# The Translational Efficacy of Adjuvant Treatment Options Targeting Pancreatic Ductal Adenocarcinoma

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*On my honor as a student, I have neither given nor received aid on this assignment.*

**Abstract**

Over the past few decades, most forms of cancer have seen upward trends in their 5-year survival rates, with the exception of pancreatic cancer. This is in part due to the fact that individuals diagnosed with pancreatic ductal adenocarcinoma have one primary option for curative treatment, surgical resection, and only a small subset of patients meet the requirements for surgery. Furthermore, many patients will later develop cancer of the liver due to hepatic micrometastases not seen previously detected, leading to a poor prognosis. As such, finding an adjuvant treatment strategy that will prolong the survival of PDAC patients is a research focal point.

Currently, many pancreatic cancer treatments center upon the use of chemotherapeutic agents and small molecule inhibitors. Here we have focused on the liver microenvironment to generate effective treatment options that will translate to the clinical setting for individuals with pancreatic ductal adenocarcinoma. Tissue samples from human PDAC tumors were obtained and CyQUANT Cell Proliferation Assays were performed to quantify the responses of various pancreatic cancer cell lines to different chemotherapies and molecular inhibitors. Flow cytometry was used to quantify the impact of various immunotherapy options in promoting macrophage engulfment of tumor cells. The results of these experiments provided insight into the heterogeneity of pancreatic tumors, and as such, the need for patient-specific treatment options. Furthermore, our studies revealed the complexity of the interactions within the tumor microenvironment, emphasizing the need for continued experimentation in this field. The bioethical implications of work are discussed with regard to the eventual costs of these treatments and equity of access to all individuals.

**Table of Contents**

## List of Abbreviations

PDAC — Pancreatic Ductal Adenocarcinoma

PanIN — Pancreatic Intraepithelial Neoplasia

MCN — Mucinous Cystic Neoplasm

IPMN — Intraductal Papillary Mucinous Neoplasm

PSCs — Pancreatic Stellate Cells

MDSCs — Myeloid-Derived Tumor Suppressor Cells

Tregs — Regulatory T Cells

TAMs — Tumor-Associated Macrophages

TGFβ — Transforming Growth Factor-β

ECM — Extracellular Matrix

CAFs — Cancer-Associated Fibroblasts

myCAFs — Myofibroblastic CAFs

iCAFs — Inflammatory CAFs

PRRs — Pattern Recognition Receptors

PAMPs — Pathogen-Associated Molecular Pathogens

EVs — Extracellular Vesicles

EMT — Epithelial-Mesenchymal Transition

E-cadherin — Epithelial Cadherin

N-cadherin — Neural Cadherin

EMT-TF — EMT-Transcription Factor

CCLs — Pancreatic Cancer Cell lines

GEMMs — Genetically Engineered Mouse Models

CDXs — Circulating Tumor Cell-Derived Xenografts

PDXs — Patient-Derived Xenografts

CD47 — Cluster of Differentiation 47

SIRPα — Signal Regulatory Protein α

MAMs — Metastasis-Associated Macrophages

α-CD47 — anti-CD47

RNAi — RNA Interference

## Introduction

### *General Introduction to Pancreatic Cancer*

During 2021, The American Cancer Society predicts that in the United States more than 60,000 individuals will be diagnosed with pancreatic cancer and nearly 50,000 individuals will die as a result of this disease.[1] While pancreatic cancer accounts for only 3% of all cancer diagnoses in the United States, its 5-year survival rate remains the lowest of all cancers, with a 5-year survival rate of just 10% from 2009-2015.[2,3] Research on other cancers has progressed significantly in recent years, leading to improved diagnoses and survival rates. Pancreatic cancer, however, remains the exception, with stagnant survival rates since the 1960s.[4]

Both endocrine and exocrine cells in the pancreas can contribute to the development of pancreatic cancer.[5,6] Nevertheless, exocrine tumors are significantly more common, comprising nearly 95% of all pancreatic cancers. Exocrine tumors can be further subdivided into two primary subtypes: pancreatic ductal adenocarcinoma (PDAC) and mucinous tumors. PDAC accounts for more than 90% of pancreatic cancer diagnoses and as such, was the main focus of this thesis.[5]

The disproportionate mortality rate associated with pancreatic cancer can be attributed to its detection and diagnosis. In its early stages, pancreatic cancer displays symptoms that can be easily overlooked including weight loss, jaundice, loss of appetite, nausea, pancreatitis, and recent-onset diabetes.[7-9] As a result of the vague symptom presentation displayed by early-stage pancreatic cancer, only about 10% of pancreatic cancers remain localized to the pancreas at the time of diagnosis.[10]

Clinically, individuals with pancreatic cancer are placed into one of four stages at the time of diagnosis: resecable, borderline resectable, locally advanced and metastatic disease.[8]

However, only 10%-15% of individuals who present with pancreatic cancer tumors are eligible for resection.[11,12] Late diagnoses limits treatment options for pancreatic cancer, leaving surgical resection of the tumor as the only potentially curative option.[10] Additionally, surgical resection does not guarantee successful outcomes in the future. Even after an individual undergoes resection (the Whipple procedure or distal pancreatectomy, as determined by tumor location), 5-year survival rates only improve to 21% for negative margin resection and 11% for positive margin resections.[10]

The lack of significant improvements in pancreatic cancer outcomes over the past few decades has drawn attention to factors that may make an individual high risk for development of this disease. Risk-factors for pancreatic cancer can be delineated between those that are non-modifiable and those that are modifiable. Non-modifiable risk factors for pancreatic cancer include: age, sex, ethnicity, blood group, gut microbiota, family history, and diabetes.[11] Age tends to be a defining characteristic of pancreatic cancer since it is typically associated with older individuals (e.g., 90% of individuals diagnosed with pancreatic cancer are over the age of 55).[11,13,14] Among ethnic groups in the US, African Americans have an approximately 50%-90% greater risk of developing pancreatic cancer than Caucasians.[11,13] Finally, the emergence of pancreatic cancer appears to be associated with familial history.[11] If two or more of an individual's relatives have had pancreatic cancer, this individual's risk of pancreatic cancer is nearly nine times higher than that of an individual who has no familial history of the disease.[11,15]

In contrast, modifiable risk factors include smoking history, alcohol use, chronic pancreatitis, obesity, and dietary factors. Multiple studies have found that individuals who smoke tobacco products have a 74% higher chance of developing pancreatic cancer compared to never smokers, making smoking an important modifiable risk factor in the development of pancreatic

cancer.[11,16] Chronic pancreatitis (i.e., inflammation of the pancreas), is associated with later emergence of pancreatic cancer with nearly 5% of patients diagnosed with the disease developing pancreatic cancer.[11,17] Finally, the impact of the obesity epidemic on pancreatic cancer risk continues to be a focus of research. A 2015 study showed that an individual's chances of developing pancreatic cancer increased by 10% with every additional 5 BMI units.[11,18] Recognition of the strength of these correlations between risk factors, both non-modifiable and modifiable, contributed to the creation of High Risk Clinics, including the High Risk Pancreatic Cancer Clinic at the University of Virginia.[19] With an understanding that pancreatic cancer is typically discovered too late into disease progression, this clinic aims to screen individuals who meet some of these risk factors in hopes of discovering pancreatic cancer prior to the emergence of symptoms.[19]

### ***Physiological Background of Cancer Development***

#### *Oncogenes and Tumor Suppressor Genes*

Cancer develops as a result of genetic mutations that alter the functionality of genes.[20] Genes with the ability to promote cancer development are divided into two broad categories: oncogenes and tumor suppressor genes.[20] According to the National Human Genome Research Institute, an oncogene is defined as, "a mutated gene that contributes to the development of a cancer. In their normal, unmutated state, oncogenes are called proto-oncogenes, and they play roles in the regulation of cell division.[21]" On the other hand, tumor suppressors are genes that, "direct the production of a protein that is part of the system that regulates cell division. When mutated, a tumor suppressor gene is unable to do its job, and as a result uncontrolled cell growth may occur.[22]"

*Physiological Background of Pancreatic Cancer Development*

Beginning in 1976, studies were conducted to determine whether lesions in the pancreatic duct predispose an individual to pancreatic adenocarcinoma.[23] Based on the findings of numerous clinical studies and genetic analyses researchers concluded that there is a sequential progression to pancreatic ductal adenocarcinoma development, with the process requiring the transformation of the normal epithelium into duct lesions which may ultimately become ductal adenocarcinoma.[23,24] Subsequent studies classified these lesions into three distinct groups: pancreatic intraepithelial neoplasia (PanIN), mucinous cystic neoplasm (MCN), and intraductal papillary mucinous neoplasm (IPMN).[25,26,27] PanINs are the most common lesion and as such, have garnered the most attention in the scientific community. Typically, these lesions develop in the small pancreatic ducts.[24,25,27] PanINs are assigned a stage from I to III; as the stage of the PanINs increase, they are more likely to progress into PDAC. Furthermore, there appears to be a correlation between the stage assigned to a PanIN and the number of genetic mutations it presents in genetic analyses.[25,28-34] As such, understanding the molecular basis behind the development of these lesions and PDAC has become a focus of future studies to enable early detection of this disease.[25]

Pancreatic cancer is a genetically heterogeneous disease, caused by mutations in various genes; the most frequently mutated genes that contribute to pancreatic cancer development include: *KRAS, CDKN2A, TP53,* and *SMAD4*.[5] Nevertheless, PDAC progression is most often associated with mutations in *KRAS* as mutations in this gene occur in more than 90% of individuals with pancreatic cancer.[5,35] Unlike the later three genes, mutations in *KRAS* lead to the creation of an oncogene. Alterations in the KRAS protein lead to a constant activation of the downstream signaling pathway, which promotes tumor growth.[5,36] Mutations in the *KRAS* gene

appear early in the progression of PDAC with more than 90% of PanIN lesions expressing this genetic alteration.[5,37] While *KRAS* mutations directly contribute to the development of PanINs, mutations in this gene must be accompanied by mutations in tumor-suppressor genes to form PDAC.[5,38-40] The inactivation of these tumor-suppressor genes enable the cell to bypass checkpoints that would normally cause the cell cycle to arrest or the cell to undergo apoptosis.[5,41] As stated previously, *KRAS* is typically the first mutation to arise in the progression of pancreatic cancer, but subsequent mutations in the aforementioned tumor suppressor genes are essential to its complete development.[5,42]

