure showing the gating strategy based on CD3 and live/dead is presented on the left. The right graph shows a significantly lower CD3$^+$ immune infiltrate in PDAC as compared to metastatic melanoma. (**B**) IHC analysis quantified the amount of CD3$^+$ infiltration in PDAC. (**C**) IHC analysis showed that >75% of the PDAC tumor tissue expresses MHC class I demonstrating HLA Class I expression. (**D**) CD8/CD4 ratio in fresh PDAC sample compared to melanoma. (**E**) Phenotypic analysis of expression of CD28, CD45RA, PD1, and BTLA on CD8$^+$ TIL present in fresh samples.

3.2.2: PDAC shows an enriched T-cell repertoire in the tumor

Enrichment of T-cell clones at the tumor site in comparison to the blood would suggest that the patient is mounting an immunogenic response to its tumor and that specific T-cell clones are migrating to the tumor and proliferating in the tissue. To determine the tissue-specific distribution of the T-cell repertoire, we sequenced the T-cell receptor beta-

chain (TCRB) CDR3 region of the T cells present in the blood, tumor and normal tissue from seven patients when available (Figure 9). This analysis, presented as productive clonality, revealed that the T-cell repertoire in the tumor is generally more clonal than in the blood (Figure 9A). Productive clonality is a measure of the degree to which one or several unique clones dominate the repertoire [245]. Linear regression analysis compared the relative frequencies of individual TCRB clones present in both the blood and the tumor (Figure 7B). All autologous blood-tumor pairs displayed slopes (m) <1, demonstrating a higher frequency of shared clones in the tumor than the blood. However, it is possible that high frequency clones present in the blood may correlate with high frequency clones present in the tumor. To determine the strength of correlation between clones in these sites, clones were partitioned and compared as follows using Spearman correlation: top clone frequency defined as ≥ 0.24% in the tumor (red circles), remaining clones partitioned in half with mid-frequency shared clones (green circles) and low-frequency shared clones (blue circles). Interestingly, Spearman correlation analysis showed weak or undefined correlation between the frequency of T-cell clones in the blood and their frequency in the tumor tissue (rs <0.5), even among the top ranking clones in the tumor (red circles). Additionally, there were a few instances where the middle-ranking clones in the tumor (green circles) had greater correlation with their frequencies in the blood. Only MP31 showed good correlation (rs = 0.61) of the frequencies of these top shared clones (Figure 9B). Linear regression analysis was also used to compare the relative frequencies of individual TCRB clones present in both the normal and tumor tissue (Figure 9C). Similar to the tumor-blood pairs, the slopes for all the tumor-normal tissue pairs were <1, indicating that individual shared clones were found at higher frequencies in the tumor than the normal. In addition, clones were partitioned and correlation

calculated in the same manner as described above. In contrast to the tumor-blood pairs, the frequencies of the T cells in autologous tumor-normal tissue pairs showed stronger correlation among the top and mid-frequency clones. Four of the five pairs (MP64B, MP75, MP81, and MP84B) had rs > 0.44 while two of them (MP64B and M75) had rs > 0.5, showing that the repertoires were very similar between the tumor and the normal tissue in these cases. Also different from the tumor versus blood comparison was that the top clones in the tumor (red circles) were often high ranking in the normal tissue as shown by clustering along the hashed line. MP31 shared very few clones overall, so it was not partitioned.

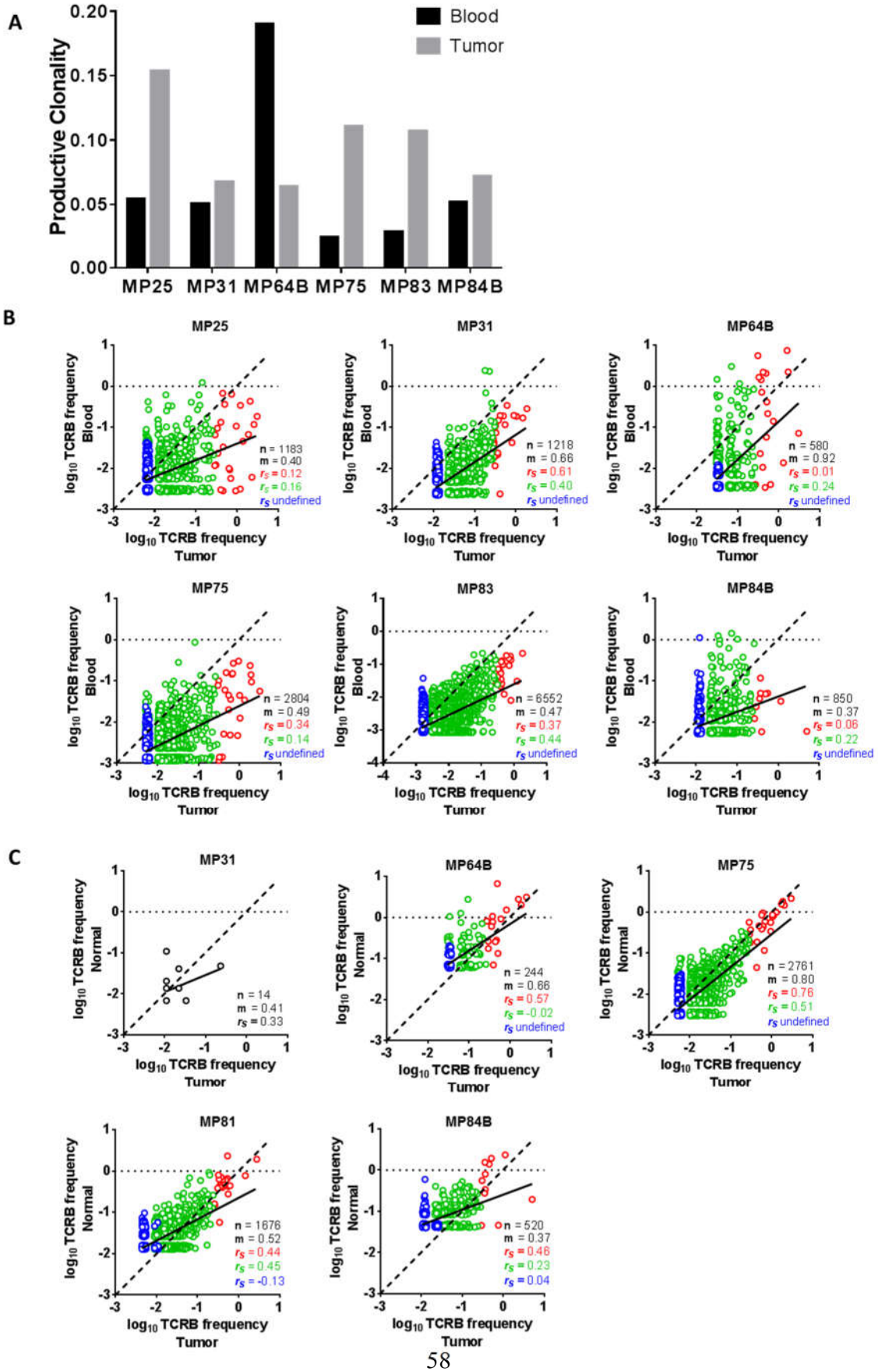
A
Productive Clonality
0.20
0.15
0.10
0.05
0.00
Blood
Tumor
MP25
MP31
MP64B
MP75
MP83
MP84B
B
MP25
n = 1183
m = 0.40
rS = 0.12
rS = 0.16
rS undefined
MP31
n = 1218
m = 0.66
rS = 0.61
rS = 0.40
rS undefined
MP64B
n = 580
m = 0.92
rS = 0.01
rS = 0.24
rS undefined
MP75
n = 2804
m = 0.49
rS = 0.34
rS = 0.14
rS undefined
MP83
n = 6552
m = 0.47
rS = 0.37
rS = 0.44
rS undefined
MP84B
n = 850
m = 0.37
rS = 0.06
rS = 0.22
rS undefined
log10 TCRB frequency Blood
log10 TCRB frequency Tumor
C
MP31
n = 14
m = 0.41
rS = 0.33
MP64B
n = 244
m = 0.66
rS = 0.57
rS = -0.02
rS undefined
MP75
n = 2761
m = 0.80
rS = 0.76
rS = 0.51
rS undefined
MP81
n = 1676
m = 0.52
rS = 0.44
rS = 0.45
rS = -0.13
MP84B
n = 520
m = 0.37
rS = 0.46
rS = 0.23
rS = 0.04
log10 TCRB frequency Normal
log10 TCRB frequency Tumor

Figure 9. TCRB clonality and frequency of shared TCRB clones higher in tumor than autologous blood and normal tissue.

CDR3 sequencing of the TCR beta-chain was done on the blood, tumor and normal tissue. (**A**) TCRB clonality is higher in the tumor than the blood for most samples. (**B**) Frequencies of shared clones in the tumor and blood and (**C**) tumor and normal tissue are shown. Top T-cell clones from the tumor that are also shared between the blood or normal tissue are highlighted in red. The remaining shared clones are split in half with the top half colored green and the bottom half colored blue. Shared clones (n), linear regression analysis with line of best fit (solid line), slope of best-fit line (m), and color-coded Spearman correlation coefficient (r_S) for each group are next to each graph in (**B**) and (**C**). Hatched lines represent the identity line, i.e. 1:1 frequency ratio. The data plotted in (**B**) and (**C**) was jittered to prevent over-plotting.

3.2.3: 4-1BB mAb increases total TIL growth, success rate, and frequency of $CD8^+$ TIL

Prior work by our group detailed how infusion of melanoma patients with a higher proportion of $CD8^+$ T cells and larger amount of TIL in general correlated with better clinical response [108]. This result coupled with our observations of a predominance of $CD4^+$ TIL and relative scarcity of $CD3^+$ infiltration in general prompted us to consider ways to generate greater TIL growth that was rich in $CD8^+$ T cells. Previous work showed that recently antigen-activated $CD3^+CD8^+$ TIL upregulate expression of the costimulatory molecule 4-1BB [55]. Furthermore, additional work by our group and others demonstrated that stimulation of this pathway through the use of an agonistic anti-4-1BB (a4-1BB) antibody could decrease time of TIL culture while increasing total TIL growth, particularly that of $CD3^+CD8^+$ T cells [241, 243]. Thus we set up samples for TIL culture where one set of fragments received only the conventional high-dose IL-2 (n=28) and the other received high-dose IL-2 plus the a4-1BB mAb (n=27) (Table 1). The addition of a4-1BB increased the average total TIL growth from 40x106 cells for IL-2 alone to 100x106 for IL-2 + a4-1BB (Figure 10A). Only cultures that grew in at least one of the two conditions are represented in Figure 10A. Additionally, a4-1BB doubled the success rate (14/27; 52%) for TIL growth

from fragments compared to IL-2 only (8/28; 29%) (Table 2). The benchmark for a successful TIL culture, 12x106 total cells, was established from scaling down the MDACC Clinical Melanoma TIL Lab's criterion for success where 20 fragments are set up for TIL expansion and 40x106 cells is considered the minimum to treat a patient. Some IL-2 only cultures did not reach the benchmark and several produced no discernible TIL growth while their companion a4-1BB culture produced ≥12x106 cells (Table 1). To that effect, our work demonstrates that use of a 4-1BB mAb could rescue cultures that would not have grown under the conventional methods.

Next, we determined whether the cells that grew out of the cultures treated with a4-1BB were enriched for $CD3^+$ TIL and if $CD8^+$ TIL now comprised the majority of $CD3^+$ T cells. In cultures treated with a4-1BB, the total number of $CD3^+$ TIL was significantly increased over IL-2 only cultures on average from 30x106 cells to 75x106 cells respectively

Table 1. TIL from PDAC are mainly $CD4^+$ but a4-1BB switches majority to $CD8^+$

TIL from Primary PDAC											
		IL-2 only					IL-2 + a4-1BB				
		Phenotype			TIL Yield		Phenotype			TIL Yield,	
Patient:	Site:	$CD3^+CD8^+$%	$CD3^+CD4^+$%	$CD3^+\gamma\delta^+$%	Total ($x10^6$)	per 20 fragments*	$CD3^+CD8^+$%	$CD3^+CD4^+$%	$CD3^+\gamma\delta^+$%	Total ($x10^6$)	per 20 fragments*
MP044	Pancreas	50	30	1	13	43.3	48	1	43.5	116	387
MP045	Pancreas	12	80	1.2	5	16.7	61	1.4	7.75	41	137
MP046	Pancreas	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G
MP047	Pancreas	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G
MP050	Pancreas	N/G	N/G	N/G	N/G	N/G	91.4	1	2.26	26	86.7
MP054	Pancreas	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G
MP056	Pancreas	N/G	N/G	N/G	N/G	N/G	25.1	70.3	3.14	15	50
MP058	Pancreas	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G
MP080	Pancreas	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G
Avg		31	55	1.1	9	30	56.38	18.43	14.16	49.5	165
TIL from Metastatic PDAC											
		Phenotype			TIL Yield		Phenotype			TIL Yield,	
Patient:	Site:	$CD3^+CD8^+$%	$CD3^+CD4^+$%	$CD3^+\gamma\delta^+$%	Total ($x10^6$)	per 20 fragments*	$CD3^+CD8^+$%	$CD3^+CD4^+$%	$CD3^+\gamma\delta^+$%	Total ($x10^6$)	per 20 fragments*
MP01	Liver	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G
MP06	Liver	7.3	91.5	0.5	45	150	N/A	N/A	N/A	N/A	N/A
MP010	Liver	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G
MP012	Liver	N/G	N/G	N/G	N/G	N/G	84.2	1	16.5	9	30
MP016	Liver	N/G	N/G	N/G	N/G	N/G	82.9	1	13.2	160	533
MP019	Liver	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G
MP025	Liver	5	93	1	77	257	70	20	3	135	450
MP031	Liver	13	83	1.37	34	113	60	1	31.4	139	463
MP055	Liver	1	99	1	29	96.7	88	2	16.4	84	280
MP064A	Liver	3.4	96	0.02	38	127	1	1	0.18	69	230
MP064B	Liver	89	61	1	237	790	99	1	0.17	234	780
MP067	Liver	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G
MP077	Liver	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G
MP063	Peritoneum	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G
MP075	Omentum	N/G	N/G	N/G	N/G	N/G	8.5	74	5.29	173	577
MP081	Liver	27	45	1	123	410	69	6	7.8	135	450
MP083	Neck LNs	22.7	9.65	53	6.8	22.7	51	1	50	69.5	232
MP084A	Liver	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G	N/G
MP084B	Liver	N/G	N/G	N/G	N/G	N/G	72	1.2	8.8	66	220
Avg		21.05	72.27	7.36	73.7	246	62.33	9.93	13.89	116	386

All TIL analyses are pre-Rapid Expansion Protocol (REP)
N/G No growth; N/A Not applicable b/c condition Not tested
*Extrapolated to the 20 fragments used during clinical scale expansion

(Figure 10B). Only cultures that grew in at least one of the two conditions are represented in Figure 10B. Since NK cells and γδ TCR^+ T cells can also express 4-1BB, we stained for their presence in primary cultures to determine if the a4-1BB antibody being used was stimulating their growth [246, 247]. Indeed we found that three cultures with a4-1BB were enriched with a $CD3^-CD56^+$ population that was a greater proportion of the culture than $CD3^+$ TIL (Figure 10C). Likewise, some cultures also showed an increase in γδ TCR^+ T cells in a4-1BB cultures versus IL-2 alone (Figure 10D, top graph). Only cultures that grew in both conditions are represented in Figure 10C and D. Neither the increase in NK cell growth (p = 0.203) or γδ TCR^+ T-cell growth (p = 0.078) due to 4-1BB co-stimulation was found to be significant overall. However, co-stimulation with a4-1BB produced a primary TIL culture that was on average 55% $CD3^+CD8^+$ TIL and 5% $CD4^+$ TIL. We observed the opposite situation in IL-2 alone cultures where 25% of $CD3^+$ TIL were $CD8^+$ and 60% were $CD4^+$. Overall, the 4-1BB mAb caused a dramatic switch in the composition of $CD3^+$ TIL towards the more favorable $CD8^+$ TIL (Figure 10D).

Table 2. Using a4-1BB increases clinically relevant growth success rate.

	IL-2 only[a]	IL-2 + a4-1BB[a]
Primary	1/9 (11%)	4/9 (44%)
Met.	7/19 (37%)	10/18 (55%)
Total	8/28 (29%)	14/27 (52%)

[a]Only cultures that reached clinically relevant number of $12x10^6$ were counted.

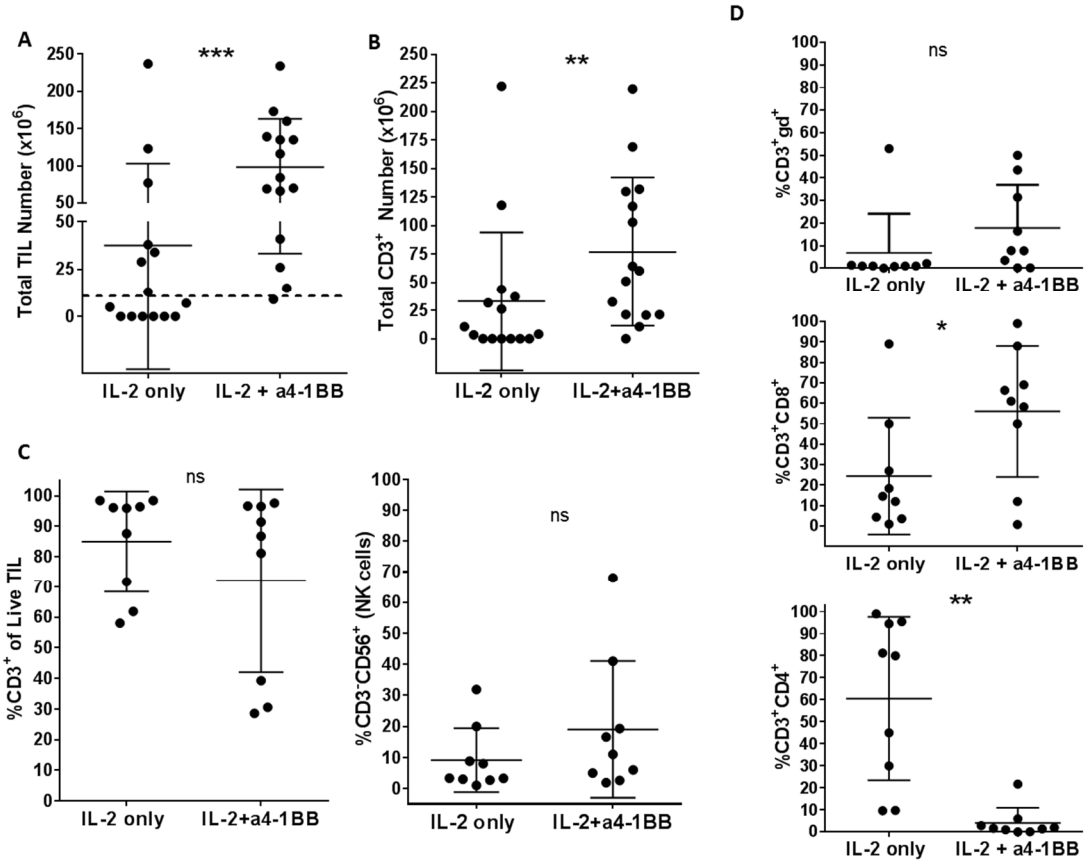

Figure 10. High numbers of CD8$^+$ TIL can be expanded from PDAC with the use of agonistic anti-4-1BB antibody.
Tumor fragments were set up with either high dose of IL-2 alone or with the addition of an agonistic anti-4-1BB (a4-1BB). (**A**) Total TIL numbers and (**B**) total CD3$^+$ TIL expanded with or without a4-1BB. Expansion was considered successful with 12x10^6 cells or more (dotted line); cultures which did not reach this number in both conditions are not presented. (**C**) Percentage of CD3$^+$ and CD3$^-$ CD56$^+$ TIL grown in each condition. Only cultures with successful growth in both conditions are presented. (**D**) Percentage of γδ TCR$^+$, CD8$^+$, and CD4$^+$ within the CD3$^+$ T cell subset. Only cultures with successful growth in both conditions are presented.

3.2.4: Addition of 4-1BB mAb does not overly differentiate CD8$^+$ TIL

To better understand what effect the augmented growth via 4-1BB mAb stimulation had on CD8$^+$ TIL differentiation, we performed phenotypic analysis of CD28, CD45RA, PD-1, and BTLA expression. Between IL-2 only and a4-1BB cultures, the only significant change was a decrease in CD45RA expression (p = 0.031) (Figure 11A). In fact, the level of

expression of these markers was comparable to that seen on $CD8^+$ TIL in the fresh tumors (Figure 6E). Further phenotyping was done using the established memory markers CD45RA, CD27, CD28, and CCR7 (Figure 11B) [248]. The vast majority of TIL, regardless of culture condition, were $CD45RA^-CCR7^-$, indicating that they are effector memory (EM) cells. Further characterization of their EM status was done by analyzing differential expression of CD27 and CD28, which has been shown to subdivide EM cells into four subsets termed EM1, EM2, EM3, and EM4 (Figure 11C) [249].The majority of TIL, again regardless of culture condition, fell in the EM3 ($CD27^-CD28^-$) subset which Romero et al. have shown to display stronger cytolytic activity [249]. We also further explored the degree of differentiation with the expression of KLRG1, CD57, Eomes, T-bet and Granzyme B. As shown in Figure 12, KLRG1 was absent from both culture conditions. Combined with the other markers, this supplementary analysis further testifies that the stimulation of 4-1BB does not overly differentiate the cells and leads to the proliferation of effector/memory. Overall, this shows that even though a4-1BB stimulates aggressive expansion of activated $CD8^+$ TIL, they do not become overly or terminally differentiated.

