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The role of myeloid cells in modulating the therapeutic effectiveness of immune checkpoint inhibitors in pancreatic ductal adenocarcinoma

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THE ROLE OF MYELOID CELLS IN MODULATING THE THERAPEUTIC EFFECTIVENESS OF IMMUNE CHECKPOINT INHIBITORS IN PANCREATIC DUCTAL ADENOCARCINOMA

by

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is a highly fatal cancer, accounting for 3.2% of new cancer cases yearly but nearly 8% of all yearly cancer mortalities. Over the past twenty years, our understanding of cancer biology has greatly improved which has resulted in vastly improved prognoses for many cancers. However, the prognosis of pancreatic ductal adenocarcinoma has not improved despite the advance in cancer treatments. This is especially apparent with cancer immunotherapies, a newer therapeutic strategy that utilizes the innate defense mechanism of the body to target malignancies. Immune checkpoint inhibitors are a type of cancer immunotherapy that act by inhibiting the PD-1/PD-L1 and CTLA-4 immune checkpoint pathways and allowing T lymphocytes to proliferate and generate an antitumor response. They have greatly improved the prognosis for many types of malignancies, but clinical studies show that immune checkpoint inhibition has had a limited effect on the prognosis of PDAC. Recent studies have demonstrated that the immune microenvironment of PDAC is highly immunosuppressive, which is a probable factor in limiting the therapeutic efficacy of immune checkpoint inhibitors. Myeloid derived suppressor cells (MDSCs) are a main component of the immune microenvironment in PDAC. They are immature cells of myeloid origin that express $CD11b^{+}Gr\text{-}1^{+}$ on their surface, making them phenotypically

distinct from mature dendritic cells. Their infiltration of the PDAC microenvironment early on in the course of the disease is promoted in a large part by the cytokine GM-CSF. MDSCs are believed to contribute to the limited efficacy of immune checkpoint inhibitor therapy both directly and indirectly. Indirect mechanisms are mediated by promoting the activity of other immunosuppressive cells in the PDAC microenvironment such as tumor associated macrophages and regulatory T lymphocytes. MDSCs induce the transformation of naïve $CD4^{+}$ T lymphocytes into protumorigenic regulatory T lymphocytes. They also promote the polarization of macrophages to the tumor associated macrophage phenotype ($IL\text{-}10^{high}$ $IL\text{-}12^{low}$) by secreting IL-10, which decreases IL-12 synthesis by macrophages present in the tumor microenvironment. On top of mediating immunosuppression through other cell types, MDSCs directly mediate immunosuppression by decreasing the amounts of amino acids necessary for anti-tumor immunity in the tumor microenvironment and disrupting the activity of antigen presenting cells and the signaling needed to initiate a cytotoxic T lymphocyte response. The decreased amount of arginine limits the ability of T cells to proliferate, resulting in a weaker cytotoxic response. These mechanisms limit the antitumor response against pancreatic ductal adenocarcinoma, resulting in the decreased response to immune checkpoint inhibitor therapy observed in clinical trials. Future attempts to strengthen the anti-tumor immune response must be combinatorial therapies that incorporate therapeutic strategies that seek to alleviate MDSC-mediated immunosuppression of T lymphocytes from the tumor microenvironment in addition to the more widely available immune checkpoint inhibitor therapy. Such therapeutics are currently being studied in murine

models and have shown promising preliminary results but have yet to have been examined in clinical trials. These therapies are an ideal avenue to explore in a search for more effective therapy for this highly lethal disease.

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LIST OF ABBREVIATIONS

Ab ..... Antibody

APC ..... Antigen Presenting Cell

CAF ..... Cancer Associated Fibroblast

CAR – T cell ..... Chimeric Antigen Receptor T cell

CD ..... Cluster of Differentiation

Cdk ..... Cyclin Dependent Kinase

CSC ..... Cancer Stem Cell

CTL ..... Cytotoxic T Lymphocyte

CTLA-4 ..... Cytotoxic T Lymphocyte Associated Protein 4

DAMP ..... Damage Associated Molecular Pattern

DC ..... Dendritic Cell

DFS ..... Disease Free Survival

EGF ..... Epidermal Growth Factor

FACS ..... Fluorescence Activated Cell Sorting

FGF ..... Fibroblast Growth Factor

FRET ..... Fluorescence Resonance Energy Transfer

FRET-SE ..... Fluorescence Resonance Energy Transfer Sensitized Emission

GM-CSF ..... Granulocyte-Macrophage Colony Stimulating Factor

H&E ..... Hematoxylin and Eosin

HGF ..... Hepatocyte Growth Factor

HIF-1 ..... Hypoxia Induced Factor

IF ..... Immunofluorescence

IHC ..... Immunohistochemistry

IL ..... Interleukin

iNOS ..... Inducible Nitric Oxide Synthase

mAb ..... Monoclonal Antibody

MDSC ..... Myeloid Derived Suppressor Cell

MFI ..... Mean Fluorescence Intensity

MHC ..... Major Histocompatibility Complex

NK ..... Natural Killer

OS ..... Overall Survival

PAMP ..... Pathogen Associated Molecular Pattern

PanIN ..... Pancreatic Intraepithelial Neoplasia

PDAC ..... Pancreatic Ductal Adenocarcinoma

PD-1 ..... Programmed Death -1

PD-L1/2 ..... Programmed Death Ligand 1/2

pMHC ..... Peptide-MHC

PNT ..... Peroxynitrite

ROS ..... Reactive Oxygen Species

SEER ..... Surveillance, Epidemiology, and End Results

TAA ..... Tumor Associated Antigen

TAM ..... Tumor Associated Macrophage

TCR ..... T cell Receptor

TME.....Tumor Microenvironment

VEGF.....Vascular Endothelial Growth Factor

INTRODUCTION

Pancreatic cancer is a rare yet highly lethal cancer. Based on data collected from 2010-2016 by the SEER Program of the National Cancer Institute, pancreatic cancer is estimated to account for 3.2% of all new cancer diagnoses and a disproportionate 7.8% of cancer fatalities in 2020 due to its dismal five-year survival rate of 10%.[1] Pancreatic cancer can be divided into two subtypes: pancreatic ductal adenocarcinoma (PDAC), which accounts for 90% of cases of pancreatic cancer and neuroendocrine tumors, which account for the remaining 10%.[1] PDAC is the fourth highest cause of cancer related deaths globally[2], making it crucial to find effective therapeutic options. Despite advances in our understanding of cancer biology, the survival rate of PDAC has remained constant for the past thirty years, as shown in **Figure 1**.

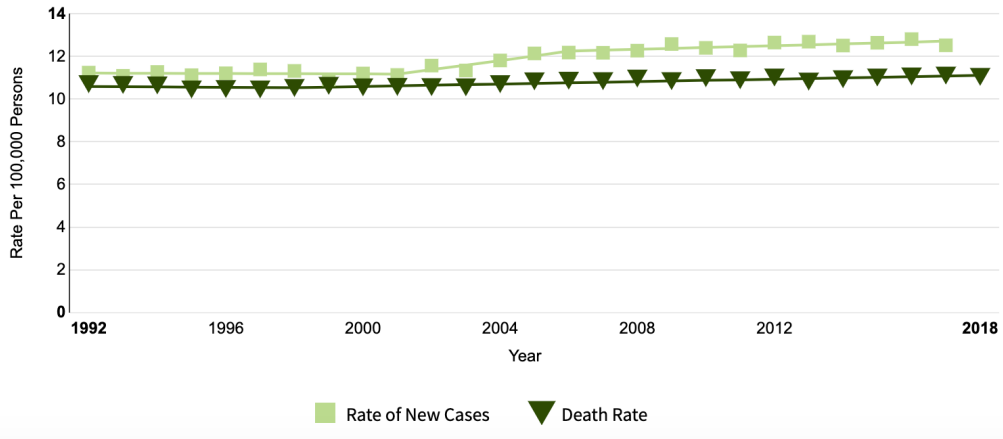

Figure 1. Rates of New Cases and Deaths. Over the past 25 years, there have been dramatic improvements in our knowledge of cancer biology and in development of therapies that utilize the biological differences between normal and malignant cells to treat cancers. For many cancers, this has resulted in a significant drop in the death rate associated with the cancer. However, in the case of pancreatic cancer, the understanding of pancreatic cancer biology has not translated into more effective therapies. Figure adapted from SEER webpage, accessed on Oct. 1, 2020.[1]

The severity of a PDAC diagnosis is largely dependent on the stage of disease when the patient is diagnosed. As a metastatic disease, it has a significantly worse five-year relative rate of survival than localized disease (2.9% survival vs 39.8% survival).[1] The treatments for metastatic disease remain largely palliative, designed to reduce the symptoms of the disease, rather than treating the malignancy itself. The lack of an improvement in the prognosis of both local and metastatic pancreatic cancer can be attributed to the highly therapeutic resistant nature of pancreatic cancer. This therapeutic resistance arises from a combination of the highly heterogeneous nature of the cancer, allowing it to garner mutations that enable it to become resistant to conventional therapies and of the highly fibrotic microenvironment of pancreatic cancer, which promotes growth of the tumor while restricting access to administered drugs and the activity of cytotoxic lymphocytes.[2] Restricting the activity of cytotoxic lymphocytes has greatly decreased the efficacy of immunotherapies that have shown promising results in many other malignancies.

In this thesis, I will address the immunosuppressive tumor microenvironment that is characteristic of PDAC. The PDAC microenvironment is responsible for enabling the progression of PDAC to metastatic disease and for limiting the efficacy of immunotherapies that have been shown to be promising therapies for other cancers. The following introduction will provide an overview of the immune response. It will emphasize the adaptive immune response, describe the immunotherapeutic strategies commonly used to treat malignancies and provide a general description of the tumor microenvironment.

An Overview of Cancer Immunotherapy

One of the newest strategies to treat malignancies that has shown great promise in pre-clinical models of cancer and in clinical trials is cancer immunotherapy. It utilizes the endogenous defense system of the body, the immune system, to target malignancies. There are several treatment options that fall under the category of cancer immunotherapies. These treatments require an understanding of the biology involved in the interactions between immune cells and an understanding of the cancer immunity cycle.