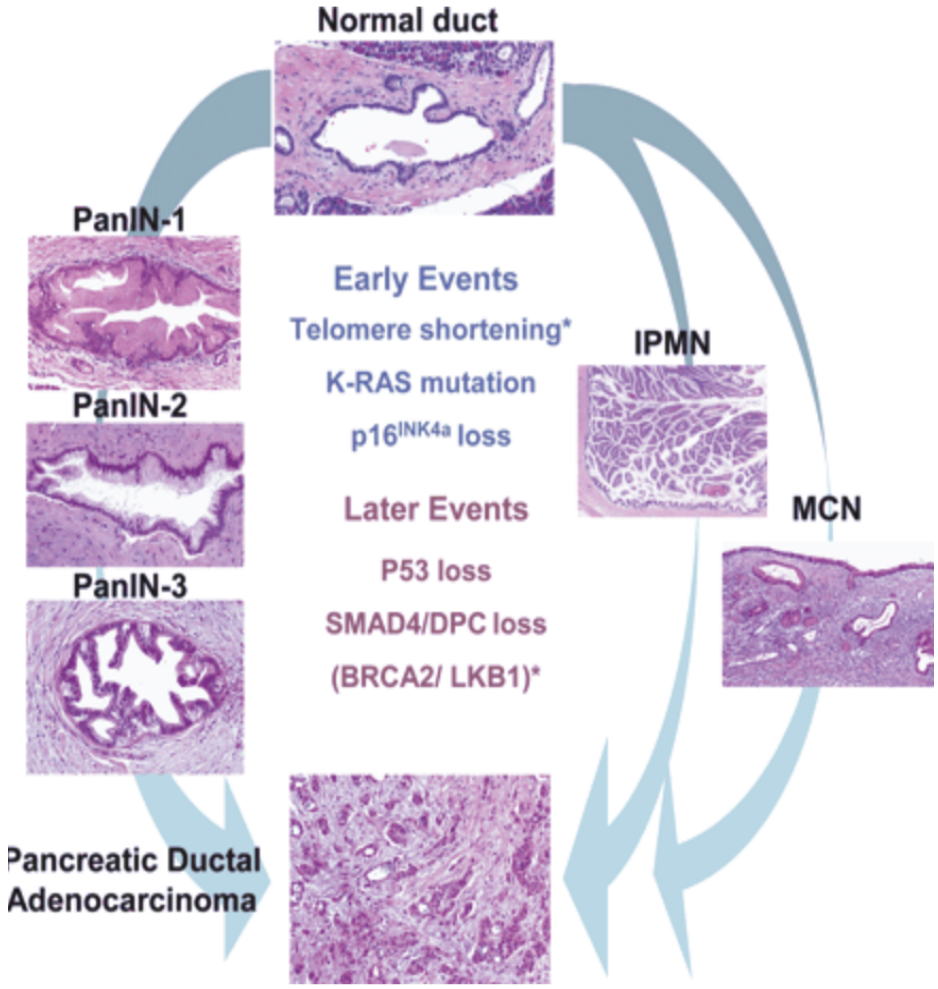


**Figure 1.** The progression of Pancreatic Ductal Adenocarcinoma Development from precursor lesions.[25]

***Role of the Tumor Microenvironment***

The tumor microenvironment, defined as the dynamic interactions that occur between cancer cells, stromal cells, and extracellular components, plays a significant role in the development of pancreatic cancer [43] Typically, tissue stroma coordinates responses to injuries in order to maintain physiological homeostasis. However, cancer has the ability to alter the regulated functioning of the stroma in an effort to generate a more favorable microenvironment for the tumor.[44,45] The contribution of stromal cells is essential to the development of pancreatic cancer; stromal cells can be divided into a number of categories including: pancreatic stellate cells (PSCs), myeloid-derived tumor suppressor cells (MDSCs), regulatory T cells (Tregs) and tumor-associated macrophages (TAMs).[43,46] Both the cancer and stromal cells present in the tumor microenvironment release extracellular components; these extracellular components include growth factors, such as transforming growth factor-β (TGFβ), and proteins that can impact interactions with the extracellular matrix (ECM).[43,47] While new immunotherapies have been generated to target the microenvironments of various cancer types, these experiments have not yielded significant results in regards to pancreatic cancer.[44,48] These dismal experimental results have led to an increased desire to understand the complex interactions that occur within the tumor microenvironment.

*Desmoplasia and Pancreatic Cancer*

A defining characteristic of pancreatic cancer is the appearance of desmoplasia, an increase in the amount of fibrotic tissue surrounding the primary tumor resulting from pancreatic stellate cell activation.[43,44,49,50] Nearly 90% of the volume of a pancreatic cancer tumor is composed of desmoplasia.[51] A desmoplastic environment is associated with a poor prognosis,

since it prevents vascularization in the tumor microenvironment, in turn limiting the effectiveness of chemotherapeutic agents and immune cell activation.[43,44,52,53] Experimental results continue to display the complexity of desmoplasia as therapeutics targeting singular agents within the extracellular matrix of tumors, such as those targeting hyaluronan, have been ineffective in clinical trials.[44] Previously, desmoplasia was only associated with pro-tumorigenic tasks. However, recent studies have shown that defining desmoplasia in this manner does not account for a number of its newly discovered functions. As such, desmoplasia is now defined as a reactive process to the development of cancer that transitions between pro-tumoral and anti-tumoral actions.[44]

As previously stated, there are a number of stromal cells that contribute to the creation of the desmoplastic environment that enhances cell growth and survival. One cell type of note is cancer-associated fibroblasts (CAFs), which typically develop from PSCs.[51,54] Previously, CAFs were believed to function only in support of tumor growth. This idea was based on evidence showing that CAFs have a direct impact on the extracellular matrix by enhancing cells' migratory abilities and survival.[54,55] There is however increasing evidence showing that some CAFs act in an anti-tumoral manner rather than a pro-tumoral manner.[54,56] This has led researchers to hypothesize that CAFs are composed of a heterogeneous population of subtypes of that have different functions in the tumor microenvironment.[54] Currently, two primary CAF subtypes have been identified: inflammatory CAFs (iCAFs) and myofibroblastic CAFs (myCAFs).[51] Unlike myCAFs, iCAFs display lower levels of αSMA and elevated levels of cytokines and chemokines that are related to cancer growth and survival.[51] It is expected that other subtypes of CAFs exist, but these two subtypes have provided researchers with new avenues to develop targeted PDAC therapies.[51]

*Immune System and Pancreatic Cancer*

Due to the considerable stromal cell activation associated with PDAC, the tumor microenvironment has the ability to suppress immune activity.[57,58] This lack of immune regulation contributes to the proliferation of tumor cells, enabling the formation of solid tumors, as seen in PDAC.[58,59] When analyzing the immune cells that partake in pancreatic cancer development, myeloid cells garner the most attention. The myeloid cells that contribute most significantly to the development of PDAC can be further subdivided into two categories: tumor-associated macrophages and myeloid-derived suppressor cells.[57]

➢ *The Role of Tumor-Associated Macrophages*

Macrophages are defined as, "specialized cells involved in the detection, phagocytosis, and destruction of bacteria and other harmful organisms. In addition, they can present antigens to T cells and initiate inflammation by releasing molecules that activate other cells.[60]" Phagocytosis describes this process through which macrophages engulf harmful organisms and cellular remnants.[61] Typically, this process occurs nonspecifically; macrophages utilize pattern recognition receptors (PRRs) which bind to the pathogen-associated molecular pathogens (PAMPs) present on the phagocyte.[62] Following this binding, the cell membrane of the macrophage wraps around the phagocyte, enabling the macrophage to take in these particles and transport them to the lysosome where they will be degraded.[60,62]

While all macrophages share a common place of origin, blood monocytes, they are a heterogenous group of cells with subpopulations that are differentiated according to the environment in which they reside; tumor-associated macrophages, which are found in the tumor microenvironment, can be classified as one such subtype.[60] Oftentimes, researchers differentiate

TAMs according to their phenotype, leading to two subdivisions: M1 macrophages and M2 macrophages. In terms of tumor development, M1 macrophages foster inflammation, which coincides with an antitumor role in the microenvironment, while M2 macrophages act in an anti-inflammatory manner and as such, promote tumor growth.[58,63] While researchers delineate between these two groups, it is important to note that the macrophages present in pancreatic cancer can transform from one subtype into the other. Nevertheless, it has been shown that macrophages associated with PDAC are predisposed to the M2 phenotype, therefore promoting the tumor's development.[58]

Despite the fact that macrophages typically conduct phagocytosis and are vital to homeostasis, it has been shown that an increased number of TAMs in tumors correlates with a worse prognosis clinically.[64-66] This can be attributed to a number of the events in which TAMs participate such as tumor initiation, chemoresistance, enhanced tumor invasiveness, and suppression of other aspects of the immune system. In the tumor microenvironment, TAMs secrete various signaling molecules including growth factors, cytokines, and chemokines that induce a progression in tumor growth.[64,67] Furthermore, both *in vivo* and *in vitro* studies have indicated that deletion of TAMs from the tumor microenvironment enhances the efficacy of chemotherapies on tumors.[64,68] In terms of metastasis, macrophages release matrix proteins that promote reorganization of the ECM, which contribute to increased tumor invasiveness.[64,69] Finally, when macrophages undergo polarization and adopt an M2 phenotype, they begin to secrete signals that suppress other immune cells such as T cells; this immunosuppression confers added barriers to the generation of successful immunotherapies.[64]

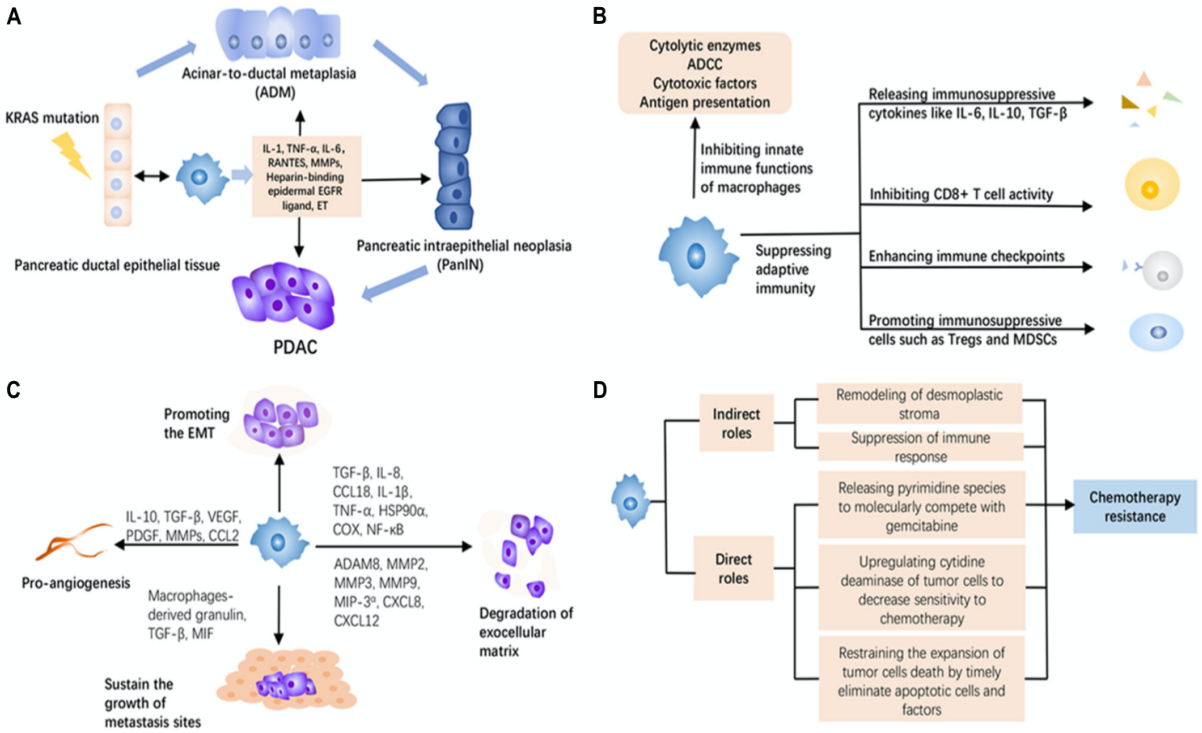


**Figure 2.** Contributions of tumor-associated macrophages (TAMs) in the development of Pancreatic Ductal Adenocarcinoma.[58]

➢ *Myeloid Derived Suppressor Cells*

Similar to TAMs, the increased presence of MDSCs in the tumor microenvironment of PDAC corresponds to higher clinical stages.[70,71] As the primary tumor site transforms the surrounding tissue into a hypoxic environment, signals are released that draw MDSCs towards the tumor microenvironment.[70,72] MDSCs prevent the activation of lymphocytes as well as the recognition of antigens, further reinforcing the immunosuppressive environment that is characteristic of PDAC.[73-75] Delineating different subpopulations of MDSCs has proven difficult due to their heterogeneity.[73] Recent studies, however, have proposed that MDSCs may differentiate into tumor-associated macrophages as a result of the hypoxic conditions present in the tumor's microenvironment.[70,76]

### *Metastasis and Pancreatic Cancer*

Frequently, pancreatic cancer is not diagnosed until the disease has started to spread to other parts of the body through metastasis. About 53% of individuals diagnosed with pancreatic cancer will present with disease that is classified as "distant," meaning that it has already undergone metastasis. This classification is associated with a 5-year survival rate of just 3%.[3] Due to the high likelihood of metastasis in pancreatic cancer, research has been conducted to understand the mechanisms through which metastasis occurs in hopes of finding treatments that can impede the spread of this disease.[77]