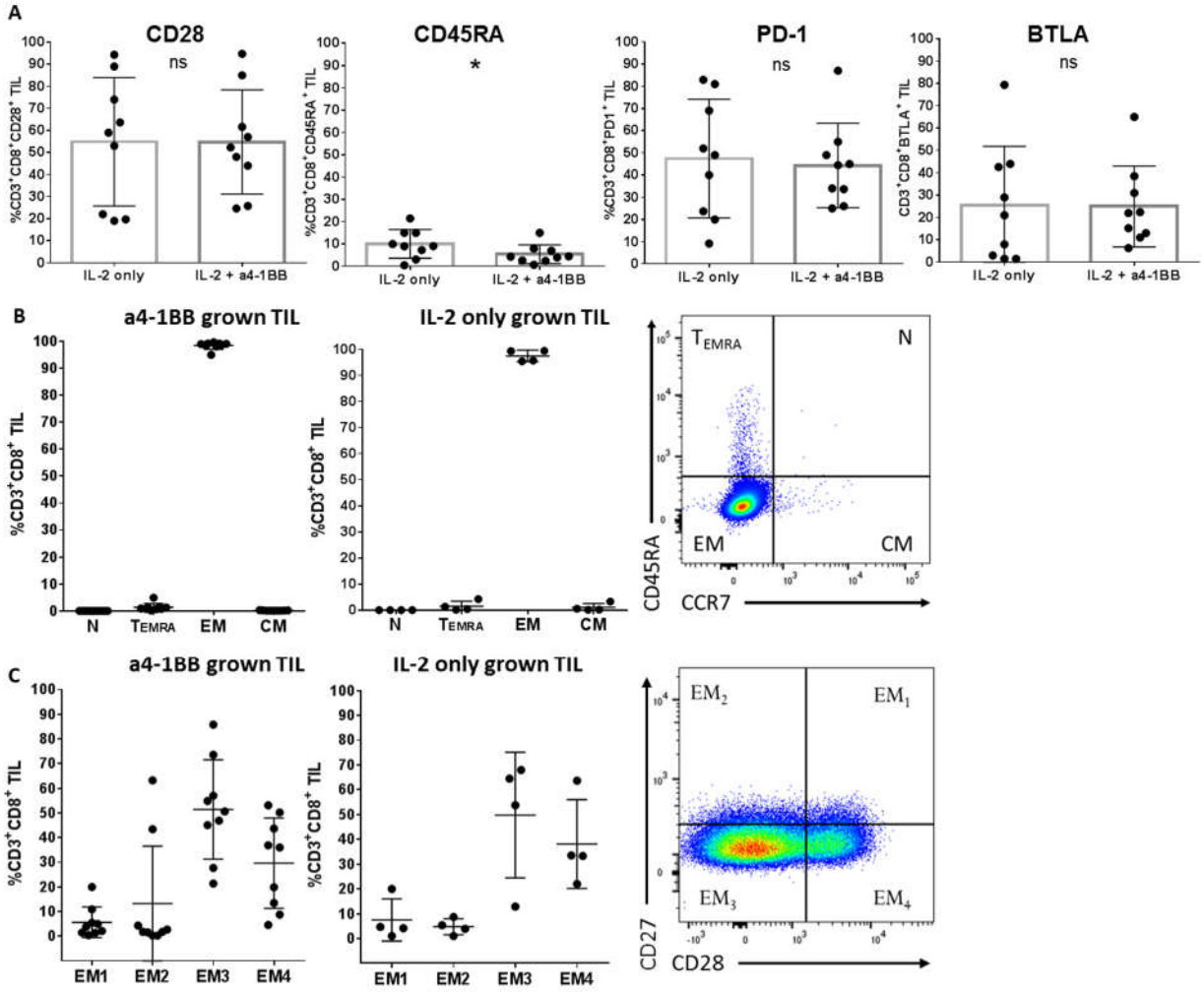

Figure 11. Addition of a4-1BB does not overly differentiate CD8$^+$ TIL.

(A) Percent expression of phenotypic markers (CD28, CD45RA, PD1 and BTLA) for T-cell activation and differentiation on CD8$^+$ TIL post-culture evaluated by flow cytometry. **(B)** Comparing expression of CD45RA and CCR7 shows whether TIL are naïve (N), central memory (CM), effector memory (EM), or terminally differentiated effector memory that re-express CD45RA (T_{EMRA}). **(C)** Comparison of CD27 and CD28 expression shows EM subsets of CD8$^+$ TIL grown in each culture condition. Representative dot plots for **B** and **C** are shown on the right.

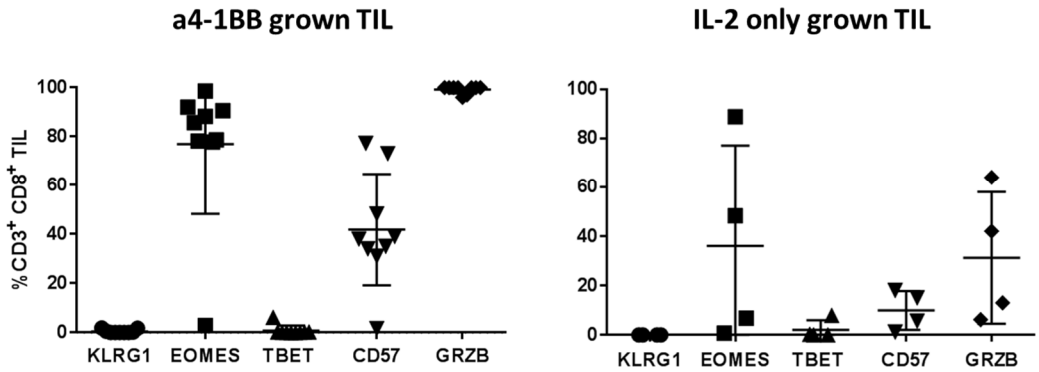

Figure 12. Addition of a4-1BB does not lead CD8$^+$ TIL into senescence.
Percent expression of phenotypic surface and intracellular markers (KLRG1, EOMES, TBET, CD57 and GRZB) for T-cell effector/memory status of CD8$^+$ TIL post-culture evaluated by flow cytometry.

3.2.5: Expansion of distinct CD8$^+$ T-cell clones favored by a4-1BB mAb compared to IL-2 alone

Next, we questioned how the culture conditions affected the repertoire of the TIL that grew out by sequencing the TCR of sorted CD8$^+$ T cells and comparing their relative frequencies to each other and to their starting frequency in the tumor (Figure 13). Many clones are shared (red lines) between the tumor and both TIL culture conditions. However, these clones are present at different frequencies between the conditions, as demonstrated by the position of the lines where further away from center denotes higher frequency. This shows that the addition of a4-1BB mAb favors expansion of unique clones as compared to IL-2 alone. This is further suggested by the presence of several T-cell clones that are shared between the tumor and only one of the culture conditions (blue lines). Finally, sequencing detected some clones that were not present in the tumor but were either present in only one culture (black lines) or shared only between the two culture conditions (green lines). Overall, in 4/5 patients, the addition of a4-1BB mAb in the culture expands a greater number of CD8$^+$ TIL but focuses their repertoire as evidenced by the smaller number of TIL clones in the a4-1BB cultures than the IL-2 alone cultures.

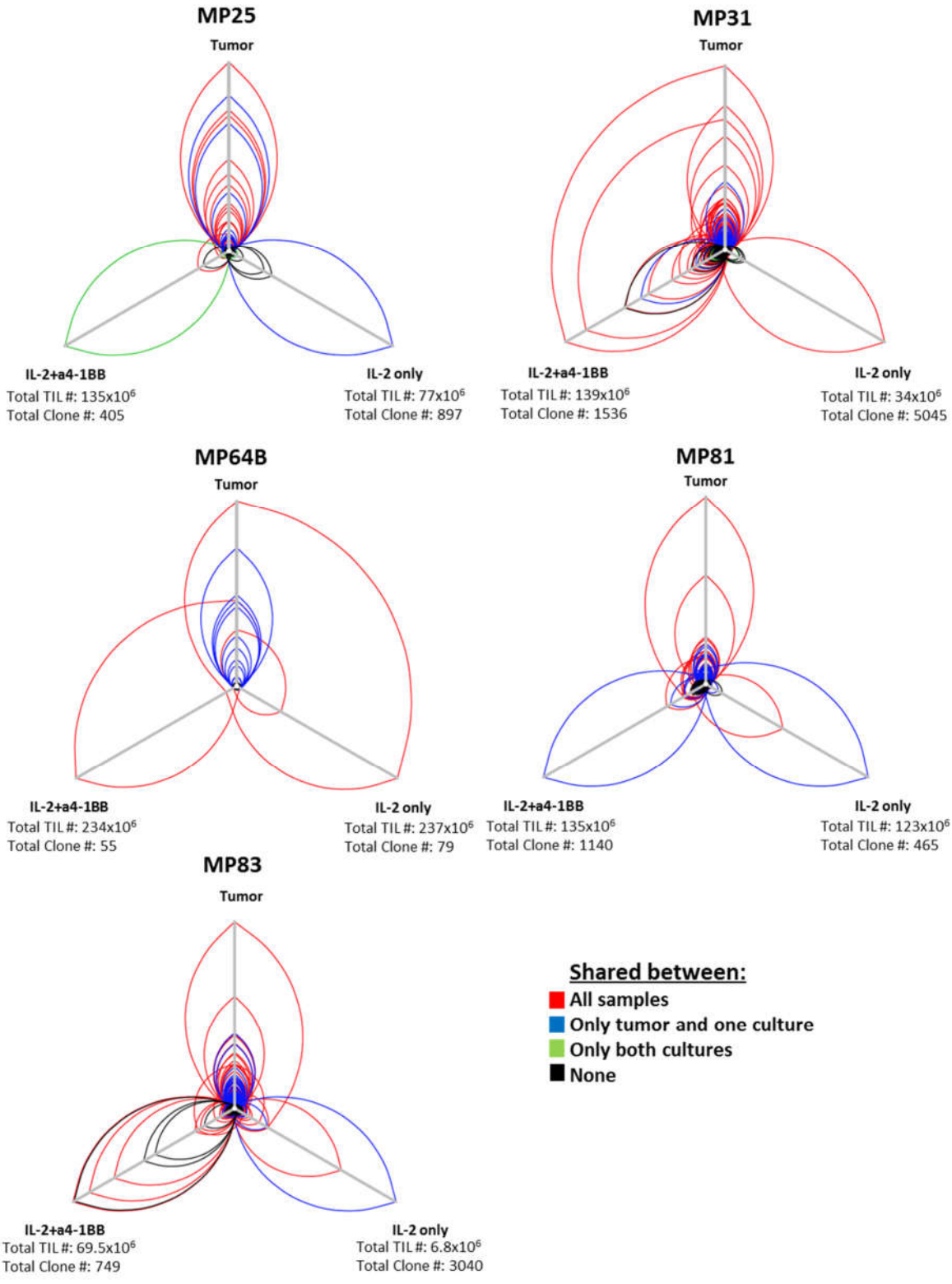
MP25
Tumor
IL-2+a4-1BB
Total TIL #: 135x10^6
Total Clone #: 405
IL-2 only
Total TIL #: 77x10^6
Total Clone #: 897
MP31
Tumor
IL-2+a4-1BB
Total TIL #: 139x10^6
Total Clone #: 1536
IL-2 only
Total TIL #: 34x10^6
Total Clone #: 5045
MP64B
Tumor
IL-2+a4-1BB
Total TIL #: 234x10^6
Total Clone #: 55
IL-2 only
Total TIL #: 237x10^6
Total Clone #: 79
MP81
Tumor
IL-2+a4-1BB
Total TIL #: 135x10^6
Total Clone #: 1140
IL-2 only
Total TIL #: 123x10^6
Total Clone #: 465
MP83
Tumor
IL-2+a4-1BB
Total TIL #: 69.5x10^6
Total Clone #: 749
IL-2 only
Total TIL #: 6.8x10^6
Total Clone #: 3040
Shared between:
All samples
Only tumor and one culture
Only both cultures
None

Figure 13. Distinct TCR repertoires are favored in each culture condition.
CDR3 sequencing of the TCR beta-chain was done on the blood, tumor and expanded TIL ($CD8^+$ sorted). Relative frequency in each sample is indicated along each axis of the hive plot with frequency increasing away from the center. Red lines indicate TCRB clones shared between all three samples, blue lines for those shared between one culture condition and the tumor, green for those shared only between the culture conditions and finally black lines indicate TCRB clones found in only one condition. Total TIL grown and number of unique clones in each condition are shown.

3.2.6: PDAC $CD8^+$ TIL recognize autologous tumor targets

Given the distinct repertoire generated by each culture condition, the anti-tumor potential of both IL-2 only and IL-2 + a4-1BB cultured PDAC $CD8^+$ TIL was assessed. To this end, sorted $CD8^+$ TIL lines from both conditions were rapidly expanded (REP) and co-cultured for 24 h with autologous tumor targets derived from patients MP81 and MP64B (Figure 14). Bulk TIL initially expanded using a4-1BB in combination with IL-2 were also put through the REP process and achieved the expected fold expansion (500x – 1500x) after 14 days, confirming that both sorted $CD8^+$ and bulk populations could exponentially grow after being propagated with a4-1BB (Figure 15). Prior to co-culture setup, autologous tumor cells and CAPAN-1, a HLA-mismatched pancreatic tumor line for MP81, were stained for MHCI expression (HLA-ABC) (Figure 16). Autologous tumor cells were found to express low, but detectable levels of MHCI as compared to the CAPAN-1 cells (MFI: MP81 467, MP64B 606 vs 5387 for CAPAN-1). In spite of this low level of MHCI expression, both IL-2 grown and a4-1BB grown MP81 TIL secreted more IFN-γ in the presence of the autologous target than with CAPAN-1 (Figure 14A). Upregulation of 4-1BB on $CD8^+$ T cells has been incorporated in tumor recognition assays previously [240, 250]. As such, both MP81 TIL lines significantly upregulated 4-1BB expression after exposure to the tumor target as compared to with the CAPAN-1 control Figure 14B and C). The upregulation of 4-

1BB was particularly high on the $CD56^+$ T-cell subset, a cytotoxic T-cell subset [251]. Given that the 4-1BB expression was higher in the $CD56^+$ $CD8^+$ MP81 TIL subset, we assessed MHCI-restricted recognition by blocking MHCI on its tumor target (Figure 14D and E, left panels). It was observed that most of the recognition in the total $CD8^+$ population was MHCI-restricted for both culture conditions. This experimental setup was repeated with an additional TIL and autologous tumor target (MP64B) with similar observations (Figure 14D and E, right panels). Taken together, the IFN-γ secretion and elevated 4-1BB expression indicate there are MHC class I-restricted tumor-reactive $CD8^+$ T cells in the PDAC TIL repertoire.

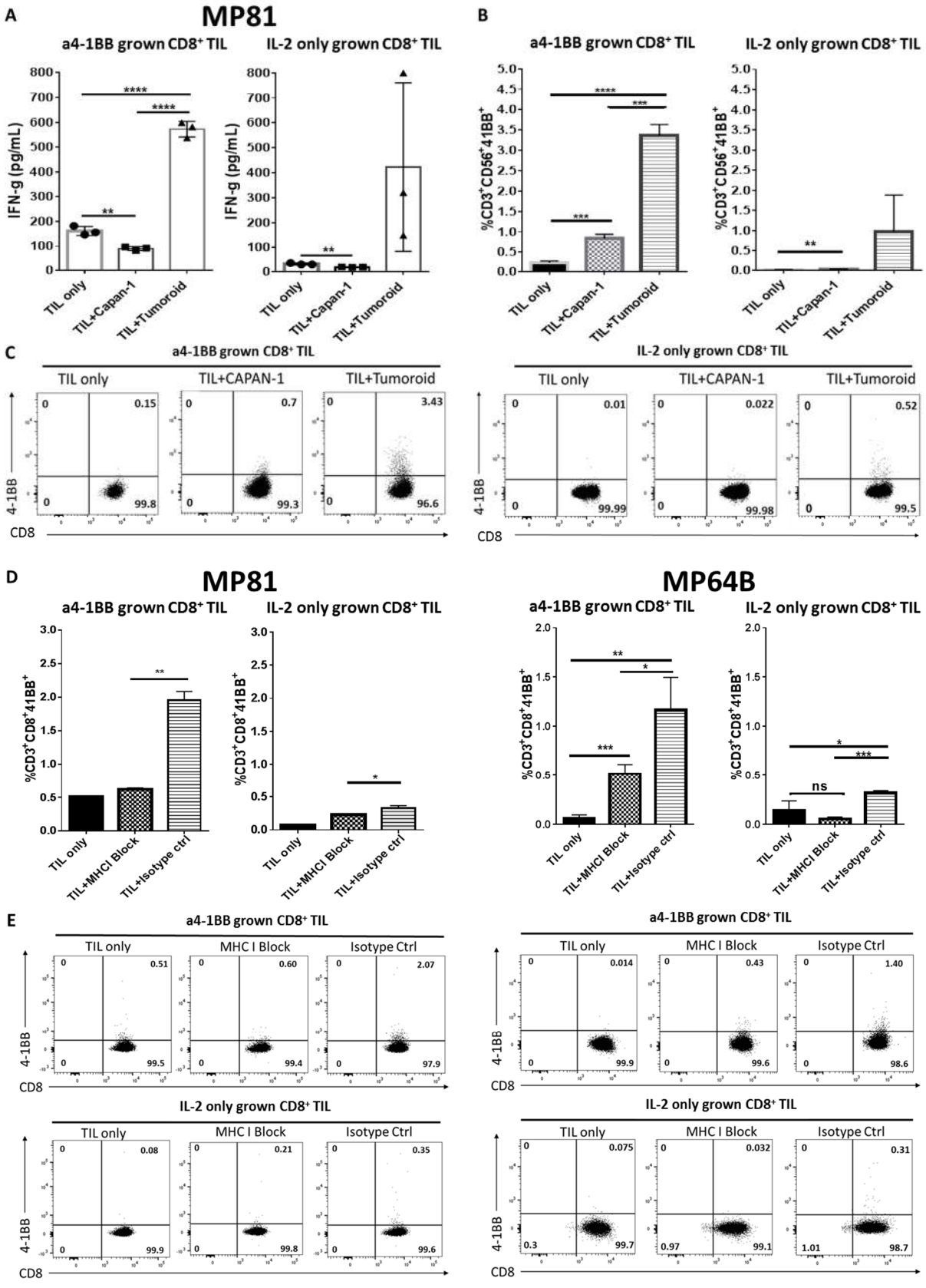
A
MP81
a4-1BB grown CD8+ TIL
IL-2 only grown CD8+ TIL
IFN-g (pg/mL)
TIL only
TIL+Capan-1
TIL+Tumoroid
B
%CD3+CD56+41BB+
C
TIL+CAPAN-1
4-1BB
CD8
D
MP64B
%CD3+CD8+41BB+
TIL+MHCI Block
TIL+Isotype ctrl
ns
E
MHC I Block
Isotype Ctrl

Figure 14. Reactivity of PDAC TIL to an autologous tumor target.
(A-C) Sorted MP81 $CD8^+$ TIL grown in IL-2+a4-1BB or IL-2 only were co-cultured for 24 h with autologous tumor, HLA-mismatched tumor line CAPAN-1, or media (TIL only). All conditions were in triplicate. **(A)** IFN-γ secretion was measured as well as **(B, C)** upregulation of 4-1BB on $CD56^+CD8^+$ TIL. Part **(B)** shows the compiled results of dot plot analysis of 4-1BB upregulation visualized by flow cytometry and **(C)** shows representative dot plots of 4-1BB upregulation. Results for a4-1BB grown TIL and IL-2 only grown TIL are from separate experiments. **(D, E)** Sorted MP81 $CD8^+$ TIL (left graphs) and **s**orted MP64B $CD8^+$ TIL (right graphs) grown in IL-2+a4-1BB or IL-2 only were co-cultured for 24 h with autologous tumor, pre-treated with MHC-I blocking antibody (W6/32) or isotype control (IgG2a), or media (TIL only). All conditions were in duplicate or triplicate. Part **(D)** shows the compiled results of dot plot analysis of 4-1BB upregulation visualized by flow cytometry for MP81 $CD8^+$ TIL (left graph) and MP64B $CD8^+$ TIL (right graph). **(E)** shows representative dot plots of 4-1BB upregulation.

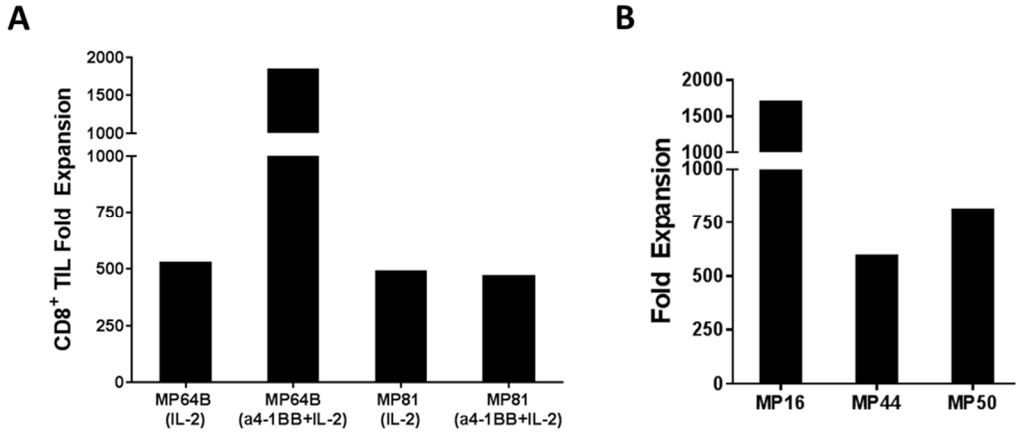

Figure 15. Fold expansion of bulk and CD8+ PDAC TIL following the REP.
(A) Sorted PDAC $CD8^+$ TIL (IL-2 alone or a4-1BB + IL-2) and **(B)** bulk a4-1BB stimulated PDAC TIL were rapidly expanded and their fold expansion calculated on Day 14.

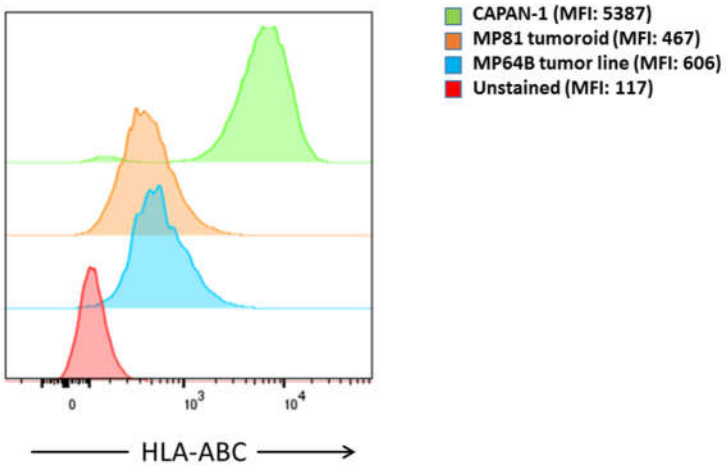

Figure 16. Level of MHC class I expression on PDAC tumor cell lines.
HLA-A,B,C expression was assessed using flow cytometry on autologous PDAC tumor cell lines as well as on a commercially available PDAC tumor cell line (CAPAN-1). Unstained tumor cells were used as a control (red histogram). The mean fluorescence intensity (MFI) of MHC class I expression for each line assessed is shown in the legend.