An Overview of the Immune System

The immune system can be subdivided into the innate immune system and the adaptive immune system. The innate immune system consists of the body's initial defense against pathogens and is composed of barriers such as the skin and the mucous linings of the respiratory tract, gastrointestinal tract, and reproductive tract, and several types of cells including granulocytes, monocytes, macrophages, dendritic cells, and natural killer (NK) cells.[3] These cells recognize pathogens by common molecular patterns referred to as pathogen associated molecular patterns (PAMPs) and respond rapidly to the presence of foreign substances in the body but do not generate memory of the pathogen. Dendritic cells and macrophages (often referred to as antigen presenting cells or APCs) engulf and process the pathogen to initiate a more specific response that is mediated by the adaptive immune system. Once APCs have completed processing the pathogen, they upregulate several molecules on their cell surface, including the major histocompatibility complex II (MHC II), the co-stimulatory molecule B7, the cell adhesion molecule ICAM-1, and

chemokines that promote cell trafficking. In order to generate the response of the adaptive immune system, dendritic cells and macrophages must migrate to lymph nodes and activate lymphocytes (T and B cells).[3] This migration is enabled by the expression of chemokines by the antigen presenting cells. Once the antigen presenting cells enter the lymph node, they interact with T lymphocytes that have T cell receptors specific for the antigen they are presenting. This interaction begins the process of activating the adaptive immune system, which provides a more specific response against a pathogen as well as a lasting memory of the pathogen in the immune system. The subsequent steps of the lymphocyte's activity are dependent on the cell surface marker expressed by the T lymphocyte. The T cell receptor on $CD4^+$ T lymphocytes binds to the MHC II molecule expressed by the antigen presenting cells, resulting in the upregulation of the co-stimulatory molecule CD28 and secretion of interleukin 2. Full T lymphocyte activation requires the co-stimulatory molecules CD28 and B7 to bind to each other. The co-stimulatory signal stabilizes the interleukin -2 mRNA, allowing it to bind to the interleukin-2 receptor expressed by T lymphocytes and promote the proliferation of memory and effector T lymphocytes. Once fully activated, the $CD4^+$ T lymphocytes follow a chemical gradient of sphingosine -1 to exit the lymph node and migrate to the site of infection, where they promote the activity of phagocytes and cytotoxic T lymphocytes by releasing pro-inflammatory cytokines. The subtype of $CD4^+$ T lymphocyte determines which cytokines are released. The primary three subtypes of $CD4^+$ T lymphocytes are T_H1 cells, T_H2 cells, and T_H17 cells. Differentiation of CD4+ T lymphocytes into T_H1 cells is promoted by the release of interleukin-12 by dendritic cells.

Interleukin 12 binds to receptors on naïve $CD4^+$ T lymphocytes and promotes the expression of the transcription factors, T-bet, STAT1, and STAT4, which promote the T_h1 phenotype.[4] T_h1 cells promote the phagocytic activity of macrophages and neutrophils by releasing the cytokines IFN- γ and TNF- α and expressing the CD40L. IFN- γ binds to a receptor expressed on macrophages and the CD40 ligand binds to the CD40 cell surface protein, further promoting the phagocytic activity of the macrophage. In addition to promoting phagocytic activity, the interactions between T_H1 cells and macrophages also cause macrophages to increase secretion of the cytokines, TNF, IL-1, and IL-12, and the expression of MHC and co-stimulatory molecules. The secretion of the cytokines further promotes the T_H1 phenotype, while the upregulation of MHC and co-stimulatory molecules enables better activation of naïve $CD4^+$ T lymphocytes. TNF- α promotes the activation of neutrophils, which enhances microbial killing. The secretion of interleukin 4 from eosinophils and mast cells results in the expression of the transcription factors GATA-3 and STAT6, which promote differentiation of naïve CD4+ T lymphocytes into T_H2 cells.[4] T_H2 cells are primarily responsible for eradicating helminthic infections. However, they can also block classical macrophage activation and inflammation by secreting interleukin 4 and interleukin 10, limiting the effectiveness of the innate immune response to disease. The last subtype of $CD4^+$ T lymphocytes are T_H17 cells. Naïve $CD4^+$ T lymphocytes differentiate into T_H17 cells when exposed to extracellular bacteria and fungi and release interleukin 17 and interleukin 22 to strengthen epithelial barriers and to promote an inflammatory response. IL-17 acts upon leukocytes, promoting the release of TNF- α, interleukin 1, interleukin 6, and colony stimulating

factorss, which are the cytokines responsible for promoting the differentiation of macrophages and the inflammatory response.

$CD8^+$ T cells, also referred to as cytotoxic lymphocytes, are also activated in the lymph node by interaction with MHC molecules. As $CD8^+$ T lymphocytes are directly responsible for cytotoxicity, their activation requires the presence of $CD4^+$ T lymphocytes in addition to antigen presenting cells. IL-2 secreted from $CD4^+$ T lymphocytes binds to IL-2 receptors on $CD8^+$ T lymphocytes, enabling them to differentiate into cytotoxic lymphocytes and memory $CD8^+$ T lymphocytes. Unlike $CD4^+$ T lymphocytes, they interact with only MHC I molecules, making them capable of monitoring the intracellular activity of a large number of cells. The T cell receptor of $CD8^+$ T lymphocytes recognizes an antigen displayed by the MHC molecule, causing the TCR to bind to the displayed antigen. This binding interaction promotes the upregulation of cellular adhesion molecules ICAM-1 by the APC and LFA-1 by the $CD8^+$ T lymphocyte, to further stabilize their interaction. Once $CD8^+$ T cells are fully activated, they promote apoptosis of the infected cells by trafficking to the location of the infected cell by following a chemokine trail, releasing Granzyme B and perforin into the infected cells, and then detaching from the cell. Perforin disrupts the cell membrane of infected cells, allowing the Granzyme B to enter the cells and degrade intracellular proteins, causing apoptosis of the infected cell.

Cancer Immunity Cycle

The cancer immunity cycle is a set of sequential events that must occur in order for an antitumor immune response to effectively eliminate cancer cells. **Figure 2** summarizes the events of the cancer immunity cycle.

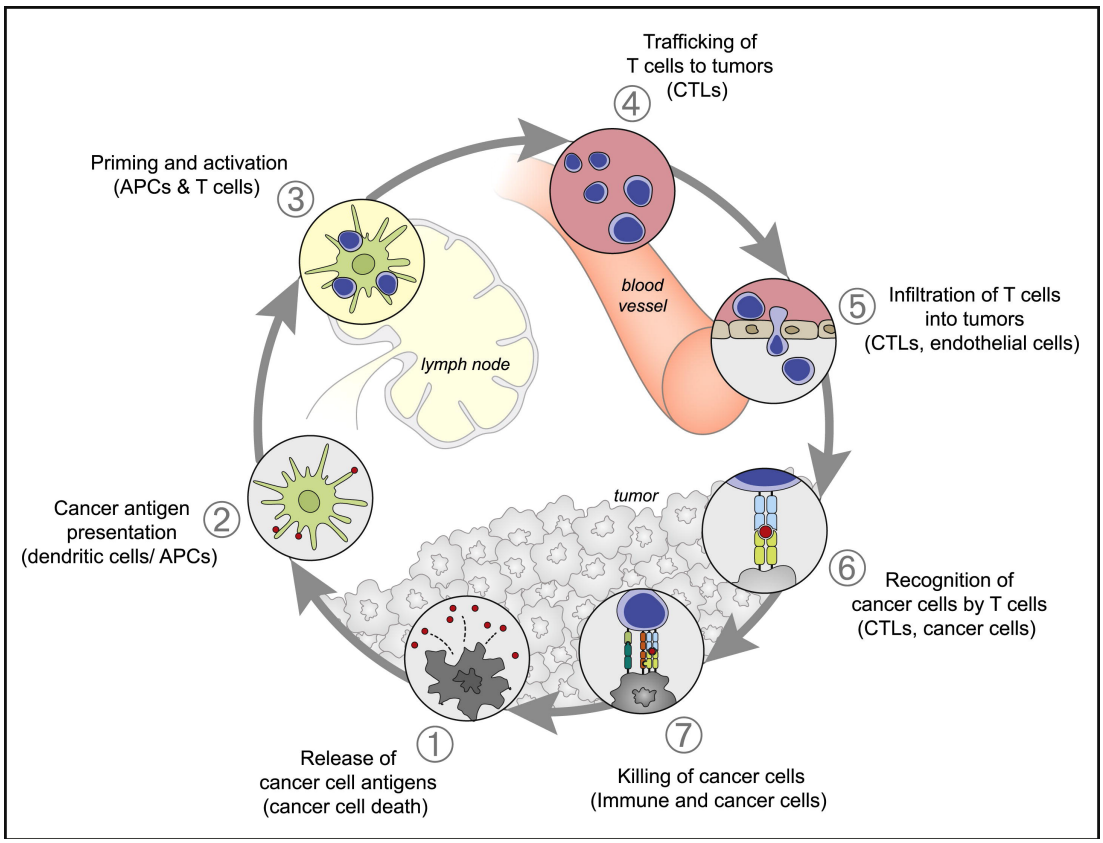

Figure 2. The Cancer Immunity Cycle. In order for the immune system to effectively kill tumor cells, there are a set of sequential steps that must occur, as illustrated above. When cancer cells die, they release a set of neoantigens that are taken up by antigen presenting cells such as dendritic cells and then presented to $CD4^+$ and cytotoxic T lymphocytes on MHC Class I and Class II molecules that are expressed on the surface of the dendritic cells. Antigen presentation by dendritic cells results in CTL priming and activation, leading to CTLs entering the bloodstream and infiltrating the tumor. Upon infiltration, T cells recognize the cancer cells expressing the tumor associated antigen and trigger apoptosis of the cancer cells, releasing additional tumor associated antigens and continuing the cancer immunity cycle. Figure taken from Chen and Mellman 2013.[5]

In many patients with cancer, the cancer immunity cycle is malfunctioning, leading to an impaired immune response in the presence of cancer cells. Cancer immunotherapies seek to restore immune activity against the malignancy by disrupting immune checkpoints, increasing recognition of tumor associated antigens, increasing the ability of CTLs to infiltrate a tumor, and increasing CTL activity against a tumor.

At each step in the cancer immunity cycle, there is a balance of regulatory signals that promote and inhibit CTL activity. In the initial step of the cancer immunity cycle, a failure of the dendritic cells to take up the tumor associated antigens will result in a failure to activate CTLs against tumor associated antigens and no immune response to the TAAs. The subsequent step, antigen presentation to CTLs, occurs in the lymph nodes and is stimulated by cytokines such as TNF-α and IFN-α and inhibited by interleukin 4, interleukin 10, and interleukin 13.[5] Upon antigen presentation, CTLs become activated to migrate to the tumor. This activation requires signaling between the co-stimulatory molecules CD28 (expressed on the T lymphocyte) and B7 (expressed on the surface of the dendritic cell). Co-stimulatory signaling can be interrupted by the immune checkpoints, CTLA-4 and PD-L1, resulting in the inhibition of activation of T cells. These immune checkpoint molecules are often overexpressed on malignant cells, suggesting that they are used as a mechanism of immune evasion.

Upon receiving co-stimulatory signals, $CD8^+$ T lymphocytes must migrate to the location of the malignancy via the vasculature and enter the tumor. In many malignancies, the cancer cells are located in epithelial tissues, and CTLs must extravasate the vasculature in order to target the cells successfully. This process is promoted by the

expression of cellular adhesion molecules ICAM-1 and LFA-1, that enable leukocytes to bind to the walls of the vasculature and exit the bloodstream. Once in the tumor, the T-cell receptor interacts with the proteins displayed by the MHC I complex on the cancer cells to initiate the release of perforin and cytotoxic granzymes to the cancer cells. One strategy malignant cells use to avoid this cytotoxic response is suppressing the expression of MHC Class I molecules on their membrane, therefore preventing the TCR from recognizing them as malignant cells and initiating apoptosis in these cells.