#### *The Pre-Metastatic Niche*

In 1889, Stephen Paget devised the "seed and soil hypothesis" to explain the process of cancer metastasis.[77,78] After analyzing the manner in which breast cancer preferentially metastasized to some organs over others, Paget speculated that tumor cells, or the "seeds," will only metastasize to organs that will nurture their growth, the "soil.[77,78]" While research conducted since this time has revealed that the process of metastasis is more complex than described by this original hypothesis, pancreatic cancer does preferentially metastasize to the liver. Liver metastasis occurs in nearly 80% of individuals diagnosed with PDAC, with metastasis to the peritoneum and lungs occurring in approximately 50% of patients.[77,79,80]

Numerous hypotheses of metastasis have been generated since Paget's work, the most prominent of which is the idea of a 'pre-metastatic niche.[81,82]' The concept of a pre-metastatic niche, or PMN, rests on the ability of tumors to promote favorable environments in other organs, prior to metastasis, that will promote the growth of these cells.[81-85] It has been proposed that both tumor-secreted factors and tumor extracellular vesicles (EVs) contribute to the priming that

occurs in the PMN.[81] These actions are thought to occur prior to events within the tumor microenvironment which prepare cells in the primary tumor to relocate to a new organ.[77,81]

*Epithelial-Mesenchymal Transition*

As defined by Kalluri et. al[86], "an epithelial-mesenchymal transition (EMT) is a biologic process that allows a polarized epithelial cell, which normally interacts with basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of ECM components." Epithelial cadherin (E-cadherin) and neural cadherin (N-cadherin) are utilized to classify the stage of EMT as E-cadherin is essential for epithelial cell structure, while N-cadherin is associated with a mesenchymal morphology.[86,87] In terms of the tumor microenvironment specifically, stromal cells release the signals necessary for an epithelial cell to undergo EMT.[87-90] Analyzing these signaling molecules has proven beneficial for pathologists, as an increase in their production is associated with increased tumor malignancy.[87,91]

Recent studies have found a correlation between EMT and metastasis. Nevertheless, research in this field has proven difficult as multiple experiments have displayed contradictory results.[87] Most notably, deletion of *Zeb1*, an EMT-transcription factor (EMT-TF), mitigated metastasis, particularly to the lungs, in a pancreatic cancer mouse model. [92] Furthermore, mice with *Zeb1* ablations displayed a lower number of PanIN-precursor lesions, which are typically correlated with pancreatic cancer development. [92] Similar experiments conducted utilizing other EMT-TF knockouts, such as *Snai1* and *Twist1*, did not impact the development of PanINs or metastasis in a manner similar to *Zeb1*.[92,93] As such, these experiments lead researchers to

conclude that EMT-TFs do not play a role in tumor progression and metastasis.[92,93] Nevertheless, researchers explained these conflicting results by claiming that each EMT-TF contributes to EMT in a distinct manner, which is further dependent on the tissue that is being analyzed.[92,93] Due to the lack of a clear consensus regarding the role of EMT in tumor metastasis, further research must be focused on understanding the mechanisms through which EMT-TFs operate in order to develop effective treatments.[92]

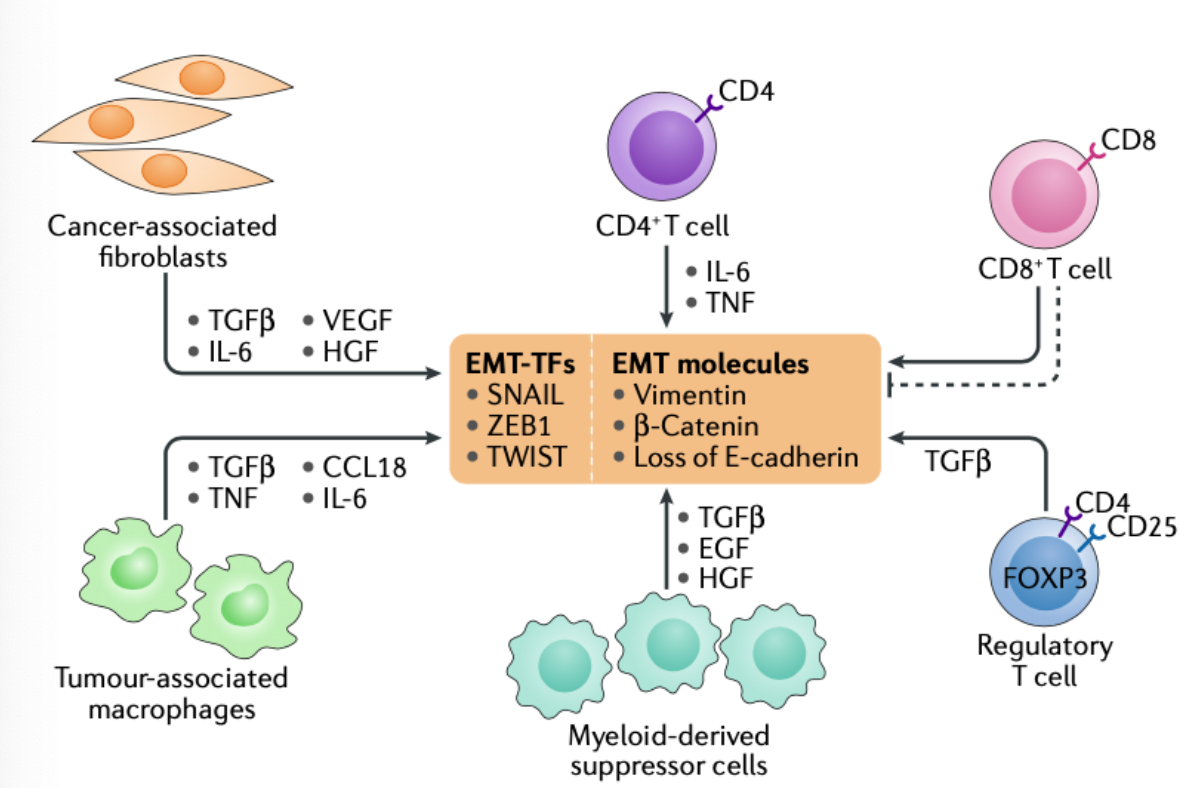


**Figure 3.** The interplay between the tumor microenvironment and carcinoma cells promotes the activation of EMT.[87]

### *Models for Pancreatic Cancer Research*

One challenge that has arisen in the search for novel treatment options targeting pancreatic cancer is the generation of models that accurately represent the microenvironment of the patient in preclinical studies.[94] Many different models have been established to mimic the microenvironment of patients with PDAC, such as pancreatic cancer cell lines (CCLs),

organoids, genetically engineered mouse models (GEMMs), circulating tumor cell-derived xenografts (CDXs), and patient-derived xenografts (PDXs).[94] CCLs have been utilized for both *in vitro* and *in vivo* experiments. However, because these cell lines are derived from the primary tumor when passaged in immune-deficient mice they begin to undergo genetic alterations. Unfortunately, alterations acquired during *in vitro* analyses cannot be recovered at a later time.[94,95] Furthermore, pancreatic cancer tumors are highly heterogeneous and the tumor microenvironment contains numerous cell types such as fibroblasts and immune cells. CCLs neglect the reciprocal interaction that occurs amongst these cell types in the tumor microenvironment, posing a limitation on the potential applications of treatments.[94]

GEMMs have been generated to represent the most commonly mutated genes found in pancreatic cancer tumors including mutations in the *KRAS* gene and tumor-suppressor genes like *SMAD4*.[94,96] Unlike CCLs, these cell models closely represent the physiological changes that occur during the development of disease in humans.[94] Nevertheless, the use of GEMMs has several limitations. As noted previously, pancreatic cancer tumors are heterogeneous, yet in the generation of these mouse models, all the cells acquire the same mutations.[94] Furthermore, the creation of these models is restricted by the current level of understanding surrounding the most impactful mutations in tumor development.[94]

Unlike the other models, PDXs have the ability to recapitulate the dynamic interactions between the heterogeneous tumor and its microenvironment.[94] As such, PDXs are most often used in the development of adjuvant treatment options for pancreatic cancer.[94] In contrast to CCLs and CDXs, which develop genetic alterations *in vitro,* PDXs have been shown to maintain between 93% to 99% of the genes expressed in the patient's original tumor.[94,97] While PDXs present unique benefits in comparison to the other models, there are still a number of limitations

with this model. PDX models are generated in immune-deficient mice so that the human tissue is not rejected by the organism, however, this process does not allow for the accurate representation of the efficacy of immunotherapy treatments.[94] Finally, elements of the tumor microenvironment from the human can be replaced by murine elements, and as such, tumor development in this model are not directly correlated to the tumor development from a human.[94]

Within the Bauer lab, orthotopic xenograft models are utilized to replicate human pancreatic cancer,[97] Unlike the CCLs and CDXs, this model of pancreatic cancer does not undergo significant genetic alterations during passages. Furthermore, orthotopic tumor generation more accurately represents the interplay between the tumor and its microenvironment that occurs in human patients.[97] Finally, understanding the process by which pancreatic cancer metastasizes to organs such as the liver is essential to developing new and effective treatments. Oftentimes, models that rely on subcutaneous injections do not present with metastatic disease.[97,98] However, this orthotopic model, which utilizes pancreatic xenografts, supports the appearance of metastases in the liver, peritoneum, and diaphragm of the mouse.[97] Nevertheless, limitations are inherent to all model systems. Of significant note is that this model is generated in immune-deficient mice which lack adaptive immunity, but retain innate immune function. As described above, these mice do not provide complete insight into the role of the immune system in the development of the tumor.[97]

### *Current Treatment Options for PDAC Patients*

For individuals with pancreatic ductal adenocarcinoma, surgical resection remains the only opportunity for a cure. [99,100] The process of generating a safe and effective surgical procedure for pancreatic cancer, however, took nearly 100 years. In 1882, Friedrich

Trendelenberg successfully removed the first tumor from a pancreas.[101] From the 1880s to the 1940s, many individuals attempted to strengthen this procedure, including Allen Whipple who reported the use of a two-stage pancreaticoduodenectomy (Whipple Procedure) in 1942.[101,102] In the years following this report, operative mortality rates for this procedure remained stagnant at nearly 33%.[101,102] Significant reductions in mortality rates associated with the Whipple procedure were not observed until the 1980s, when the rate improved to 2%.[101,103]

While surgical resection of pancreatic cancer has improved significantly, the disease returns in more than 90% of patients if they do not undergo additional treatments following resection.[99,100] As such, the American Society of Clinical Oncology recommends that individuals continue treatment for 6 months after their resection.[101] Currently, the standard of care for patients with resectable tumors involves adjuvant chemotherapy, typically with gemcitabine.[104-106] However, a clinical trial conducted in 2018 comparing a modified FOLFIRINOX regimen to gemcitabine and found a significant increase in disease-free survival for those patients who received the modified FOLFIRINOX regimen.[104] Unfortunately, this regimen compounded the toxic effects of the traditional gemcitabine therapy.[104] When patients present with metastatic disease at the time of diagnosis, FOLFIRINOX and nab-paclitaxel-gemcitabine are the primary lines of treatment. Nevertheless, each of these options generate considerable toxic effects.[99,107,108]

Over the last two decades, extensive research has been focused on determining the potential benefits of neoadjuvant therapy for pancreatic cancer treatment.[109,110] Unlike adjuvant therapy, which focuses on preventing relapse after surgical resection, neoadjuvant therapy aims to generate a tumor response prior to surgery.[109] The primary goal of neoadjuvant therapy is to make surgical resection a reality for individuals with borderline resectable or locally advanced

tumors.[99,111,112] While data from neoadjuvant therapy trials is still lacking, there are currently several research institutions conducting phase III clinical trials.[99,113]

