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Neutrophils in Pancreatic Cancer Progression

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NEUTROPHILS IN PANCREATIC CANCER PROGRESSION

by

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ABSTRACT

Pancreatic cancer (PC) remains a challenge to modern-day cancer therapeutics, with a dismal five-year survival rate of 10%. Due to the pancreas's location and desmoplasia surrounding it, patients receive late diagnoses and fail to respond to chemotherapy regimens. Tumor-promoting inflammation, one of the emerging hallmarks of cancer, contributes to tumor cells' survival and proliferation. This inflammation is often the result of infiltrating leukocytes and pro-inflammatory cytokines released into the tumor microenvironment (TME).

Neutrophils, one of the most prominent immune cells in our body, play an essential role in sustaining this smoldering inflammation observed in the TME. Previously, our group has shown that these neutrophils are complicit in breast cancer progression and even metastasis. With a similar rationale in mind, this study focuses on how neutrophils invading the TME, also known as tumor-associated neutrophils (TAN's), correlate with disease progression in pancreatic cancer. Our data demonstrated that TAN infiltration is associated with disease progression.

Furthermore, to understand this TAN infiltration, we theorized that the TME plays a significant role in TAN recruitment and TAN proliferation. Our previous work elucidated TAN recruitment by showing increased expression of chemokines in the TME. We also

examined TAN and tumor cell interaction *in vitro* and observed increased tumor cell survival and decreased neutrophil survival. This is theoretically explained by the increased propensity of neutrophils to undergo NETosis and form neutrophil extracellular traps, which have also been shown to correlate with disease progression. Our data suggested neutrophil differentiation in the TME leads to the upregulation of multiple chemokines and, in theory, explains the high TAN infiltration observed in the TME. Together, these data suggest the critical role of TAN and tumor cell interaction in the TME.

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List of Abbreviations

1. **ATRA:** All-trans retinoic acid
2. **CTC:** Circulating Tumor Cells
3. **DMSO:** Dimethyl sulfoxide
4. **ECM:** Extracellular matrix
5. **EMT:** Epithelial to mesenchymal transition
6. **EPCAM:** Epithelial cell adhesion molecule
7. **FBS:** Fetal bovine serum
8. **G-CSF:** Granulocyte colony-stimulating factor
9. **GM-CSF:** Granulocyte-macrophage colony-stimulating factor
10. **GMP:** Granulocyte-monocyte progenitors
11. **HSC**: Hematopoietic stem cell
12. **ICAM:** Intracellular adhesion molecule
13. **IFN-β:** Interferon-beta
14. **IL17:** Interleukin 17
15. **IL23:** Interleukin 23
16. **iNOS: I**nducible nitric oxide synthase
17. **MDSC:** Myeloid-derived suppressor cell
18. **MET:** Mesenchymal to epithelial transition
19. **MMP:** Matrix metalloproteinase
20. **MPO:** Myeloperoxidase
21. **NET:** Neutrophil extracellular trap
22. **NK Cells:** Natural killer cells

23. **NLR:** Neutrophil to lymphocyte ratio
24. **PAMP:** Pathogen associated molecular pattern
25. **PDAC:** Pancreatic ductal Adenocarcinoma
26. **PGE:** Prostaglandin E2
27. **PRR:** Pathogen recognition receptor
28. **RNS:** Reactive nitrogen species
29. **ROS:** Reactive oxidative species
30. **SNAP:** Soluble NSF attachment protein
31. **TAN:** Tumor-associated neutrophil
32. **TGF-β:** Transforming growth factor-beta
33. **TIMP:** Tissue inhibitor of metalloproteinase
34. **TLR:** Toll-like receptor
35. **TME:** Tumor-microenvironment
36. **TNF-α:** Tumor necrosis factor-alpha
37. **VEGFR:** Vascular endothelial growth factor receptor

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INTRODUCTION

The War on Cancer

In 1971, U.S. President Richard Nixon announced the 'War on Cancer' by signing the National Cancer Act. The purpose was to find a cure for cancer. Although the holy grail of cancer therapeutics remains elusive to modern science, we have made outstanding progress in the last 50 years of research. The mortality rate for cancer has significantly declined, with noticeable improvements in lung, prostate, and breast cancer[71]. Although rising cancer awareness worldwide plays a part in this success, the advent of modern screening techniques for cancer detection and new cancer therapeutics has made considerable inroads in cancer detection and treatment. However, our war on cancer is far from over. Cancer remains the second most leading cause of death in the United States, behind cardiovascular diseases (Center for Disease and Control Prevention, https://www.cdc.gov/cancer/dcpc/data/index.htm).

Cancer is described as the uncontrolled growth of host cells, which can invade other parts of our body. Weinberg and Hanahan eloquently detailed specific characteristics a tumor cell develop during its life cycle in their landmark article in 2000[1]. These characteristics, which they call hallmarks, provide a solid foundation to create new targets for modern cancer therapeutics. Briefly, the six hallmarks of cancer were classified as 1) sustained proliferative signaling, 2) evading growth suppressors, 3) ability to metastasize, 4) resisting cell death, 5) enabling replicative immortality, and 6) induced angiogenesis[1]. Since then, Weinberg and Hanahan have broadened their horizon and added two additional hallmarks, 'dysregulating cellular metabolism' and 'avoiding immune destruction' [23].