3.2.7: Further improvement of TIL culture process for PDAC with a 3-signal approach

As just shown above, the manipulation of 4-1BB through agonistic stimulation (Urelumab, BMS), increased $CD8^+$ TIL proliferation and improved culture success in pancreatic cancer. Additional work to further improve the TIL culture method was performed by our group which led to the development of a new methodology utilizing an anti-CD3 antibody (OKT3) in the early TIL culture as well as transitioning to TIL culture in a gas-permeable culture flask (GREX) to expand melanoma TIL [218]. The application of this novel strategy, named TIL 3.0, to PDAC TIL culture led to the expansion of a greater number of $CD3^+$ TIL ($200x10^6$ average) compared to IL-2 only and IL-2+a41BB culture methods (Figure 17A). This increase in $CD3^+$ TIL number is characterized by a consistent maintenance of the high percentage of $CD8^+$ TIL (Figure 17B) and low percentage of $CD4^+$ TIL (Figure 17C). The TIL 3.0 method also consistently reduced the time of culture from a

period of 3-5 weeks for IL-2 only and IL-2+a41BB to 2-3 weeks (Figure 17D). Finally, TIL 3.0 drastically increased the overall success rate of establishing an PDAC TIL culture from 29% (10/34) for IL-2 only and 48% (16/33) for IL-2+a41BB to 95% (19/20) for the IL-2+OKT3+a41BB method (Figure 17E). The benchmark for a successful TIL culture, $12x10^6$ total cells, was established from scaling down the MDACC Clinical Melanoma TIL Lab's criterion for success where 20 fragments are set up for TIL expansion and $40x10^6$ cells is considered the minimum to treat a patient [252].

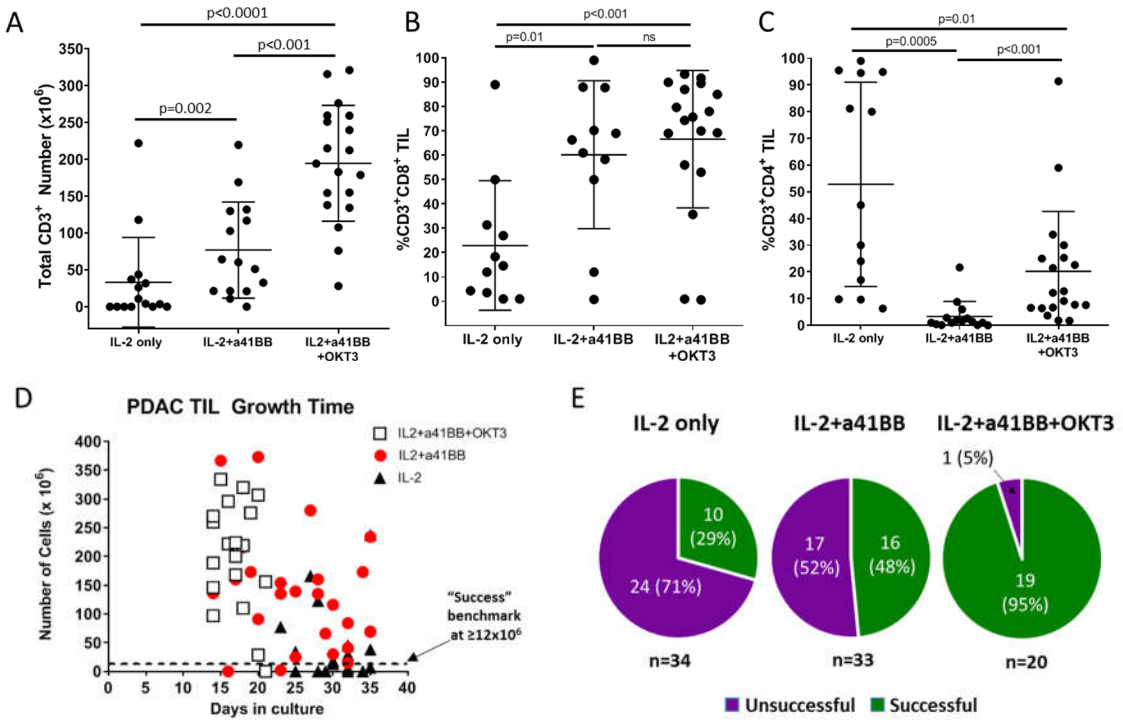

Figure 17. Characterization of PDAC TIL growth across culture conditions.
Comparison of the (**A**) total $CD3^+$ TIL number, (**B**) percentage of $CD3^+CD8^+$ TIL, and (**C**) percentage of $CD3^+CD4^+$ TIL generated between the different culture methods. Comparison between culture methods of the (**D**) time of culture and (**E**) success rate of growth for all attempted cultures.

3.2.8 Synchronous stimulation of 4-1BB and CD3 prevents CD8$^+$ TIL over differentiation

To understand how the different culture methods affected the CD8$^+$ TIL, the expression of CD28, BTLA, and PD-1 were explored as surrogates for T-cell activation and differentiation (Figure 18). These 3 markers were strategically selected for the power of assessment they provide individually as well as together. BTLA$^+$CD8$^+$ TIL have been reported by our group to be less-differentiated cells, capable of prolong persistence and serial killer capacities [188, 189]. Expression of CD28 is also a trait of lesser differentiation, while the expression of PD1 is increased with chronic antigen exposure and exhaustion, thus signifying later stage of differentiation [249, 253]. When comparing expression of the 3 markers on PDAC TIL expanded with IL-2 only or IL-2+a41BB, no significant difference was observed. However, CD8$^+$ TIL grown with the TIL 3.0 method were comprised of a significantly greater percentage of cells expressing BTLA and CD28, and a significantly smaller percentage of CD8$^+$ TIL expressing PD-1, altogether suggesting a less differentiated profile (Figure 18).

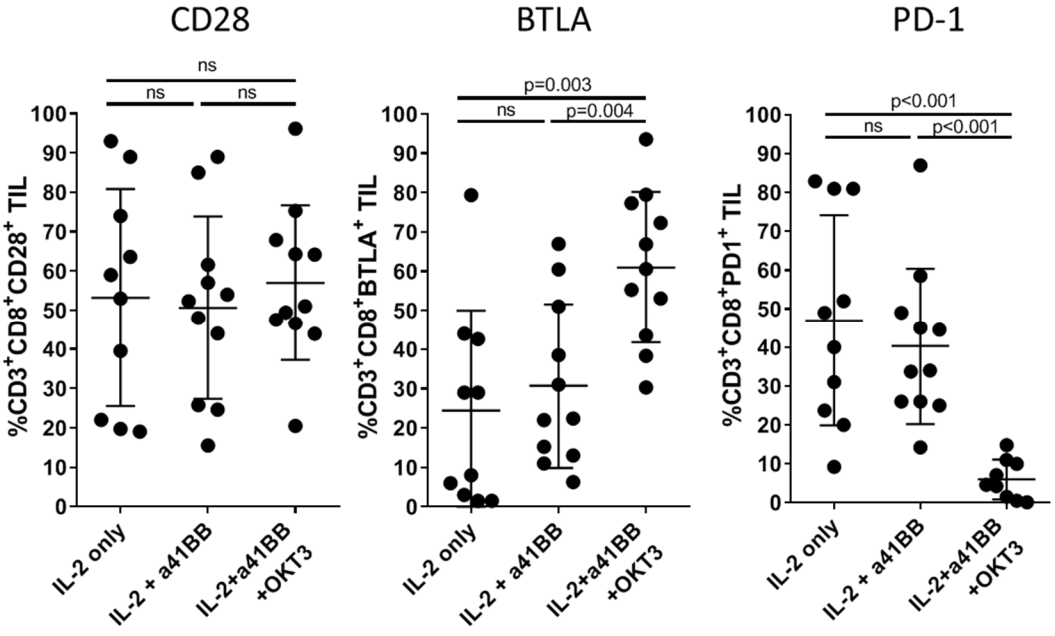

Figure 18. Phenotyping of grown PDAC TIL across culture conditions.
Comparison between the different culture methods of the percentage of CD28, BTLA, and PD-1 expressing $CD8^+$ TIL generated.

3.3: Summary

In this chapter, it was shown that PDAC has a scarce, yet activated $CD8^+$ TIL infiltrate that is preferentially expanded with the addition of an agonistic 4-1BB mAb to the TIL culture. The a4-1BB mAb consistently augmented total TIL numbers and doubled the success rate of TIL growth without overly differentiating them in spite of the aggressive proliferation spurred by 4-1BB costimulation. Finally, despite the fact that the a4-1BB mAb favored expansion of distinct $CD8^+$ T-cell clones from the tumor in comparison to IL-2 alone, TIL derived with either culture condition showed tumor recognition via IFN-γ secretion and 4-1BB upregulation. While only two TIL lines and paired autologous tumor targets were able to be tested, it was observed that there was a higher frequency of anti-tumor reactive $CD8^+$ TIL in the a4-1BB grown cultures compared with TIL grown in IL-2 alone. Further improvements to the culture process by the addition of an agonistic 4-1BB mAb and OKT3, a novel 3-signal approach, increased the ability to grow TIL from PDAC, improves the total yield, and stimulates the proliferation of activated $CD8^+$ T cells. Using the TIL 3.0 methodology to grow PDAC TIL brought about several major improvements; 1) reduce the manufacturing time, 2) increase the success rate to over 90%, 3) require less tumor tissue (only 5 tumor fragments, or the equivalent of 2 core needle biopsies). In the context of a clinical trial, these improvements would make it possible to grow enough TIL to treat over 90% of the enrolled patients with a product consistently high in CD8 content. These results suggest that the a4-1BB mAb can facilitate TIL ACT for PDAC by increasing the final yield of the desirable, anti-tumor $CD8^+$ T cells clones present in PDAC.

Chapter 4: Heterogeneity of Pancreatic Cancer Tumor-Infiltrating Lymphocytes at the Single-Cell Level

4.1: Introduction

While exploring the TCR repertoire in PDAC in the previous chapter, high frequency T-cell clones overlapping between the tumor and uninvolved-tissue were observed, potentially suggesting the presence of tissue-resident memory T cells (T_{RM}). T_{RM} are a unique population of T cells that don't recirculate between the tissue and periphery, and their importance has only been elucidated within the past decade [254]. Instead, they are small population of memory T cells that take up residence in peripheral tissues (e.g. skin, lungs, GI tract) sites after trafficking there as a part of an immune response. By doing so, they can function as a rapid response arm of the adaptive immune system to quickly engage any re-challenge. After pathogen clearance, signals in the local tissue environment, such as TGF-β or IL-15, help promote a change in transcriptional programming that results in engraftment of long-lived T_{RM} population [255]. This T_{RM} profile is typically characterized by upregulation of genes associated with tissue retention (CD103, CD69, CD49a/ITGA1) and longevity (Bcl-2, CD127/IL7Ra) coupled with downregulation of genes associated with tissue egress (S1PR1/5, L-selectin/CD62L, CCR7, KLF2) [256].

The role of T_{RM} was first appreciated in the context of infection as they were found to mediate a novel mechanism of immunity that involved non-migratory T cells in barrier tissues [257-259]. However, in recent years the importance of T_{RM} in cancer immunity has also been uncovered [260]. Several reports have demonstrated that the presence of T_{RM}, particularly $CD103^+CD8^+$ T_{RM}, is correlated with improved survival in several epithelial cancers [261-267]. The reason this correlation has been found predominantly in epithelial

cancers is due to the fact that epithelial tissues express TGF-β, which induces CD103 expression on T cells, and the only known ligand for CD103 is the epithelial-associated protein E-cadherin and is expressed on epithelial cells [260, 268]. The interaction between CD103 on T cells and E-cadherin on tumor cells could play an important role in tumor control as *in vitro* studies have shown that it is important for immune synapse formation and subsequent release of cytolytic granules [269, 270]. Additionally, these T_{RM} cells have also been shown to harbor increased cytotoxicity, be composed of the tumor-reactive $CD8^+$ TIL, and have higher expression of checkpoint markers [261, 262, 264, 271-273]. In pancreatic cancer, an *in vitro* study showed that $CD103^+CD8^+$ T cells more efficiently adhered to E-cadherin expressing tumor cells, thus allowing for increased cytolysis [274]. Given the emerging importance of T_{RM} in tumor control as reported in the literature as well as the hint at their presence in our own study, we wanted to further explore T_{RM} in PDAC.

To capture the breadth of the T_{RM} phenotype in PDAC, we turned to multi-parameter flow cytometry and single-cell RNA-sequencing (scRNAseq) for their ability to provide a detailed understanding of diverse cell populations. scRNAseq in particular has emerged as a powerful tool for immunological studies given the extreme heterogeneity of immune cells. T cells are no exception to this, as their heterogeneity is governed by not only various transcriptomic profiles that direct their development and differentiation, but also the diversity of their TCRs. scRNAseq provides the means to asses a complex, and yet-to-be-fully-defined, cell population like T_{RM} cells in an unbiased manner. Several studies have been published in the last few years showing the power of scRNAseq to identify novel T-cell phenotypes in different cancers [262, 275-280]. This approach is particularly attractive for exploring T_{RM} in PDAC due to the lack of a defined phenotype in this cancer type as well as

PDAC's overall low-level of immune infiltration. Therefore, we sought to explore the PDAC T_{RM} population by combining high-order flow cytometry, scRNA transcriptomic and TCR sequencing.

4.2: Results

4.2.1 Exploring tissue-resident memory T cells in PDAC via CD103 expression

To initially determine the presence of T_{RM} in PDAC, a subset of primary PDAC samples (n=10) were analyzed using multi-parameter flow cytometry (Figure 19). In the literature, the T_{RM} population is typically characterized by expression of integrin α_E (ITGAE, also known as CD103), which is a well-characterized marker for T_{RM} cells, as well expression of several activation and checkpoint markers (CTLA4, PD-1, LAG3, TIM3 ICOS, and Ki67). The majority of CD103 expression was found on $CD8^+$ TIL with almost no expression found on $CD4^+$ TIL in freshly disaggregated primary tumor tissue (Figure 19A and 19B). This is similar previously published data in OvCa showing that the $CD103^+$ T_{RM} population is mainly within the $CD8^+$ TIL compartment [266]. To better understand the relative abundance of CD103 expression in PDAC, OvCa TIL were used as a point of comparison due to the reported presence of $CD103^+$ T_{RM} in the literature (Figure 19C) [261]. Based on the percentage of $CD103^+$ expression, PDAC was found to have about half as much T_{RM} on average as OvCa (21% vs. 45% respectively). The $CD8^+$ TIL showed a large fraction of PD-1^+ and $Ki67^+$ cells compared to the other markers (Figure 19A). To determine if these markers were preferentially co-expressed with CD103, the differential expression between $CD103^+$ and $CD103^-$ $CD8^+$ TIL was explored (Figure 19D). Compared to $CD103^-CD8^+$ TIL (non-T_{RM}), the CD8 T_{RM} population showed only a trend for higher PD-1expression but nothing statistically significant.

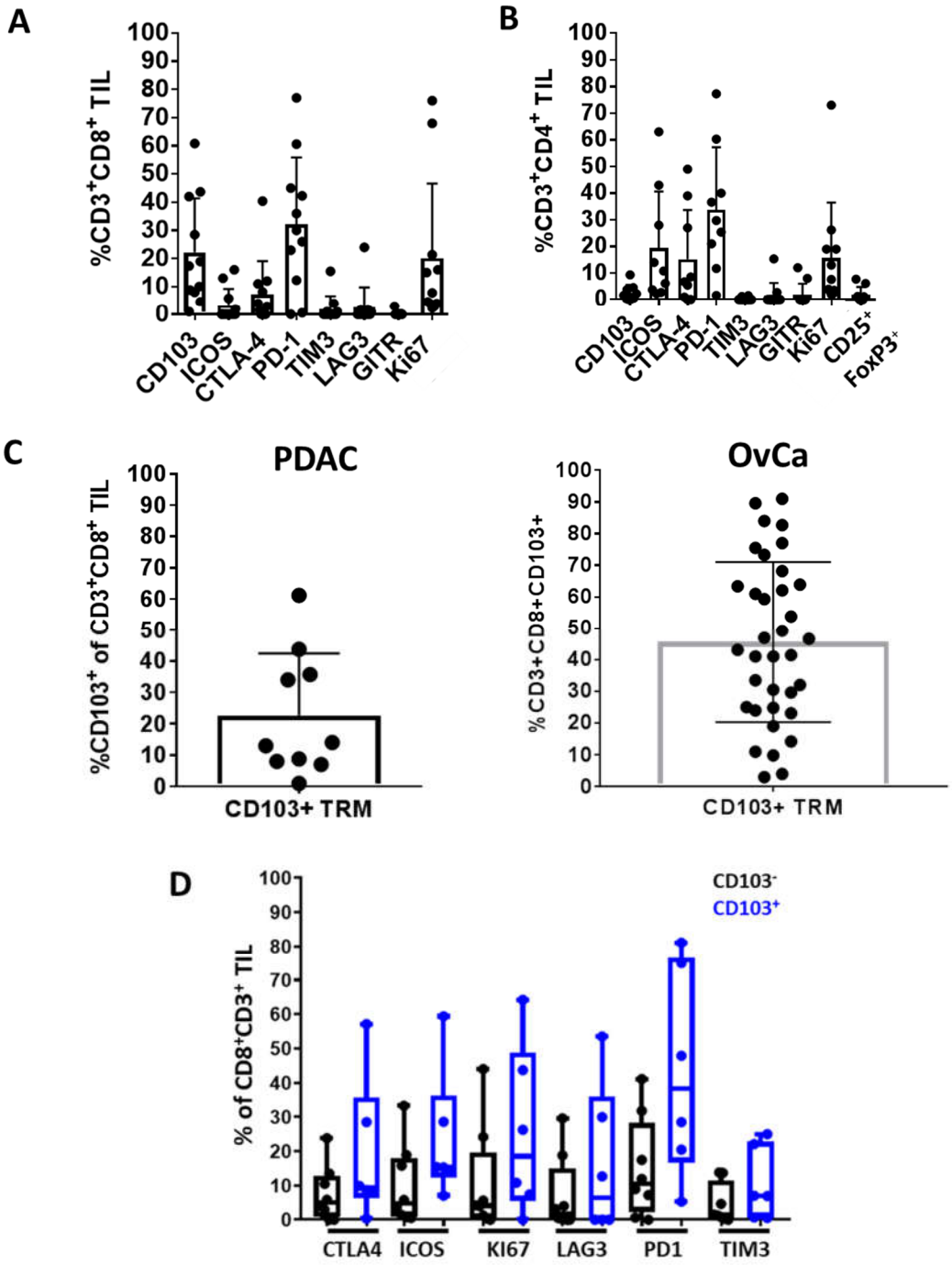

Figure 19. Flow cytometry analysis of tissue-resident memory T cells in PDAC. Expression of the T_{RM} marker CD103 and several activation/inhibitory markers on **(A)** $CD8^+$ TIL and **(B)** $CD4^+$ TIL. **(C)** Comparison of the $CD8^+CD103^+$ TIL fraction in PDAC and OvCa. **(D)** Comparison of activation and inhibitory markers on $CD8^+CD103^+$ or $CD8^+CD103^-$ TIL.

4.2.2 Transcriptomic characterization of TIL isolated from primary pancreatic tumors

The lower proportion of of T_{RM} based on CD103 expression in PDAC could be due to the fact that it reportedly marks this T cell population mainly in barrier or mucosal tissues (such as found in OvCa). Moreover, the literature indicates that T_{RM} do not always express CD103 [281]. This fact coupled with the self-limitation of flow cytometry panel using a small number of selected markers prompted us to turn to an unbiased and more comprehensive approach to elucidate the phenotype of T_{RM} in PDAC. To interrogate this phenotype in an unbiased manner, single-cell RNA sequencing was performed on 1,616 $CD3^+$ TIL (922 $CD8^+$ and 694 $CD4^+$) freshly isolated from 6 primary PDAC samples (Figure 20). The patient characteristics for sequenced samples are presented in Table 3.

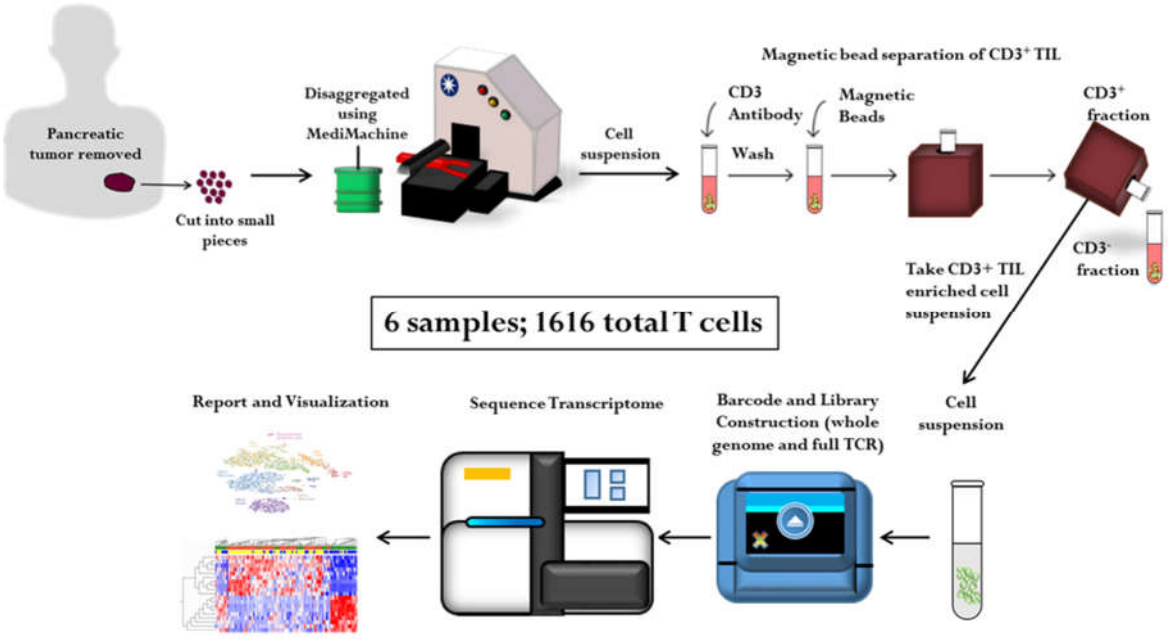

Figure 20. Schematic of sample processing for single-cell RNA sequencing.