## Future Directions for PDAC Treatment

### *Immunotherapy*

While cancer incidence has decreased by nearly 30% since the 1990s, in sharp contrast, the incidence of pancreatic cancer diagnoses has slightly increased over the past 20 years.[114,115] This discrepancy can be attributed to numerous factors, among the most important are the invasiveness of the disease and the fact that it presents with vague symptoms at early stages.[116] Nevertheless, to understand the lack of improvements in pancreatic cancer outcomes, one must consider the absence of targeted therapeutic options for this disease as well.[116] Due to the stark statistics on incidence and survival associated with pancreatic cancer, researchers have shifted their focus to the generation of novel treatments for this disease. As a result, immunotherapy, which is currently the fourth most commonly used treatment option for cancer following surgery, chemotherapy, and radiotherapy, has become a promising route through which improved outcomes can be achieved in PDAC treatment.[117,118]

The general premise of immunotherapy treatments is to take advantage of the anti-tumor role of the immune system, therefore preventing tumor proliferation and recurrence.[117] There are two primary forms of immunotherapy treatments: active and passive. These immunotherapies can be differentiated according to the manner in which they impact the immune system.[118] Active immunotherapies drive the host's immune system to generate a response to specific tumor-associated antigens.[117,118] Treatment options that utilize an active immunotherapy include cancer vaccines and cell-based therapies.[118] Passive immunotherapies facilitate an immune

response by administering small molecule drugs, utilizing monoclonal antibodies (mAbs), or by introducing immune cells, such as T cells.[117,118]As such, passive immunotherapies act in contrast to active immunotherapies as they do not directly alter the host immune cells throughout the therapeutic process.[118,119] While not categorized as an active or passive immunotherapy, immunomodulators also play an important role in cancer treatment. Immunomodulators, such as interferon and IL, are non-specific molecules utilized to promote the innate immune systems response to cancer cells.[114]

A protein that has garnered significant attention within PDAC immunotherapy research is CD47 (Cluster of Differentiation 47).[120,121] CD47 is overexpressed on many cancer cells where it inhibits phagocytosis by macrophages through an interaction with Signal Regulatory Protein α (SIRPα) on the macrophage.[120-125] Overexpression of CD47 on PDAC cells negatively impacts the ability of macrophages to phagocytose tumor cells *in vitro.*[120] Studies focusing on the primary tumor site had shown that in a subcutaneous model of PDAC, treatment with an anti-CD47 monoclonal antibody, in addition to chemotherapy, significantly slowed tumor growth whereas anti-CD47 treatment alone did not yield significant results.[126,127]

Based on these previous experiments, the Bauer lab analyzed whether targeting CD47 would promote phagocytosis of PDAC micrometastases by macrophages in the liver (Kupffer cells) in an attempt to generate an adjuvant treatment option for individuals with PDAC.[126] While the role of TAMs within the microenvironment of the primary tumor has been well established, the impact of metastasis-associated macrophages (MAMs) has not been as stringently analyzed.[126] Studies have shown that Kupffer cells employ phagocytosis to rid the liver of tumor cells.[126,128] Based on these findings, it was proposed that CD47 is the mechanism by which PDAC micrometastases in the liver avoid phagocytosis, making CD47 a reasonable

target for PDAC treatment.[126] Unlike the results from the previous studies, which had shown that isolated anti-CD47 (α-CD47) treatment would not significantly slow tumor growth, the α-CD47 treatment alone was sufficient in targeting PDAC micrometastases *in vivo*.[126] Differences between the physiological features of the liver and pancreas have been suggest to responsible for the apparent discrepancy in these experimental results.[126] Overall, this experimental data supports the use of α-CD47 treatment in PDAC clinical trials for patients following surgical resection.[126]

*Genomic Targets*

As previously described, pancreatic cancer is primarily associated with mutations in four genes: *KRAS*, *P53*, *SMAD4*, and *CDKN2A*. Due to the prominence of these mutations, particularly those in *KRAS,* researchers have attempted to target the proteins that act downstream in these signaling pathways, utilizing agents such as MEK inhibitors.[114,129] However, these inhibitors utilized in combination with standard chemotherapeutic agents have not generated significant results in clinical trials.[114,129] To explain these results, which appear to contradict the biological understanding of this disease, researchers have referenced the interconnectedness of the pathways that contribute to PDAC development. Although proteins downstream of the KRAS pathway may be effectively inhibited by these agents, the redundancy found in this disease enables its continued proliferation.[114]

While these treatment options did not succeed in trials, new developments have gained traction in recent years. Despite the prevalence of *KRAS* mutations in PDACs, no treatment has been successful in targeting this protein. Such lack of progress can be explained by the high affinity of the mutated protein for GTP and GDP.[130-132] Nevertheless, RNA interference (RNAi)

has provided researchers with a possible route to inhibit KRAS production.[130] After preclinical models of PDAC found significant reductions in tumor growth following the addition of a KRAS-specific RNAi agent, researchers have begun phase 2 trials to illustrate the potential benefits of this therapy in combination with chemotherapeutic regimens.[114] Additional personalized treatment options have generated notable results in recent studies. AMG510 is an RNAi based therapy that specifically inhibits the G12C mutation of KRAS. Current trials utilizing AMG510 have displayed clinical effectiveness in other solid tumors.[114] Due to the individualized nature of this medication, its impact will be limited to populations in which this genetic alteration commonly occurs, such as the Japanese population which exhibits this mutation in nearly 60% of PDAC cases.[114,133]

*Stromal Targets*

Due to the prominence of stromal cells in the pancreatic cancer tumor microenvironment, the stroma has garnered attention as a potential therapeutic target.[114] Researchers first hypothesized that depletion of CAFs would strengthen the potential impact of chemotherapeutic agents.[134] However, the deletion of α-SMA from mouse models led to undifferentiated tumors with an increased metastatic potential.[134,135] When FAP-expressing cells were depleted from pancreatic cancer mouse models, researchers witnessed the opposite phenomenon as this deletion resulted in decreased tumor proliferation.[134] Following this preclinical success, a FAP-specific antibody was developed and found to be successful in Phase I trials but did not increase survival rates for patients with metastatic colorectal cancer in Phase II trials. As a result of the dismal results in the treatment of colorectal cancer patients, trials utilizing this agent to treat PDAC have been suspended.[134]

Another approach adopted to target the stroma focuses on the interplay between CAFs and the remainder of the tumor microenvironment.[134] Multiple studies have reported the possible benefits brought about by targeting IL-1 or TGF-β, signaling molecules released by cancer cells to activate CAFs.[134] By inhibiting release of the signaling molecules, researchers hope to generate a reduction in the number of CAFs that have the ability to promote metastasis in PDAC.[134] The interactions between CAFs and the ECM are also of significant interest to researchers. One of these, Hh signaling promotes reorganization of the ECM resulting in increased communication between CAFs and cancer cells.[134,136] Inhibitors of this signaling pathway have provided benefits to small populations of patients when utilized in combination with chemotherapy.[134,137] The other is HA expression. Patients that display a higher proportion of HA expression in their tumors have benefitted from HA depletion, yet these approaches have not shown broad clinical significance.[134,138] Researchers have concluded that a further understanding of the subtypes of CAFs as well as their interactions in the tumor microenvironment are essential to generating clinically significant treatment options.[134]

### *Goals of this Study*

Pancreatic cancer lacks treatment opportunities that are vitally important to the improvement in cancer survival rates over the last two decades. As such, it is essential that future research in this field accounts for the unique challenges that pancreatic cancer poses in terms of the translatability of treatment options from the laboratory setting to the clinical setting. For this reason, I analyzed the efficacy of new, personalized therapeutic approaches for individuals with pancreatic cancer utilizing tumor cell lines generated from patient-derived PDAC tumors and models that recapitulate the microenvironment that is characteristic of PDAC. The results of

these studies provide new insight into the potential benefits of chemotherapies, small molecule therapies, and immunotherapies as options to patients currently undergoing treatment in the clinic.

## Methodology

### *Materials*

The following materials were utilized in our experiments. Treatments are categorized into four subsections: chemotherapies, molecular inhibitors, flow cytometry reagents, and other.

1. Chemotherapies

   Gemcitabine (2',2'-difluoro deoxycytidine), is a chemotherapy commonly utilized to target pancreatic, lung, and bladder cancers. It is a nucleic acid analog that prevents the activity of ribonucleotide reductase and terminates DNA replication leading to cell death.[139,140] Doxorubicin is an anthracycline antibiotic that interferes with DNA replication by inserting itself into the helix and inhibiting the activity of topoisomerase II.[141] It is often used to target lymphomas, leukemia, and solid organ cancers.

2. Molecular Inhibitors

   Trametinib (GSK1120212) inhibits mitogen/extracellular signal-related kinase (MEK1/2). This treatment option has been shown to interfere with the phosphorylation of MEK by RAS.[142] Foretinib (GSK1363089) is a small molecule inhibitor that targets receptor tyrosine kinases including c-Met and vascular endothelial growth factor receptor-2 (VEGFR-2). Foretinb has generated positive results in the treatment of head and neck squamous cell cancer, breast cancer, and non-small cell lung cancer.[143,144]

3. Flow Cytometry Reagents

CypHer 5 is a pH-sensitive cyanine dye derivative that is not fluorescent at a neutral pH, but becomes fluorescent in an acidic environment. As such, CypHer can be utilized to analyze the movement of receptors into acidic endosomes.[145] F4/80 is a glycoprotein that can be utilized to signal macrophage expression. Most macrophages express F4/80, yet the presence of this molecule differs in different environments.[146] DAPI is a blue-fluorescent DNA stain that binds to A-T rich regions in DNA. DAPI has the ability to cross the membranes of dead cells, but not viable cells, therefore enabling its use as a sign of cell death in flow cytometry.[147] Annexin V staining enables the identification of apoptotic cells. Annexin V is a phospholipid-binding protein that binds phospholipid phosphatidylserine, the protein that translocates to the plasma membrane when a cell is undergoing apoptosis.[148]

4. Other

   Bumetanide is a diuretic utilized to treat high blood pressure, edema, and kidney disease.[149] Furthermore, bumetanide has been shown to inhibit the transporter function of SLC12A2.[150]

*Generation of Patient-Derived Cell Lines*

Following surgical resection, PDAC tumor samples 366, 395, 449, 608, 188, and 738 were produced from human tumor surgical pathology specimens. The collection of these samples relied on a collaboration with the University of Virginia Biorepository and Tissue Research Facility and occurred in accordance with the University of Virginia Institutional Review Board for Health Sciences Research guidelines, which required that each patient provided written informed consent prior to the collection of their remnant human tumor surgical pathology

specimens.[126] Each specimen was orthotopically implanted into the pancreas of immunocompromised mice. Cell lines were then generated from these tumors and firefly luciferase lentivirus (KeraFAST) was added.[126] In order to preserve these samples, the cells were placed into RPMI containing 10% FBS and penicillin/streptomycin.[151] These animal experiments followed a research protocol that had been reviewed and approved by the UVA Institutional Animal Care and Use Committee.

*Cell Culture*

Tumor cells were cultured in Roswell Park Memorial Institute (RPMI) Medium containing L-glutamine (300 mg/L) and phenol red (5 mg/L) and without HEPES.[152] Because RPMI lacks proteins, lipids, and growth factors, 10% FBS was added prior to its addition to the cell culture plates.[152] Cells were plated at a density of approximately $2x10^5$ tumor cells per $cm^2$ and the volume of media added was adjusted in accordance with this value.[153] Cells were incubated at 37°C under 5% $CO_2$. Every other day, the culture dishes were analyzed. If the media in the plates turned yellow, the existing media would be aspirated, the plate would be washed with PBS, and replenished with fresh media. In the absence of a color change, the media was replaced after 4-5 days.[153] When the cells reached confluence, the existing media was aspirated and the plates were washed with PBS. After washing with PBS, TrypLE was added to the plates; TrypLE contains cell-dissociation enzymes that can be utilized as a substitute for Trypsin.[154] The plates were then placed in the incubator for 5-10 minutes, or until the cells were no longer adherent to the plate. Following incubation, the TryLE was neutralized with media and the cells were resuspended. The existing cells were then split, typically following a 1:3 ratio but this value

varied depending on the prior confluence of the cells. After transfer to a new plate, the cells were resuspended in an additional volume of media in accordance with the plate size.