One of the enabling characteristics of these hallmarks is tumor-promoting inflammation[23]. Cancer is often considered the 'wound which never heals' and behaves as a constant source of inflammation. This results in the recruitment of a plethora of different immune cells into the tumor region, which further instigates inflammation, creating a positive feedback loop. Many articles have shed light on the link between inflammation and tumor development, particularly in the context of pancreatic cancer. Inflammation in pancreatic cancer derives from the crosstalk between immune cells and the tumor, which is carried out through various pro-inflammatory cytokines[61].

Pancreatic Cancer

Although the future looks promising with most types of cancer, the threat of pancreatic cancer still looms on the horizon. Despite being a relatively rare type of cancer, accounting for only 3% of the total cases in the United States, it causes about 8% of the total estimated deaths. In fact, by 2030, the American Cancer Society has suggested that pancreatic cancer will be the second most leading cause of cancer-related deaths.

As the name suggests, pancreatic cancer originates from the pancreas, a vital organ involved in digestive and endocrine functions. Most of these cancers originate from exocrine cells (85%) and are thus called exocrine cancers. These cancers are challenging to diagnose early, and most patients end up being diagnosed when cancer has metastasized to various organs, such as the liver. Unfortunately, at this point, surgical intervention is no longer a viable option. The patient must undergo chemotherapy-based regimens, which are not very effective in the context of pancreatic cancer. The delayed diagnosis and absence of efficacious treatment options have held the 5-year overall survival rate of pancreatic cancer to less than 10%.

As is the case for most cancers, the advent of tumorigenesis in pancreatic cancer stems from oncogenic mutations in the exocrine cells. Almost 90% of these tumors have the KRAS oncogenic mutation, which is involved in downstream signaling pathways enhancing survival[3]. Like most cancers, the p53 tumor suppressor gene is commonly found to be mutated in pancreatic cancer[4]. P53 expression is frequently associated with cell cycle arrest by blocking the G1/S checkpoint upon sensing DNA damage and subsequently initiating apoptosis.

Chemoresistance in Pancreatic Cancer

The ability of a tumor to resist chemotherapeutic drugs is called chemoresistance and is commonly observed in PDAC[62]. It is broadly defined under two categories; intrinsic or acquired resistance[5]. Intrinsic resistance in pancreatic cancer is seen as the presence of a characteristically dense stroma derived from pancreatic stellate cells that secrete collagen, one of the building blocks of the extracellular matrix (ECM)[3]. This stroma acts as a physical barrier preventing chemotherapeutic drug entry into the tumor, diminishing the effectiveness of chemotherapy regimens. Acquired resistance or resistance that is conferred after prolonged treatment with chemotherapeutic drugs is attained by multiple pathways. A common pathway involves the efflux of hydrophobic drugs through the cell membrane by utilizing ATP binding cassette transporters[6]. Another cancer cell trait showing an interesting role in chemoresistance is epithelial to mesenchymal transition (EMT), a crucial step towards metastasis in cancer[7]. A study published in 2015 showed that inhibiting EMT through deletion of vital transcription factors Snail and Twist resulted in increased sensitivity for gemcitabine treatment in their *Pdx1-cre; LSL-Kras^{G12D}; P53$^{R172H/+}$* and *Ptf1a (P48)-cre; LSL-Kras^{G12D}; Tgfbr2$^{L/L}$* mouse models[8]. A recent article

highlighted the elusive link between chemoresistance and neutrophils, which suggested that the expression of CD16 on the neutrophil surface was linked to a decreased efficacy of chemotherapeutic drug capecitabine in colorectal cancer patients[63]. Low expression of CD16 is commonly seen on immature neutrophils and potentially plays an immunosuppressive role by suppressing natural killer (NK) cells and cytotoxic T- cells.

Neutrophils

Neutrophils are white blood cells of the granulocytic lineage. They are derived from hematopoietic stem cells and mature in the bone marrow. About 10^{11} neutrophils are generated every day, which makes up for their short life spans of less than 24 hours[91]. Once fully matured, they circulate in the bloodstream and subsequently home in on sources of inflammation in the tissue. They are highly efficient at extravasation and are often dubbed as first responders to a pathogenic invasion. Neutrophils are largely pro-inflammatory in nature and can rapidly recruit other immune cells like dendritic cells and T-cells to the site of infection through the release of cytokines like CCL3[9] and CXCL12[10,] respectively. These cells are also very potent phagocytes and will usually assist in the clearance of pathogenic invasions themselves. Neutrophils are intertwined with cancer progression and are known to be involved in tumorigenesis, angiogenesis, immunosuppression, and even metastasis in cancer[2].