Therapeutic Strategies

Several therapeutic strategies have been developed to increase immunoreactivity to malignant cells. They seek to increase immune recognition of proteins overexpressed in tumors and to decrease the inhibition of the immune system by immune checkpoints. Increased immune recognition of cancer antigens is promoted by the use of cancer vaccines and CAR-T cell therapy. Cancer vaccines employ dendritic cells to present tumor associated antigens to T lymphocytes, activating the T lymphocytes with a T-cell receptor that recognizes the specific neoantigens displayed on the MHC Class II molecule on the surface of the dendritic cells. This increased recognition of tumor associated antigens promotes an increase in cytotoxic activity against malignant cells. Another strategy, adoptive T cell therapy, aims to increase immune recognition of malignant cells by extracting immature T lymphocytes from a patient, genetically engineering them to have TCRs that are specific for the tumor associated antigens present in the patient's tumor, and administering chemical signals to mature the T cells to promote cytotoxic activity against the cancer cells. Immune checkpoint inhibitor therapies aim to prevent

the natural immune regulatory mechanisms, immune checkpoints from suppressing the activity of the adaptive immune system's antitumor response. To gain a more detailed understanding of how these therapies function *in vivo*, it is critical to examine the interactions between the cells in the tumor environment.

An Overview of the Tumor Microenvironment

As our understanding of cancer biology has progressed over the past fifty years, it has become apparent that cancer cells are dependent on their environment to successfully undergo transformation into lethal malignancies.[6] The surrounding environment is referred to as the tumor microenvironment and is composed of a heterogeneous group of cells. These cells form an intricate signaling network with the malignant cells present in the tumor, resulting in reciprocal interactions which further promote the growth of the cancer. **Figure 3** is a schematic of how the reciprocal interactions between the tumor microenvironment (TME) and the malignant cells drives the progression of cancer.

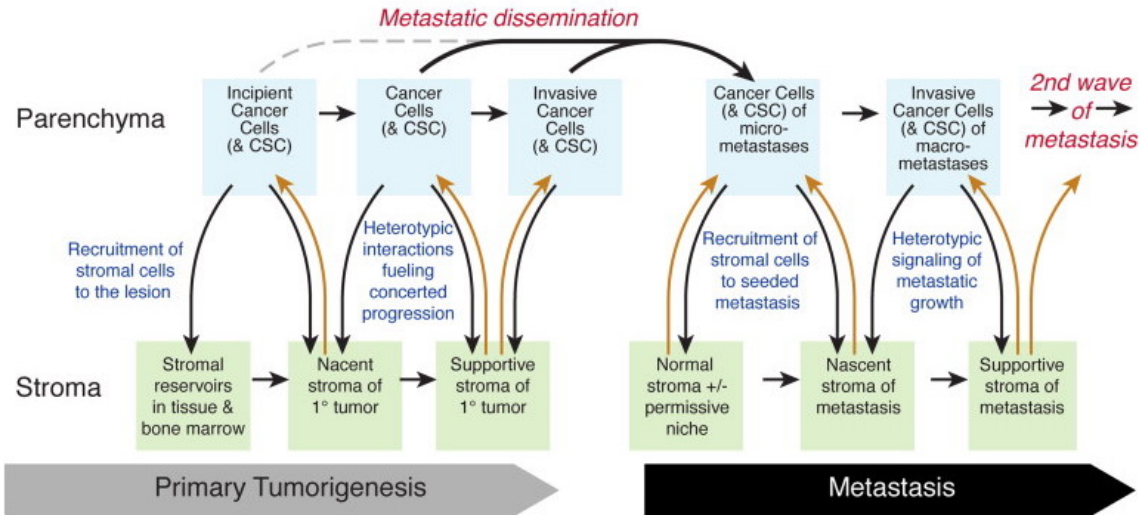

Figure 3. Reciprocal Interactions between Cancer Cells and Stromal Tissue. During tumorigenesis, the malignant cancer cells recruit supportive stromal cells to provide a

favorable microenvironment to allow the tumor to grow. As the cancer progresses, reciprocal signaling allows the surrounding stromal cells to provide the cancer cells with favorable conditions for proliferation, causing the cancer to increase its recruitment of stromal cells. Upon the dissemination of cancer cells and the formation of a micrometastatic site, malignant cells interact with the already existing stroma to promote a favorable environment for the new metastasis of the cancer to proliferate and maintain an invasive phenotype. Figure adapted from Hanahan and Weinberg 2011.[6]

As mentioned previously, the tumor microenvironment is a highly heterogeneous group of cells. **Figure 4** is a summary of the cell types present within the tumor microenvironment and their roles in the progression of malignancies.

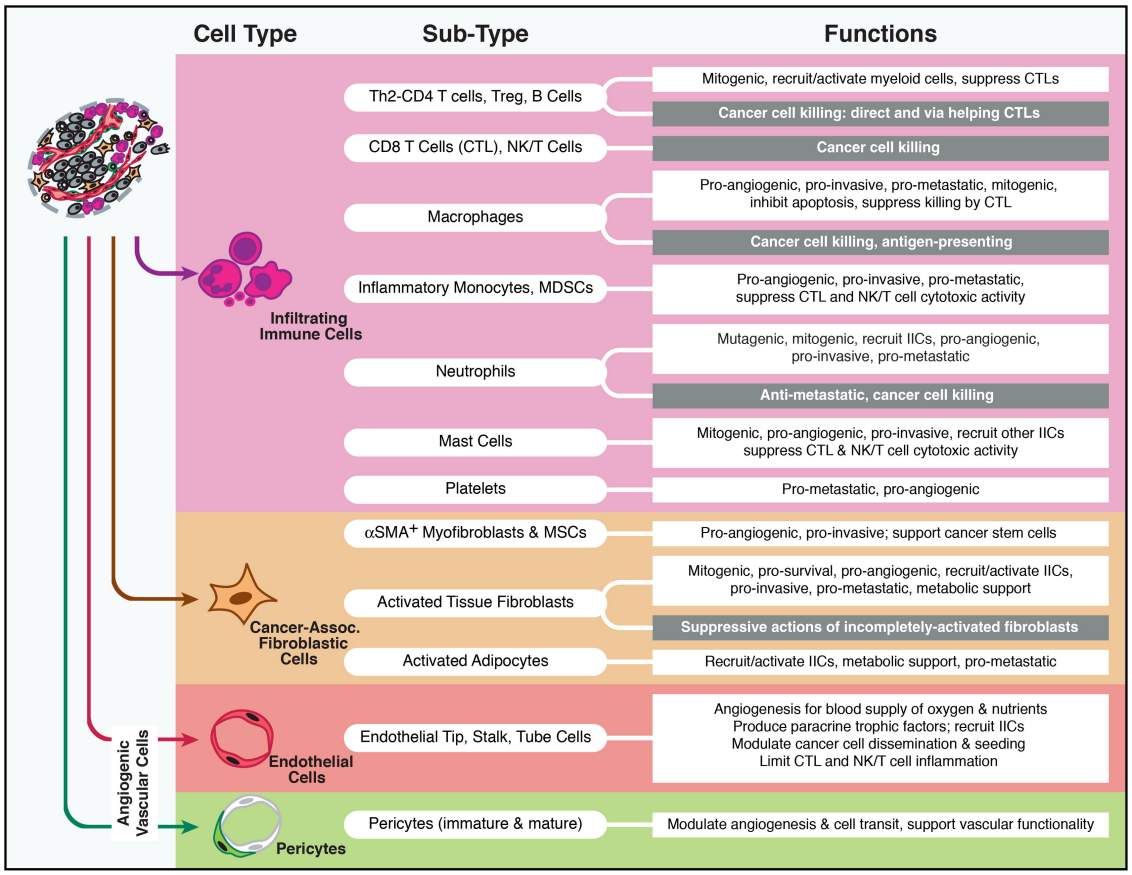

Figure 4. Cell Types Present Within the Tumor Microenvironment and their Functions. The cells present within a tumor microenvironment can be divided into four categories: infiltrating immune cells, cancer associated fibroblasts, endothelial cells, and pericytes. These cells are often recruited by the cancer cells and "reprogrammed" to become protumorigenic. Their roles in promoting the progression of the tumor are often overlapping, utilizing redundant signaling pathways that are ideal therapeutic targets. Figure taken from Hanahan and Coussens 2012.[7]

The cell types within the tumor microenvironment that play a large role in promoting the progression of a malignancy can be divided into three broad categories: Angiogenic vascular cells, infiltrating immune cells, and mesenchymal cells. Angiogenic cells are primarily composed of endothelial cells that line the walls of the vasculature. Endothelial cells respond to hypoxia within the tumor microenvironment by producing hypoxia inducing factor (HIF-1) and vascular endothelial growth factor (VEGF). VEGF and HIF-1 promote angiogenesis, allowing the tumor increased access to nutrients and oxygen necessary for its survival, as well as creating a conduit that enables malignant cells to enter the bloodstream and form distant metastases.[8]

Mesenchymal cells in a tumor microenvironment are referred to as cancer associated fibroblasts (CAFs), and they are a diverse cell population that is widely implicated in promoting tumorigenesis, metastasis, and immunosuppression. They are derived from a variety of cell types (normal fibroblasts, surrounding endothelial cells, pericytes, stellate cells, bone-marrow derived mesenchymal cells, and adipocytes), and the precursor cell type determines the function of the CAF.[9] Normally, fibroblasts are in a quiescent state, but when activated by wound healing processes or fibrosis, fibroblasts differentiate to myofibroblasts. Once activated, myofibroblasts in normal tissue express various growth factors, chemokines, and cytokines that promote wound healing, and then undergo apoptosis after wound healing is completed. As inflammation persists in cancer, activated myofibroblasts fail to undergo apoptosis, instead contributing to carcinogenesis by releasing growth factors and promoting immunosuppression.

Infiltrating immune cells consist of several distinct cell types that can have both pro-tumorigenic and anti-tumorigenic effects. Immune cells that are anti-tumorigenic include cytotoxic ($CD8^+$) T lymphocytes, $CD4^+$ T lymphocytes, and dendritic cells. $CD8^+$ T lymphocytes recognize the cancerous cells by the expression of mutated proteins by MHC I molecules on the membrane of the cells. The activity of the CTLs is aided by the support of $CD4^+$ Th1 cells, which secrete the cytokines IL-2 and IFN-γ to promote cytotoxicity. Dendritic cells indirectly promote anti-tumorigenic activity, aiding the CTL recognition of tumor associated antigens by displaying TAAs on MHC II molecules that are expressed on the surface of dendritic cells. Protumorigenic immune cells within the tumor microenvironment include tumor associated macrophages (TAMs), myeloid derived suppressor cells (MDSCs), and Th2 and T regulatory $CD4^+$ T-cells.[10] Tumor associated macrophages consist of several subpopulations of cells and are derived from circulating monocytes in a process regulated by GM-CSF. TAMs suppress the cytotoxic activity of $CD8^+$ T cells by several mechanisms, preventing them from recognizing and killing malignant cells. Other cells that have an immunosuppressive function within the TME include MDSCs and two classes of $CD4^+$ T cells: Th2 $CD4^+$ T cells and T_{reg} cells. MDSCs prevent activation of $CD8^+$ T lymphocytes indirectly by recruiting other immunosuppressive cells to the TME and directly by activating signaling pathways that suppress CTL activity. Both T_H2 and T_H17 $CD4^+$ T lymphocytes secrete cytokines such as IL-10 that inhibit CTL activity. The role of myeloid derived suppressor cells in promoting immunosuppression in PDAC, leading to decreased efficacy of cancer immune checkpoint inhibitor therapy will be explored in depth in this thesis.