Table 3. Patient characteristics for sequenced tumor samples.

Patient	Location in Pancreas	Age	Gender	Prior Treatment
MP108	Head	71	M	Treatment naïve
MP109	Tail	68	M	Pembrolizumab, Vitamin D, and Gemcitabine/Abraxane
MP112	Head	70	M	Gemcitabine/Abraxane
MP115	Body/tail	67	M	Gemcitabine
MP120	head	76	F	Gemcitabine/Abraxane, Xeloda, Radiation
MP121	head	73	M	FOLFIRINOX, Radiation

After sample processing, the data was clustered unbiasedly using t-distributed stochastic neighbor embedding (t-SNE) in collaboration with Aislyn Schalck in Dr. Nick Navin's lab (Figure 21 and 22). Four $CD8^+$ TIL clusters (CD8_C1 EOMES, CD8_C2 ZNF683, CD8_C3 GZMB, and CD8_C4 STAT1) and 3 distinct $CD4^+$ TIL clusters (CD4_C1 T_{CM}, CD4_C2 Treg, and CD4_C3 T_{EM}) were identified (Figure 21A and 22A). While 3/6 of the samples provided the majority of the cells, the relative proportions of each cluster type within each sample were similar to each other (Figures 21B and 22B). This indicated that the clusters were comparable across the 6 samples.

When focusing on the $CD8^+$ clusters, the CD8 TIL seems to be composed of several activated or cytotoxic populations based on expression of several markers (Figure 21C). CD8_C1 has expression of several MHC Class II alleles plus *GZMK*, which are markers of T cell activation. CD8_C2 has high expression of *CD69*, a well-known activation marker. CD8_C3 appears to be a cytotoxic effector cell population based on expression of *GZMB*, *GNLY*, *PRF1*, and *NKG7*. Finally, CD8_C4 has high expression of Type I interferon (IFN-I) response genes (*IFIT1, IFIT3, IRF7, STAT1*), suggesting exposure to IFN-1 which has been shown directly and indirectly promote activation and proliferation of $CD8^+$ T cells. As

hypothesized, based on the low CD103 expression found by flow cytometry, no T_{RM} cluster defined by CD103 appeared within the CD8 population. Instead, we found a potential T_{RM} cluster that was defined by expression of *ZNF683* and *CD69* (Figure 21C). While *CD69* is primarily thought of as a marker of activation, it has also been found to mark T_{RM} T cells. More importantly, however, is the expression of the transcription factor *ZNF683* (also known as Hobit, or homolog of Blimp-1 in T cells) which is reported to be important for T_{RM} formation. Furthermore, high expression of *CD69* and *ZNF683* was coupled with low expression of *KLF2*, *S1PR5*, and *ZEB2* (Figure 21C). This expression profile is similar to that described for T_{RM} cells in the literature [255, 278, 282]. Finally, expression of checkpoint molecules *PD-1* or *TIM3* were not associated with the T_{RM}, which is in contrast to published observations from other cancer types.

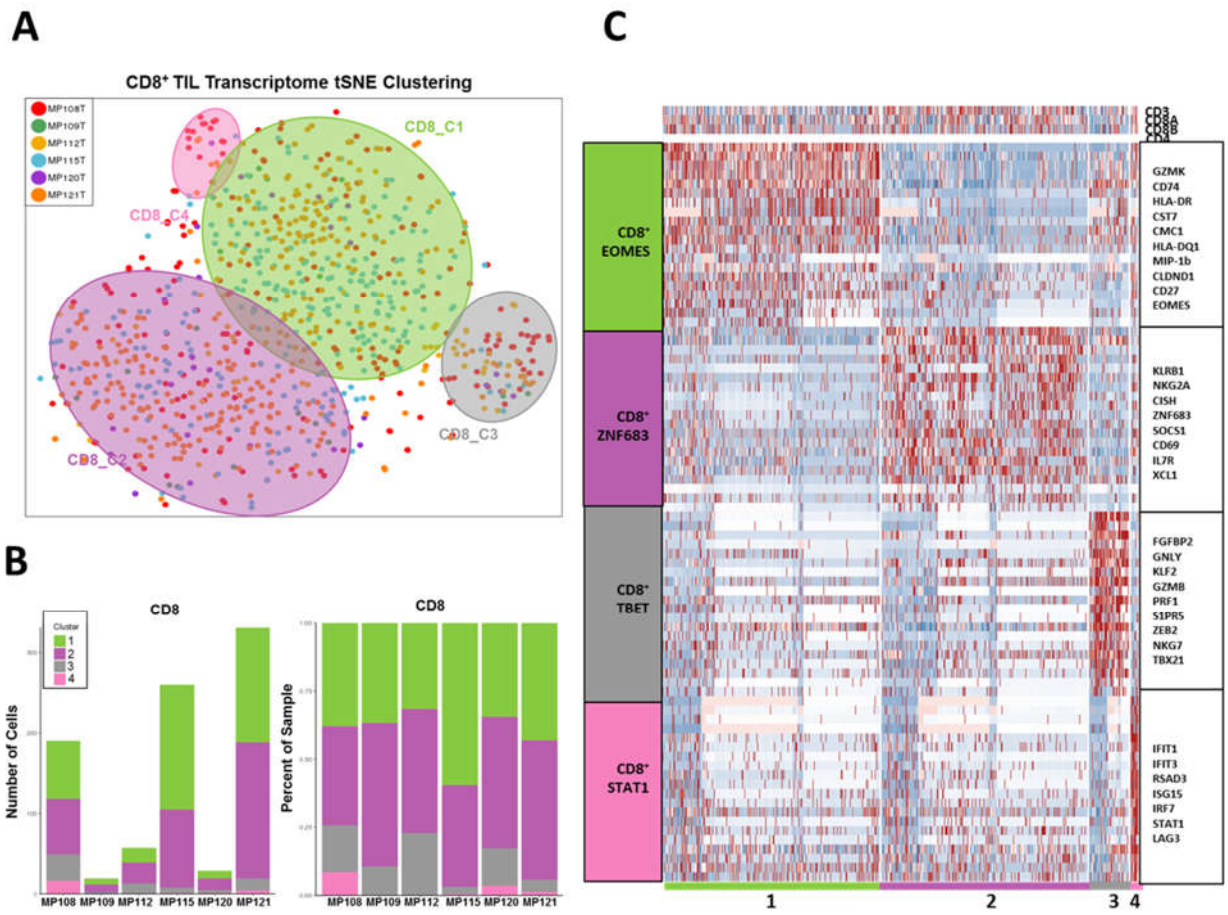

Figure 21. Characteristics of CD8⁺ TIL populations in primary PDAC
(A) t-SNE plot of 922 CD8⁺ TIL colored by patient and demarcated by colored circles indicating four distinct clusters based on gene expression differences. **(B)** Bar graphs showing the cell number contributed by each sample to a cluster (left) and its relative proportion (right) **(C)** Heatmap from single-cell RNAseq unsupervised clustering analysis defines 4 clusters as indicated by colored bars along the x-axis. Figure generated in collaboration with Aislyn Schalck and Dr. Nick Navin and used with their permission.

Within the CD4 population, CD4_C1 surprisingly had expression of *CCR7* and *TCF7* which are indicative of a central memory T cell population (Figure 22C). Given that T_{CM} are not frequently found at tumor sites, this population is likely contamination from circulating CD4 T cells in the blood that may have been passing through the tumor. While Tregs were not detected at a high level by flow cytometry (Figure 19B), the CD4_C2 population appears

to be a robust and activated Treg population based on expression of the classical Treg markers *FOXP3* and *CD25* as well as the activation markers *GITR*, *OX40*, *TIGIT*, *CD39*, and *4-1BB.* Interestingly, the Treg population also had high expression of *IL-32*, typically known as a pro-inflammatory cytokine. Finally, CD4_C3 contained an activated Th1 CD4 subset as evidenced by expression of *CD40LG*, *CD69*, and *IL-2* (Figure 22C). In addition, the cells from this cluster may harbor some effector cell functions based on expression of *granzyme A* and *K*.

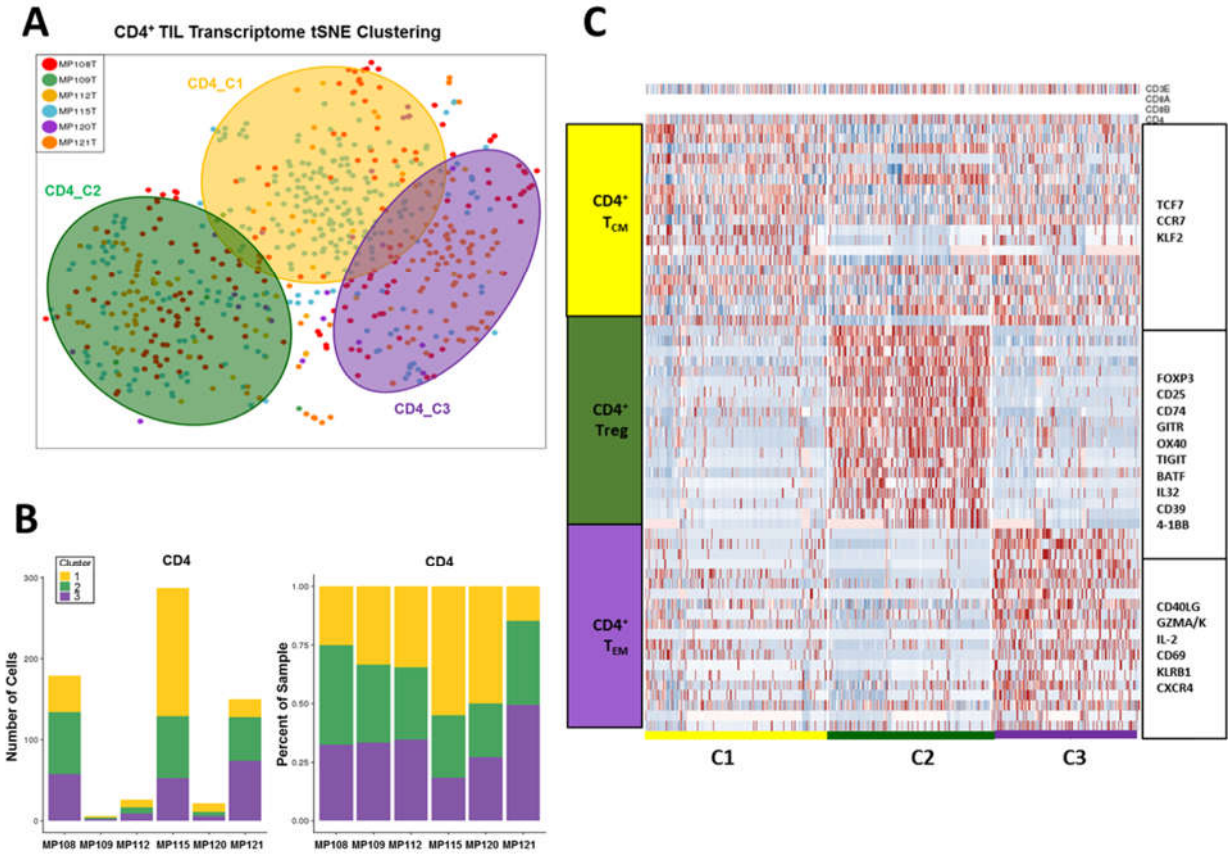

Figure 22. Unsupervised clustering of CD4$^+$ single cell transcriptomes.
(A) t-SNE plot of 694 CD4$^+$ TIL colored by patient and demarcated by colored circles indicating three distinct clusters based on gene expression differences. **(B)** Bar graphs showing the cell number contributed by each sample for each cluster (left) and its relative proportion (right) **(C)** Heatmap from single-cell RNAseq unsupervised clustering analysis reveals 3 clusters as indicated by colored bars along the x-axis. Figure generated in collaboration with Aislyn Schalck and Dr. Nick Navin and used with their permission.

4.2.3 Characterization of TIL clonal repertoire in the tissue

In addition to transcriptomic sequencing of the TIL, single-cell TCR sequencing was performed in order to compare the frequency of clonal populations with the transcriptomic profile they have. Enrichment of a certain clonal population at a tumor site in indicative of that population participating in an immune response. To that end, Circos plots were generated for the CD8 and $CD4^+$ TIL separately (Figure 23 and 24). The inner ring is color-coded by sample, the next ring is color-coded by transcriptomic cluster, and the grey outer ring contains bars that indicate the relative frequency of that TCR clone. The $CD8^+$ TIL Circo indicates that the clones that are enriched at the tumor site (found in numerous copies, delineated by the tall grey bars on the outer circle) mainly come from clusters CD8_C1 EOMES and CD8_C2 ZNF683, while the other two clusters show little to no enrichment of clones within them (Figure 23). On the other hand, the $CD4^+$ TIL Circo plot shows little clonal expansion of clones overall (Figure 24). Of the clones that show some measure of expansion, the majority are in the CD4_C2 Treg cluster followed by a few in the CD4_C3 T_{EM} cluster. While only one or two samples seem to have most of the clonal CD8 expansion detected, the CD4 clonal expansion appears to be spread more evenly among the samples.

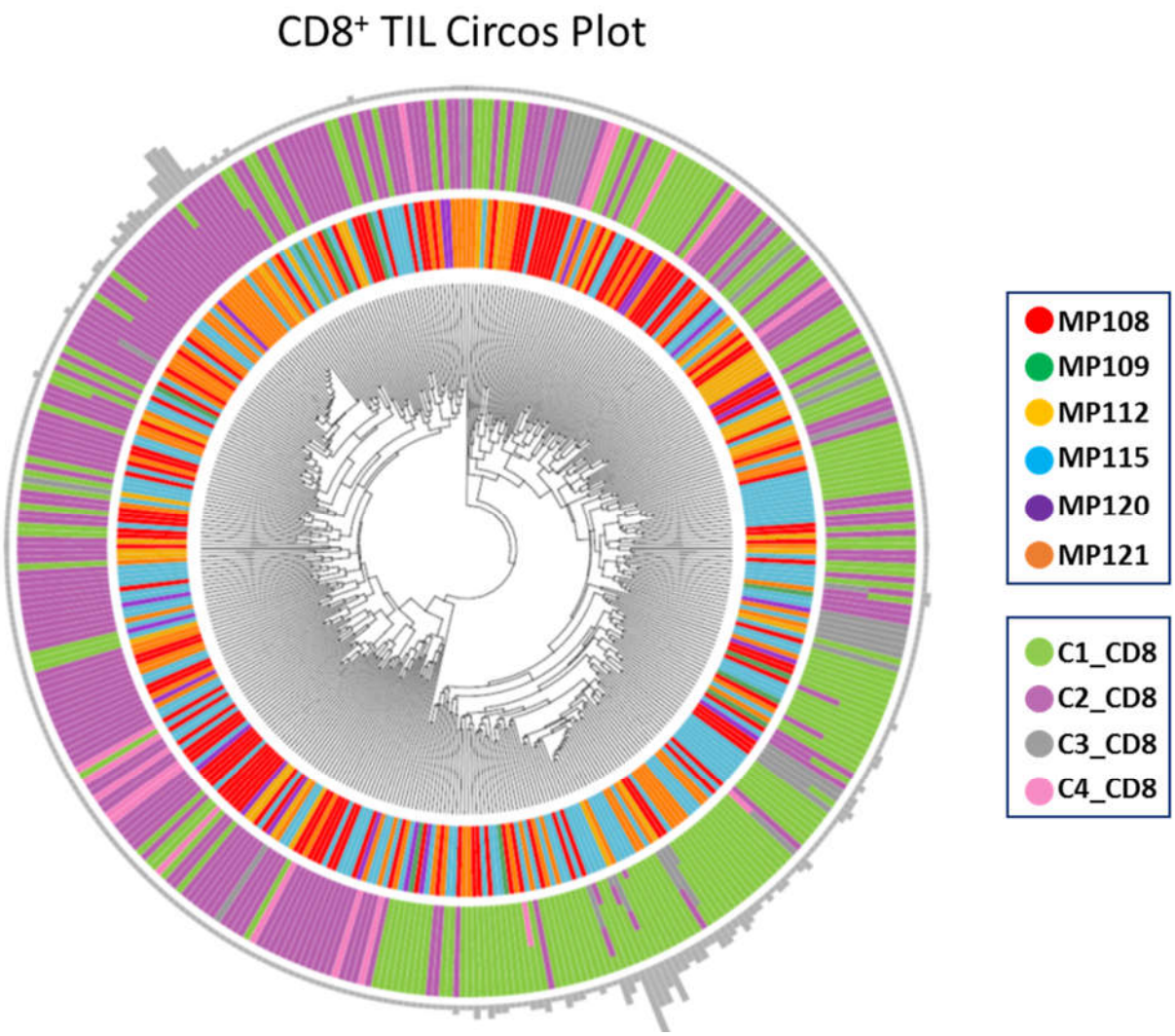

Figure 23. CD8$^+$ TIL Circos plot combining transcriptomic profile with TCR frequency. The inner ring is color-coded by sample, the next ring is color-coded by transcriptomic cluster, and the outer ring contains bars that indicate the relative frequency of that TCR clone. Figure generated in collaboration with Aislyn Schalck and Dr. Nick Navin and used with their permission.

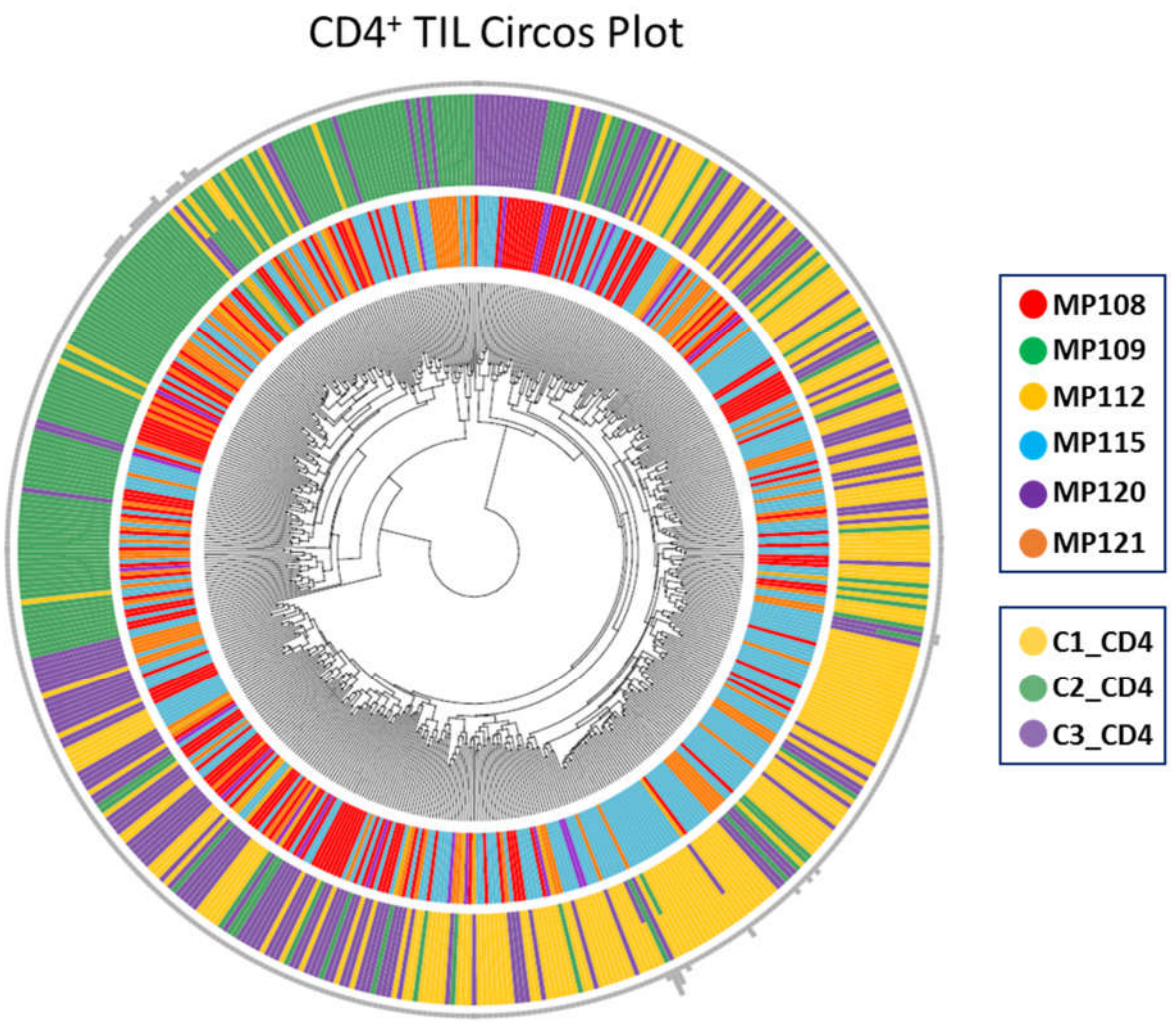

Figure 24. CD4$^+$ TIL Circos plot combining transcriptomic profile with TCR frequency. The inner ring is color-coded by sample, the next ring is color-coded by transcriptomic cluster, and the outer ring contains bars that indicate the relative frequency of that TCR clone. Figure generated in collaboration with Aislyn Schalck and Dr. Nick Navin and used with their permission.

4.2.4 TIL 3.0 produces a large proportion of cytotoxic and proliferative CD8+ TIL

To better understand the effect of the TIL 3.0 culture method on the TIL generated, expanded TIL products from the same tissues that were initially sequenced were also sent for transcriptomic and TCR scRNAseq (MP108, MP109, MP112, MP115, MP116, and MP121; Figure 25A). Initial tSNE anlaysis revealed 7 populations, which were classified based on unsupervised heatmap clustering: C1_CD4-OX40, C2_CD8-GZMB, C3_CD3-CYCLING, C4_CD3-DYING, C5_γδ-TCELL, C6_CD4-MHCII, and C7_CD8-HSP (Figure 25B and 25C). Based on the tSNE clustering, the major populations present were a cytotoxic CD8 cluster (CD8_GZMB) defined by expression of *GZMB* and *PRF1*, and a proliferative T cell population (CD3_CYCLING) defined by expression of cell-cycle related genes like *KI67, TUBB,* and *TOP2A* (Figure 25C). Furthermore, it is apparent that the proliferating cell population is mainly coming from the cytotoxic CD8 cluster based on proximity and shape of the CD8-GZMB and CD3-CYCLING clusters (Figure 25B). The fact that these are the two main populations meshes well with the previous observation that TIL 3.0 produces a large TIL product that consists mainly of activated $CD8^+$ TIL (Figure 17 and 18). In addition to the major activated CD8-GZMB cluster, a smaller CD8 cluster (C7_CD8-HSP) was found that is curiously characterized by expression of several heat-shock proteins (HSPs) which are chaperone proteins known for promoting cell survival under stress conditions.