Neutrophil Structure

Neutrophils are relatively small cells with a diameter of around 15 micrometers. Mature neutrophils consist of a well-defined multi-lobed (3-5) nucleus, inter-connected by chromatin through histone proteins**.** As is typical of granulocytic cells, their cytoplasm

contains a multitude of different granules, each able to release potent anti-microbial enzymes upon activation.

Neutrophil Development and Life Span

Neutrophils originate from hematopoietic stem cells in the bone marrow. After subsequent downstream signaling, these hematopoietic stem cells (HSC's) eventually develop into granulocyte-monocyte progenitors (GMPs). Granulocyte colony-stimulating factor (G-CSF), a type of growth factor/glycoprotein produced in the bone marrow, is essential in the further differentiation of GMPs into myeloblasts, which eventually become mature neutrophils[11].

In order to preserve self-tolerance and homeostasis, the release of fully matured neutrophils is highly regulated. Many chemotactic factors such as CXCL1, CXCL2, complement factors like C3A and C5A, and even G-CSF can regulate the release of these neutrophils through the bone marrow and into the bloodstream. These neutrophils follow this chemokine trail into the inflammatory source, where they extravasate into the tissue. Here, they recognize pattern recognition receptors (PRR's) on the pathogen, which activate them to subsequently clear the pathogen by phagocytosis and the release of other pro-inflammatory cytokines. In most cases, neutrophils, upon activation, shortly undergo apoptosis and are cleared by the resident tissue macrophages. However, in the context of cancer, we have observed that the neutrophil life span in the tumor is abnormally prolonged, increasing the possibilities of crosstalk between neutrophils and cancer.

Neutrophil Methods of Activation

Depending on a multitude of different factors, the neutrophil can undergo three different methods of activation:

1. **Phagocytosis**

Phagocytosis is the process by which cells engulf other cells or materials through various receptor-mediated signaling processes. Neutrophils are often referred to as 'professional phagocytes' and readily internalize and subsequently destroy pathogens through anti-microbial mechanisms. They have specialized receptors such as toll-like receptors (TLRs), which recognize conserved molecular patterns known as pathogen-associated molecular patterns (PAMPs). They also recognize opsonized particles through specific Fc receptors. Once the pathogen is recognized, it is subsequently internalized into the neutrophil, with a specialized vacuole known as a phagosome forming around it. This phagosome undergoes extensive remodeling events and eventually matures into a vesicle with a more anti-microbial composition, killing the pathogen trapped inside[44].

In the context of cancer, neutrophils readily phagocytose opsonized tumor cells through a process known as antibody-dependent cellular phagocytosis (ADCC)[64]. However, tumor cells are usually larger than neutrophils, posing a challenge to the complete phagocytosis carried out by neutrophils[45].

2. **Degranulation**

Neutrophils can release anti-microbial enzymes from their granules into the TME upon stimulation. As is expected from a cell of the granulocytic lineage, neutrophils host

a vast arsenal of different granules, most of which are anti-microbial. They are four types of granules expressed by the neutrophil. Azurophilic granules consist of bactericidal enzymes like myeloperoxidase, hydrolases, cathepsin-G, and defensins[92]. Secondary granules consist mainly of lactoferrin, an integral part of mucose and neutrophil gelatinase-associated lipocalin[92]. Tertiary granules contain matrix metalloproteinase 9, which is often linked with ECM remodeling[92]. Finally, secretory granules contain various pathogen recognition receptors as well as complement receptors[92]. The degranulation process is very tightly regulated through intracellular molecules B-arrestins and soluble NSF attachment protein (SNAP)[46].

Several enzymes released by neutrophils are linked to cancer progression. Matrix metalloproteinase 9 (MMP9) primarily cleaves the ECM around the TME, which paves the way for new blood vessel formation, called angiogenesis, which is a critical step towards tumor progression. Arginase, also released by neutrophils, is widely known to inhibit T-cell function and replication, preventing effective immune responses towards the tumor.

3. **NETosis**

Neutrophil extracellular traps (NETs) result from a process termed as NETosis, which culminates in the release of web-like structures composed of DNA fibers and granular proteins and usually results in neutrophil death. NETs have had a controversial history since their discovery in 2004[47]. Initially thought to only bind to pathogens, impairing their movement and eventually degrading them, the past 15 years have shed some light on their involvement in various inflammatory diseases such as cancer[48].

The process of NETosis primarily involves the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which provokes nuclear membrane disintegration and chromatin decondensation through neutrophil elastase, myeloperoxidase, and various histones, respectively[49,50]. Citrullinated histone 3 is commonly used as a NET bio-marker. Neutrophil elastase and myeloperoxidase work synergistically together by degrading various histones, causing chromatin decondensation[49]. This leads to the mixing of neutrophil granular proteins with chromatin, and eventually, this amalgamation swells and ruptures the plasma membrane, resulting in the violent expulsion of NETs. Neutrophils can partake in a milder, rapid form of NETosis, known as vital NETosis, which remarkably retains some neutrophil functions such as chemotaxis and phagocytosis and is independent of NADPH oxidase formation[51,52].