PUBLISHED STUDIES

Targeting Immune Checkpoints in Pancreatic Ductal Adenocarcinoma

The immune system utilizes a highly effective strategy to identify and eradicate pathogens. However, prolonged activation of the adaptive immune system can result in autoimmunity, where the immune system recognizes and targets cells expressing self-antigens, and subsequent tissue damage. In addition to the multiple mechanisms of peripheral and central tolerance that prevent autoimmunity, there are redundant signaling pathways that prevent the prolonged activation of the immune system. Two of the most well characterized such signaling pathways are the PD-1/PD-L1 pathway and the CTLA-4/B7 pathway. These pathways are referred to as immune checkpoints, as they prevent the over-activation and prolonged activation of T lymphocytes in response to a foreign antigen.

PD-1 is the programmed death receptor expressed by T cells in response to their activation by signaling from the T cell receptor.[11] The upregulation of PD-1 on the surface of T lymphocytes allows the PD-1 receptor to bind to its ligand, PD-L1. PD-L1 is expressed on the surface of several different cell types, including on the surface of tumor cells. This interaction has been shown to decrease T lymphocyte proliferation and the effectiveness of the cytotoxic T lymphocyte response. **Figure 5** illustrates the effects of

the PD-1 – PD-L1 interaction on the proliferation of $CD8^+$ T cells.

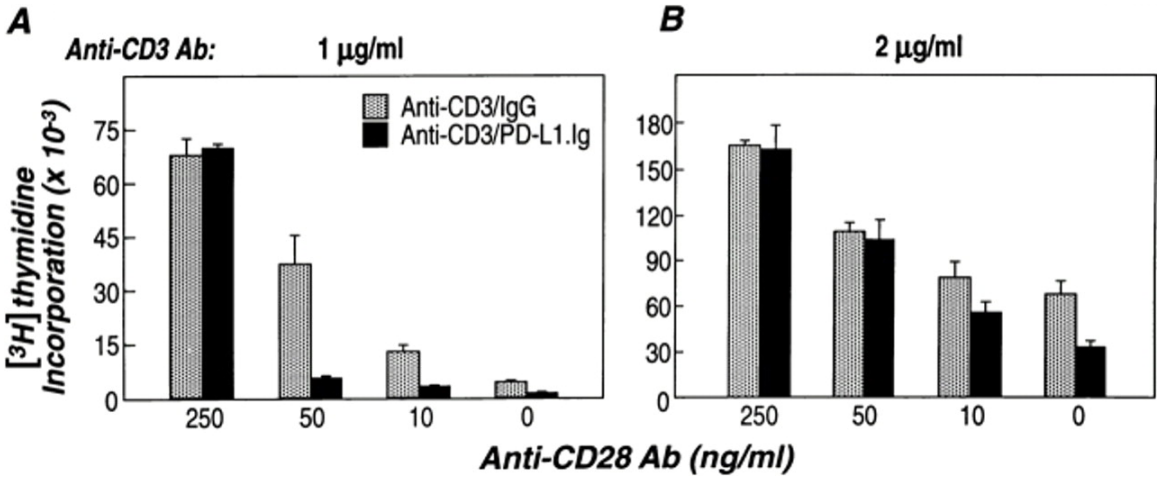

Figure 5. The Effect of PD-L1 Signaling on the Proliferation of $CD8^+$ T Lymphocytes. [^{3}H] thymidine incorporation was used to measure the rate of proliferation of $CD8^+$ T lymphocytes under varying conditions of antibodies against CD3, CD28, and treatment with one of two cross-linked antibodies, either Anti-CD3/IgG or Anti-CD3/PD-L1.Ig. The cross linked Anti-CD3/IgG was used as a control to establish the effects of treating $CD3^+$ T lymphocytes with increasing amounts of Anti-CD28 Ab. Increasing the concentrations of the anti-CD28 antibody resulted in an increase in [^{3}H] Thymidine incorporation, indicating a significant increase in proliferation of $CD8^+$ T cells. The same result occurred when the amount of Anti-CD3 Ab was increased. In contrast to treating the cells with the anti-CD3/IgG antibody, treatment with the anti-CD3/PD-L1.Ig resulted in decreased [^{3}H] Thymidine incorporation, indicating a significant decrease in T lymphocyte proliferation resulting from PD-L1.Ig signaling. Figure taken from Freeman et al., 2000.[12]

The decrease in T lymphocyte proliferation resulting from PD-1 – PD-L1 signaling may result from the combined effect of several signaling pathways that are activated upon TCR activation. A summary of the mechanisms by which PD-L1 prevents activation of CTLs is shown in **Figure 6**.

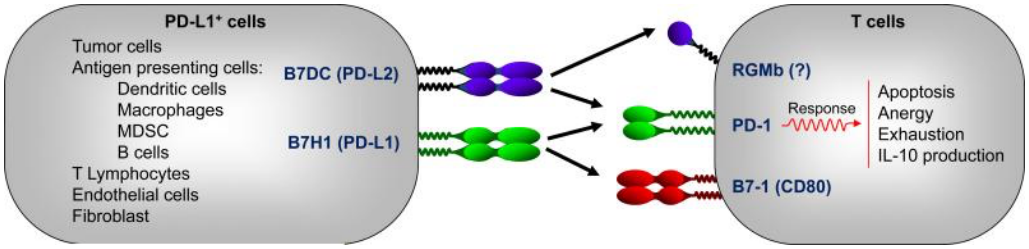

Figure 6. The Mechanism of PD-L1/2- PD-1 Immunosuppression. Many cells within the TME express the proteins PD-L1 or PD-L2. These proteins are the ligand for the PD-1 receptor expressed on T lymphocytes, and a binding interaction between PD-L1/2 and PD-1 results in a failure of the T lymphocytes to activate, instead resulting in apoptosis, functional exhaustion, anergy, or the release of immunosuppressive cytokines such as interleukin 10. In addition to interactions with PD-1, the PD-L1 ligand can also interact with the B7 molecule expressed by the T-cell, preventing CD28 from binding and activating the T-cell. Figure taken from Zou, Wolchok, and Chen 2016.[13]

Increased PD-L1 expression on malignant cells has been shown to be one of the many mechanisms by which cancers escape anti-tumor activity. As PD-1 – PD-L1 interactions often result in anergy and apoptosis of T-cells, increasing the frequency of these interactions results in immunosuppression of the anti-tumor immune response. PD-1 – PD-L1 induced immunosuppression promoted by MDSCs has been observed in pancreatic ductal adenocarcinoma. Zhang, et al., created a transgenic mouse model by crossing CD11b-DTR with mice that had either a mutation in iKras or iKras and p53. These mice were then treated with diphtheria toxin to cause depletion of myeloid CD11b+ cells, and analyzed for gene expression of various genes, including *Pdcdlg1* and *Pdcdlg2***,** which encode the PD-L1 expressed on myeloid and tumor cells and PD-L2 expressed by myeloid cells.[14] It was found that depletion of myeloid cells leads to decreased expression of PD-L1 on tumor cells, which results in increased activation of cytotoxic lymphocytes and a stronger antitumor response. This increased activation of $CD8^+$ T lymphoctyes is further demonstrated by the increased secretion of IL-2 and IFN-

γ. However, PD-1 blockade alone was insufficient to recreate the results of myeloid cell inhibition, as inhibition of the PD-1/PD-L1 pathway leads to upregulation of CTLA-4, another immune checkpoint molecule, suggesting that myeloid derived suppressor cells contribute to several mechanisms of immune evasion.

CTLA-4 is an immune checkpoint that is also upregulated in response to the activation of T lymphocytes. **Figure 7** illustrates the mechanism of CTLA-4 immunosuppression by using anti-CD3 antibodies to activate T lymphocytes and analyzing the effects of treating activated T lymphocytes with anti-CD28, anti-B7-1, and anti-CTLA-4 antibodies, either individually or in combination.

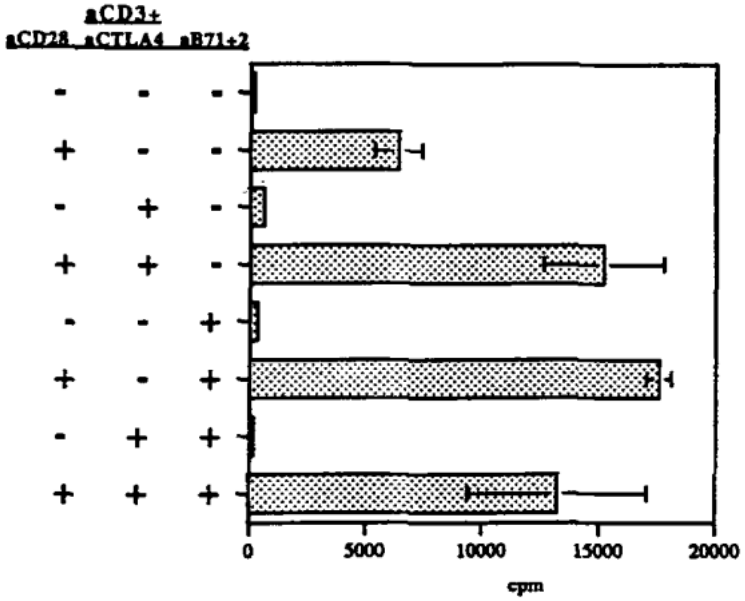

Figure 7. The Effects of treating activated T cells with anti-CD28, anti-CTLA-4, and anti-B7-1/2 Antibodies. An anti-CD3 antibody was used to activate T lymphocytes and their rate of proliferation was observed after treating them with anti-CD28, anti-CTLA-4, and anti-B7-1/2 antibodies. The anti-CD28 antibody was proven to be necessary for T cell proliferation, as expected due to CD28's role as a required co-stimulatory molecule. Addition of anti B7-1/2 antibodies dramatically increased the rate of T cell proliferation,

as did addition of anti-CTLA-4 antibodies, suggesting that B7 molecules are part of the CTLA-4 inhibitory pathway. However, anti-B7-1/2 antibodies were insufficient to increase T cell proliferation alone, suggesting that they are not expressed on T-cells in numbers large enough to act as a co-stimulatory signal. Figure taken from Krummel and Allison 1995.[15]

CD28 is well known as the co-stimulatory signal that is required for T lymphocyte activation. As expected, the combination of anti-CD3 and anti-CD28 resulted in increased proliferation of T lymphocytes. As shown, both anti-CTLA-4 and anti-B7 antibodies increased the rate of proliferation of T lymphocytes that had received the necessary co-stimulatory signals, indicating that CTLA-4 competes with CD28 for binding to the B7 co-stimulatory molecule. Subsequently, it has been shown that CTLA-4 has a higher affinity for B7-1 and B7-2 than CD28, making it a strong inhibitory molecule for T lymphocyte activation.

CTLA-4 blockade results in the diminished growth of tumors, as demonstrated by Leach, Krummel, and Allison.[16] Leach, et al. injected BALB/c mice with tumor cells that did not express B7 molecules and treated the tumors with anti-CTLA-4 Ab, anti-CD28 Ab, or no antibody. The mice treated with no antibody or the anti-CD28 antibody experienced continued progression of the malignancy and eventually required euthanization 35 days post injection. In stark contrast, mice injected with the tumor cells, and subsequently treated with the anti-CTLA-4 antibody did not experience progressive tumor growth.[16] In addition to suppressing tumor growth, Leach, et al. showed that treatment with the anti-CTLA-4 antibody resulted in protection against a secondary

tumor challenge. **Figure 8** demonstrates the difference in tumor growth between control mice and mice treated with the anti-CTLA-4 Ab prior to the second tumor challenge.