While the majority of TIL produced by TIL 3.0 are CD8+ T cells, there were also two small clusters of activated of CD4+ TIL (C1_CD4-OX40 and C6_CD4-MHCII) as well as a γδ T cell cluster (C5_γδ-TCELL) (Figure 25B). The larger of the CD4 clusters (C1_CD4) had, surprisingly, an activated phenotype as defined by expression of *CD40LG, TNFRSF4* (OX40), and *TNFRSF18* (GITR) (Figure 25C). The minor CD4 cluster (C6_CD4) was

defined by high expression of several MHCII alleles as well as expression of *IL5* and *IL13*. Since γδ T cells can be stimulated by a41BB, a small component of these TIL were detected in the TIL products. This γδ T cell cluster, C5_γδ-TCELL, was defined chiefly by expression of *TRDC* (γδ TCR) but also had expression of genes associated with activation like *GNLY, CCL3* (MIP1a)*, XCL1,* and *XCL2*. Finally, a cluster of cells was detected (C4_CD3-DYING) that had increased expression of mitochondrial genes, which indicates they are likely undergoing apoptosis.

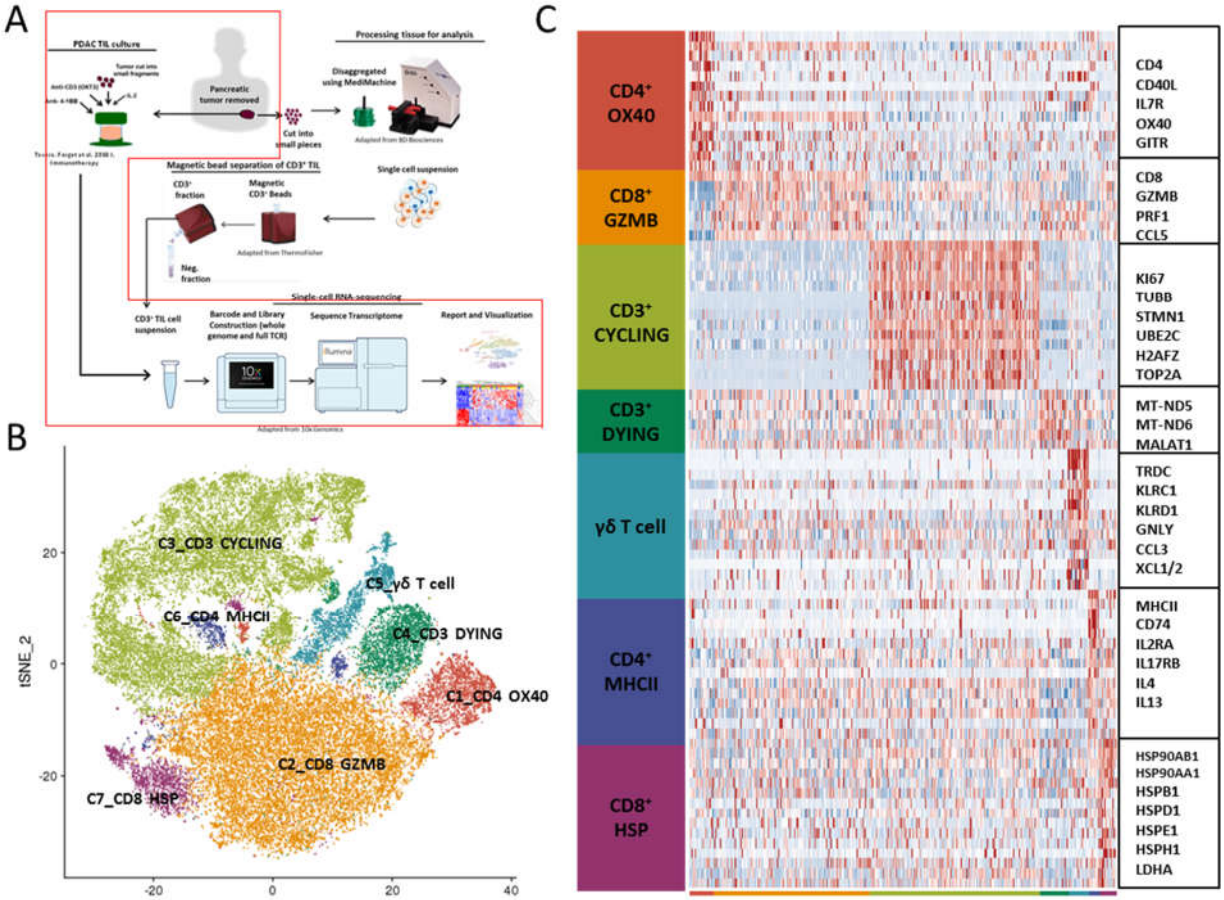

Figure 25. Transcriptomic scRNAseq of grown PDAC TIL.

(A) Tissue from the same samples that were sent for scRNAseq of fresh TIL were used to generate a TIL product using the TIL 3.0 protocol. **t**SNE clustering **(B)** and unsupervised clustering **(C)** were generated from the transcriptomic data, revealing 7 transcriptomic clusters. Figure generated in collaboration with Aislyn Schalck and Dr. Nick Navin and used with their permission.

4.2.5 TIL 3.0 maintains high frequency clones from activated TIL populations

Previous work presented in Chapter 3 Section 3.2.5 looked at the frequency of clones in the fresh tumor in comparison to their frequency in the TIL product after culture with IL-2+a41BB. This TCR sequencing analysis revealed that high frequency clonotypes in the tissue did not necessarily expand to a proportionally high frequency in the in the TIL product and *vice versa*. To explore how this relationship might change with TIL 3.0, which now includes an additional stimulation with OKT3, the frequency of clonotypes in the fresh tumor (Fresh TIL, black) and TIL product (Grown TIL, blue) were compared (Figure 26). Analysis of sequencing data found TIL 3.0 is able to maintain the original frequency of clones found in the tumor tissue. In other words, if a clonotype was at a high frequency in the tissue it was also a high proportion of the grown TIL.

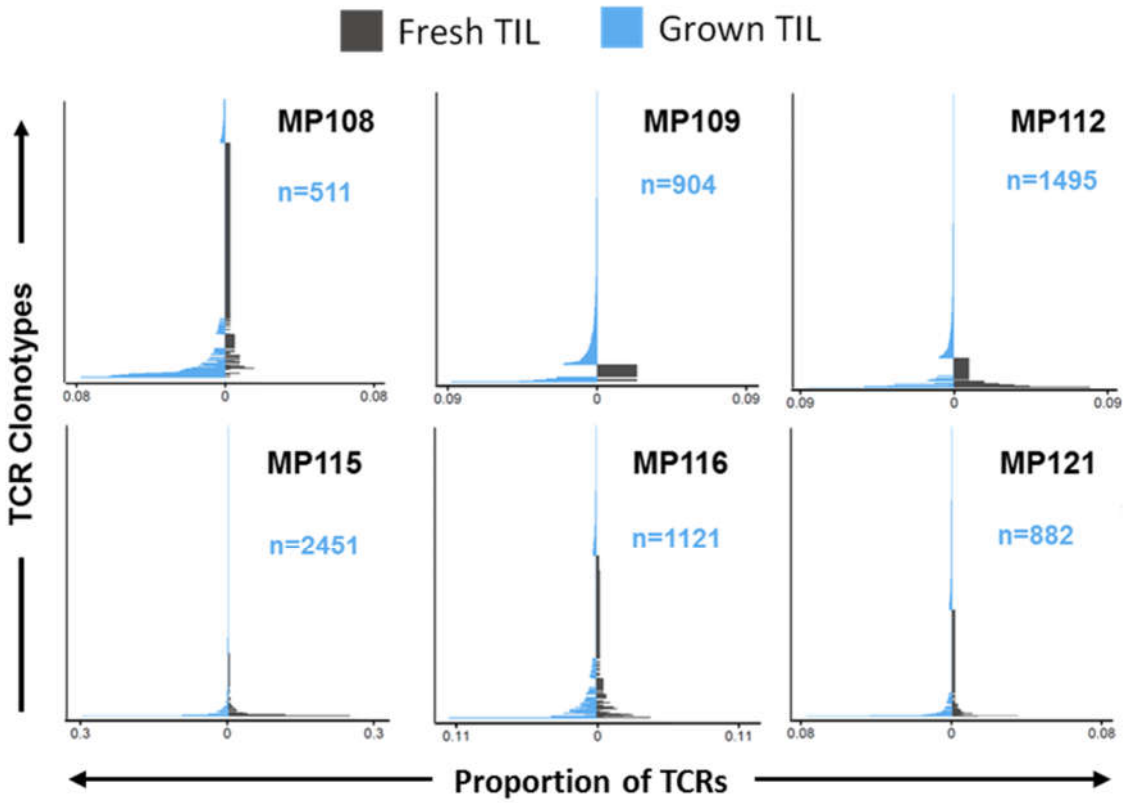

Figure 26. Preservation in the TIL product of the initial clonal dominance in fresh tissue.
Six TIL products were TCR scRNAseq'd and the relative frequency of their clonotype repertoire was compared to that found in the tissue. The clonotypes from the fresh TIL are in black and the grown TIL are in blue. The number of clonotypes in each increases up the y-axis. Along the x-axis, starting in the middle at 0 in either direction, the relative frequency in the grown TIL (blue) or fresh TIL (black) increases. The number of clonotypes detected in the grown TIL product for each sample is also shown. Figure generated in collaboration with Aislyn Schalck and Dr. Nick Navin and used with their permission.

The process of TIL expansion alters the transcriptome of TIL. To track TIL transcriptomic features through expansion, we made use of the TCR sequence information to link transcriptomes pre and post expansion (Figure 27). Figure 27A represents the expanded clones, mapped to their initial cluster in the fresh tissue (Figure 27B), where solid circles represent clones that expanded and shaded circles represent clones that did not expand (i.e., were not found in expanded TIL). The CD8 clusters in the tSNE plot are color coded to correspond with the phenotypic groups in the heatmap. Expanded clones were found to come from all four phenotypic clusters that were identified, particularly the CD8-EOMES and CD8-ZNF683 clusters (Figure 27A). In Figure 27A and 27B, the same tSNE clustering and heatmap are shown as in Figure 21C.

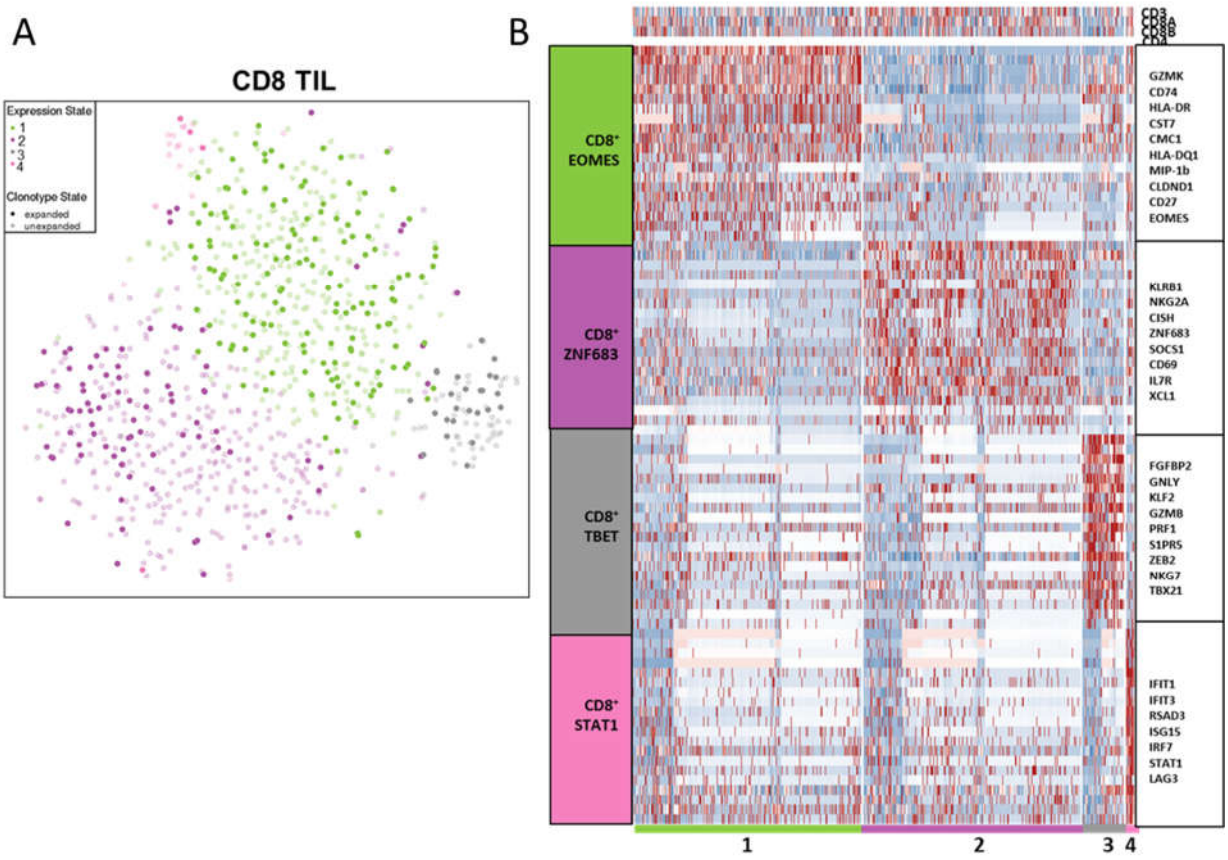

Figure 27. Comparison of the phenotypic profile from which the grown TIL were generated.
(A) tSNE clustering of fresh CD8 TIL, where closed circles indicate the clone was detected in the grown TIL product and open circle indicates it was not detected. The clusters are also color coded to match the phenotypic profiles displayed in **(B)** the heat map. Figure generated in collaboration with Aislyn Schalck and Dr. Nick Navin and used with their permission.

4.3: Summary

In summary, initial flow cytometry on several fresh, primary PDAC samples data did not detect a substantial tissue-resident memory T cell population as classically defined by having expression of CD103 and checkpoint markers. However, single-cell RNA transcriptomic sequencing allowed for unbiased analysis of the TIL heterogeneity in these samples. Transcriptomic sequencing revealed 4 distinct $CD8^+$ TIL clusters and 3 distinct $CD4^+$ TIL clusters. While there is variability in the TIL infiltration, the relative proportion of

these clusters is similar across patients. Additionally, scRNAseq was able to identify cell populations that were not appreciated by flow cytometry, such as a $CD69^{+}ZNF683^{+}$ TIL population that may represent the true phenotype of T_{RM}-like $CD8^{+}$ T cells found in PDAC as compared to the classically-defined phenotype with CD103. Furthermore, sequencing revealed a highly activated Treg population that would have otherwise been overlooked if the analysis was based solely on flow cytometry data. Finally, paired single-cell TCR sequencing revealed that, although there was not a substantial amount of clonal TIL expansion, the majority of this expansion belonged the potentially important $CD8^{+}$ TRM and the $CD4^{+}$ Treg populations. Using this paired TCR-transcriptome sequencing data to analyze the TIL grown with TIL 3.0 showed the major populations that compose the TIL product are an activated and cytotoxic CD8 population that is proliferating. Additionally, the TCR sequencing data shows that, notably, TIL 3.0 is able to expand $CD8^{+}$ TIL from the high frequency and activated clonotypes. Overall, this analysis shows the potential of combining multi-parameter flow cytometry and scRNAseq in defining the heterogeneity of TIL in PDAC.

Chapter 5: Potential clinical application of tumor-infiltrating lymphocyte therapy for ovarian epithelial cancer prior or post-resistance to chemotherapy

This chapter is based on the original research article "Potential clinical application of tumor-infiltrating lymphocyte therapy for ovarian epithelial cancer prior or post-resistance to chemotherapy" published by Sakellariou-Thompson *et al.* in *Cancer Immunology, Immunotherapy* on October 10th, 2019 (DOI: 10.1007/s00262-019-02402-z). It is presented with permission from Springer as per sections 4a and 4c of the "Authors' Retained Rights" section of the Copyright Transfer Statement document which states, in part:

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5.1: Introduction

Epithelial ovarian cancer (OvCa) is the deadliest gynecological cancer, and is estimated to account for almost 14,000 deaths in 2018 [283]. Although prognosis of early-stage OvCa is favorable, 70% of patients are diagnosed with advanced and metastatic disease [284]. Traditional management of advanced stage OvCa includes tumor reductive surgery and adjuvant platinum-taxane chemotherapy, which results in high rates of initial complete response. However, nearly 90% of patients recur and the 5-year survival rate for late-stage disease is only 28% [285, 286].

Nevertheless, OvCa possesses a strong immune infiltrate that could provide an avenue to greater treatment efficacy and better long-term survival in the context of immunotherapy. Several groups have shown that the presence of $CD8^+$ TIL is associated with a greater 5-year survival in OvCa, suggesting that $CD8^+$ TIL exert some degree of tumor

control [85, 216, 287-291]. This provides a rationale for the use of immunotherapy to harness the anti-tumor potential of this immune infiltrate. Checkpoint blockade immunotherapy has already made a tremendous mark in the treatment of cancer. Its success was first observed in the treatment of metastatic melanoma with agents that block the CTLA-4 and PD-1 axis [43, 226, 227, 292, 293]. This approach was later transposed to non-small cell lung cancer and renal cell carcinoma [228-230]. Unfortunately, the success of checkpoint blockade has not been reproduced in OvCa to date [210, 294, 295].

Since *in vivo* manipulation of the TIL through checkpoint blockade does not seem to be sufficient to generate a strong clinical response, approaches involving *ex vivo* manipulation of immune cells, such as adoptive cell therapy (ACT) using autologous TIL, might be able to provide a large quantity of anti-tumor T cells needed for tumor control. Our group and others have demonstrated the effectiveness of TIL ACT in metastatic melanoma [94, 95, 108, 237]. With objective response rates (ORR) of 40-50% in metastatic melanoma, TIL ACT is among the best treatment options for this patient population. In the 1990s, several groups attempted to transpose TIL ACT to OvCa either alone or in combination with chemotherapy, or in the adjuvant setting after debulking surgery [296-300]. These early trials had some promising results but suffered from a few limitations. Later studies demonstrated the need for lymphodepleting pre-conditioning regimens for long term TIL engraftment and improved cell generation methodologies that resulted in the infusion of patients with greater cell numbers [301]. Furthermore, improvements in techniques for TIL enrichment and activation, mainly for $CD8^+$ TIL as they have been correlated with clinical response in melanoma, may increase favorable clinical outcomes in OvCa TIL trials [95, 108].

As stated previously, the manipulation of 4-1BB/CD137 through agonistic stimulation (Urelumab, BMS), increased $CD8^+$ TIL proliferation in melanoma, triple negative breast cancer and pancreatic cancer [220, 243, 252]. Additional work to further improve the culture method was also done by adding an anti-CD3 antibody (OKT3) to the early TIL culture as well as transitioning to TIL culture in a gas-permeable culture flask [218]. Here, the addition of an agonistic 4-1BB mAb and OKT3, a novel 3-signal approach, increases the ability to grow TIL from OvCa, improves the total yield, and stimulates the proliferation of activated $CD8^+$ T cells. In addition, these $CD8^+$ TIL displayed HLA-restricted tumor recognition. These results support the use of TIL expanded with a41BB and OKT3 in ACT strategies for patients with OvCa.

5.2 Results for OvCa TIL

5.2.1 Primary and metastatic OvCa TIL infiltrate is predominantly $CD8^+$ T-cell rich

The lymphoid immune infiltrate was assessed at the onset of the culture by performing flow cytometry on manually disaggregated primary and metastatic tumor samples (n=84). The proportion of $CD3^+$ TIL among live cells recovered varied widely among samples (median 3%, range 0.1%-20%) (Figure 28A). Infiltration by NK cells was overall less (median 1% range 0.1%-35%) (Figure 28A). However, $CD3^+$ TIL were a significantly greater portion of the infiltrate than NK cells in most cases ($p<0.0001$).

Because TIL ACT for OvCa would most likely target patients who progressed on standard of care and thus would be administered to chemo-refractory patients likely to be metastatic, the contribution of those parameters on the T-cell infiltration was investigated. When patients were stratified by chemotherapy exposure and surgery site, metastatic tumors

were found to have more $CD3^+$ TIL than primary tumors (median 4% vs. 1.5%, p=0.029) as a component of live cells (Figure 28B). No significant difference in the proportion of $CD3^+$ TIL was found between pre-chemotherapy and post-chemotherapy samples (Figure 28C).

Within the $CD3^+$ TIL compartment, the mean CD8:CD4 ratio was 1.5 demonstrating that OvCa is predominately infiltrated by $CD8^+$ TIL (Figure 28D). No difference in the CD8:CD4 ratio was found in the context of primary/metastasis and pre/post-chemotherapy (Figure 28E and 28F). As a point of comparison, both the $CD3^+$ TIL infiltration and CD8:CD4 ratio were found to be similar to what our group has previously reported in metastatic melanoma [252], suggesting that OvCa tumors are relatively well infiltrated by T cells.