An abundance of various neutrophil activators in the TME, such as IL8 and CXCR1/ CXCR2 ligands[54,93], often creates a hospitable environment for NET formation. Much like their precursor neutrophils, NETs have been implicated in both pro-tumor as well as anti-tumorigenic capabilities[48]. They were shown to potentially orchestrate circulating tumor cell extravasation by binding to them[53]. This partnership also serves as a mechanical barrier between tumor cells and other cytotoxic immune cells like natural killer cells and cytotoxic T-cells, preventing physical contact between them and thereby preventing tumor cell degradation[54].

Neutrophil to lymphocyte ratio (NLR) in cancer

Inflammation, one of the seven hallmarks of cancer[23], is a key factor in instigating tumorigenesis in cancer. This systemic inflammation results in the invasion of many types

of immune cells into the tumor. Subsequently, these immune cells are activated and release their cytokines, which causes even more inflammation in the tumor micro-environment, forming a positive feedback loop.

The NLR ratio is often described as an indicator for systemic inflammation and is commonly used as a prognostic biomarker for tumor progression. The relative ease of obtaining the NLR ratio from patients makes it an attractive biomarker. A high NLR ratio often portrays large amounts of circulating neutrophils in the bloodstream, characteristic of systemic inflammation, and can often result in elevated tumor-associated neutrophil levels. Tumor-associated neutrophils (TANs) are often complicit in angiogenesis and tumorigenesis through the release of MMP's and ROS radicals, respectively. They can also inhibit lymphocyte maturation by releasing arginase, thus creating an immunosuppressive environment. These reasons could potentially explain why there is a relationship between high NLR ratios and the poor prognosis of patients.

Recent studies have shown that a higher NLR ratio leads to worse overall survival in breast cancer patients[12] and PDAC patients after surgical resection[13]. Xiang et al. evaluated the efficacy of the NLR ratio in multiple PDAC patients who had undergone surgical resection and suggested that this ratio can be used as a possible clinical biomarker for PDAC. Similarly, Iwai et al. evaluated the NLR ratio in patients with unresectable pancreatic cancer and derived similar conclusions[65].

Neutrophil Recruitment in Cancer

As previously explained, HSC's possess the ability to differentiate into neutrophils. Once these neutrophils are fully matured in the bone marrow, they are subsequently

released into the bloodstream and are destined to circulate our bodies until they die. However, through the complex interactions between selectins, integrins, and chemokines, neutrophils are recruited to sites of inflammation in the tissue.

The smoldering inflammation around the tumor microenvironment is often primarily responsible for recruiting neutrophils in the tumor area. A myriad of different chemokines is involved in neutrophil recruitment observed in cancer. CXCL-8, commonly known as the neutrophil recruitment factor, plays a well-established role in the chemotaxis of neutrophils. Our lab has previously linked interleukin (IL)-8 secreted by breast tumors with neutrophil recruitment. CXCL-8 binds to CXCR1, and CXCR2 receptors commonly expressed on neutrophils, thus facilitating their movement into the tumor. IL-8 has also been shown to induce angiogenesis, which is a critical step towards tumorigenesis[14]. Himmel et al. showed that CXCL8 is also produced by regulatory T cells, which could be another potential source for neutrophil recruitment[15].

CXCL1 and CXCL2, highly expressed by tumor cells, are also potent chemokines involved in neutrophil recruitment through the CXCL1-CXCR1 axis **(Fig 1.1)**. Previous studies have linked G-CSF with this axis and postulate that the combined effect stimulates chemotaxis in neutrophils[16]. It is also interesting to note that tumor-associated neutrophils also release CXCL1 and CXCL2 and can potentially attract circulating neutrophils into the tumor themselves, thus creating a positive feedback loop.

Neutrophil Polarization

TANs are highly plastic and are known to polarize into different phenotypes depending upon what signals they receive in the TME. They are polarized into either a pro-inflammatory N1 phenotype or an anti-inflammatory N2 phenotype. This

nomenclature was derived from the M1/M2 classification used for macrophages. The N1/N2 dichotomy is defined by their functional phenotypes, as no specific cell markers have been discovered to date. This polarization depends on cytokine signals received in the TME. Mishalian et al. suggest that the tumor stage (early or established) plays a role in establishing the neutrophil phenotype[55].

1. **N1 Phenotype**

Similar to neutrophils under normal homeostatic conditions, N1 neutrophil subpopulations are short-lived, contain hyper-segmented nuclei, and are highly pro-inflammatory. This phenotype is primarily induced by the expression of interferon-beta (IFN)-β in the TME[56]. They are pro-inflammatory in nature[11] and are also known to display direct tumor cell cytotoxicity by releasing potent anti-microbial substances like peroxidases and nitric oxide or through ADCC[25]. Because of these reasons, N1 neutrophils are widely regarded as anti-tumorigenic in nature.