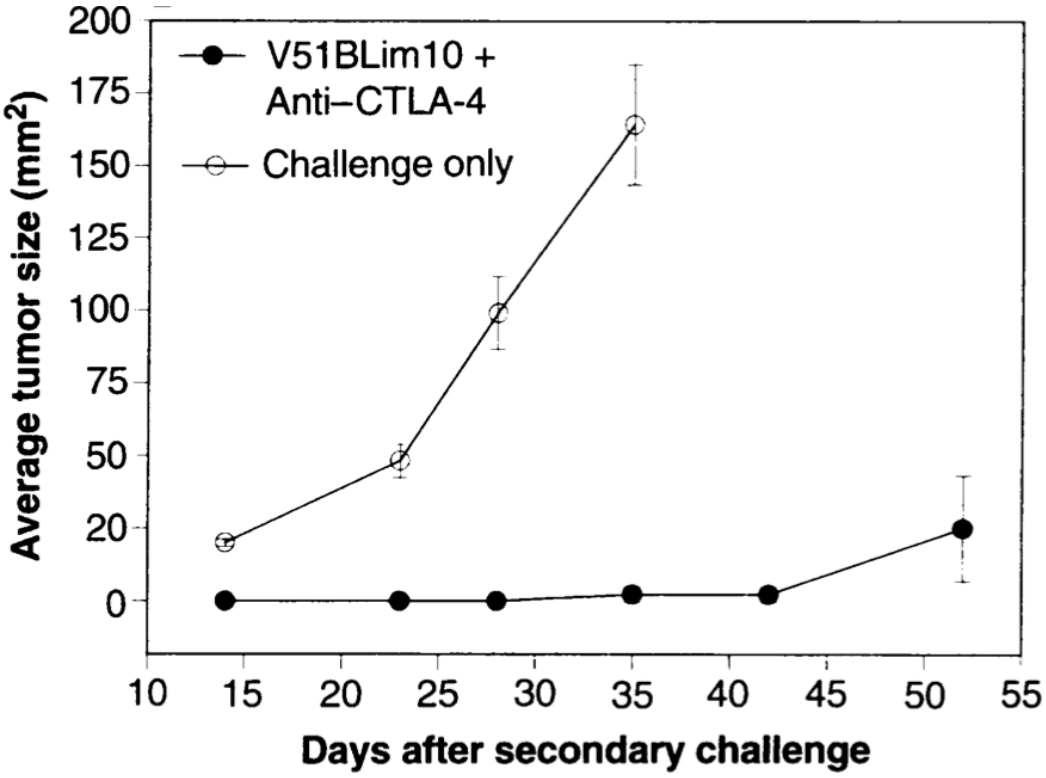

Figure 8. Effect of Treating Mice with Anti-CTLA-4 prior to a Secondary Tumor Challenge. Leach et al., injected mice with tumor cells that did not express B7 and treated one group of these mice with anti-CTLA-4 antibodies, causing them to completely reject the tumor cells. Seventy days later, the mice that had rejected the tumor cells were rechallenged via injection of tumor cells in the opposite flank. The response of the mice treated with anti-CTLA-4 prior to the rechallenge was compared to the response of naïve mice upon injection. The naïve mice experienced progressive tumor growth while the mice previously treated with the anti-CTLA-4 antibody were able to prevent the tumor from progressing. Figure taken from Leach, Krummel, and Allison 1996.[16]

Due to their role in immunosuppression, both the PD-1 – PD-L1 interaction and the CTLA-4 checkpoint have been targeted by cancer immunotherapy, with promising preclinical results in several malignancies. **Table 1** shows the drugs targeting PD-1 and PD-L1 that are at various stages in clinical development.

Table 1. Drugs in Clinical Development that Target PD-1 or PD-L1. There are many drugs targeting the PD-1/PD-L1 immune checkpoint to induce an immune response against a malignancy that have been effective in pre-clinical studies at producing an antitumor response. These drugs are now in various stages of clinical development and are being used to treat many types of cancer, with varied degrees of effectiveness. Adapted from Topalian, Drake, and Pardoll, 2015.[17]

Target	Drug Name	Other Names	Source	Isotype and Characteristics	Clinical Testing
PD-1	MEDI 0680	AMP-514	MedImmune/ Astrazeneca	Information not available	Phase I
	Nivolumab	Opdivo, BMS-9365558, MDX-11006. ONO, 4538	Bristol-Myers Squibb, Ono Pharmaceuticals	Fully human IgG4	Approved; treatment-refractory unresectable melanoma and squamous NSCLC
	pembrolizumab	Keytruda, MK-3475, Iambrolizumab	Merck	Humanized IgG4	Approved, treatment-refractory unresectable melanoma
	Pidilizumab	CT-011	CureTech	Humanized IgG1	Phase I-II
PD-L1	BMS-936559	MDX-1105	Bristol-Myers Squibb	Fully human IgG4	Phase 1
	MEDI 4736	None	MedImmune/ AstraZeneca	Fc-modified human IgG1	Phase I-III
	MPDL 3280A	RG7446	Genentech/Roche	Fc-modified human IgG1	Phase I – III
	MSB0010718C	None	EMD Serono	Fully human IgG1	Phase I - II

Despite the clinical success of immune checkpoint inhibitor for other malignancies,

PDAC has been resistant to immune checkpoint inhibitor therapy. **Table 2** summarizes

the results of clinical trials testing the effectiveness of immune checkpoint inhibitor therapeutics as treatments for PDAC.

Type of Immunotherapy	**Molecules**	**Trial**	**Phase**	***n***	**Population**	**Main Results**
Immune Checkpoint Inhibitors	PD-L1 (BMS-936559)	Brahmer *et al*[18]	I	14	Advanced PDAC Pre-treated	No Objective Response
	PD – L1 (atezolizumab)	Herbst *et al*[19]	I	1	Advanced PDAC Pre-treated	No Objective Response
	PD-1 (pembrolizumab)	Patnail *et al*[20]	I	1	Advanced PDAC Pre-treated	No Objective Response
	CTLA-4 (ipilimumab)	Royal *et al*[21]	II	27	Advanced PDAC Pre-treated	No Objective Response

Table 2. Summary of Clinical Trial Results of Immune Checkpoint Inhibitor Therapy for Pancreatic Ductal Adenocarcinoma. Several trials have been done to test the efficacy of the immune checkpoint inhibitors PD-1, PD-L1, and CTLA-4. As shown, these trials have not progressed past stage I of the trial due to a lack of objective response, suggesting that despite the strong response in pre-clinical studies, there are other factors involved in the clinical response. Table adapted from Hilmi, Bartholin, and Neuzillet 2018.[22]

There are several possible explanations for why the effectiveness of immune checkpoint inhibitor therapy was limited. One of the main explanations is centered around how other cells present within the PDAC microenvironment control the response to immunotherapy.

The Microenvironment of Pancreatic Ductal Adenocarcinoma

The microenvironment of pancreatic ductal adenocarcinoma has been described as highly dense and fibrotic, which greatly limits the ability of cells to infiltrate the tumor. The cells that are unable to reach the malignant cells in the tumor are often T cells, limiting their ability to target malignant cells. **Figure 9** demonstrates the pattern of CD3+ T lymphocyte infiltration in human PDAC.

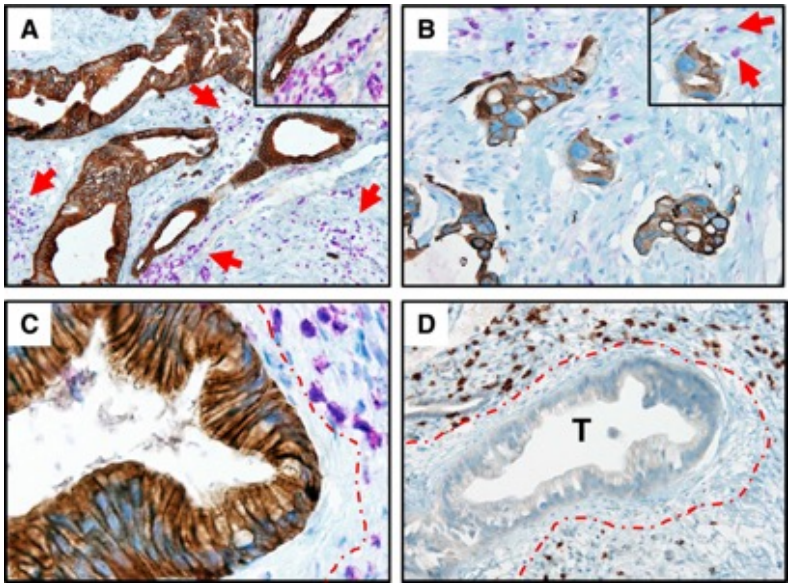

Figure 9. $CD3^+$ T lymphocyte Infiltration Pattern in Human PDAC. Sections of tissue taken from human PDAC were treated with immunohistochemical staining to show the locations of $CD3^+$ T cells with respect to the location of the cancer cells. The stains used were a light blue nuclear stain and a hematoxylin counterstain. Red arrows illustrate the location of $CD3^+$ T cells in Panels A & B, and the insets show a magnified view of the tissue, enabling more clear visualization of the localization of the $CD3^+$ T cells in relation to the malignant cells (stained for CK19 in brown). C & D utilize a dotted red line to illustrate the physical separation of $CD3^+$ T cells from the tumor cells. The

physical separation of $CD3^+$ T cells from malignant cells shown in these figures is a possible reason for the limited efficacy of immune checkpoint inhibitor therapy, as CTLs must bind to MHC molecules on target cells in order to induce the cytotoxic response. Figure taken from Beatty, Eghbali, and Kim 2017.[23]

In direct contrast to the limited ability of $CD3^+$ T lymphocytes to infiltrate tumors, myeloid cells have been shown to successfully infiltrate tumors and interact with the malignant cells. There are several types of myeloid cells that have been observed in the TME of PDAC. A study by Ino et al. analyzed over two hundred patient samples of PDAC for the presence of myeloid cells using immunohistochemistry to examine the effect of the presence of myeloid cells on the prognosis of PDAC.[24] Staining was done for the cell markers CD68, CD163, CD204, CD66b, CD4, CD8, FOXP3, and HLA-DR. The CD68 cell marker indicated the presence of macrophages, and the expression of CD163 or CD204 was used to detect the presence of M2 (alternatively activated macrophages) while the expression of HLA-DR was used to detect the presence of M1 (classically activated macrophages). The cell marker CD66b was used to detect neutrophils, and the CD4, CD8, and FOXP3 markers were used to differentiate between different subsets of T cells, with FOXP3 staining indicating the presence of T_{reg} cells. Analysis of the stained tissue showed that M2 macrophages predominated areas of the tumor that were necrotic while T cells remained on the periphery of the tumor. Ino et al. also analyzed the clinical impact of the presence of immune infiltrating cells on overall survival (OS) and disease-free survival (DFS)[24], and **Figure 10** shows the Kaplan Meier curves resulting from the analysis.