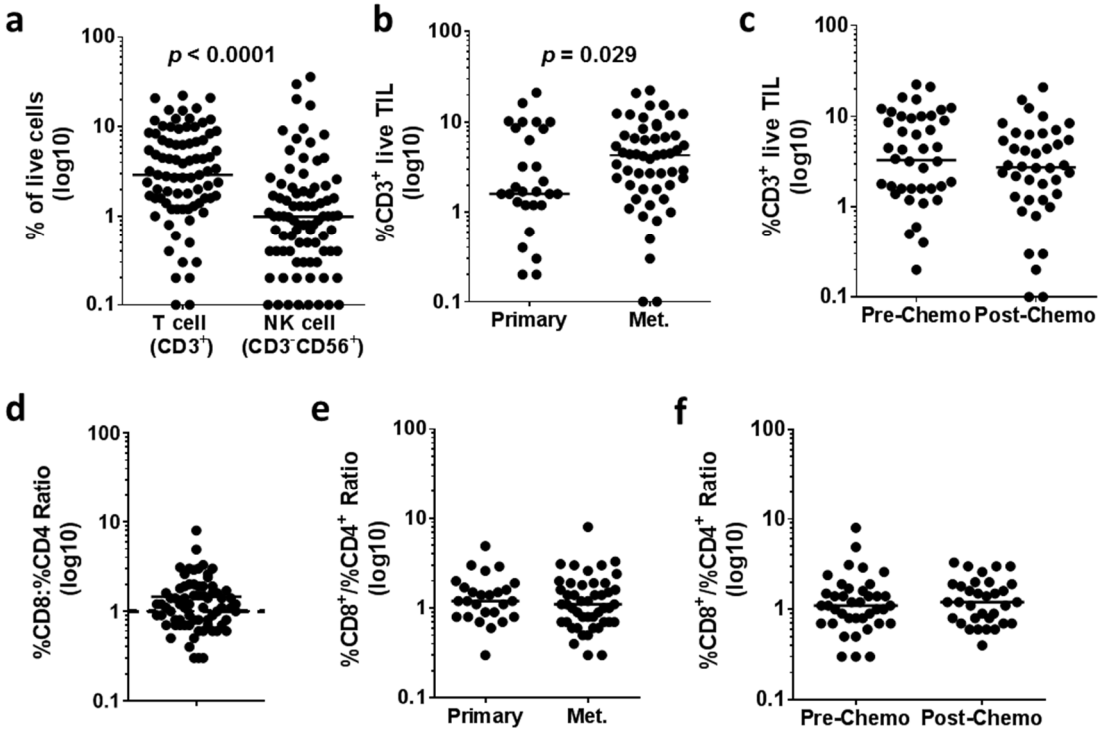

Figure 28. Characterization of lymphocyte infiltrate in primary/metastatic and pre/post-chemo OvCa samples.
(**A**) T cell ($CD3^+$) and NK cell ($CD3^-CD56^+$) infiltration is compared within the live lymphocyte population using flow cytometry, with the horizontal bar representing the median value of each population (n=84). T cell ($CD3^+$) infiltration is compared between (**B**) primary and metastatic sites, and (**C**) pre- and post-chemo samples, with the horizontal bars indicating the median value (n=84). (**D**) The ratio of $CD8^+$% to $CD4^+$% T cells within the $CD3^+$ compartment is displayed. The dotted line indicates where a ratio of 1 is in relation to the average ratio which is represented by the solid horizontal line (n=72). The CD8/CD4 ratio is compared between (**E**) primary and metastatic sites and (**F**) pre- and post-chemo samples, with the horizontal bars indicating the median value (n=72). Samples that had less than 100 cells within the CD8 and CD4 gates were excluded from analysis.

5.2.2 Use of agonistic 4-1BB mAb and anti-CD3 increases TIL growth and success rate

Prior work by our group detailed how infusion of melanoma patients with a higher proportion of $CD8^+$ T cells and larger amount of TIL in general correlates with clinical response [95, 108]. It was reasoned that $CD8^+$ TIL could also be important in other solid tumor types as their presence correlates with improved survival. Thus, the two new methodologies, which aimed at facilitating the expansion of $CD8^+$ TIL from ovarian cancer tissue, were tested next (Figure 29).

Fresh tumor samples were set up for TIL culture using the different expansion methods. The first method compared IL-2 only versus IL-2 plus an agonistic 4-1BB mAb (a41BB). Use of a41BB significantly increased the average total $CD3^+$ TIL growth from $30x10^6$ cells for IL-2 alone to $50x10^6$ for IL-2 + a41BB (Figure 29A). Additionally, a41BB greatly increased the average percentage of $CD8^+$ TIL in culture from 30% for IL-2 alone to 65% for IL-2 + a41BB (Figure 29B). A corresponding decrease in the average percentage of $CD4^+$ TIL from the IL-2 only condition to the IL-2 + a41BB condition was also observed (Figure 30). The mean $CD4^+$ percentage dropped from 50% to 5% (Figure 30A) and the median CD8-to-CD4 ratio increased from 0.4 to 60 respectively (Figure 30B).

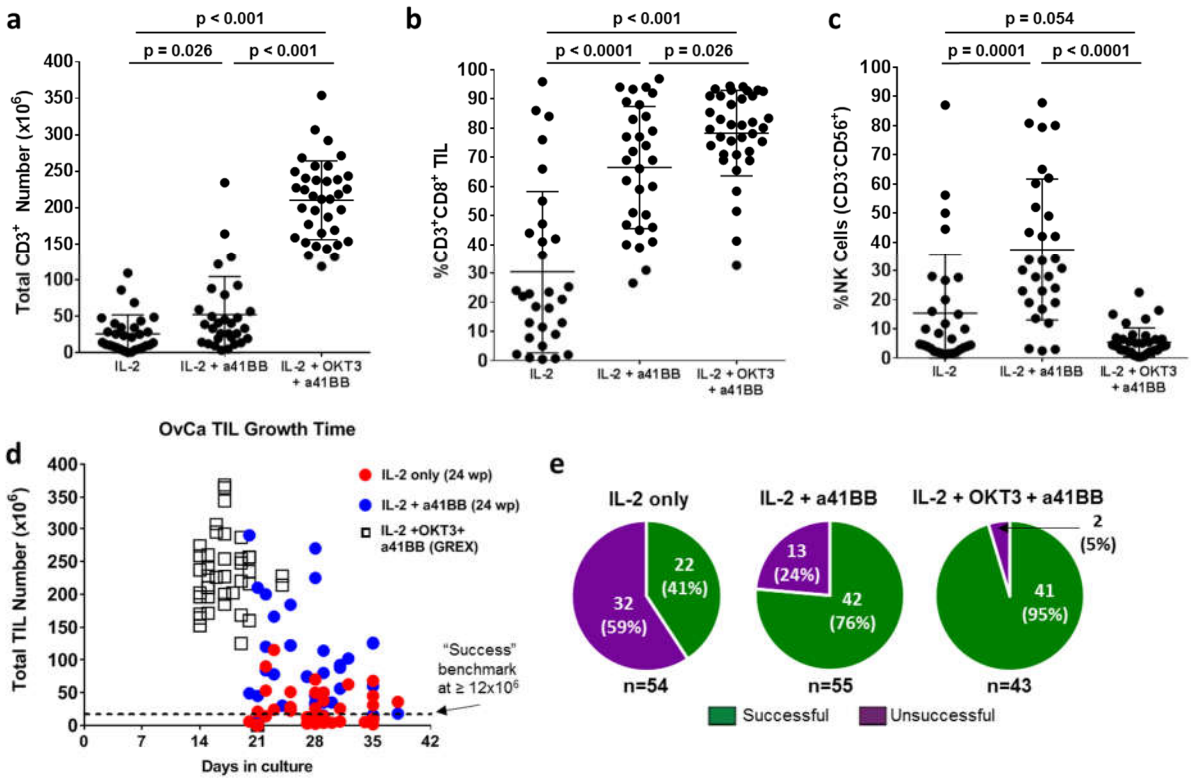

Figure 29. Characterization of OvCa TIL growth across culture conditions.

Comparison of the (**A**) total $CD3^+$ TIL number, (**B**) percentage of $CD3^+CD8^+$ TIL, and (**C**) percentage of NK cells generated between the different culture methods. Samples in IL-2 and IL-2+a41BB conditions are paired (n=30) while samples in IL-2+OKT3+a41BB are unpaired (n=36). For (**A**), (**B**), and (**C**), only samples that grew are shown. Some samples are not shown due to dropout after QC. The horizontal bars indicate the mean value and SD are shown for each population. Comparison between culture methods of the (**E**) time of culture and (**E**) success rate of growth for all attempted cultures. In **E**, IL-2+a41BB has 55 samples because one sample was set up without an IL-2 only counterpart culture.

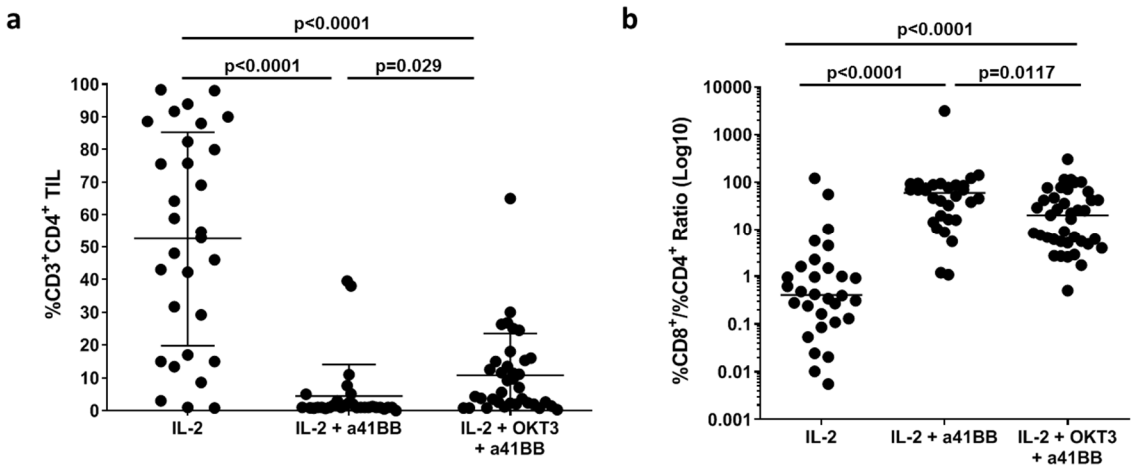

Figure 30. Characterization of OvCa CD4+ TIL growth.
Comparison between the different culture conditions of (**A**) percentage of $CD4^+$ TIL and (**B**) the $\%CD8^+/\%CD4^+$ ratio from successful cultures. For (**A**), The horizontal bars indicate the mean value and SD, and in (**B**) the horizontal bars indicate the median value**.**

However, since NK cells can also express 4-1BB, the addition of the a41BB mAb increased their growth as well from 15% to 40% of the expanded culture on average (Figure 29C). To avoid expansion of NK cells, a 3rd culture strategy using an agonistic stimulation of CD3 with an anti-CD3 mAb (OKT3) was implemented. Similar to previous work for metastatic melanoma TIL expansion, this strategy (named TIL 3.0) led to a high percentage of $CD8^+$ TIL (80% average, Figure 29B) with a low percentage of $CD4^+$ TIL (10% average, Figure 30), and NK cells (5% average, Figure 29C) [218]. Protocol-induced changes in $CD4^+$ Tregs were not assessed due to prior observations that high-dose IL-2 does not allow Treg growth and abrogates their function [243, 302]. To show the benefit of a41BB, a sub-set of 10 samples were cultured with IL-2 + OKT3 (no a41BB) and compared with TIL 3.0 (Figure 31). Without a41BB, the total number of $CD3^+$ TIL and the percentage of that which were $CD8^+$ TIL were both diminished while NK cells remained low (Figure 31A).

TIL 3.0 also produced a greater number of OvCa TIL (mean of $200x10^6$ cells) after a period of 2-3 weeks, as opposed to the 3-5 weeks required for the other two methods (Figure 29A and 29D). The consistent reduction in culture time was lost when IL-2 + OKT3 without a41BB was used and the yield of TIL was inferior (Figure 31B). The time of expansion did not appear to depend on chemotherapy exposure (Figure 32A), although $CD3^+$ TIL from post-chemotherapy samples grew slightly, but significantly, less ($225x10^6$ vs. $200x10^6$, p=0.028) using TIL 3.0 (Figure 32B). Regardless, all TIL 3.0 cultures produced markedly more TIL than IL-2 only or IL-2+a41BB.

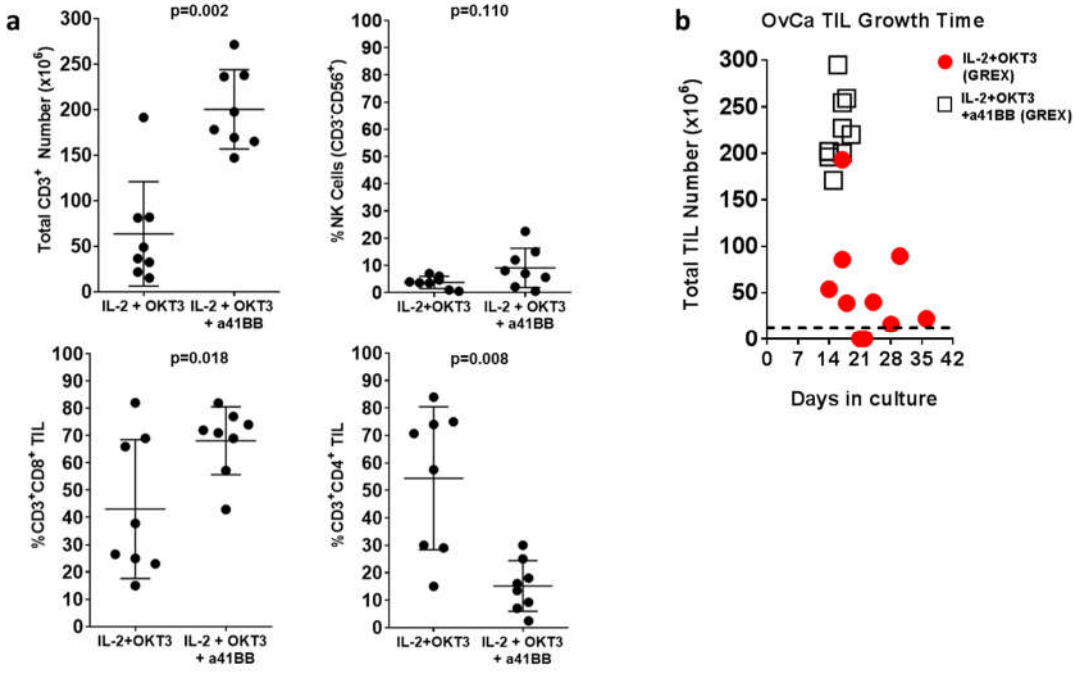

Figure 31. Characterization of OvCa TIL growth using IL-2+OKT3 without a41BB. Comparison of (**A**) total $CD3^+$ TIL number, percentage of NK cells, percentage of $CD3^+CD8^+$ TIL, and $CD3^+CD4^+$ TIL between IL-2+OKT3 and TIL 3.0. Only samples where both conditions produced TIL are shown (n=8). The horizontal bars indicate the mean value and SD is shown for each population. Comparison of the (**B**) time of culture and success rate between culture methods (n=10 for both).

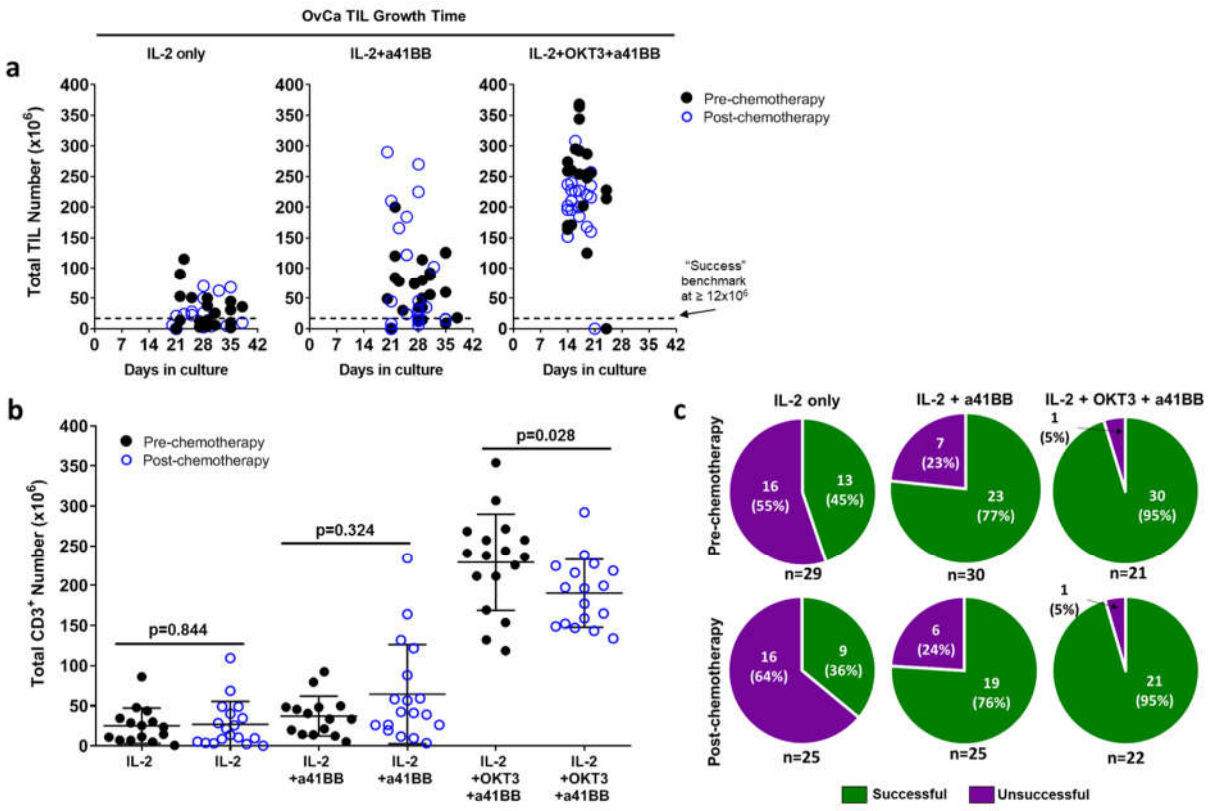

Figure 32. Chemotherapy exposure does not affect OvCa TIL growth.
Comparison of (**A**) the days in culture, (**B**) total $CD3^+$ TIL growth, and (**C**) growth success rate for all three conditions between pre- and post-chemotherapy samples. (**A**) and (**C**) show all attempted cultures and (**B**) shows only cultures that grew. In (**B**), the horizontal bars indicate the mean value and SD are shown for each population.

The overall success rate of establishing an OvCa TIL culture was greatly increased from 41% (22/54) for IL-2 only and 76% (42/55) for IL-2+a41BB to 95% (41/43) for the IL-2+OKT3+a41BB method (Figure 29E). The benchmark for a successful TIL culture, $12x10^6$ total cells, was established from scaling down the MDACC Clinical Melanoma TIL Lab's criterion for success where 20 fragments are set up for TIL expansion and $40x10^6$ cells is considered the minimum to treat a patient [252]. Chemotherapy exposure only affected the success rate of expansion for the IL-2 only condition, with the success being 45% (13/29) for

pre-chemotherapy and 36% (9/25) for post-chemotherapy (Figure 32C). This discrepancy was negated for the IL-2+a41BB and TIL 3.0 methods.

It was assessed if the presence of TIL in the fresh sample affected the success of growth (Figure 33). When comparing the percentage of $CD3^+$ TIL in the fresh sample vs the number grown, there was a strong correlation ($r_S > 0.5$) for the IL-2 only culture method where more $CD3^+$ TIL grew if there were a higher percentage of $CD3^+$ TIL present in the initial tumor tissue (Figure 33A). Moreover, it was apparent that cultures with less than 2% $CD3^+$ TIL in the initial tumor tissue cultured with IL-2 alone did not grow, but cultures initiated with as little as 0.2% $CD3^+$ T-cell infiltrate could yield appreciable number of TIL when cultured with IL-2+OKT3+a41BB. However, while the IL-2+a41BB and TIL 3.0 culture conditions did not show as strong of a correlation with T-cell infiltration ($r_S < 0.5$), the overall trend was the same as indicated by the positive slopes of the linear regression lines (Figure 33A). There was no strong correlation with respect to the $CD8^+/CD4^+$ ratio of fresh TIL and the amount of $CD8^+$ TIL generated, particularly for the IL-2 only condition due to the lack of $CD8^+$ TIL generated by this method (Figure 33B). Overall, there was a positive trend towards growing more $CD8^+$ TIL if there was a higher $CD8^+/CD4^+$ ratio initially as indicated by the slopes of the regression line for IL-2+a41BB and IL-2+OKT3+a41BB.

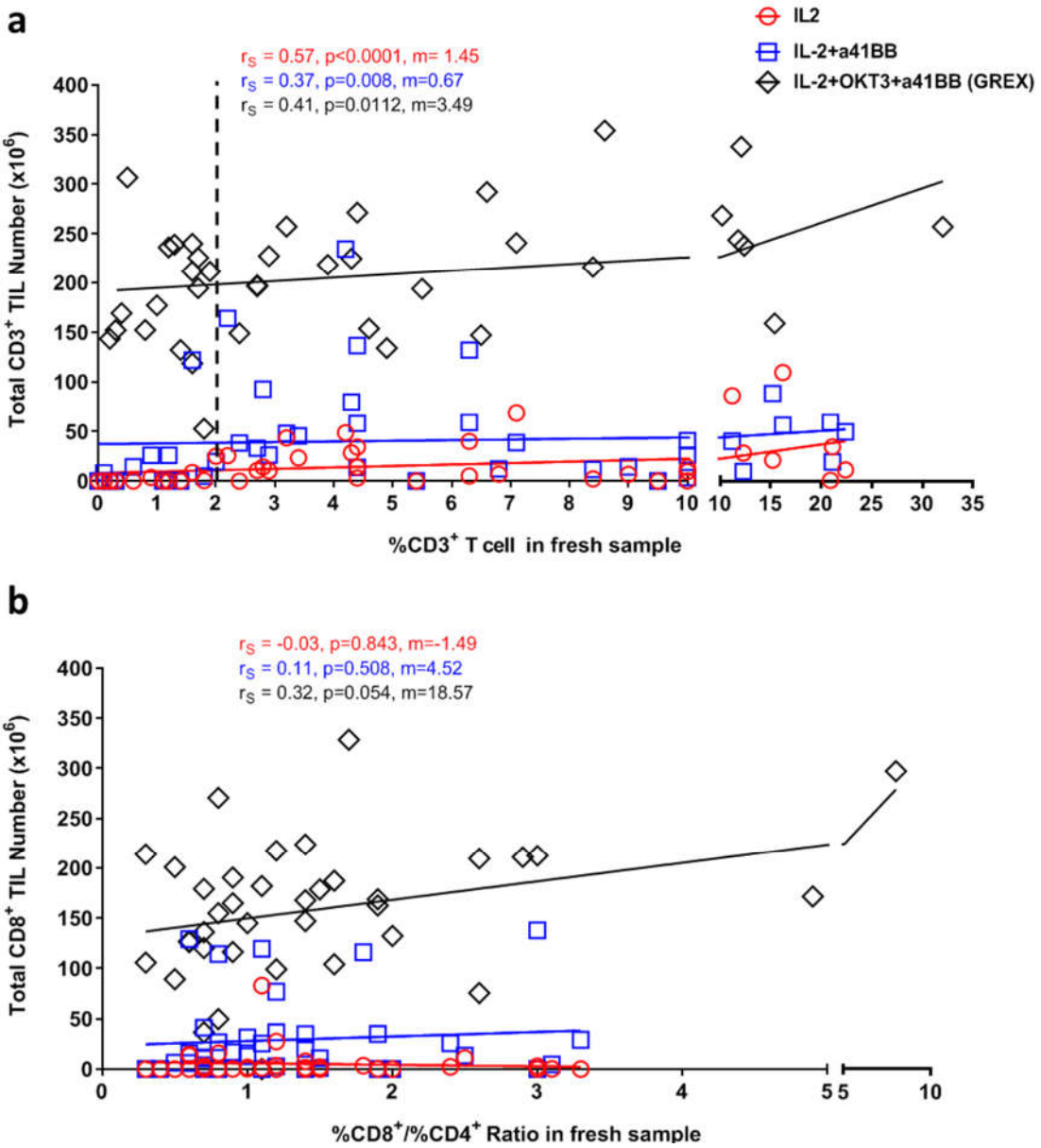

Figure 33. Correlation between amount of OvCa TIL present in fresh tissue and magnitude of TIL growth.