2. **N2 Phenotype**

In contrast, the N2 neutrophil phenotype is comparatively long-lived, has circular nuclei, and is primarily immuno-suppressive. Transforming growth factor-beta (TGF-β) is widely known to induce naïve TAN's into this phenotype. Coincidentally, TGF-β is highly expressed in many tumor microenvironments, skewing TAN's distribution towards the N2 phenotype. The N2 neutrophil subpopulation is notoriously known to stimulate tumor progression and growth. Its immunosuppressive abilities and increased arginase expression prevent T-cell recruitment and maturation, allowing tumors to grow unchecked[66,67].

ROS and RNS radicals produced by these neutrophils have been shown to cause genetic instability, promoting tumorigenesis in some cancer models[68]. They also release MMP8 and MMP9, which cleave the ECM and activate VEGF, paving the way for new blood vessels to form, which are crucial for developing tumors. These neutrophils are also complicit in metastasis by releasing NE, which stimulates EMT in tumor cells.

The N1/ N2 classification model, although convenient, remains a controversial classification. This is largely because of the absence of specific surface receptors and difficulty to observe these neutrophil subtypes in human[94]. Moreover, secretory substances released by these neutrophils, such as ROS, possess a dual role in cancer. They can play an anti-tumorigenic role or a pro-tumorigenic role depending on their concentrations[95].

Neutrophil Survival in the TME

Neutrophils are universally considered to have a half-life of around 5.5 hours in the bloodstream[69]. However, in the context of cancer, various reports advocate that they persist in the TME for extended periods of time[70]. It has been suggested that tumor-derived cytokines may be responsible for the attenuation of neutrophil apoptosis[57,58,59]. TANs and tumors may work in tandem to increase each other's survival, as it was shown that human and neck squamous adenocarcinomas increased neutrophil survival *in vitro*. In contrast, these neutrophils expressed MMP-9, a well-established pro-angiogenic factor[60].

Tumor-Associated Neutrophil Induced Immunosuppression

TAN's are widely known to have immunosuppressive abilities. These qualities account for the tumor's unperturbed growth in the body. Ironically, neutrophils hinder other cells in the innate immune system, such as antigen-presenting cells like macrophages and dendritic cells. It was also reported that the crosstalk between myeloid-derived suppressor cells (MDSCs) and macrophages was able to stimulate M2 polarization[17]. TANs also debilitate NK cell function, which, together with the activity of cytotoxic T-cells, constitute effective tumor cell destruction[18]. Despite sharing the same myeloid precursors, MDSCs are always considered as pro-tumorigenic while neutrophils display both pro-tumorigenic and anti-tumorigenic abilities[96]. MDSCs are also usually less dense than neutrophils[96]. Youn et al. showed that tumor-bearing murine MDSC's highly expressed CD115 and CD244 relative to neutrophils and may be used as potential surface receptors for the same[97].

Although neutrophils have been shown to impair other innate immune cells, the biggest blow of their immunosuppressive abilities is felt by T-cells, which play a critical role in the adaptive immune system. Arginase-1, which is upregulated in N2 neutrophils, has been shown to impair T-cell function. Degrading arginine also leads to cell cycle arrest in T-cells, thus preventing replication.

Neutrophils are capable of recruiting regulatory T-cells known for their immunosuppressive qualities and are also commonly associated with the downregulation of other effector T-cells. Previous studies have shown that CD40, a receptor expressed on MDSCs, interacts with regulatory T-cells and promotes their accumulation[19]. Another

study showed that the activation of MDSCs through PGE2 reduced the proliferation of $CD4^{+}CD25^{-}$ T cells.

Activation of neutrophils through their specific surface Fc receptors often results in ROS release through NADPH oxidase complex formation. ROS has been found to be highly upregulated in MDSCs localized in murine tumors, often resulting in the suppression of T-cell response[20]. It was also recently shown that co-culture assays of T helper cells and neutrophils conditioned with tumor cell supernatant derived from gastric cancer cells displayed a decrease in T cell proliferation[21], which could likely be a result of ROS expression by the neutrophils.

Neutrophils Complicit in Metastasis

Metastasis occurs when tumor cells originating from the primary tumor spread to different organs of the body. Tumor cells detach from the primary tumor site, enter the bloodstream, and subsequently extravasate into other organs[98]**.** This advanced stage of cancer is the leading cause of death in cancer-related deaths and is especially relevant in the context of pancreatic cancer[99].

Metastatic Cascade

The metastatic cascade is a marathon of complex and challenging events that a tumor cell must overcome to progress towards the metastatic phase, including the movement of the tumor cell from the primary site to the metastatic site, the second being the colonization of the tumor cell at the metastatic site[22]. Although metastasis is a highly inefficient process, eventually, tumor cells will colonize distant organ sites. These cells may develop into a secondary tumor, considered metastasis, resulting in a progressively

worse prognosis. However, various immune cells are complicit in supporting these tumor cells through the cascade. We will be talking about neutrophils in this regard.