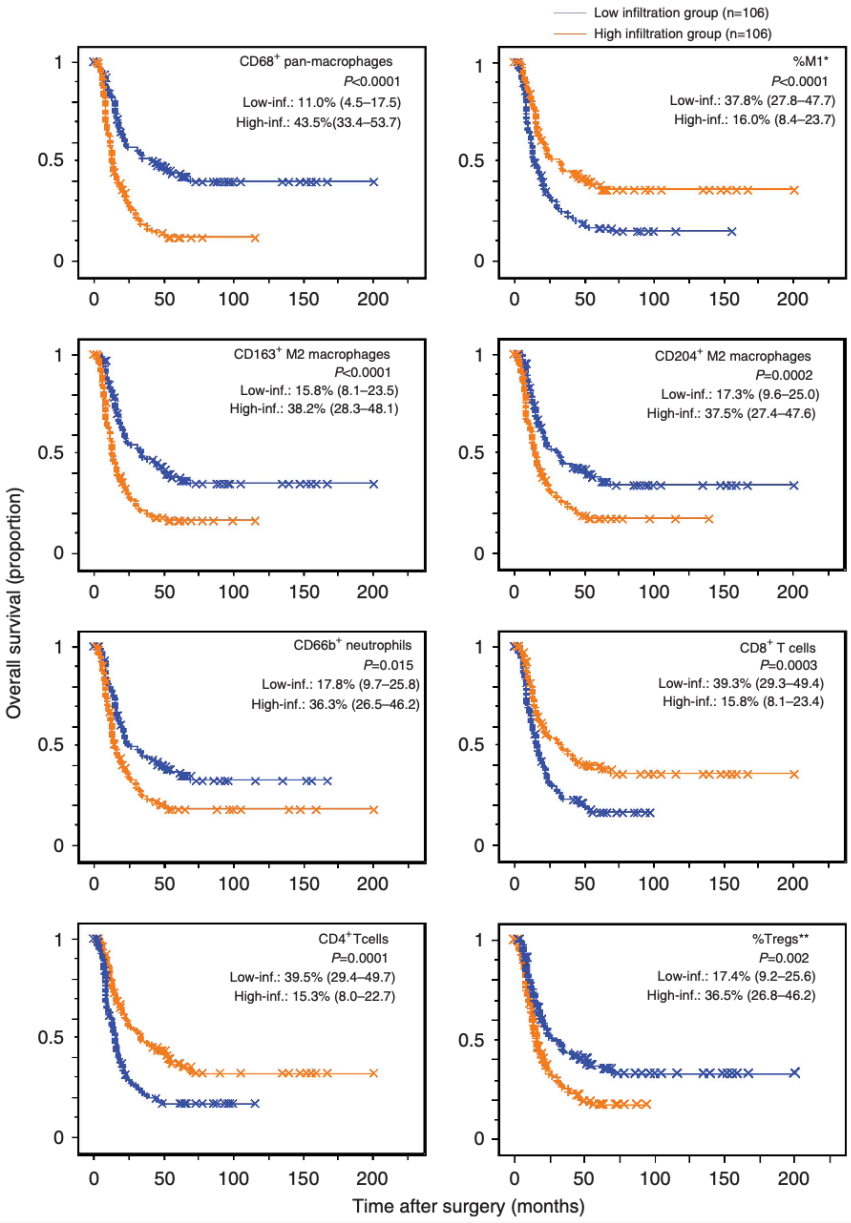

Figure 10. Differential Overall Survival in Patients with Low and High Infiltration of Various Immune Cell Types. Kaplan-Meier analyses of the overall survival of human PDAC patients with resectable cancer shows a correlation with the types of immune cells infiltrating the tumor. Low infiltration of $CD68^+$ macrophages was shown to have a correlation with a greater proportion of patients surviving for a longer period of time. When the macrophage population was broken down into M1, $CD163^+$ M2, and $CD204^+$ M2 macrophages, it became apparent that while high infiltration of M1 macrophages was correlated to a longer overall survival, high infiltration of both $CD163^+$ M2 and $CD204^+$ M2 macrophages was correlated to a decreased overall survival. Similarly, while the

presence of $CD4^+$ and $CD8^+$ T lymphocytes was correlated with increased overall survival, high infiltration of T_{reg} cells results in a decrease in overall survival. Figure taken from Ino et al. 2013.[24]

As shown by the figure, alternatively activated M2 macrophages and regulatory T cells are associated with decreased overall survival in human PDAC. A greater understanding of the origin and the difference in phenotype between M1 and M2 macrophages as well as $CD4^+$ and $CD8^+$ T lymphocytes and T_{reg} cells will help illustrate the mechanisms by which these cells promote the progression of PDAC.

Macrophages are derived from monocytes circulating in the blood upon extravasation into the tissue. They vary in phenotype from M1 "classically activated" macrophages to M2 "alternatively activated" macrophages (also referred to as tumor associated macrophages or TAMs). M1 macrophages are tumoricidal and are activated by high levels of IFN-γ. They express high levels of interleukin 12 and low levels of interleukin 10. In direct contrast to M1 macrophages, M2 macrophages are pro-tumorigenic and express high levels of interleukin 10 but low levels of interleukin 12. Differentiation into the M2 phenotype is promoted by the cytokines interleukin 4, interleukin 10, and interleukin 13 along with glucocorticoid hormones.[25] M2 macrophages exert their protumorigenic activity in many ways, one of which is by secreting the immunosuppressive cytokine IL-10 which reduces cytotoxic T cell activation. Another mechanism by which M2 macrophages generate an immunosuppressive microenvironment is by impairing the antigen presenting ability of macrophages, thus limiting T cell recognition of tumor associated antigens. Due to the stark contrast in the activity of M1 and M2 macrophages, many recent investigations are centered around the

differing signals that result in monocyte differentiation into classically activated M1 macrophages or TAMs. A class of myeloid cells called myeloid derived suppressor cells are partially responsible for polarizing the phenotype of macrophages within the tumor microenvironment to tumor associated macrophages. MDSCs decrease macrophage expression of IL-12 in a contact dependent manner, causing polarization towards the tumor associated macrophage phenotype. Additionally, signaling between MDSCs and macrophages promotes both cell types to increase secretion of IL-10, leading to a dramatic increase in immunosuppression.[26]

Similarly to M2 tumor associated macrophages, regulatory T cells represent a class of T cell without the typical antitumorigenic activity that is characteristic of $CD4^+$ and $CD8^+$ T lymphocytes. Regulatory T cells express the FOXP3 transcription factor and the cell surface markers CD4 and CD25. Their primary function in normal tissue is to regulate the adaptive immune response to prevent autoimmune reactions from occurring. They do so by employing several mechanisms including competing for binding to IL-2 by expressing the higher affinity heterotrimeric IL-2R (composed of the α chain, β chain, and γ chain), increasing the expression of the CTLA-4 immune checkpoint, secreting granzyme A and perforin to kill T cells, and inducing the expression of B7-H4 in antigen presenting cells, which promotes immunosuppression.[27] In malignancies, these regulatory strategies enable tumor cells to evade the immune system, making T_{reg} cells protumorigenic. It is important to understand how normally tumoricidal $CD4^+$ T cells are induced into becoming T_{reg} cells, as targeting these interactions could enable a reversal of the immunosuppressive activities of T_{reg} cells.

One main source of T_{reg} cells is a distinct lineage of $CD4^+$ T lymphocytes that originate in the thymus, where the expression of the main markers of T_{reg} cells, FOXP3 and CD25, are upregulated. In addition to the thymus derived T_{reg} cells, naïve $CD4^+$ T lymphocytes can also be induced into becoming T_{reg} cells by myeloid derived suppressor cells. Pan et al. analyzed the role of CD40 expression by MDSCs in inducing the activation of T_{reg} cells in the tumor microenvironment.[28] IFN-γ released by antigen activated T cells causes the upregulation of CD40 and MHCII molecules on myeloid derived suppressor cells.[23] The upregulation of CD40 was proven to be essential to the activation and proliferation of T_{reg} cells, as shown in **Figure 11.**

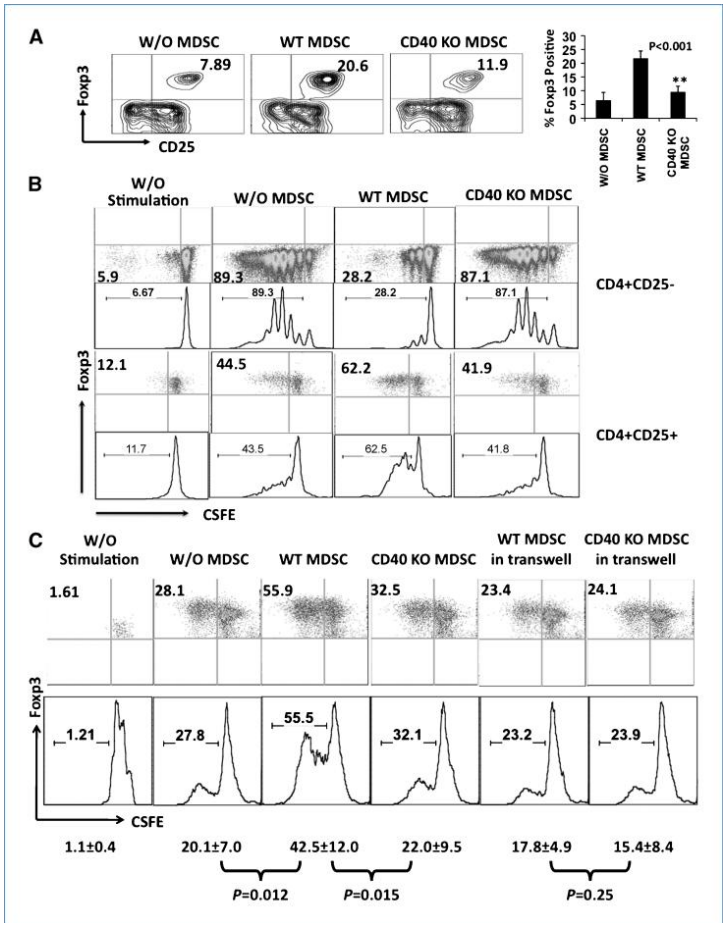

Figure 11. The Importance of CD40 Expression by MDSCs on the Proliferation and Activation of Regulatory T Cells. Purified naïve $CD4^+$ T lymphocytes were cultured alone, or cocultured with either wild type MDSCs or CD40 knockout MDSCs and subsequently analyzed for gene expression of the regulatory T cell markers, CD25 and FOXP3 using flow cytometry. When naïve $CD4^+$ T lymphocytes were co-cultured with WT MDSCs, they expressed much higher levels of both FOXP3 and CD25, suggesting that a greater percentage of the naïve $CD4^+$ T lymphocytes had become T_{reg} cells. In direct contrast, when the naïve $CD4^+$ T lymphocytes were cultured with MDSCs that had CD40 knocked out, the expression level of both CD25 and FOXP3 was much lower, indicating that the development of T_{reg} cells from naïve $CD4^+$ T lymphocytes is dependent on the expression of CD40. This is further confirmed by the proliferation assay that was subsequently performed where naïve $CD4^+$ T lymphocytes cultured under the same conditions previously described were stained with the proliferation marker CFSE, stimulated with anti-CD3/anti-CD28 antibodies for three days before the assessment of proliferation using flow cytometry. The proliferation of $CD4^+$ T lymphocytes was compared to the proliferation of T_{reg} cells by gating on two cell populations: $CD4^+$ cells and $FOXP3^+$ cells. Naïve $CD4^+$ T lymphocytes that were cocultured with WT MDSCs resulted in suppressed proliferation of $CD4^+$ T lymphocytes while the proliferation of regulatory T cells was promoted. The loss of CD40 expression in the CD40 knockout MDSCs resulted in the opposite occurring, proliferation of $CD4^+$ T lymphocytes but not regulatory T cells, emphasizing the importance of CD40 expression by MDSCs in promoting the proliferation of the immunosuppressive regulatory T cells. Figure taken from Pan et al. 2010.[28]

MDSCS have been shown to mediate their immunosuppressive effects indirectly, through the actions of tumor associated macrophages and T_{reg} cells, as they promote the prevalence of these protumorigenic cell types in the tumor microenvironment. However, myeloid cell derived suppressor cells also directly promote an immunosuppressive microenvironment in malignancies through several mechanisms, making them an important aspect of the immune microenvironment.