*Cell Count*

In order to determine the amount of cells present in a sample, 100µL of the desired cells and 100µL of Trypan Blue were added to a 96 well plate and mixed gently.[155] This mixture was then placed into the hemocytometer which was analyzed under the 10X objective of a light microscope. The unstained cells in the top two sets of 16 squares were counted and this value was recorded in the live cell tally.[155]

*CyQuant Cell Proliferation Assay*

On the first day of this experiment, existing media was removed from cell culture plates, which were then washed with PBS. Cells were then treated with trypsin and incubated. Once the cells were no longer adherent to the cell culture dish, they were placed into a 10mL tube and equalized with cell culture media. The number of cells present in the sample was determined based on the hemocytometer procedure described above.

Utilizing the information acquired from the cell count, 5000 cells were plated in each well along with 10% FBS containing RPMI media. Furthermore, approximately 230,400 cells from each cell line were placed into a centrifuge tube and spun down. The supernatant was removed following centrifugation and these samples were placed at -80℃ until the day of analysis to be utilized in the standard curve. The remaining plates were incubated overnight at 37℃ and 5% $CO_2$, giving the cells enough time to attach to the plate. The following day, the desired treatment was added to the wells at various concentrations. Two days after treatment,

100µL of media was removed from each well and replaced with fresh media. Drug was then added for a second time. Following two days of incubation, all the remaining media was removed from the wells and each well was washed with PBS. To ensure that the plates were completely dry, they were pounded against a flat surface on top of a paper towel. The plate was then wrapped in parafilm and stored overnight in the -80°C freezer.

To quantify the proliferation of the samples, the plates were thawed at room temperature. 200µL of the CyQUANT® GR dye and cell-lysis buffer (Invitrogen) were prepared for each sample well and the standard curve pellets according to the Thermofisher Protocol.[156] A standard curve was generated from the pellets that were frozen on the first day of this experiment following the Thermofisher Protocol.[156] This process resulted in a standard curve dilution of 1:3. After the CyQUANT GR dye and cell-lysis buffer was added to each sample well, the plates were incubated for 2-5 minutes without exposure to light.[156] A fluorescence reader was used to analyze the plates, with the appropriate filter sets (Biotek, Winooski, VT).

*Annexin V Staining*

Utilizing cell counts acquired from the hemocytometer procedure described above, $1x10^6$ cells were plated from each desired cell line. Throughout the experiment, various doses of drugs including gemcitabine and doxorubicin were added at different time points. On the day of analysis, an Annexin V Apoptosis Detection kit was utilized to stain the cell lines. Each sample required 100µL of an Annexin V and Binding Buffer solution, generated in a 1:20 ratio. After preparing this solution, the cell lines were harvested and placed into flow cytometry tubes. The Annexin V and Binding Buffer solution was added to each sample and then incubated for 30 minutes on ice and protected from light. While these samples were incubated, a solution was

generated from DAPI and Binding Buffer in a 1:1000 ratio. Following the incubation period, 250µL of the DAPI and Binding Buffer solution was added to each sample. The cell samples were then analyzed by flow cytometry.[157]

*Isolation of Macrophages and In Vitro Engulfment Assay*

1. *IgG and CD47 Engulfment Assay*

On the first day of this experiment, macrophages were isolated. To obtain macrophages, 5mL of PBS containing 2% FBS was added to the peritoneal cavity of mice.[126,158] The fluid obtained from the peritoneum was then placed in a centrifuge and spun down. Following centrifugation, these cells were resuspended in X-VIVO 10 serum-free hematopoietic cell medium and plated at a density of 500,000 cells in a 24-well plate (Lonza Group).[126] The X-VIVO media on the macrophages was changed daily.

Three days after the macrophages were plated, 608 cells were harvested and counted utilizing the cell count procedure described above. Based on the cell count, $9x10^6$ cells were filtered into a tube. 10mL of PBS was then added to these cells and CypHer was added at a ratio of 1:10,000. These samples were placed in the incubator at 37℃ for 30 minutes. Following this incubation period, the samples were centrifuged at 1500 RPM for 5 minutes, the cell pellet recovered and then resuspended in an X-VIVO solution containing either IgG, IgG1, or anti-CD47 antibodies. The samples were incubated in their respective antibody on ice for 45 minutes. X-VIVO media was then added to the six sample tubes to ensure each sample had a final concentration of 1.5mL. 500µL of the treatment cells or control cells were added to each well of macrophages. To initiate contact between the cell samples and the macrophages, the plates were placed into a centrifuge and spun for 1 minute. The samples were harvested by adding 2x trypsin

to each well and incubating at 37°C for 15 minutes. After undergoing incubation, PBS was added to neutralize the trypsin and each sample was transferred into a FACS tube to be spun in a centrifuge.

After centrifugation, 775µL of supernatant was removed from the FACS tubes, leaving nearly 25µL of supernatant and the cell pellet. There were four control samples in this experiment: DAPI only 608 cells, CypHer only 608 cells, F4/80 only macrophages, and macrophages spiked with CypHer stained 608 tumor cells and F4/80. 25µL of F4/80 was added to the remaining 20 sample tubes and 250µL of DAPI was added to these samples as well just prior to analysis at the Flow Cytometry Core.

2. *Bumetanide Engulfment Assay*

On the first day of this experiment, macrophages were acquired from the peritoneum of mice as described above. 366 tumor cells were also placed in a 12-well plate this day. The following day, 1µg/mL of gemcitabine was added to the 366 tumor cells and the media on the macrophages was replaced.

On day 5 of this experiment, the macrophages were treated with 10µM bumetanide for 1 hour. While the macrophages underwent treatment with bumetanide, the tumor cells were harvested. After harvesting these cells, all of the tumor samples except the unstained and Annexin V control samples, were stained with CypHer for 45 minutes at 37°C. Destaining was performed in serum free RPMI for 15 minutes and the tumor samples were then centrifuged and the supernatant discarded. The macrophages were then resuspended in X-VIVO serum-free hematopoietic cell medium at $2x10^6$/mL. 250µL of this solution was added to each of the tumor cell lines. To ensure contact between the tumor cells and macrophages, the plates were centrifuged for 2 minutes and then placed in an incubator for 30 minutes at 37°C. Following

incubation, the media containing the cells was removed from the wells and placed into a centrifuge tube on ice. To remove the macrophages, 2x Trypsin was added. Following a 10-minute incubation, PBS containing 2% (w/v) BSA was added to neutralize the Trypsin. These samples were placed into the centrifuge tubes, the cells and debris pelleted, and the supernatants aspirated off. 25µL of a staining solution consisting of 1:11 (v/v) EPCAM PE, 1:50 (v/v) F4/80 PEcy7 and 290µL of a buffer solution was added to each of the 12 samples. The samples were incubated on ice for 30 minutes following this addition. The controls were then prepared; F4/80 was added to the macrophage only sample, EPCAM was added to the tumor cells, and a tumor cell only sample was set aside to be stained with DAPI in the flow cytometry core. These samples, including the tumor cell and CypHer samples that were collected earlier, were spun, aspirated, and transferred to FACS tubes. A portion of the macrophage and F4/80 sample was combined with the tumor and CypHer and EPCAM sample, the macrophage and F4/80 sample was then added to the tumor and CypHer only sample, and the macrophage and F4/80 sample was combined with the tumor and EPCAM only sample. All the samples were then taken to the Flow Cytometry Core Facility for analysis; DAPI and binding buffer were added to each sample prior to analysis.

## Results

### *Cell Proliferation in the Presence of Gemcitabine*

Due to the unique characteristics associated with the cell lines utilized in the Bauer lab, we examined the response of seven patient-derived PDAC cell lines to gemcitabine treatment.

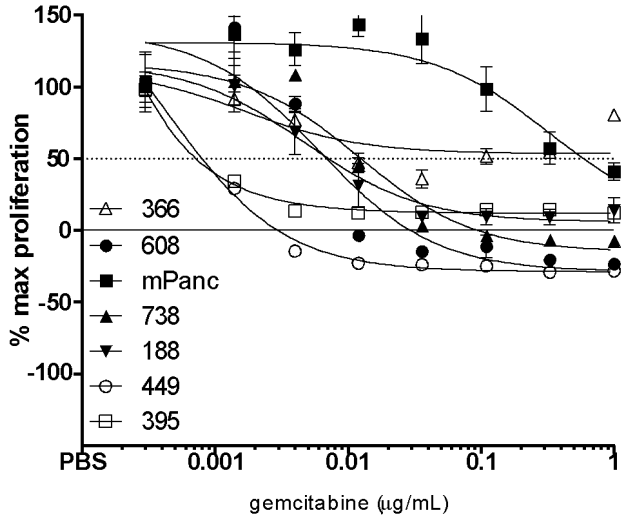


**Figure 4.** Gemcitabine Sensitivity Curves. Seven cell lines derived from PDAC tumor samples were dosed with various concentrations of gemcitabine. This graph is representative of a number of CyQuant Cell Proliferation Assays that were performed. Each cell line displayed different capacities for proliferation in the presence of this chemotherapeutic agent.

As shown in Figure 4, cell proliferation in the presence of gemcitabine varied among cell lines. The responses of each line were consistent between experiments. In each of the repeat experiments, mPanc (ATTC) had an increased resistance to gemcitabine while cell lines 449 and 608 displayed sharp decreases in their proliferative capacities in the presence of this chemotherapeutic agent.

*IgG and CD47 Engulfment Assay*

In order to further analyze the efficacy of a CD47 blockade, an engulfment assay was performed that compared the response of tumor cell line 608 to various IgG treatments in combination with an α-CD47 treatment.[126]

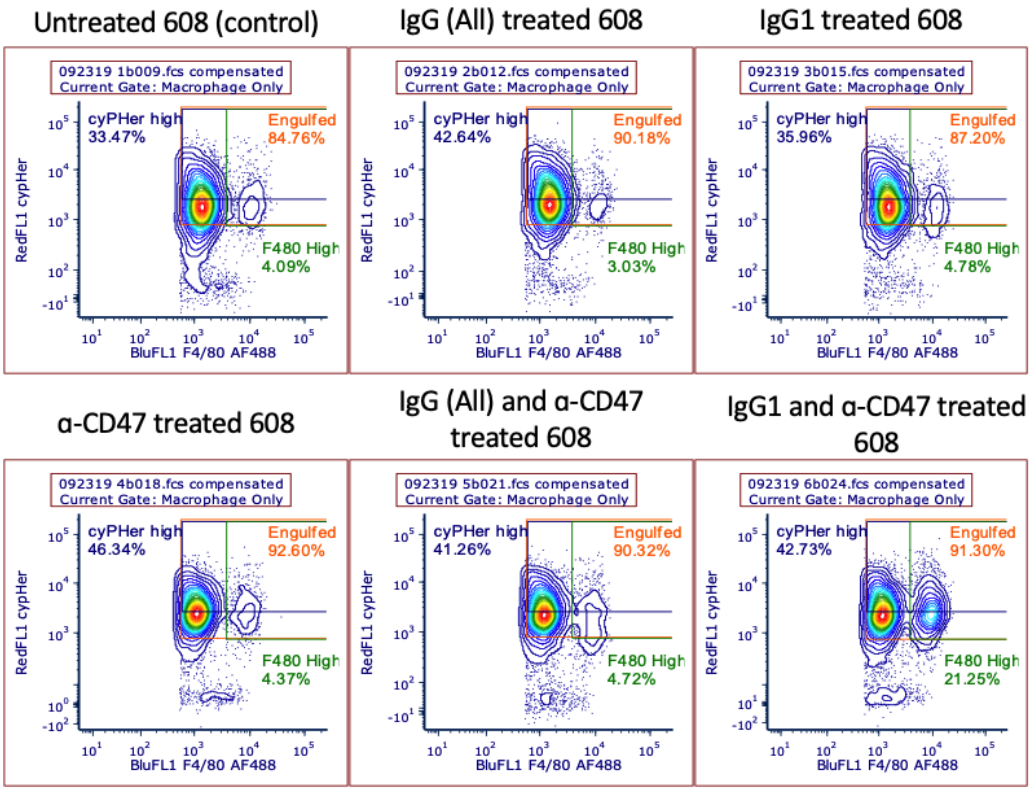


**Figure 5A.** α-CD47 and IgG Macrophage Engulfment Assay Flow Cytometry Analysis. The cell plots display the percentage of macrophages that had engulfed tumor cells, the percentage of CypHer high tumor cells, and the percentage of F480 cells in the samples of six treatment groups. This experiment was repeated in triplicate, but the data above is representative of the other data points.