Proliferation and Angiogenesis

Angiogenesis, or the formation of new blood vessels, is an essential process for tumor cell proliferation. It feeds the tumor with a steady supply of oxygen and nutrients from the blood. The angiogenic switch governs the extent of angiogenesis in our body and typically maintains a healthy equilibrium between pro-angiogenic and anti-angiogenic factors. However, in the context of tumor development, this switch remains in a perpetual 'on' state, skewing towards the formation of blood vessels. This is, in fact, so critical for tumor development, it is often labeled as one of the hallmarks of cancer[1,23].

There is a very significant relationship between angiogenesis and metastasis. Not only does angiogenesis support tumor growth, accelerating its progression towards the advanced metastatic stage, it also provides a critical pathway for detached tumor cells from the primary site to enter the bloodstream. There have been many studies that have linked metastasis with angiogenesis[24].

TANs aid in the process of angiogenesis mainly in multiple ways. They are a major source of vascular endothelial growth factor (VEGF), commonly known as a pro-angiogenic factor, which binds to resident epithelial cells, maintaining the 'on' state of the angiogenic switch. They also release MMPs, which are notoriously known for remodeling the ECM, giving the new blood vessels much-required space to grow[25].

EMT of Tumor Cells

Perhaps the defining feature of the metastatic cascade is the seemingly transient change of tumor cell phenotype, from epithelial to mesenchymal. Initially, this pathway was used by newly developed cells during embryonic development[100]. However, in the context of metastasis, this transition is hijacked by tumor cells, allowing them to enter a more mobile state, essentially giving them 'wings of freedom'.

This EMT transition is bought upon by various transcription factors such as Snail and Twist[101]. Upon further downstream signaling, it results in a dwindling expression of E-cadherin on the cell membrane[26]. Proteins responsible for strengthening the basement membrane and tight junctions and gap junctions around the tumor cells are downregulated because of these transcription factors, stimulating the transition from epithelial to mesenchymal phenotype[27]. Correspondingly, this transition is characterized by the up-regulation of proteins such as vimentin and N-cadherin[28].

Many studies have linked TANs with the EMT transition observed in tumor cells. It was recently shown that expression of specific mesenchymal markers was increased when gastric cancer cells were treated with neutrophils *in vitro*[29]. They proposed that neutrophils instigate the JAK2/STAT3 pathway in tumor cells through the release of IL-17, resulting in EMT. In another study, a similar co-culture assay was performed using neutrophils with human epithelial ovarian cancer cells, which resulted in a decrease of E-cadherin expression on the cancer cell[30].

Circulating through the Bloodstream

Tumor cells that have successfully escaped from the confines of their primary organ site and enter the bloodstream are called circulating tumor cells (CTCs). This long

and arduous journey through the bloodstream is where most of these CTCs meet their demise. A recent study even highlighted the possibility of using CTCs as a prognostic biomarker in patients with metastatic breast cancer[31]. Current techniques for measuring CTCs involve using epithelial cell adhesion molecule (EPCAM) as a surface marker, which may underrepresent mesenchymal CTCs. In the context of PDAC, neutrophils were found to be clustered around CTCs, potentially behaving as a physical protective barrier against other cytotoxic immune cells like NK and cytotoxic T-cells[32].

Extravasation

Extravasation is another crucial process involving the escape of the CTC into distant tissue. CTCs may undergo a similar approach like leukocyte extravasation, involving the complex process of selectin-mediated rolling, adhesions with the capillary bed through integrin and cadherin expression[33]. The homing of CTCs to a distant metastatic site is not considered to be a random process. Most metastatic colonies in PDAC are formed in the liver. This can be explained by Stephan Paget's legendary seed and soil hypothesis, which suggests that interactions between the 'seed' (circulating tumor cell) and the soil (existing microenvironment around the metastatic site) are responsible for determining the secondary metastatic site[102].

Relatively recent literature suggests that neutrophils help in CTC extravasation by protecting them from natural killer (NK) cells, which are very effective tumor cell killers[34]. Through intravital microscopy, another group of scientists showed that neutrophils might assist in circulating tumor cell adhesion in their murine model for liver metastasis[35]. Neutrophils may also possibly behave as a 'chaperone' to CTCs through the interaction between tumor cell-expressed ICAM-1 and various neutrophil receptors[36,37].