Myeloid Derived Suppressor Cells

Myeloid derived suppressor cells are a phenotypically and morphologically diverse group of cells that express the cell surface proteins CD11b and Gr-1.[29] There are two major subtypes of MDSCs that are distinguished based on the expression of cell surface

markers. The expression pattern of granulocytic MDSCs (Gr-MDSCs) in humans is either $CD11b^{+}CD14^{-}CD15^{+}$ or $CD11b^{+}CD14^{-}CD66b^{+}$ whereas the expression pattern of monocytic MDSCs (M- MDSCs) is $CD11b^{+}CD14^{+}HLA\text{-}DR^{-/lo}CD15^{+}$.[29] These cells originate from a common precursor cell, the common myeloid progenitor cell, and are able to differentiate into dendritic cells, macrophages, and other granulocytes. Myeloid derived suppressor cells along with other immunosuppressive cells were found to infiltrate the tumor microenvironment of pancreatic cancer and the precursor lesions very early on in the progression of the disease. Flow cytometric analysis for the presence of MDSCs was performed and the results are shown in **Figure 12.**

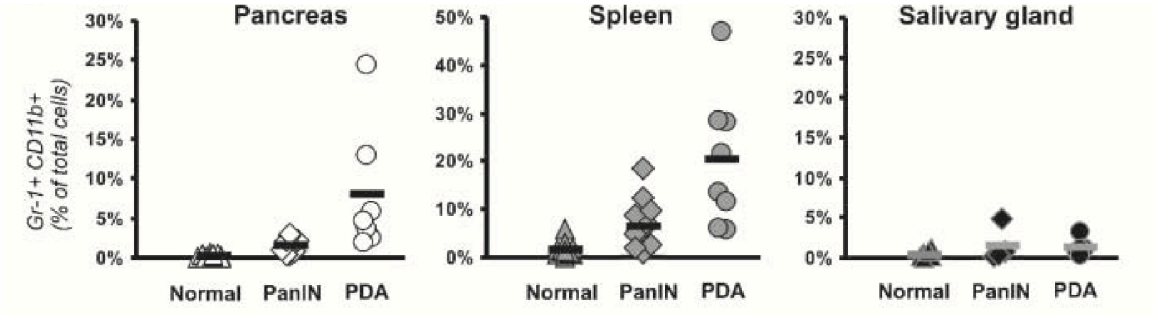

Figure 12. Presence of Myeloid Derived Suppressor Cells Over the Progression of Pancreatic Ductal Adenocarcinoma. Flow cytometric analysis was used to quantify the presence of MDSCs at various stages of disease. The cell marker $Gr\text{-}1^{+}CDllb^{+}$ was used to detect the presence of MDSCs in normal tissue, PanIN tissue, and PDAC, using tissue samples from the pancreas, the spleen, and the salivary gland. PanIN (pancreatic intraepithelial neoplasia) is an early stage of the disease. While the number of myeloid derived suppressor cells infiltrating the lesion does not increase in the early stages of the disease, there is an increase in MDSCs in the spleen, as shown by the second graph. When the disease progressed to PDAC, the percentage of MDSCs increased in both the pancreas and in the spleen, suggesting increased infiltration in the microenvironment but also increase in the production of MDSCs. Figure taken from Clark et al. 2007.

The increased percentage of MDSCs as the disease progresses is indicative of their prominent role in the immunosuppressive microenvironment in pancreatic cancer. In

contrast to T lymphocytes, myeloid derived suppressor cells are able to enter the tumor microenvironment of pancreatic cancer and are found to be in close proximity to malignant cells. They are driven to enter the microenvironment by a range of cytokines, and one of the cytokines most noted for its role in promoting the differentiation and accumulation of MDSCs in the tumor microenvironment is GM-CSF. Bayne et al. demonstrated that upregulation of GM-CSF by PDAC promotes the differentiation of MDSC precursor cells into MDSCs and the subsequent proliferation of myeloid derived suppressor cells.[30] **Figure 13** depicts the significance of GM-CSF plays in promoting the presence of myeloid derived suppressor cells.

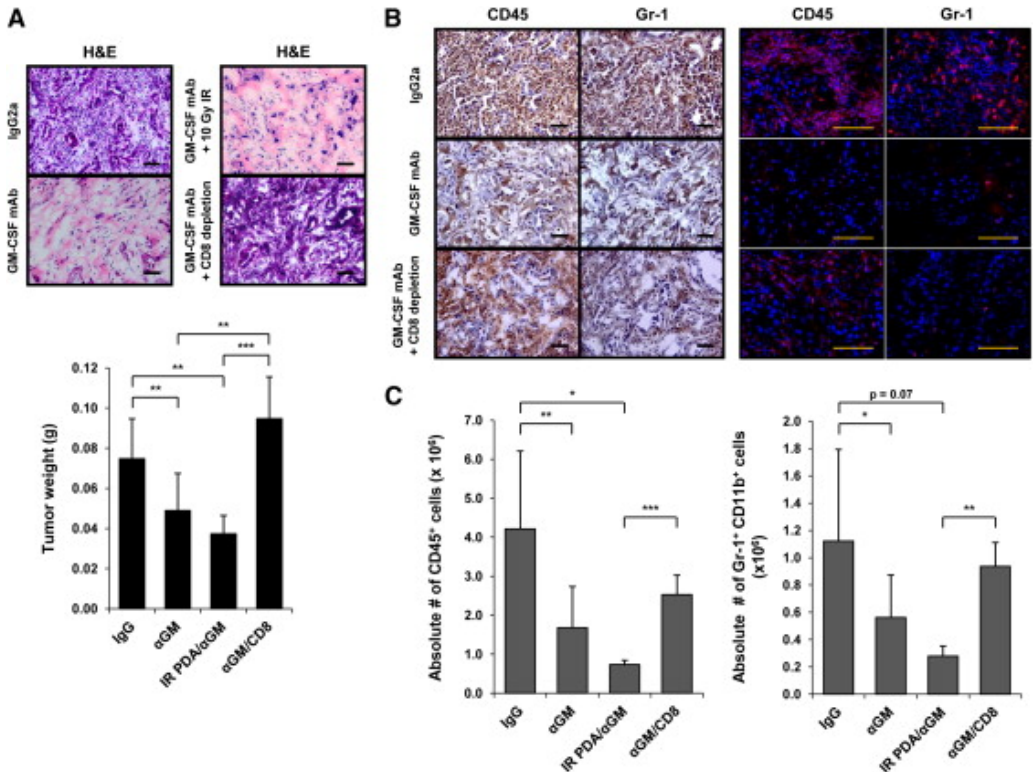

Figure 13. Effects of anti-GM-CSF Ab on the Proliferation of Gr-1$^+$CD11b$^+$ cells and Tumor Growth. Hematoxylin and eosin staining of mouse PDAC samples treated with control IgG2α Ab, anti-GM-CSF mAb, irradiation with 10 Gray and treatment with anti-GM-CSF mAb, or anti-GM-CSF mAb and CD8$^+$ T lymphocyte depletion showed a

decrease in the presence of tumor cells when treated with GM-CSF blockade. The combination of irradiation and GM-CSF blockade also decreased the presence of tumor cells. However, the depletion of $CD8^+$ T cells reversed the effects of GM-CSF blockade, suggesting that the GM-CSF promotes suppression of cytotoxic T cell activity. Tumor growth was also measured under these four conditions, with results that are consistent with the H&E staining: that GM-CSF blockade alone or in combination with irradiation resulted in a significant decrease in tumor growth in comparison to treatment with the IgG control antibody, but depletion of $CD8^+$ T lymphocytes reversed this effect. Immunohistochemistry and immunofluorescence were used to measure the effects of GM-CSF blockade on the presence of both $CD45^+$ and $Gr\text{-}1^+$ positive cells within a tumor. IHC staining and IF for CD45 and Gr-1 was done under three conditions: treatment with control IgG2α Ab, anti-GM-CSF mAb, and anti-GM-CSF mAb with $CD8^+$ T cell depletion. CD45 is a cell surface protein expressed by all cells descended from the hematopoietic lineage, and Gr-1 is a protein expressed by MDSCs. Treatment with the control antibody did not affect the expression levels of either CD45 or Gr-1, but GM-CSF blockade decreased expression levels of both markers. $CD8^+$ T cell depletion partially reversed the effects of GM-CSF blockade. Flow cytometry analysis of murine PDAC tumors enabled quantification of the number of cells that expressed CD45 and Gr-1 under the conditions previously described: treatment with a control IgG2α Ab, anti-GM-CSF mAb, irradiation with 10 Gray and treatment with anti-GM-CSF mAb, or anti-GM-CSF mAb and $CD8^+$ T cell depletion. The results were consistent with the results of the IHC and IF. The number of $CD45^+$ and $Gr\text{-}1^+$ cells decreased in response to treatment with a GM-CSF blockade either alone or in combination with irradiation, but this effect was reversed by depletion of $CD8^+$ T cells. Figure taken from Bayne, et al. 2012.[30]

The reversal of the effects of GM-CSF blockade on tumor growth after $CD8^+$ T cell depletion indicates that elevated GM-CSF levels found within the PDAC microenvironment promote immunosuppression by increasing the proliferation of MDSCs.

Myeloid derived suppressor cells mediate immunosuppression through several direct mechanisms in addition to promoting the activity of other immunosuppressive cells. They decrease the needed amino acids for the antitumor response and disrupt T cell activation and signaling. MDSCs express the enzymes arginase and inducible nitric oxide synthase. Arginase metabolizes the amino acid arginine present in the tumor

microenvironment, and the depletion of arginine results in decreased proliferation of T cells.[31] Rodriguez et al. showed that arginine depletion leads to T lymphocyte arrest in the $G_0 – G_1$ phase of the cell cycle through decreased expression of cyclin D3 and cyclin dependent kinase 4.[32] Downregulation of cyclin D3 and Cdk 4 results in decreased phosphorylation of the Rb complex, which is needed for activation of the transcription factor E2F-1and the expression of genes that promote DNA synthesis and replication. Decreased proliferation of T cells resulting from the loss of arginine within the TME results in a weakened antitumor response.