(**A**) Comparison between %CD3$^+$ TIL (in live cell population) in fresh sample versus the total CD3$^+$ TIL grown for IL-2 only (red), IL-2+a41BB (blue), and TIL 3.0 (black). The dashed vertical line indicates the cutoff at which IL-2 cultures don't grow if initial CD3% is <2%. (**B**) Comparison between %CD8/%CD4 ratio in fresh sample versus the total CD8$^+$ TIL grown for each condition. Each sample is paired between the fresh sample and the TIL number generated from the same sample. r_S = Spearman correlation coefficient, p value is for the significance of the correlation, m = slope of the linear regression line (solid lines that represent general trend of the population).

5.2.3 Concurrent engagement of 4-1BB and CD3 prevents $CD8^+$ TIL over differentiation

To understand how the different culture methods affected the $CD8^+$ TIL, the expression of CD28, BTLA, and PD-1 expression were explored as surrogates for T-cell activation and differentiation (Figure 34). These 3 markers were strategically selected for the power of assessment they provide individually as well as together. $BTLA^+CD8^+$ TIL have been reported by our group to be less-differentiated cells, capable of prolong persistence and serial killer capacities [188, 189]. Expression of CD28 is also a trait of lesser differentiation, while PD1 is often associated with exhaustion when highly expressed [249, 253]. When looking at expression of the 3 markers on OvCa TIL expanded with IL-2 only and IL-2+a41BB, no significant difference was observed between both groups. Nonetheless, both showed a spread in CD28 and PD1 expression (Figure 34, left and right graph) with a low expression of BTLA (Figure 34, middle graph). However, TIL products grown with the TIL 3.0 method had significantly greater percentage of TIL expressing BTLA and CD28, and a significantly smaller percentage expressing PD-1 altogether suggesting a less differentiated profile (Figure 34).

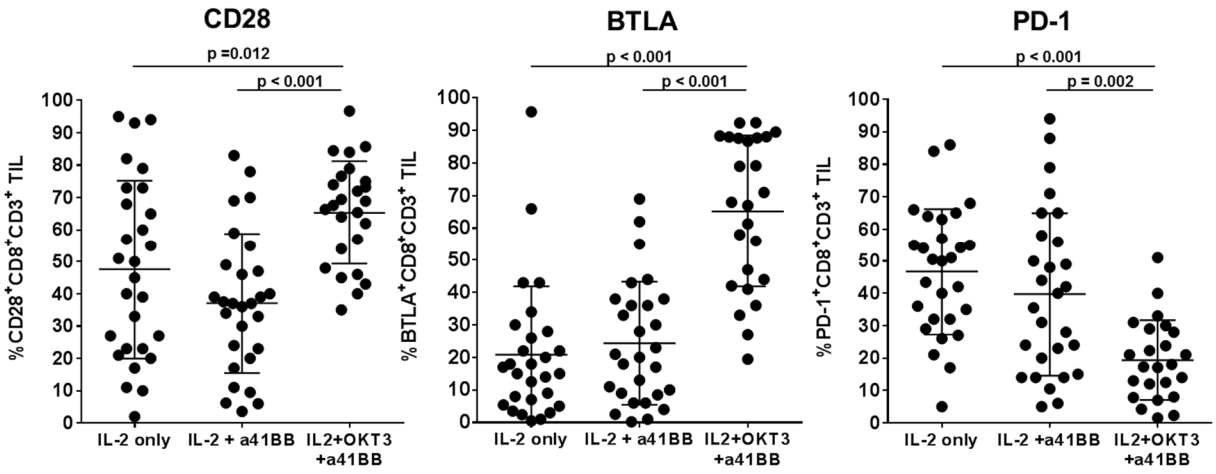

Figure 34. Phenotyping of activation and differentiation of grown OvCa TIL.
Comparison between the different culture methods of the percentage of CD28, BTLA, and PD-1 expressing $CD8^+$ TIL generated. Samples in IL-2 and IL-2+a41BB conditions are paired (n=28) while samples in IL-2+OKT3+a41BB are unpaired (n=25). Samples not passing QC were excluded from analysis. The horizontal bars indicate the mean value and SD are shown for each population.

5.2.4 OvCa $CD8^+$ TIL show HLA-matched tumor reactivity

In order for TIL ACT to be an effective therapy, the TIL generation process needs to expand anti-tumor T cells. Since the three different growth methods used could potentially change the expanded TIL repertoire, tumor reactivity was used as a marker to validate preservation of tumor-reactive TIL clones. Since our main focus is reactivity of cytotoxic $CD8^+$ T cells, each TIL line was sorted for $CD8^+$ TIL and further expanded using the rapid expansion protocol to achieve sufficient cell numbers. Eight TIL lines were chosen to assess HLA-matched tumor reactivity that were representative of the different culture methods, surgery sites, and prior chemotherapy exposure, which is summarized in Table 4.

Table 4. Tumor targets for each TIL line and the HLA alleles for which they match.

TIL Line	Surgery site	Prior Chemo	TIL Culture Method	Tumor Target	TIL:Tumor HLA match
14-530A	Primary	N	IL-2 only and IL-2+a41BB	SKOV3	A*03:01
15-033	Pelvic Tumor	Y		COV362	B*40:01
15-094	Omentum	Y		COV318	A*02:01
16-025B	Omentum	N		SKOV3	B*18:01, B*35:01, C*04:01
17-309	Omentum	N	IL-2+a41BB and IL-2+a41BB+OKT3	SKOV3	A*68:01, C*04:01
17-378	Omentum	Y		SKOV3	C*05:01
18-073A	Primary	Y		COV318	A*02:01
18-121 #1*	Primary	N		COV318	A*02:01, B*07:02, C*07:01
18-121 #2*				SKOV3	B*18:01

*18-121 is one TIL line that was tested against two tumor targets.

For the first set of four lines, reactivity was assessed comparing initial growth with IL-2 only or IL-2+a41BB (Figure 35A). The second set of four lines had reactivity compared between those expanded with IL-2+a41BB or with TIL 3.0 (Figure 35B). Prior to co-culture setup, all tumor lines were found to express robust levels of HLA class I by flow cytometry (SKOV3, MFI: 2962; COV318, MFI: 5343; COV362, MFI: 2182; Figure 36). As shown in Figure 35A, upon co-culture of the $CD8^+$ TIL with tumor targets, 2/4 of the first set (15-094 and 16-025B) demonstrated HLA-restricted anti-tumor reactivity via secretion of IFN-γ. The reactivity was split between one line grown with IL-2+a41BB (15-094) and one with IL-2 only (16-025B). Likewise, in Figure 35B, 3/4 of the second set of TIL lines were reactive against their HLA-matched target. One TIL line (18-121) even recognized two different tumor lines. Two TIL lines in this group (17-309 and 18-121) showed reactivity regardless of culture method while one showed reactivity only from TIL grown with IL-2+a41BB (17-378). Overall, TIL recognition of HLA-matched tumor targets was observed across the multiple expansion platforms.

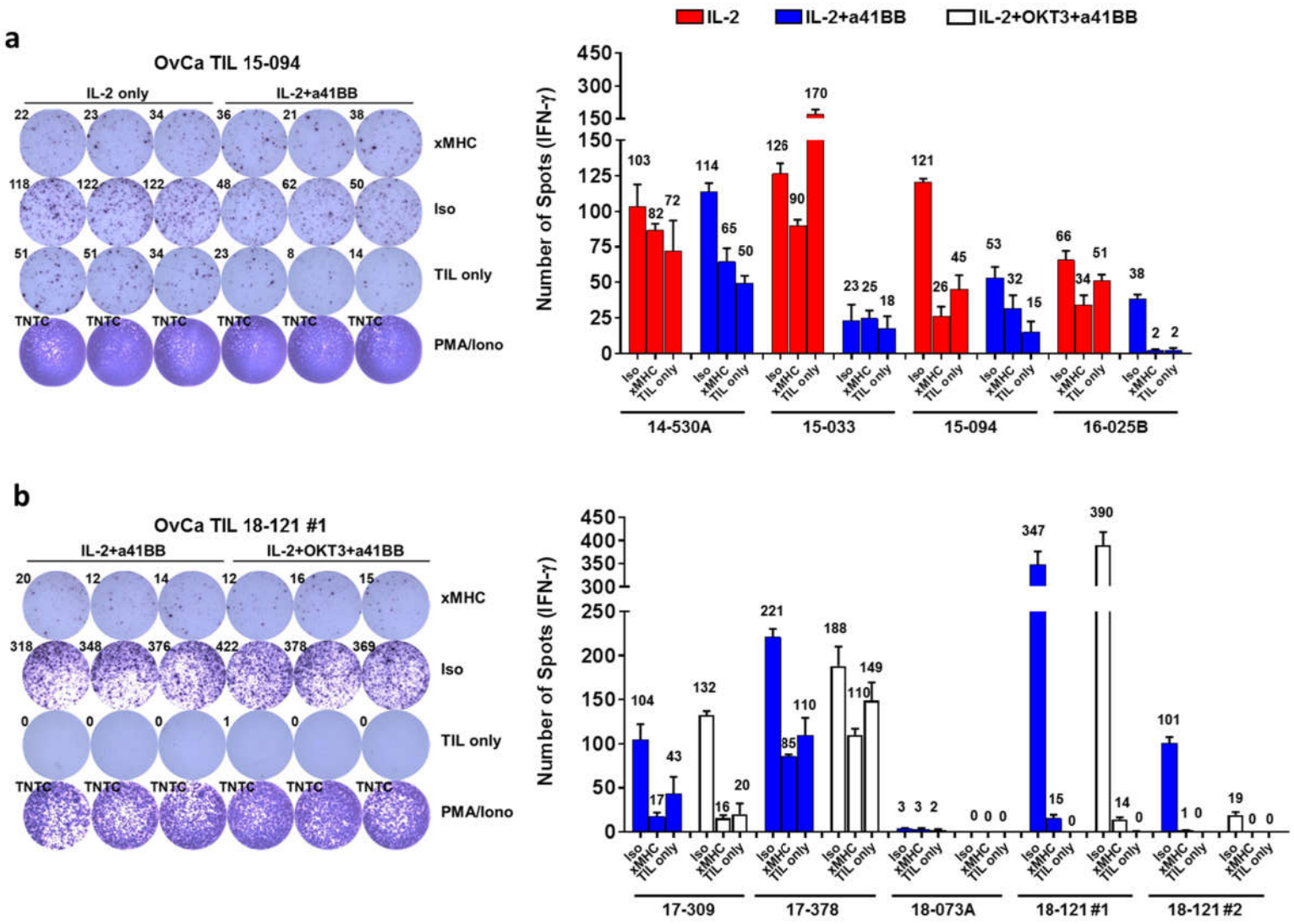
a
OvCa TIL 15-094
IL-2 only
IL-2+a41BB
xMHC
Iso
TIL only
PMA/Iono
IL-2
IL-2+a41BB
IL-2+OKT3+a41BB
Number of Spots (IFN-γ)
14-530A
15-033
15-094
16-025B
b
OvCa TIL 18-121 #1
IL-2+a41BB
IL-2+OKT3+a41BB
17-309
17-378
18-073A
18-121 #1
18-121 #2

Figure 35. Testing reactivity of CD8$^+$ OvCa TIL via IFN-γ ELISPOT.
(**A**) Comparison of reactivity of TIL grown with either IL-2 only (red) or IL-2+a41BB (blue) for four different lines, with a representative ELISPOT image shown. (**B**) Comparison of reactivity of TIL grown with either IL-2+a41BB (blue) or IL-2+OKT3+a41BB (white) for four different lines, with a representative ELISPOT image shown. Bar graphs show average of 3 replicate wells with the SD and average spot value for each condition above the bars. Reactivity was considered positive if the average was ≥ twice the xMHC condition. xMHC = MHCI block, Iso = isotype control for MHCI blocking Ab, TIL only = T cells with media only, PMA/Iono = PMA and Ionomycin for positive control, TNTC = too numerous to count.

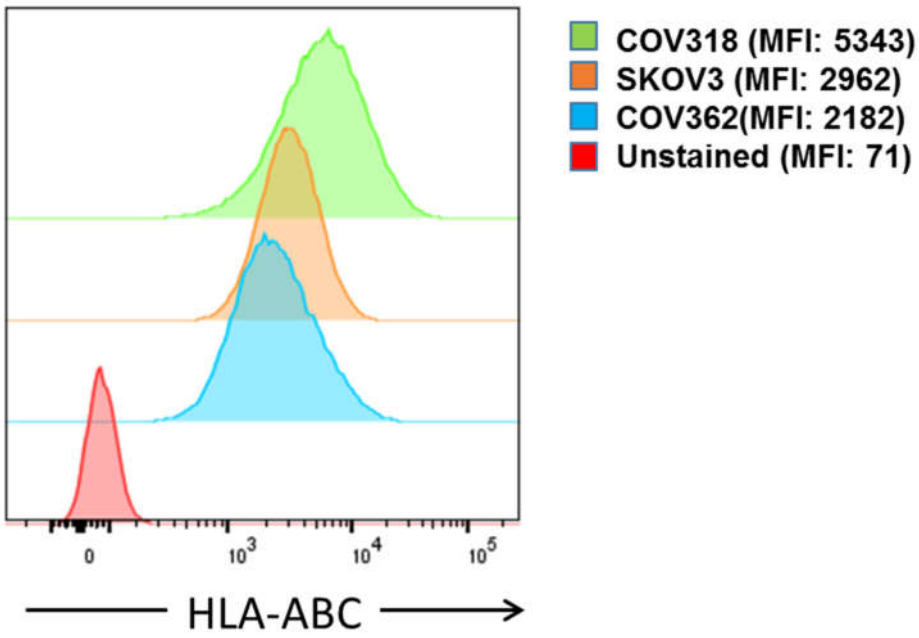

Figure 36. HLA-ABC expression of OvCa cell lines.
The expression of MHCI alleles A, B, and C is shown in comparison to an unstained control. The mean fluorescence intensity (MFI) is shown in the legend to indicate the magnitude of expression.

5.3 Summary for OvCa TIL

It was observed that OvCa has a robust and activated $CD8^+$ TIL infiltrate that is not significantly impacted by surgery site or resistance to chemotherapy. This infiltrate is greatly expanded with the addition of an agonistic 4-1BB mAb to the TIL culture. Furthermore, specific growth of $CD3^+CD8^+$ TIL is augmented by adding the anti-CD3 Ab OKT3. The 4-1BB mAb alone and together with OKT3 consistently improved the success rate of reaching a clinically relevant number of TIL. Finally, OvCa $CD8^+$ TIL derived with either culture method showed tumor recognition via IFN-γ secretion in response to HLA-matched ovarian cancer cell lines. These results suggest that enhanced culture method can facilitate TIL ACT for OvCa by increasing the yield of a TIL product containing anti-tumor $CD8^+$ T cells regardless of primary or metastatic site, and in a chemotherapy refractory setting.

Chapter 6: Discussion

Immunotherapy has drastically remodeled the landscape of cancer therapy by harnessing the power of our own immune system. The most prominent example of this is the development of checkpoint blockade therapy, which inhibits immune regulatory checkpoints in order to reinvigorate anti-tumor T cell activity. This concept has delivered great improvements in terms of response rates and survival to several solid tumor types such as advanced melanoma, non-small cell lung cancer, renal cell carcinoma, and all MSI-high cancers. While much success has been found for these cancer types, which are often highly immunogenic [206-208], there are many others (e.g. pancreatic, ovarian, breast, MSS colorectal, prostate cancers) which have not yet derived benefit even though they are in great need of improved therapy options. Given the heterogeneity of cancers, the immune contexture of some tumor types may not be conducive to CBI which relies on *in vivo* manipulation of the immune response. Therefore, certain tumors may benefit most from an immunotherapy that approach the issue from another angle such as TIL-based ACT.

TIL ACT can take advantage of antigen-specific TIL by expanding them *ex vivo* in an environment conducive to their activation and proliferation. TIL ACT has already found success in metastatic cutaneous melanoma where it provides overall response rates around 40-50% [94, 95, 109, 152, 238]. Despite the lack of efficacy of CBI for several of these cancer types (pancreatic, ovarian, breast, MSS colorectal, prostate), there is still evidence for the role TIL in control of these tumors [27]. Therefore, the success of TIL ACT in melanoma could be translated to other tumor types. As such, in this dissertation, I sought to assess the feasibility of this therapy in other solid tumors that still have an unmet need. Specifically, I was guided by the hypothesis that tumor-specific TIL exist in solid tumors beyond melanoma

and their potential for tumor clearance can be harnessed through TIL ACT. In order to address this question, pancreatic ductal adenocarcinoma (PDAC) and high-grade serous ovarian cancer (OvCa) were chosen as models. Both PDAC and OvCa are often diagnosed at a late stage and face poor prognoses with 5 year survival rates of <10% and 28% respectively. CBI has failed to provide improved clinical benefit for either [209-211, 294, 295]. Therefore, patients still rely on surgery and/or chemotherapy as treatment options, but the majority recur due to the limited effectiveness. Despite the lack of efficacy for CBI so far in PDAC and OvCa, the presence of T cells in the tumor tissue (TIL) is correlated with increased survival in both [85, 216, 290, 291]. Therefore, over the course of investigating transposing TIL ACT to PDAC and OvCa, I determined that both harbored tumor-reactive $CD8^+$ TIL that could be expanded *ex vivo* for the potential application of TIL ACT. I also had the opportunity to improve the TIL expansion to increase the feasibility for PDAC and OvCa by using the three core signals needed to proper T-cell expansion and activation. Finally, I was able to use single-cell RNA sequencing to deeply interrogate the heterogeneity of PDAC TIL in an unbiased manner. This analysis revealed a potentially prognostically relevant TIL population as well as showing that the improved culture process expanded the high-frequency and activated T cell clones. In this chapter, these results will be discussed within the greater context of the field as well as potential future directions.

Understanding the TIL compartment of a tumor type is key to assessing the feasibility of TIL ACT as success is likely to rely, in part, on the quantity and quality of TIL present. Flow cytometry analysis provided insight into the general makeup of the TIL component while helping to highlight the heterogeneity of cancers. Given the classification of PDAC as an immunologically “cold” tumor compared to melanoma, it was no surprise to find the level

of $CD3^+$ TIL infiltration was significantly less. However, it was interesting to note that the $CD3^+$ TIL component was primarily composed of $CD4^+$ TIL, which has been reported by others [303, 304]. Given that response to anti-PD1 CBI is correlated with increased CD8+ TIL at baseline, potentially the lack of response to CBI for PDAC could be attributed to the scarcity of $CD8^+$ TIL [305, 306]. However, the reason for the predominance of $CD4^+$ TIL is unclear.

One potential mechanism that was elucidated in a mouse model of pancreatic cancer is the effect of pancreatic stellate cells (PSCs). Activated PSCs in the surrounding stroma were found to sequester $CD8^+$ TIL peritumorally via secretion of CXCL12 [307]. The same disparity was observed in PDAC liver metastases and could be due to the same mechanism. The liver also contains hepatic stellate cells (HSCs) which become activated after livery injury and can secrete CXCL12 as well [308]. Inhibition of the effects of stellate cells could be a strategy to improve the effectiveness TIL ACT. One such method could be use of Plerixafor (Mozobil/AMD3100) to inhibit CXCR4, the receptor for CXCL12, thus preventing T-cell exclusion in the tumor [309, 310]. Another potential option is administration of the antifibrotic compound halofuginone, which has been shown to inhibit PSC activation in addition to making the TME more amenable to immune infiltration by reducing the tumor stromal content [311, 312].

In addition to cellular mechanisms of $CD8^+$ TIL exclusion, genomic factors could be important as well. Several groups have conducted genomic analyses and identified different molecular subtypes of PDAC [313-317]. Furthermore, meta-analysis of these studies resulted in a putative consensus nomenclature where there are two broad types of PDAC: Squamous-like and Classical-Pancreatic. The Classic-Pancreatic is further subdivided into an Exocrine-

like type, Classic-Progenitor type, and an Immunogenic type [318]. Based on these analyses, the majority of PDAC cases have a poor immune signature as well as poor prognosis compared to the immunogenic subtype. The fact that PDAC has a low mutation burden overall and the majority of PDAC subtypes are weakly immunogenic could explain the lack of $CD8^+$ TIL component. Moreover, understanding of these molecular subtypes could help guide selection of those patients that would have a better chance to respond.