Establishment of metastasis in the secondary site

The final obstacle faced by tumor cells in the metastatic cascade is to establish the secondary metastatic site. According to Paget's seed and soil hypothesis, the soil (the tumor micro-environment) is a major factor in determining the destiny of an invading tumor cell at the secondary site. The establishment of this 'pre-metastatic niche' is considered to take precedence even before CTC invasion[38]. Paget et al. showed that bone marrow-derived hematopoietic cells were linked with metastasis through the expression of VEGFR1. Similar to the primary tumor site, a largely immunosuppressive microenvironment persists in pre-metastatic niches. MDSC's, which suppress T-cell activity, are commonly found in these niches[39]. Surprisingly enough, TIMP1, primarily released by tumor cells, was also involved in niche formation in the liver through neutrophils[40]. G-CSF, a potent growth factor of neutrophils, was also seen to be upregulated in certain tumors and subsequently involved in premetastatic niche formation in the lung, again through the recruitment of neutrophils[41]. Although EMT is a crucial step in metastasis, it is also theorized that MET, mesenchymal-to-epithelial transition, also occurs during this colonization phase[42]. The TME hosts a range of infiltrating immune cells and resident stromal cells interacting with these tumor cells. Previous literature suggests that myeloid cells stimulate tumor cell proliferation by releasing versican, a large proteoglycan[43].

Conclusion

Although science has made significant improvements in fighting pancreatic cancer, a long road lies ahead of us. Due to their short lifespans, neutrophils have usually been

ignored in cancer progression in the past. However, the last decade of research elucidates a fresh new role of neutrophils in cancer. Tumor cells stimulate the polarization of naïve TANs into the pro-tumorigenic state. Immunosuppressive abilities characterize this state, thwarting other cytotoxic immune cells in destroying the tumor cells and promoting angiogenesis, crucial for tumorigenesis and eventually metastasis. TANs also have a longer lifespan and are able to persist in the TME for long periods. They may also undergo NETosis, and become NET's, which are linked with cancer progression. Neutrophils have also been implicated in being complicit in metastasis and assisting tumor cells in each step of the metastatic cascade.

Due to their critical involvement in tumor progression, neutrophils turn into attractive targets for novel immunotherapeutic strategies. Fridlender et al. have shown that blocking TGF-β in the TME resulted in an increase in neutrophils of the anti-tumorigenic phenotype[67]. Jablonska et al. showed that inactivating the IFN-β gene in their murine melanoma model led to increased tumor proliferation and angiogenesis, caused by the increased infiltration of pro-tumorigenic neutrophils[75]. Thus, by steering the polarization of naïve TANs in the TME, we can potentially improve cancer therapeutics.

The working hypothesis of my thesis is that pancreatic ductal adenocarcinoma (PDAC) cells prime the neutrophils to become pro-tumorigenic. Three specific objectives are **Objective 1:** Is PDAC progression associated with neutrophil infiltration? **Objective 2:** Why do we see an increase in neutrophil infiltration in the tumor microenvironment? **Objective 3:** What roles are neutrophil extracellular traps playing in the tumor microenvironment? To achieve these objectives, we performed immunofluorescence experiments on PDAC tissue from murine mouse models, using MPO, cathepsin-G, and

citrullinated histone 3 as markers. We also performed co-culture assays and measured survival using the WST assay. Finally, we examined the expression of multiple secretory factors in undifferentiated and differentiated neutrophils treated with tumor cell supernatant. We hope that our work here can further the understanding of neutrophil involvement in pancreatic cancer progression.