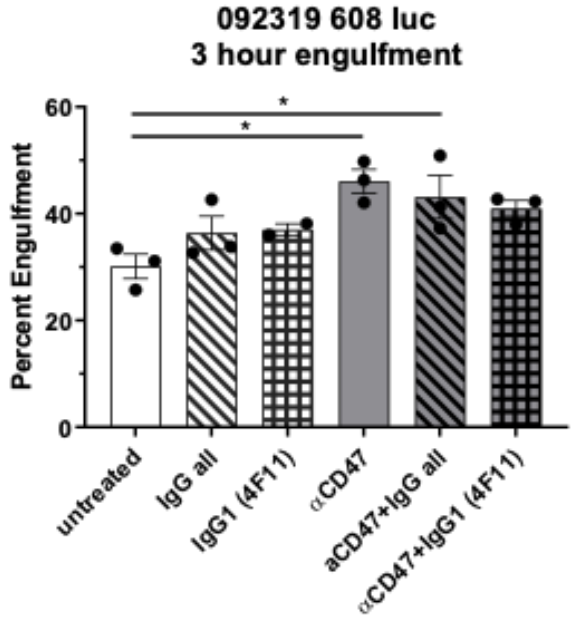


**Figure 5B.** Percent Engulfment of 608 tumor cells by macrophages in six groups. Treatment groups include: IgG (all) only, IgG1 only, α-CD47 only, α-CD47 and IgG (all), α-CD47 and IgG1. Significant increases in engulfment were observed in the α-CD47 only treatment group and the α-CD47 and IgG (all) treatment group.

Figure 5A displays the cell plots that were generated from flow cytometry analysis. In order to conduct this analysis, FCS was utilized to generate a "scatter gate" that disposed of the background signals from the flow cytometry machine. After gating out this background signal, the DAPI only control was utilized to generate a "live cell" gate, to ensure the dead cells would not be accounted for in the data analysis. Next, a "tumor cells" gate, "engulfed" gate, "CypHer high" gate, and "macrophage only" gates were generated on the control sample, with F4/80 on the x-axis and CypHer on the y-axis. The "engulfed" gate in this figure refers to the percentage of macrophages within the sample that had engulfed tumor cells as determined by the F4/80 signal and the "CypHer high" gate indicates the presence of tumor cells in an acidic environment, such as a phagosome. The presence of α-CD47 treatment appeared to slightly increase the percentage of macrophages that phagocytosed tumor cells. The addition of either

IgG treatment to α-CD47 had nearly the same level of macrophage engulfment as the α-CD47 treatment alone.

Figure 5B displays the percentage of engulfment in each of these samples as determined by the "CypHer high" gate on the cell plots in Figure 5A. Utilizing this "CypHer high" gate, it was possible to differentiate between populations of tumor cells that had been engulfed by macrophages, and therefore gave off a CypHer positive signal, and those that had not been engulfed by macrophages. As seen in this figure, treatment with α-CD47 and α-CD47 in combination with IgG (all) lead to a significant increase in tumor cell engulfment when compared to the control group. Treatment with IgG (all) or IgG1in isolation, however, did not significantly augment the baseline engulfment of tumor cells.

*Annexin V Flow Cytometry Analysis*

An initial engulfment assay that utilized bumetanide treated macrophages and untreated tumor cells generated insignificant results. Based on the ineffectiveness of these conditions, we concluded that the tumor cells needed to reach at least 30%-50% apoptosis to increase phagocytosis by macrophages. Therefore, we performed multiple trials of an Annexin V flow cytometry experiment using six cell lines: 366, 188, 395, 608, 738, and 449. Each of these cell lines were treated with varying concentrations of either gemcitabine or doxorubicin, another chemotherapy drug, at different time points. Figure 6A shows the data from the 395 cell line treated with gemcitabine and doxorubicin and Figure 6B shows the results from the 366 cell line treated with gemcitabine.

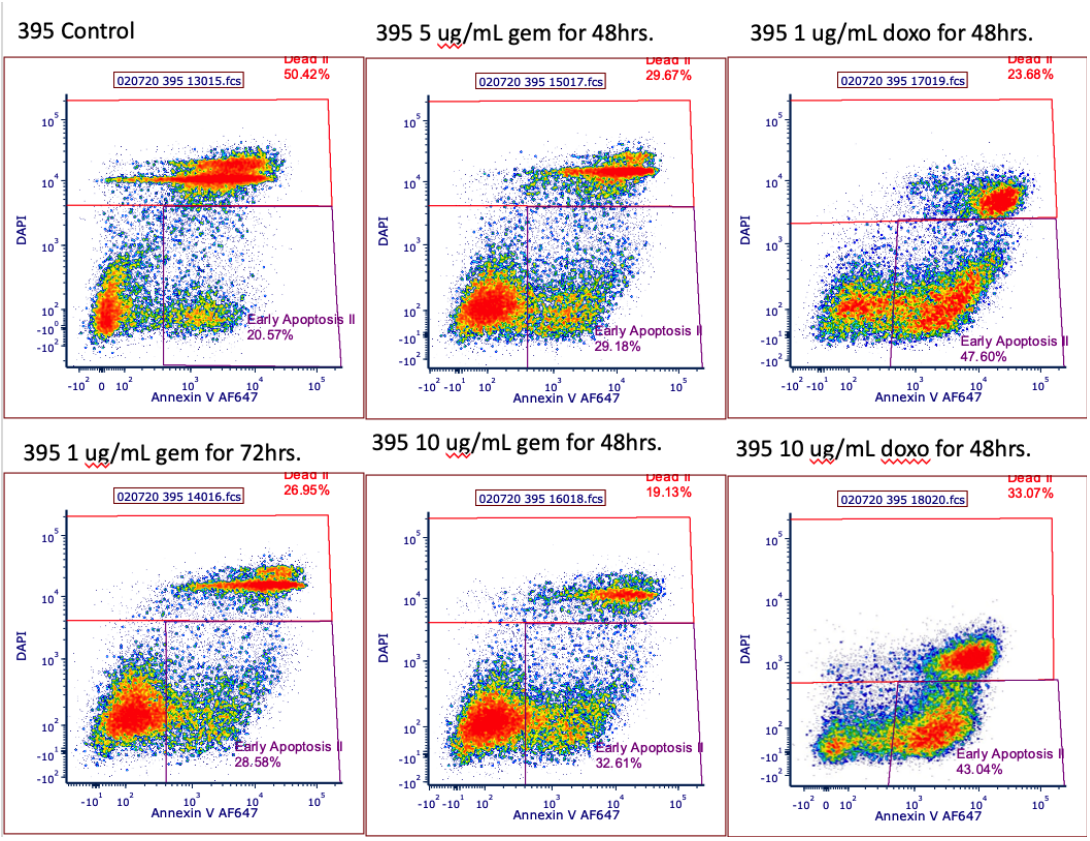


**Figure 6A.** Annexin V Flow Cytometry Analysis of 395 at varying concentrations of gemcitabine and doxorubicin treatment. Six groups were included in this experiment: control (no drug), 1µg/mL gemcitabine for 72 hours, 5µg/mL gemcitabine for 48 hours, 10µg/mL gemcitabine for 48 hours, 1µg/mL doxorubicin for 48 hours, and 10µg/mL doxorubicin for 48 hours. Scatter gates were included to gate out background signals from the flow cytometry machine and gates were generated to differentiate between cells undergoing early apoptosis and those that were dead. This experiment had been repeated multiple times with additional cell lines, yet these results are representative of the other data collected.

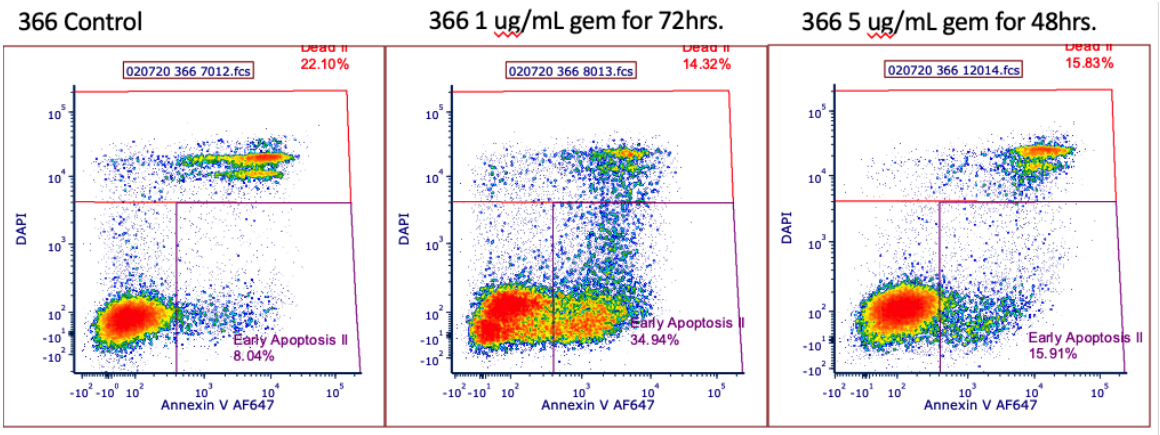


**Figure 6B.** Annexin V Flow Cytometry Analysis of 366 at varying concentrations and time points of gemcitabine treatment. Three groups were included in this experiment: control (no drug), 1µg/mL gemcitabine for 72 hours, and 5µg/mL gemcitabine for 48 hours. Scatter gates were included to gate out background signals from the flow cytometry machine and then gates were generated to differentiate between cells undergoing early apoptosis and those that were dead. This experiment had been repeated multiple times with additional cell lines, yet these results are representative of the other data collected.

Figure 6 illustrates the extent of apoptosis generated in 395 and 366 with varying doses and time points of chemotherapy treatment. Utilizing FCS Express, a scatter gate was added to the forward scatter vs. side scatter cell plots of each cell line to ensure that the background signal from the flow cytometry machine was not included in the analysis. The DAPI control sample was included to isolate the Annexin V positive samples and the dead cells. Placing Annexin V conjugated to the AF647 fluorophore on the x-axis and DAPI on the y-axis, it was possible to differentiate between cells in these samples that were undergoing apoptosis and those that were dead.

Figure 6A shows separate populations in the 395 cell line at 6 different concentrations and time points of treatment. In this Figure, the greatest amount of apoptosis was generated in the 1µg/mL doxorubicin for 48 hours treatment group. Nevertheless, this treatment group also generated a substantial percentage of dead cells. As seen in Figure 6B, the data differs between each cell line. In 366, treatment with a lower concentration of drug for a longer duration of time,

the 1µg/mL gemcitabine for 72 hours treatment group, resulted in a greater amount of apoptosis with less cell death.