In addition to reducing the number of T lymphocytes present within the microenvironment, MDSCs weaken the antitumor response by disrupting T cell activation. One mechanism they use is by releasing the superoxide anion, which then reacts with nitrous oxide present in the tumor microenvironment, forming a compound called peroxynitrite (PNT). Peroxynitrite is a free radical that has been previously shown to bind to the T cell receptor and the CD8 molecule, resulting in the loss of antitumor activity.[33] The mechanism by which nitrosylation of the T cell receptor induces a loss of antitumor immunity was described by Lu et al. by employing cytotoxicity assays to determine the effect of PNT treatment on antigen specific cytotoxicity. Treatment of tumor cells with PNT prior to addition of the antigen that the effector T cells had specificity for resulted in a loss of specific cytotoxic T cell response. However, treating the tumor cells with PNT after adding antigen resulted in a T cell response, suggesting that PNT interactions with the MHC I molecule disrupted the recognition of the antigen by the MHC I molecule and were responsible for the loss of the antigen-specific T cell

response.[33] This was further confirmed by measuring the differential formation of the peptide-MHC complex in tumor cells that were treated with PNT before and after being exposed to antigen using flow cytometry. Treating tumor cells with PNT prior to exposing them to the peptide resulted in minimal formation of the peptide-MHC I complex while treatment with PNT after peptide exposure resulted in normal formation of peptide – MHC complexes. Direct nitration of a tyrosine residue on the peptide disrupted the formation of the pMHC complex, suggesting that nitration of either component of the pMHC complex could interfere with binding of the peptide to the MHC molecule. [33] Disrupting the formation of the peptide-MHC complex prevents T cell recognition of antigens, eliminating the potential of an antitumor response.

Disruption of the peptide- MHC I complex is not the only way that MDSCs disrupt T cell activation. An analysis of T cells from PDAC patients who failed to respond to respond to immunotherapy showed a loss of the CD3 ζ chain that is required for the intracellular signaling needed for T cell activation.[34] Nagaraj et al., showed that MDSCs are responsible for the loss of the CD3ζ chain by comparing the effects of co-culturing dendritic cells and MDSCs with T cells on the expression of various tyrosine kinases involved in T cell activation.[35] T cells cocultured with dendritic cells had increased levels of phosphorylation in several molecules that are indicative of T cell activation such as the CD3ζ chain and ERK1/2 that were not increased in T cells cocultured with MDSCs. Co-culturing T-cells with MDSCs did not result in a decrease in the level of CD3ζ chain expression, but it did disrupt the association between the TCR

and the CD3ζ chain, as shown in **Figure 14.**

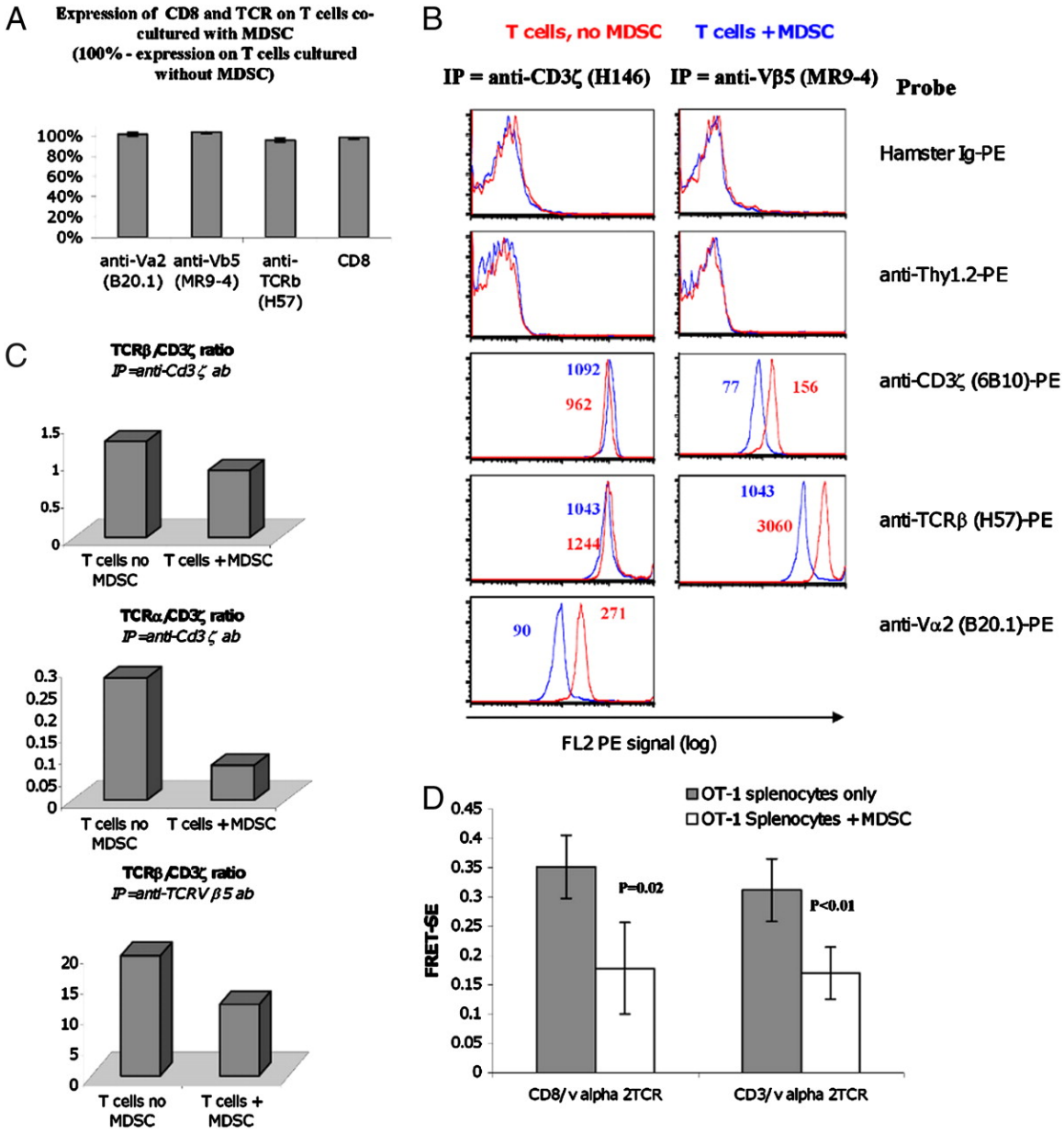

Figure 14. Myeloid Derived Suppressor Cells Disrupt the Interaction between the T Cell Receptor and the CD3ζ Chain. Panel A displays the results of FACS analysis of cell surface proteins expressed by T cells cocultured with MDSCs. T lymphocytes cocultured with MDSCs showed no changes in the overall expression levels in the genes encoding the T cell receptor or the CD8 molecule on cytotoxic T lymphocytes relative to $CD8^+$ T cells cultured without MDSCs. Panel B contains the results of an immunoprecipitation-flow cytometry experiment used to isolate CD3-TCR complexes

and examine the association of the CD3 ζ chain with the TCR subunits. When T cells were immunoprecipitated using an antibody against the β chain of the TCR complex, the mean fluorescence intensity of cells treated with the anti-CD3ζ - PE decreased. Panel C shows the relative amount of CD3 ζ, which was calculated by dividing the MFI of the TCR α or β chain probes by the MFI of the CD3 ζ probe. Co-culturing T cells with MDSCs did not cause a decrease in expression of the CD3 ζ chain but resulted in a decrease in the relative amount of CD3 ζ chain association with the T cell receptor. Panel D shows the result of FRET-SE analysis of OT-1 splenocytes cultured separately and with MDSCs. The splenocytes were then stained with an antibody against either the CD3 or CD8 cell surface marker and with an antibody against the α chain of the TCR complex. The antibodies against CD3 and CD8 acted as electron donors and were conjugated to PE and the antibody against the α chain of the TCR complex acted as an electron acceptor and was conjugated to APC. Analysis using confocal microscopy showed that there is decreased FRET between CD8 and the TCR α chain and between CD3 and the TCR α chain in OT-1 splenocytes co-cultured with MDSCs. This decrease in FRET-SE indicates a decrease in physical association between CD8 and the TCR α chain and CD3 and the TCR α chain. Figure taken from Nagaraj et al. 2010. [35]

The CD3 ζ chain is required for the intracellular signaling that leads to the full activation of T lymphocytes. Therefore, the loss of association between the TCR and the CD3 ζ chain mediated by myeloid derived suppressor cells would result in suppression of the antitumor immune response.

DISCUSSION

Myeloid derived suppressor cells are a heterogenous group of cells present within the tumor microenvironment of pancreatic ductal adenocarcinoma. They promote immunosuppression within the tumor microenvironment of PDAC both directly and indirectly. Indirect mechanisms include promoting an immunosuppressive phenotype in the other cell types within the microenvironment: tumor associated macrophages and T regulatory cells by the secretion of immunosuppressive cytokines. TAMs then disrupt the cytotoxic activity of T lymphocytes by secreting the immunosuppressive cytokine IL-10. Regulatory T cells also inhibit cytotoxic activity by several mechanisms.

In addition to the indirect mechanisms that MDSCs use to suppress the antitumor response of the adaptive immune system, MDSCs also use several direct mechanisms to promote immunosuppression within the tumor microenvironment of pancreatic cancer. They produce enzymes that deplete the microenvironment of amino acids that T lymphocytes require for proliferation, produce reactive oxygen and nitrogen species that react with the peptide-MHC complex essential to antigen recognition in T lymphocytes and eliminating the ability of the T lymphocyte to recognize and generate a cytotoxic response against tumor associated antigens. They also impair the intracellular signaling associated with the activation of the T lymphocyte by causing the CD3ζ chain to dissociate from the T cell receptor. The combination of these mechanisms creates a highly immunosuppressive microenvironment in pancreatic ductal adenocarcinoma, which greatly limits the effectiveness of current immunotherapies. One such immunotherapy, immune checkpoint inhibitors, acts by preventing the engagement of the

PD-L1/PD-1 signaling pathway between tumor cells and T lymphocytes or by binding to the CTLA-4 molecule expressed on T lymphocytes, which eliminates the competition between CTLA-4 and CD28 for binding to the co-stimulatory molecule B7. These immunotherapies, while promising in other cancers, have had limited success in treating PDAC. However, a depletion of myeloid derived suppressor cells has been shown to unmask PDAC to adaptive immunity in a murine model of pancreatic cancer.[36] While these results are untested in clinical trials, combinatorial therapies that seek to decrease the immunosuppression within the tumor microenvironment while removing the activities of immune are promising avenues to explore for PDAC, as current therapies are largely ineffective. Therefore, future studies of PDAC could employ GM-CSF inhibition in combination with immune checkpoint inhibitor therapy, as GM-CSF is one of the cytokines that is most responsible for promoting the entry of MDSCs into the PDAC microenvironment. Translating depletion of MDSCs from a murine model of PDAC to human PDAC is a foreseeable challenge for the clinical application of such combinatorial therapies.

LIST OF JOURNAL ABBREVIATIONS

Br J Cancer	British Journal of Cancer
Cancer Res	Cancer Research
Cancer Immunol Res	Cancer Immunology Research
Clin Cancer Res	Clinical Cancer Research
Immunol Invest	Immunological Investigations
Int J Mol Sci	International Journal of Molecular Sciences
J Allergy Clin Immunol	Journal of Allergy and Clinical Immunology
J Clin Invest	Journal of Clinical Investigations
JI	Journal of Immunology
J Immunol	Journal of Immunology
J Immunother	Journal of Immunotherapy
J Exp Med	Journal of Experimental Medicine
Nat Rev Immunol	Nature Reviews. Immunology
N Engl J Med	New England Journal of Medicine
Sci Transl Med	Science Translational Medicine
World J Gastroenterol	World Journal of Gastroenterology

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