Despite the low amount of $CD8^+$ TIL infiltration in PDAC, further phenotyping shows that these $CD8^+$ T cells found in the fresh tumors bear an activated phenotype, suggesting anti-tumor activity. The low percentage of CD45RA (10% ± 6%) in combination with the CD28 (50% ± 20%) and PD-1 expression (45% ± 12%) suggests the population is mainly effector memory with low to no terminally differentiated cells [319]. In order to dive deeper into PDAC TIL populations, initial bulk TCR-sequencing of the T cells found in the blood, tumor, and normal adjacent tissue was performed. TCR-sequencing allows for analysis of the repertoire of a T cell population. As laid out in section 1.1.3 of the Introduction, antigen-specific T cell clones will expand at the tumor site if they recognize their target. TCR-sequencing showed that 5/6 PDAC samples sequenced showed enrichment of select T cell clones at the tumor in comparison to the blood, suggestive of an immune response. This data corroborates work by Poschke *et al.* which also found that the T-cell repertoire in primary PDAC tumors was enriched versus the blood [303]. Only a subset of the T-cell clones found in the blood are found in the tumor, and the frequency of a clone in the blood does not correlate with its frequency in the tumor tissue. Furthermore, T-cell clones found both in the tumor and in adjacent uninvolved tissue tended to be present at higher frequency in the tumor tissue than the uninvolved, suggesting local proliferation or active

enrichment, again indicative of an anti-tumor immune response (Figure 9). However, while looking particularly at the high-frequency clones shared between the tumor tissue and adjacent uninvolved tissue, four patients exhibited similarity between the frequencies of shared TIL clones. This was highlighted by the fact that the top clones appeared to cluster along the 1:1 frequency line, suggesting that high frequency clones were shared between the tumor and adjacent uninvolved tissue (Figure 9). A possible explanation of this would be the contribution of a local immune response as opposed to a systemic one coming from the blood. This potentially indicates participation of tissue-resident memory T cells.

The idea of the participation of T_{RM} cells in an immune response for PDAC was particularly intriguing given then wealth of recent data suggesting this population of cells harbors tumor-reactive T cells or are correlated with improved survival in several tumors types, including potentially pancreatic cancer [262, 264-267, 320]. Given the complexity and variability of not only the T_{RM} cell phenotype, but other TIL populations as well, scRNAseq was employed to assess the phenotypes in an unbiased manner. scRNAseq of 6 PDAC samples revealed four $CD8^+$ TIL groups (CD8_C1 EOMES, CD8_C2 ZNF683, CD8_C3 GZMB, and CD8_C4 STAT1) and three $CD4^+$ groups (CD4_C1 T_{CM}, CD4_C2 Treg, and CD4_C3 T_{EM}). While initial flow cytometry analysis indicated that the majority of $CD8^+$ TIL were effector memory cells, scRNAseq revealed the true heterogeneity of this T_{EM} population that would have otherwise gone unnoticed. Of particular note is the CD8_C2 ZNF683 group which appears to be a T_{RM}-like group based on the expression of *ZNF683, CD69*, and *IL-7R* as well as the lack of expression of *KLF2, S1PR5,* and *ZEB2* as reported in the literature [254, 278]. Interestingly this population does not have high expression of *ITGAE* (aka CD103) or checkpoint molecules which have also been repeatedly reported in the literature

for the T_{RM} population. This could be an indication of a unique phenotype for PDAC T_{RM} cells or a reflection of T cell anergy given the reported lack of proper T-cell stimulation which is reported to be necessary for CD103 upregulation [271, 304, 321, 322]. A recent report found that expression of *ZNF683* and *IL7R* was associated with persistence of infused TIL clones in the peripheral blood of a treated colorectal cancer patient after TIL therapy [323]. This correlation suggest that the subset cells found within our CD8_C2 ZNF683 group may also have increased persistence in PDAC patients treated with TIL therapy. The results presented in this study coupled with the data in this dissertation point to the potential benefit of using scRNAseq to track TIL populations from tumor resection to infusion product to post-treatment in order improve TIL ACT for solid tumors. Finally, another phenotypic feature of note for the CD8_ZNF683 population is the expression of *XCL1*, which has been shown to be important for recruiting cross-presenting DCs as well as stabilizing their interaction with $CD8^+$ T-cells [324]. It's possible then that this subpopulation not only participates in the immune response but also helps propagate it.

The other two major CD8 effector cell populations, CD8_EOMES and CD8_TBET appear to be on opposite ends of the spectrum based expression of EOMES and *TBX21* (aka TBET). The CD8_TBET appears to be a full-fledged cytotoxic effector population based on expression of the cytotoxic T cell markers *GZMB, NKG7, GNLY* and *PRF1* while CD8_EOMES merely appears to be an activated CD8 TIL population based on expression of MHCII genes, *MIP1-b* and *GZMK*. The key to distinguishing their fate lies with the differential expression of the *EOMES* and *TBX21*, which are important transcription factors for determining the balance between terminal differentiation of effector cells and the self-renewal of central memory cells. It has been shown that $CD8^+$ T cells expressing *EOMES* are

able to compete the memory cell niche and lacking *EOMES* results in defective long-term persistence and secondary expansion after rechallenge [325]. On the other side of the coin, expression of *TBX21* is known to drive differentiation of effector T cells instead of central-memory T cells [326]. This brings up a potential model where the $CD8^+$ effector T cells go down one differentiation path to the CD8_ZNF683 T_{RM} population or down a different path which splits into either the cytotoxic, short-lived CD8_TBET population or the CD8_EOMES population that leads to more effector memory cells or recirculation of central memory cells. This is potentially supported by integration of the TCR sequencing shown in the $CD8^+$ TIL Circos plot (Figure 23). Expanded TCR clones, which are clustered at the 5 o'clock and 11 o'clock positions, tended to be found in multiple transcriptomic populations instead of staying within one cluster. This could indicate that some of the clones are expanded due to differentiation into the different subtypes, particularly those at the 5 o'clock position where the CD8_EOMES population is concentrated. Altogether, the scRNAseq data reveals an interesting insight into the dynamic cycle of $CD8^+$ TIL participating in an immune response.

On the other side of the T-cell coin from the $CD8^+$ TIL are the $CD4^+$ TIL which have two interesting populations: CD4_Treg and CD4_T_{EM}. The Treg population had distinct expression of *GITR, OX40, TIGIT, CD39*, and *4-1BB*, which are known to be expressed by activated Tregs. However, their most intriguing feature is their expression of *IL32* which is nominally a pro-inflammatory cytokine and does not seem to fit with the immunosuppressive function of Tregs [327]. In fact, there does not appear to be any literature linking secretion of IL-32 to Tregs. Instead, there are several reports on the effects of IL-32 in other contexts that could give clues to role of IL-32 secreting Tregs. It has been shown to induce expression of

the immunosuppressive factors IDO and ILT4 in macrophages, DCs, and Tregs [327]. As a result, in the context of HIV, it can actually dampen the antiviral immune response [328]. Additionally, IL-32 can induce IL-6 production which is associated with a more metastatic phenotype in pancreatic cancer [329]. Since little is known about the role of IL-32 expressing Tregs, this could serve as a novel Treg population worthy of exploration in pancreatic cancer.

The other CD4 population, CD4_T_{EM} is potentially interesting given its activated phenotype, particularly its expression of IL-2 and CD40LG, the ligand for CD40. Interim results for an ongoing clinical trial using chemotherapy plus an agonistic CD40 mAb with or without anti-PD1 (Nivolumab) has shown very promising results: the ORR among 30 patients has been greater than 50% [330]. Given this data, it's conceivable that an activated $CD40LG^{+}CD4^{+}$ T cell population could engage CD40 on antigen-presenting cells and help effector T cell activation to ultimately provide some clinical benefit. Apart from activating CD4 T cells, an agonistic OX40 mAb can abrogate Treg suppression [62]. Therefore, one possibility could be the addition of an agonistic OX40 mAb to target the CD4_T_{EM} population while simultaneously depleting the highly activated $OX40^{+}$ CD4_Treg population.

Given the fact that there are TIL present with a desirable phenotype, a major step towards developing TIL ACT for tumor types beyond melanoma is showing the feasibility of TIL culture. One of the main criticisms of TIL ACT has been the length of culture needed to produce a cellular product ready to treat patients. For metastatic melanoma, in which the therapy was pioneered, the pre-REP (fragment culture) can take up to 5 weeks using the "traditional" method of high-dose IL-2 in 24-well tissue-culture plates [331]. This is in addition to the 2 week REP process needed expand enough TIL to be able to treat a patient, producing a delay of up to 7 weeks. This prolonged wait time would result in some patients

no longer being eligible for treatment in the case of a rapid progression. This would be particularly true in the case of PDAC and OvCa patients, who are often dealing with late-stage cancer, and do not have the ability to wait almost 2 months for treatment. Furthermore, on top of the lengthy wait time is the fact that a successful TIL culture is not guaranteed. Even for a highly infiltrated tumor type like melanoma, the historical success rate for a successful TIL culture is 60-70%, which is to say that a sufficient number of TIL was expanded in the pre-REP phase in order to be able to treat a patient [96, 154, 156, 332]. The issues of extended culture time and less-than-ideal success rate were recapitulated to even worse degree in attempting TIL culture for PDAC and OvCa. Using the traditional culture method, I observed that culture time was consistently 4-5 weeks with success rates of 29% for PDAC and 41% for OvCa. In addition, this issue of prolonged TIL culture was also found in early OvCa TIL ACT trials using the traditional TIL culture method, which likely contributed to the poor results observed along with low cell numbers overall and lack of lymphodepletion before TIL infusion [296-300]. The question of why some PDAC and OvCa TIL cultures do not grow with IL-2 is still mostly an open one and several factors could be at play.

PDAC is generally characterized as an immunologically “cold” tumor that is defined by a low T-cell infiltrate, an immunosuppressive TME, and a dense desmoplastic component that excludes immune cells [304]. As it has been observed by me and others, PDAC has a much lower TIL presence than melanoma [119, 241, 304]. The poor growth observed with IL-2 only could simply be a function of low T-cell infiltration that is below a threshold amenable to outgrowth. Another possible explanation is the presence of inhibitory immune cells that increase the hurdle for T cells to expand, such as the Tregs as shown in the by

scRNAseq and flow cytometry analysis. The inhibition of TIL in pancreatic cancer has been frequently attributed to the presence of Tregs [333, 334]. Likewise, other immunosuppressive factors within the PDAC TME such as IL-10, TGF-b, IDO, myeloid-derived suppressor cells (MDSCs) also likely contribute [335].

In the context of OvCa TIL culture, a lack of TIL infiltration would not seem to be an apparent reason for the failure of 59% of cultures as we observed that the level of infiltration is quite similar to melanoma. However, we did observe a correlation with a lack of TIL growth with IL-2 when the percentage of $CD3^+$ TIL in the fresh tumor sample was below 2% (Figure 33). Due to the variability of immune infiltration in all cancers, not just OvCa, it stands to reason that some sample will not produce TIL outgrowth simply due to insufficient TIL numbers. Beyond that, a more complex cellular mechanism could be involved. Recent work pinpointed an inhibitory population of NK cells that could at least partially explain the increased difficulty to grow TIL with IL-2 in OvCa [336]. Inhibition of $CD3^+$ TIL expansion was correlated with concurrent expansion of NK cells ($CD3^-CD56^+$) within IL-2 only cultures. Furthermore, slow-growing TIL cultures were observed to contain more NK cells than fast-growing cultures. While we observed that NK cells did sometimes expand at the expense of $CD3^+$ TIL growth, we did not find a correlation between NK cell presence and lack of TIL growth. Similarly to PDAC, OvCa can harbor the same inhibitory immune populations like MDSCs and Tregs, as well as immunosuppressive factors like IDO that could contribute [337].

Beyond the poor success rate and lengthy time of TIL culture with the traditional method of PDAC and OvCa TIL culture, I also observed that predominantly $CD4^+$ TIL were produced as opposed to the CD8+ TIL which are correlated with response to TIL ACT. All

of these factors greatly reduced the feasibility of TIL ACT for PDAC and OvCa, thus the implementation of an improved culture method (TIL 3.0) which addressed all these issues. TIL 3.0 uses 4-1BB and CD3 stimulation to selectively and robustly expand $CD8^+$ TIL with a 95% success rate for both cancer types. The TIL 3.0 method was helpful for OvCa TIL culture given the fact that 4-1BB stimulation alone caused extensive NK cell proliferation in some cases, perhaps facilitating their ability to inhibit T-cell growth. The addition of OKT3 to specifically trigger T-cell expansion overcomes the issue of unwanted NK cell growth. Overall, TIL 3.0 also allowed OvCa cultures to grow that were below the 2% live $CD3^+$ TIL threshold that prevented many IL-2 only cultures from growing. Importantly, TIL 3.0 allowed for massive $CD8^+$ TIL proliferation in the absence of over differentiation as indicated by a high percentage of the TIL product expressing CD28 and BTLA, and a low percentage expressing PD-1.

Apart from being a key co-stimulatory molecule for T cells, CD28 also serves as a measure of differentiation as it is lost as a T cells becomes more differentiated, particularly at the terminal differentiation stage. Loss of expression of CD28 on TIL is associated with weak antigen responsiveness in melanoma as well as a key factor leading to senescence, while maintained expression is associated with prolonged persistence in ACT patients [161, 338, 339]. A higher percentage of BTLA expressing $CD8^+$ TIL in the infusion product was correlated with persistence and response in metastatic melanoma TIL treated patients in previous work here at MDACC [108]. Further work by Haymaker *et al.* and Ritthipichai *et al.* laid out respectively a possible explanation for this by showing that, compared to $CD8^+$ TIL lacking BTLA expression, $BTLA^+CD8^+$ TIL are less differentiated ("younger") and able to serially kill cancer cells [188, 189]. Finally, we consistently observed that the use of TIL

3.0 leads to a decreased expression of PD-1 in the expanded TIL (Figures 18 and 34). A low percentage of PD-1 expressing cells in the TIL product may help lower the risk for T cells dysfunction due to the PD-1/PD-L1 axis. These results suggest that TIL 3.0 can facilitate TIL ACT for OvCa and PDAC, and it could likely be applied to other cancer types, including melanoma.

While producing a phenotypically desirable TIL product is important, they key result is demonstrating that the TIL culture process produces a large number of tumor-reactive TIL. $CD8^+$ TIL were tested against tumor targets to demonstrate the anti-tumor potential. For PDAC, we were able to show reactivity against two autologous tumor targets, demonstrating the presence of anti-tumor activity in expanded TIL, as also reported by other groups [120, 241, 303]. Only a comparison of reactivity between IL-2 only and IL-2 + a4-1BB grown TIL was done for PDAC due to the lack of a good tumor target for TIL generated using TIL 3.0. Both TIL lines, regardless of culture method, showed anti-tumor reactivity. Interestingly, bulk TCR-sequencing of the TIL products revealed that the top clones detected were not always the high frequency TIL clones in the tumor and often were low frequency initially. This is notable given the fact that that the high-frequency clones within the tumor are often thought to be enriched due to expansion after antigen recognition in melanoma [340]. However, our data indicate that low-frequency TIL also have anti-tumor potential in PDAC. This was also observed in other GI cancers as well where mutation-reactive TIL were found at very low frequency [250]. The fact that there are tumor-reactive T cells clones in PDAC that have not undergone major clonal expansion indicates again the lack of proper stimulation which is likely related to the dearth of mutations and inhospitable TME. Further analysis of the T-cell repertoires in IL-2 only or IL-2+a4-1BB culture conditions revealed

that they produced distinct repertoires. This result stands to reason given the fact that the a4-1BB mAb selects recently antigen-activated $CD8^+$ TIL expressing 4-1BB whereas IL-2 only provides a non-specific T-cell growth signal. Consistent with this premise is the observation that the IL-2+a4-1BB grown TIL appear to harbor a higher percentage of tumor reactive TIL compared to the IL-2 only grown TIL as shown by the increase in 4-$1BB^+$ CD8+ TIL and IFN-g secretion. Since adding extra stimulation like a4-1BB increased $CD8^+$ TIL growth, this indicates the a4-1BB grown TIL product contains a larger number of anti-tumor T cells.

For testing OvCa TIL reactivity, well-established tumor cell lines that were at least partially HLA-matched to the TIL were used due to the difficulty of establishing autologous tumor lines. Not all the TIL lines showed reactivity against their HLA-matched tumor target, which could be an indication that they may recognize a private mutation specific to that patient as opposed to a shared TAA. Therefore, this does not preclude the fact that the unreactive TIL products would show anti-tumor reactivity if paired with an autologous target, which would be the case in the clinical setting as TIL ACT is meant to treat patients with autologous TIL. Similarly to PDAC, it appears that the improved culture method resulted in an OvCa TIL product that contained a greater number of tumor reactive TIL as indicated by the increased number of spots on the IFN-g ELISPOT (Figure 35). While TIL 3.0 grown OvCa TIL showed increased number of tumor reactive cells (max 0.2% of total), it was less in comparison to melanoma TIL grown using the same method (max 0.8% of total) [218]. Further single-cell TCR sequencing was done on PDAC TIL grown with TIL 3.0, showing that the initial frequency of TIL in the tumor was maintained in the cultured product. For example, this means that high-frequency clones in the tumor were expanded and remained the dominant clones in the TIL product. Therefore, given the fact that tumor-

reactive TIL can be found within the high-frequency clones in the tumor, one could assume that the increased reactivity of the OvCa TIL 3.0 grown cells could be coming from this compartment.

In addition to TCR sequencing, transcriptomic scRNAseq was performed for TIL 3.0 grown PDAC TIL in order to gain insight into the phenotype of the cultured TIL as well as from which transcriptomic cluster in the fresh samples were the TIL expanded. Not surprisingly, the TIL 3.0 cell product is made up predominantly of an activated $CD8^+$ TIL cluster (CD8_GZMB) and a cycling cluster (CD3_cycling) that's derived from the CD8 cluster. A small activated CD4 cluster (CD4_OX40) and γδ T cell cluster are also present. With regards to the $CD8^+$ TIL in the cultured product, it seems the four transcriptomic groups observed in the fresh TIL have mainly collapsed into one activated CD8 cluster. Given the fact that $CD8^+$ TIL were expanded from all four groups (including the TRM-like population) according to TCRseq data, it remains to be seen if any of the features seen in the fresh TIL are still imprinted on them and would re-emerge *in vivo* after TIL administration. This is a particularly interesting question given the previously mentioned report of *ZNF683* and *IL7R* expression being associated with persistence of TIL in the peripheral blood after TIL therapy [323]. scRNAseq could also be used to track the infused TIL for these characteristics as well as look for characteristics associated with persistence and trafficking to the tumor site. The activated CD4_OX40 cluster retained expression of CD40LG and, given the importance of $CD4^+$ T cell help to $CD8^+$ T cell function, administration of an OX40 mAb after TIL infusion could be beneficial.

While the reactivity displayed gives a strong indication of the feasibility of TIL ACT, it can also be used as a springboard for identifying specific tumor antigens that could be

targeted with cellular therapy. OvCa TIL reactivity against HLA-matched tumor lines indicates recognition of shared TAA(s). Moreover, some of the TIL lines are matched on the same HLA allele. This is particularly interesting in the case of a highly prevalent HLA allele like HLA-A*02:01, which is shared by 20-50% of the population in the USA, Europe, and China [341]. Identifying the TCR specific for a shared TAA restricted to this HLA could be an attractive therapeutic option due to its broad applicability. Endogenous T-cell reactivity against various TAA has been shown in OvCa such as mesothelin [342], wild-type or mutated p53 [136, 343, 344], NY-ESO-1 [345], and Her2/neu [346]. The same has been done for PDAC TAAs such as mesothelin, survivin, NY-ESO-1 [120]. Identifying tumor-specific mutated antigens (neoantigens) as immunotherapeutic targets is also a big area of interest due to expression being restricted to only the tumor. There is some indication of the potential of this therapeutic avenue as showed by isolation of T cells from two OvCa patients that were specific for the same p53 neoantigen [136]. Likewise, neoantigen reactive TIL were found in 9/10 GI cancer patients, including a pancreatic cancer patient [250]. The ability to target mutated KRAS, PDACs most prevalent driver mutation, could be possible given results showing response to mutant KRAS vaccines in PDAC patients as well as detection of mutant KRAS reactive TIL in colorectal cancer [115, 347]. In fact, a colorectal cancer patient that was treated with a TIL product containing mutant KRAS-specific TIL showed clinical efficacy [115]. While the TIL product contained TIL with other antigen-specificities, the KRAS-mutant specific TIL likely played a major role as indicated by the patient recurring after having lost expression of the HLA for which the mutant KRAS peptide was restricted. However, targeting neoantigens beyond a prevalently mutated genes like TP53 for OvCa (95% of cases) and KRAS for PDAC (94% of cases) could be challenging due to neither

having a high mutation burden [206]. However, therapies targeting a particular antigen could be difficult to use given the possibility of target antigen loss. TIL ACT could potentially avoid these issues by providing antigen specificity against a broad range of TAA.

In conclusion, my work has demonstrated the feasibility of TIL ACT for PDAC and OvCa by showing that they harbor an activated, anti-tumor $CD8^+$ T-cell infiltrate that can be robustly and reliably expanded using an improved 3-signal culture method consisting of a CD3 stimulation, an agonistic 4-1BB mAb, and high-dose IL-2. Additionally, these results show that TIL ACT for PDAC and OvCa is feasible regardless of primary or metastatic site, and, in the case of OvCa, in a chemotherapy refractory setting as is often the case. Furthermore, our understanding of the TIL heterogeneity in PDAC has been increased through the use of single-cell RNA transcriptomic and T-cell receptor sequencing, revealing novel TIL populations with potential prognostic implications. It was one of my biggest dreams that the results of my work could provide some benefit to patients in dire need of improved therapy options. So, it is with much hope and great expectation that I conclude my dissertation with the fact that a Phase II clinical trial partly based on this work is ongoing at MDACC to evaluate the feasibility of the adoptive transfer of autologous tumor-infiltrating lymphocytes in OvCa and PDAC.

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Vita

Donald Anastas "Donastas" Sakellariou-Thompson was born in St. Louis, Missouri, the son of Scott Thompson and Maria Sakellariou. A lifelong curiosity about the world was directed into scientific research after an influential summer spent in the chemistry lab of John R. Bleeke at Washington University in St. Louis as part of the 2008 Students and Teachers as Research Scientists (STARS) program. After graduating from Chaminade College Preparatory School (St. Louis, MO) in 2009, Donastas attended the University of North Carolina at Chapel Hill. While there, Donastas performed research in the labs of Drs. Oliver Smithies, Scott Bultman, and Bernard Weissman. In May 2013, Donastas graduated with a Bachelor of Science in Biochemistry. Donastas entered the Ph.D. program at The MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences in August 2013, where he carried out this dissertation work under the mentorship of Dr. Chantale Bernatchez and Dr. Patrick Hwu.

Publications

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