*Bumetanide Engulfment Assay*

Following the generation of the data described above, another macrophage engulfment assay was performed. To limit the possibility of errors, only the 366 cell line was utilized in this experiment. Four different treatment groups were examined in this experiment: untreated macrophages and untreated 366 cells, bumetanide treated macrophages and untreated 366 cells, bumetanide treated macrophages and gemcitabine treated 366 cells, and untreated macrophages and gemcitabine treated 366 cells. Due to the results from the previous Annexin V experiments, 1µg/mL gemcitabine for 72 hours was utilized to treat the tumor cells.

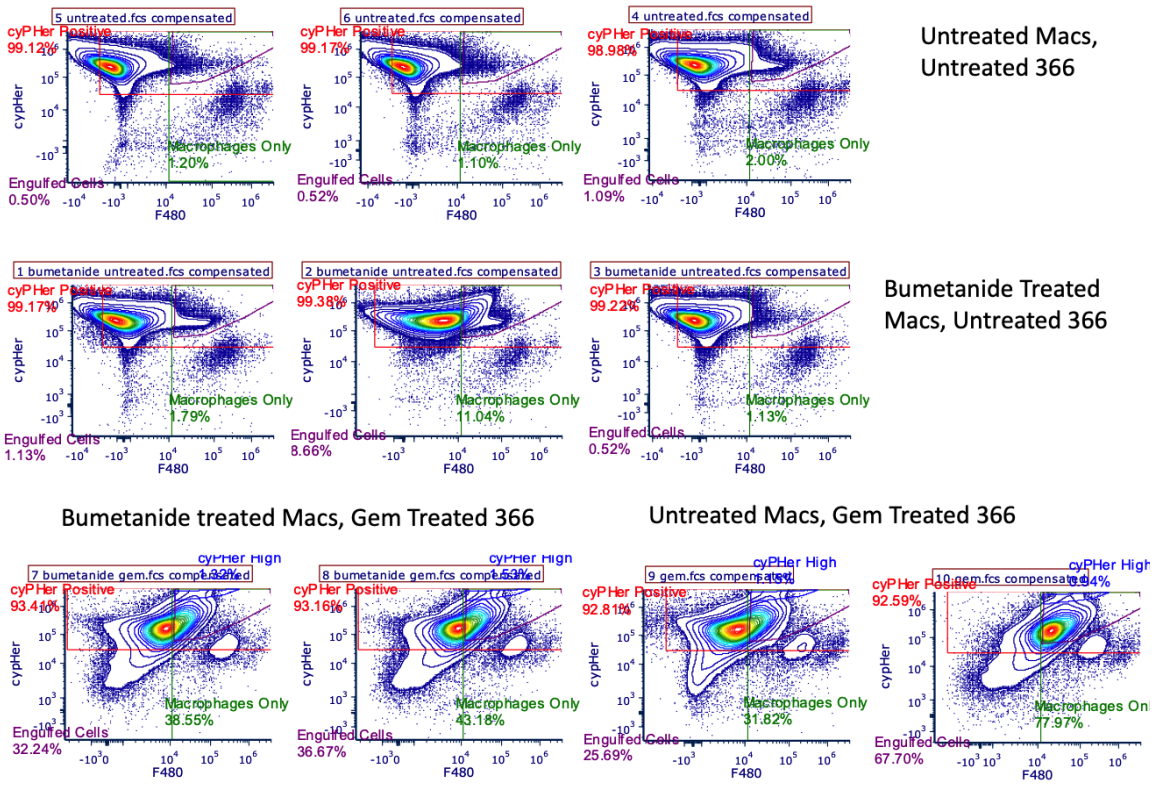


**Figure 7.** *In Vitro* Macrophage Engulfment Assay. A four-arm experiment was performed to examine whether there would be a significant increase in phagocytosis when macrophages were treated with bumetanide and tumor cells were treated with gemcitabine.

As seen in Figure 7, a flow cytometry analysis was performed to differentiate between engulfed cells and macrophages in the samples. Utilizing FCS Express, scatter gates were generated on the forward scatter vs. side scatter cell plots to diminish background signals from the flow cytometry machine. Gating on the DAPI control sample allowed for the analysis of alive cells only. The F480 control enabled a gate to be placed around "macrophages only." Furthermore, the "CypHer positive" gate and "engulfed cells" gate were generated on the tumor cell control sample.

A lack of treatment for the macrophages and the tumor cells resulted in the lowest percentage of engulfed cells in the treatment groups. The bumetanide treated macrophage and untreated tumor cell group had a relatively similar percentage of engulfed cells as well. In

comparison, the remaining treatment groups included 366 tumor cells that were treated with gemcitabine, and these groups had the highest percentage of engulfed tumor cells in this analysis. Whether the macrophages in these groups were treated with bumetanide did not significantly impact the percentage of tumor cell phagocytosis.

*Cell Proliferation in the Presence of Trametinib and Foretinib*

To better understand the potential of Trametinib and Foretinib as therapeutics, CyQuant Cell Proliferation Assays were conducted on four 366 cell lines. The first cell line shown in Figure 8 is a 366 control sample. The 366R sample had been previously made resistant to trametinib treatment *in vitro* through repeated exposure. The remaining cell lines, 366R I8 and 366R I9, were made resistant to trametinib *in vivo*. This assay enables quantification of the resistance that each of these cell lines acquired to trametinib treatment. Furthermore, these results provide insight into the effectiveness of foretinib in rescuing the sensitivity of these cells to treatment. Trametinib was added to these cell lines in a 1:3 series dilution and foretinib was added at .3μM.

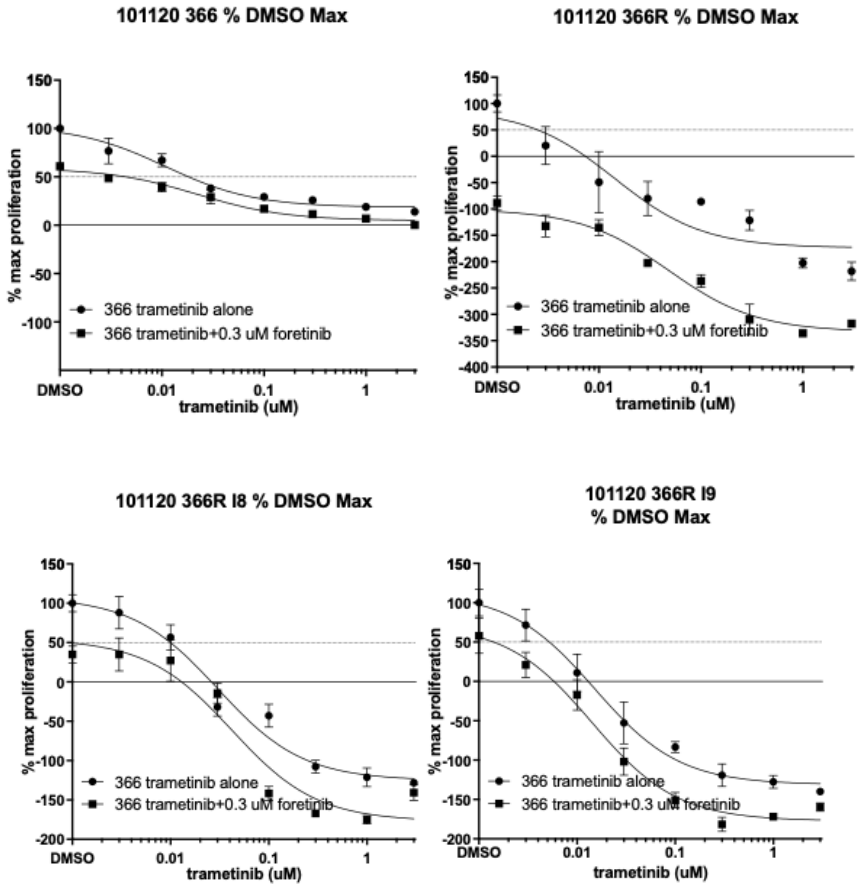


**Figure 8.** Trametinib and Foretinib CyQuant Cell Proliferation Assays. These graphs display the proliferation of four 366 cell lines in the presence of various concentrations of trametinib and foretinib. Proliferation in the presence of trametinib and foretinib was decreased in comparison to trametinib treatment alone.

As seen in Figure 8, the 366 resistant cell lines no longer displayed complete resistance to trametinib treatment; this can be seen in the fact that proliferation decreased in all of the cell lines as the concentration of trametinib increased. Furthermore, the 366R cell line had the greatest decrease in percentage of maximum proliferation in the presence of trametinib. Nevertheless, this percentage is fairly similar to those observed in the 366R I8 and 366R I9 groups. In all four of the cell lines, foretinib treatment garnered a greater decrease in proliferation, showing that treatment of resistant cells with foretinib can rescue the sensitivity of these cells to therapeutic agents. The impact of this treatment, however, appears to be slightly

more effective in inhibiting 366R proliferation than the other resistant cell lines, 366R I8 and 366R I9.

## Discussion

### *Differences Between Cell Lines*

Pancreatic cancer is characterized by heterogeneous tumors, a characteristic that was supported by the findings of this study. Although each cell line was exposed to similar conditions prior to the start of this experiment, maximum proliferation in the presence of gemcitabine varied between cell lines. The results of this experiment provide insight into the manner in which treatment options can differentially impact patients in the clinical setting. As such, the underlying mechanism that contributes to the distinct responses of these cell lines to gemcitabine necessitates further research.

The results from this experiment resemble data that has been published by the Bauer lab. In this publication, five of the cell lines that were included in the gemcitabine sensitivity experiments were analyzed *in vivo*. This analysis focused on the time it took for tumors to progress in a mouse model after conducting splenic injection of PDAC cell lines. This study supports an idea that is central to PDAC research: the heterogeneity of tumors.[126] While each cell line had been grown in identical conditions prior to this experiment, some cell lines, such as 395 and 608, had progressed within 50 days of injection, while 449 had not progressed within 400 days. The varied responses of these cell lines contribute to the understanding that no two patients with pancreatic cancer will have identical tumors. Delineating the differences between these cell lines will enable the development of patient-specific therapeutic targets, thus increasing the potential benefits for patients in the clinical setting.[126]

*Engulfment Assays*

An increase in understanding of the immune system has caused immunotherapies to be the fourth-line treatment option for various forms of cancer. Pancreatic cancer, however, provides a unique challenge in the generation of immunotherapy options, largely due to its dense desmoplasia and the pro-tumoral role that TAMs play in disease progression. Due to the lack of immunotherapy options in this field, the Bauer lab focused on a treatment that has shown a potential to be utilized after surgical resection of PDAC, α-CD47. Further analyses of literature regarding CD47 led to the belief that IgG would be a suitable target to augment the therapeutic benefit produced by α-CD47 treatment alone. This hypothesis relied upon the understanding that macrophages are activated to conduct phagocytosis through an interaction between IgG and FcγR.[159,160] As such, inhibiting CD47, which typically interferes with a macrophages ability to phagocytose tumor cells, while strengthening IgG, which promotes macrophage engulfment, was deemed a suitable target for an *in vitro* engulfment assay.

As expected, α-CD47 significantly increased the percentage of engulfment in the samples consistent with previous studies.[126] However, the addition of IgG to α-CD47 treatment did not amplify the amount of phagocytosis occurring in the samples. This result was due to a lack of a complete understanding regarding the mechanism of IgG as a therapeutic option. Furthermore, this experiment posed significant challenges in terms of analysis. We also observed different results when comparing the "engulfed" gate and the "CypHer high" gate. The nature of this experiment did not provide a straightforward manner with which to approach this data analysis and therefore, generated complications in the future applicability of this treatment.

Based on these results, the focus of immunotherapy research in the Bauer lab shifted to a target with significant results in previous publications. In 2019, Perry et. al published a study

proposing the role of the SLC12A2 protein in the process of phagocytosis. Treating macrophages with 10μM bumetanide for 1 hour, these researchers discovered an increased uptake of apoptotic Jurkat cells, that were analyzed using CypHer staining.[150]

Based on the results of this experiment, SLC12A2 appeared to be a reasonable target for pancreatic cancer treatment. As such, a macrophage engulfment assay was performed prior to the Annexin V Flow Cytometry Analysis displayed in Figure 6. The treatment of the macrophages with bumetanide in this assay did not generate a significant increase in the percentage of engulfed tumor cells. Upon reevaluation of the experimental procedure, it was determined that the tumor cells should be driven towards apoptosis, as Perry et al.[150] found an increase in phagocytosis while utilizing an apoptotic cell model.

Since the results of the Perry et al. study utilized apoptotic cells, further experimentation in the lab consisted of optimizing apoptosis of pancreatic tumor cells without killing the cells. While the Perry et al. study induced apoptosis by treating Jukart cells with ultraviolet C irradiation, the experiments shown in Figure 6 included treatment of tumor cells with gemcitabine, due to the clinical relevance of this agent. As such, Annexin V Flow Cytometry experiments were conducted in hopes of generating approximately 30%-50% apoptosis in each cell line, using different dosages and time points of gemcitabine treatment.[150] However, the first Annexin V experiments displayed a large degree of variability in the effects of gemcitabine treatment, sometimes leading to apoptosis within the desired range yet failing to accomplish this in the repeat experiment. The lack of consistency in these early experiments correlates with a known characteristic of cancer cells, their ability to evade apoptosis.[161] Apoptosis is often utilized as a means to control unregulated cell growth, yet as a cancer cell continues to proliferate, the level of control exerted by the apoptotic mechanism diminishes over time.

Without this element of regulation, cancer cells survive longer and thus accumulate an increased number of mutations, which further promote its growth.[161]

Furthermore, within the Flow Cytometry Analyses in Figure 7, there appeared to be an upward shift in the density curves. Due to this upward shift, almost all of the graphs displayed large populations of "CypHer positive" cells and made it difficult to differentiate between "CypHer positive" populations and those that would be considered "CypHer high", which are more indicative of true macrophage engulfment as this pH sensitive dye emits more fluorescence when in an acidic pH, such as the environment within the phagosome.[162] The lack of separation between these cell populations may have been due to an inaccurate dilution of the antibody. In flow cytometry analyses, a lack of antibody will not generate as bright of a stain in the population of interest, therefore making differentiation of the positive and negative cell populations more difficult. An overabundance of antibody, however, produces non-specific binding thus extending the apparent presence of the negative cell populations.[163]

As described by the Perry et al. publication, which provided the background knowledge that prompted this experiment, the mechanism through which apoptotic clearance by macrophages occurs is still being elucidated.[150] Although the data in Figures 6 and 7 display the potential for a therapeutic benefit in the future, they also underscore the need for a more complete understanding of the mechanism through which pancreatic tumor cells undergo phagocytosis by macrophages. In doing so, more specific targets can be identified in the future that will not only garner significant results *in vitro*, but *in vivo* as well.

*Trametinib and Foretinib*

While immunotherapy options have garnered significant attention in recent years, genomic targets have also proved beneficial in the treatment of a vast number of cancers. Pancreatic cancer, however, remains an exception to this trend. As described in the introduction, the inability to successfully target KRAS has posed a challenge in the development of molecularly-based therapeutic options. Nevertheless, the idea of targeting proteins downstream of KRAS has gained traction in PDAC research recently. One inhibitor of interest is trametinib.

Previously in the Bauer lab, cell lines were made resistant to trametinib treatment *in vitro* and *in vivo* through repeated exposure to the drug. These experiments were conducted to analyze the mechanisms through which patients would develop resistance to these agents if they were utilized in the clinical setting. When *in vitro* experiments were later conducted with the cell lines that had been generated *in vivo (*366R I8 and 366R I9), the resistant samples and the control sample displayed similar responses to the presence of trametinib, thus indicating that the resistant samples had lost some of their acquired resistance when taken from the *in vivo* setting and analyzed *in vitro*. Following re-exposure to trametinib *in vitro,* however, 366R I8 and 366R I9 cell lines had recovered their resistance quickly.

These experiments provided beneficial insights into the clinical translatability of these treatment options. The loss of resistance displayed by 366R I8 and 366R I9 when they were cultured *in vitro* underscores the contribution of the tumor microenvironment in acquiring drug resistance. When considering the future effectiveness of pancreatic cancer treatments in patients, *in vitro* models alone do not sufficiently replicate the tumor microenvironment and thus should be analyzed with an understanding of this limitation. Furthermore, patients often acquire resistance to treatment after prolonged exposure in the clinical setting. The results of this

experiment exhibit the potential benefits of foretinib treatment for patients following recurrence of disease and resistance to trametinib.

*Limitations*

As stated in the introduction, limitations are inherent to all models of human pancreatic cancer. Nevertheless, the results of this thesis were primarily generated *in vitro.* While it is essential to perform experiments *in vitro* to display a strong potential for therapeutic benefit prior to performing *in vivo* studies utilizing animal models, these systems neglect to account for the role of the surrounding tumor microenvironment in pancreatic cancer. The data generated from the gemcitabine sensitivity curves, for example, does not take into consideration the role that CAFs have in imparting chemotherapeutic resistance on tumor cells. The cell lines that were utilized in this analysis display particular morphological differences when cultured *in vitro,* such as varying concentrations of fibroblasts, yet these stromal cells were removed from the samples prior to performing the proliferation assay. Although the results of this experiment lend to further consideration and experimentation, these *in vitro* analyses are not entirely representative of the interactions that would occur within a human PDAC tumor.

Furthermore, a large portion of the data presented in this thesis relied on flow cytometry analyses. This method of analyzing cell proteins, while beneficial in distinguishing between cell populations, coincides with an opportunity for errors in analysis. As described in Figure 5, there was a degree of subjectivity in producing the gates for the cell populations within these cell plots. The manner in which gates were constructed in FCS Express can lead to vastly different interpretations of the data. For example, the data in Figure 5A was not significant when it described the percentage of engulfment utilizing data from the “engulfed” gate. Nevertheless, the

data in Figures 5A and 5B were significant when the “CypHer high” gate was utilized to distinguish between tumor cells that had been engulfed and those that had not undergone engulfment. As such, these results generated concerns about the proper way to differentiate between cell populations in these analyses. Furthermore, an improper spread in the cell populations, as seen in Figure 7, can also make it difficult to identify where exactly one cell population separates from another. While improper dilutions of antibodies may have contributed to this spread, subjectivity remains inherent to flow cytometry analyses, thus posing a limitation in our studies.

*Future Directions*

As briefly stated in the discussion of the gemcitabine sensitivity curves, a greater understanding of the differences between the cell lines utilized in the Bauer lab are of utmost importance. This is due to the data in Figure 4 as well as previously published data. An understanding of the varied responses of each cell line, both in regards to chemotherapeutic efficacy as well as time to disease progression, is essential in generating patient-specific treatment approaches in the future.

For this reason, an RNA Sequencing experiment has been conducted on six cell lines and their respective liver metastases. This data derived from this experiment will enable an analysis of the genes that are upregulated and those that are downregulated in each respective cell line. Furthermore, this information can be used to compare groups of cell lines to each other. For example, cell lines such as 449 and 608, which responded more effectively to gemcitabine treatment at lower doses can be compared to 395 and 366, which displayed an increased resistance to gemcitabine treatment. Utilizing this information, the Bauer lab intends to target the

selected genes *in vivo* in an effort to elucidate the mechanism through which the cell lines utilized in the lab derive their distinct characteristics, such as gemcitabine sensitivity or resistance. This data will not only provide future directions with which to generate *in vitro* and *in vivo* experiments within the laboratory, but provides a potential applicability within the clinical setting; this study will enable further the discussion surrounding the heterogeneity of pancreatic cancer tumors, thus providing insight into the treatment approach that would be most beneficial to individual patients based on the upregulation or downregulation of particular genes in their tumor.

Although the engulfment assays that utilized bumetanide treated macrophages did not produce the anticipated results, there are still future plans for immunotherapy analyses within the lab. The results generated in Figures 5, 6, and 7 have each contributed to a greater understanding of immunotherapy options for pancreatic cancer. Of particular interest for future experiments is a treatment option that can act in a synergistic manner with α-CD47 treatment. Prior to the bumetanide macrophage engulfment assays, it was thought that bumetanide would be one such therapeutic option. While bumetanide may no longer be a suitable target for this experiment, this overarching goal remains the same. As such, future research will focus on a mechanism through which more consistent apoptosis can be generated in the patient-derived cell lines, utilizing a treatment that is still clinically relevant to pancreatic cancer treatment. Furthermore, to accomplish this goal, the pathway through which apoptotic pancreatic cancer cells are recognized and therefore engulfed by macrophage also necessitates further experimentation.

While a significant amount of research must continue to be conducted in order to determine the next mechanistically significant target to be utilized in combination with α-CD47 treatment, these beginning experimental results provided insight into the complexities of

immunotherapy research and the potential roadblocks that may arise in these analyses. In order to successfully generate immunotherapy approaches that have the potential to translate to the clinical setting, an *in vivo* model must be employed in the future after generating significant *in vitro* results. This is due to the unique tumor microenvironment characteristic of pancreatic cancer. Utilizing an orthotopic xenograft mouse model will enable a proper analysis regarding the efficacy of these future therapeutic approaches in overcoming the immunosuppressive tumor microenvironment. By continuing to make use of patient-derived cell lines and orthotopic xenograft mouse models, future research will remain focused on creating treatment plans that hold promise in the clinical setting.

In regards to the genomic inhibitor analyses, the mechanism through which tumor cells acquire resistance to trametinib and the manner in which foretinib counteracts this has not yet been elucidated. As such, western blots are currently being conducted in the lab to provide insight into the proteins that the resistant cell lines upregulate in response to trametinib treatment, yet no longer active in the presence of foretinib. In performing these experiments, the alternate pathways that cells initiate in the presence of either of these drugs will be more comprehensively understood, thus enabling the development of more clinically relevant therapeutic options in the future.

*Ethical and Societal Considerations*

The manner in which pancreatic cancer manifests differs from one patient to another. As such, pancreatic cancer lends itself to the rapidly growing field of precision medicine. The Institute for Precision Medicine at UPMC has defined precision medicine as, “a medical approach that proposes to prevent and treat disease based upon a person’s unique genetic makeup

and their lifestyle habits.[164]" Precision medicine provides an avenue through which the patient-specific factors that contribute to PDAC development can be targeted. Nevertheless, the development of these technologies has generated questions regarding their equitable access due to their costs. To determine which patients are eligible for precision medications, genomic profiling must be completed. In a study with pediatric oncology patients, genomic profiling costs totaled nearly $6,000.[165] While data regarding the costs associated with precision medicine use in cancer treatment are limited, recent studies have shown a higher cost of treatment in patients given precision medicine compared to those given the standard of care treatment.[166] These experiments display limitations, however, as they do not control for the extended survival of patients who received precision medications.[166] For these reasons, future experiments should be conducted to clearly expand upon the cost-effectiveness of these treatments while ensuring that disparities in healthcare are not exacerbated by these costs.

As described in the Introduction of this thesis, African Americans are disproportionately diagnosed with pancreatic cancer in comparison to Caucasians.[167] In a study that sought to distinguish differences in care between these individuals, it was discovered that African Americans were less likely to undergo surgical resection, despite presenting with the same stage of disease. The differences in the rates of surgical resection based on race have been attributed to insufficient communications between the physician and patient, as well as a lack of trust in the relationship.[167,168] Furthermore, pancreatic cancer presents a challenge in diagnosis due to vague symptom presentation, which is exacerbated by a lack of access to health care.[167] As such, interventions that overcome the barriers in diagnosis have the ability to reduce the disparities observed in this disease. Nevertheless, the existing inequalities in pancreatic cancer burden

according to race must be addressed through targeted interventions and research in this field must be conducted with an adequate understanding of this issue.

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