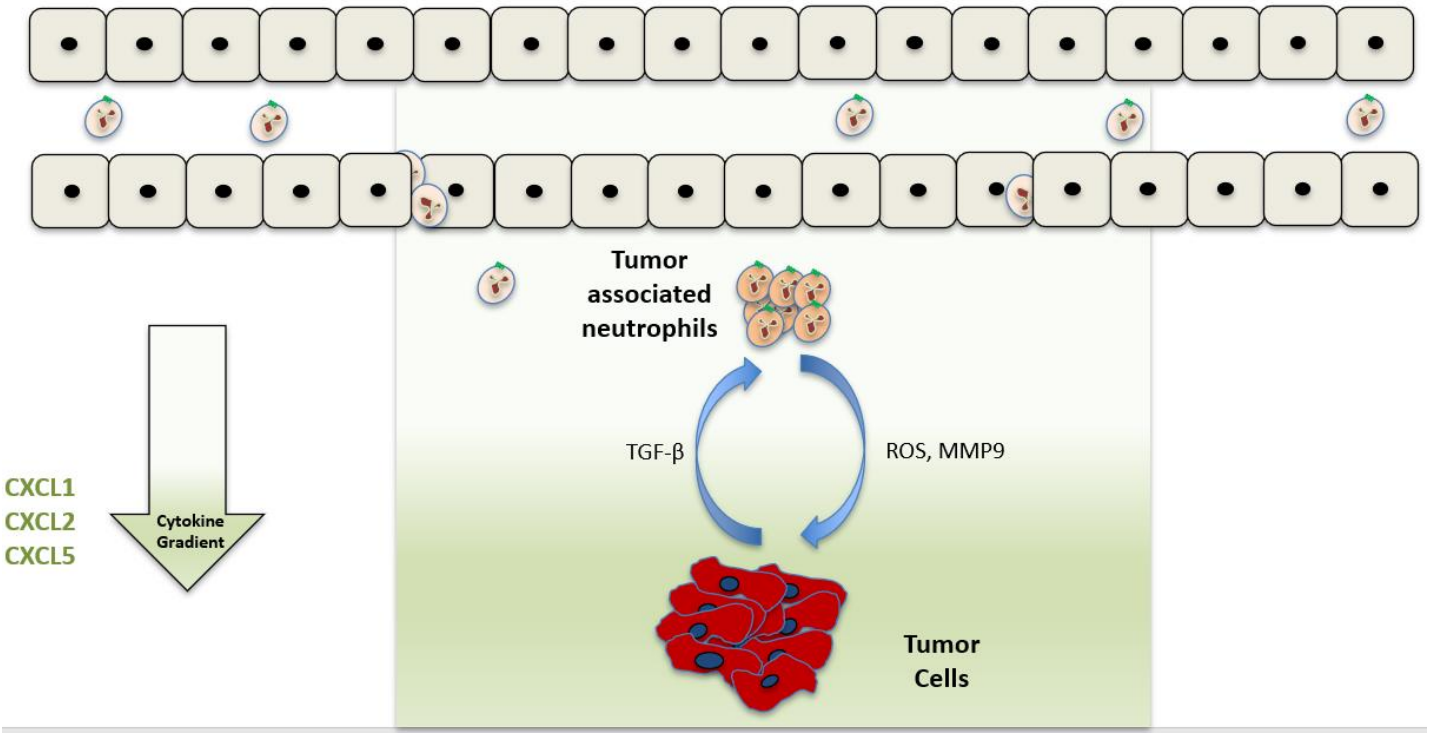

Tumor associated neutrophils
TGF-β
ROS, MMP9
Tumor Cells
CXCL1
CXCL2
CXCL5
Cytokine Gradient

Figure 1.1: Neutrophil recruitment into the TME

Neutrophils in the bloodstream extravasate into the TME by following the chemokine gradient set by the tumor cells.

MATERIALS AND METHODS

Cell Lines

We selected multiple human pancreatic adenocarcinoma cell lines derived from either the primary tumor or metastatic sites. This criterion was utilized to investigate whether the metastatic potential of a particular tumor cell line had any significant effect on our results.

Human PDAC cell lines L3.3, L3.6pl, AsPC3, T3M4, and CD18/HPAF, were cultured in the recommended media consisting of Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 5% fetal bovine serum (F.B.S.) (Sigma-Aldrich), 2-mM L-glutamine (Mediatech, Herdon, VA, U.S.A.), 1% vitamins (Mediatech), and 0.08% gentamycin (Invitrogen, Carlsbad, CA, U.S.A.)[89].

Additionally, the BXPC3 cell line, which was derived from the primary adenocarcinoma of a human patient, was cultured in Roswell Park Memorial Institute media 1640 (Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 5% F.B.S. (Sigma-Aldrich), 2-mM L-glutamine (Mediatech, Herdon, VA, U.S.A.), 1% vitamins (Mediatech), and 0.08% gentamycin (Invitrogen, Carlsbad, CA, U.S.A.).

Tumor cell supernatant was collected by plating 6 million cells in a 100 mm petri dish in serum-containing media. This media was subsequently replaced by serum-free media the next day, and the supernatant was collected 24-72 hours after.

The human leukemia cell line HL60, which displays promyelocytic characteristics, was derived from a patient suffering from acute promyelocytic leukemia and was cultured in identical media as the BXPC3 cell line. This cell line was subsequently differentiated using the protocol described by Gupta et.al[90], where they used 1.25% DMSO and 1 μM

ATRA dissolved in trans-retinoic acid for five days. Morphology of the differentiated cells was observed by cytospinning 5×10^4 cells in the Cytopro on glass slides, which were subsequently visualized through the Wright-Giemsa stain.

The murine MPRO Cell Line, Clone 2.1 (MPRO) (murine promyelocytes from ATCC, Manassas, VA, U.S.A.) was cultured in Iscove's Modified Dulbecco's Medium (IMDM, Sigma Aldrich, St. Louis, MO, U.S.A.) with 4 mM L-glutamine, 10 ng/mL murine granulocyte-macrophage colony-stimulating factor (GM-CSF, Peprotech, Pittsburgh, PA, U.S.A.), and 20% heat-inactivated horse serum (Sigma Aldrich)[58].

Live murine neutrophils were received as a generous gift from Dr. Leah Cook's lab, University of Nebraska Medical Center.

Cell Viability Assay

MPRO, HL60 differentiated, and HL60 undifferentiated cells (3×10^5 per well in a 96-well plate) were treated with supernatants derived from T3M4, CD18/HPAF, ASPC3, BXPC3 cell lines, and serum-free media for 24 hours in various dilutions of supernatant (87.5%, 75%, 50%, 25%) using serum-free media as a control. After overnight treatment, W.S.T. (Sigma Aldrich, Milwaukee, WI, U.S.A.) was added to each well as recommended by the manufacturer for four hours, and the plate was subsequently measured for absorbance at a wavelength of 450 nm using an ELx800 (Bio-Tek, Winooski, VT, U.S.A.) plate reader. Percent growth was calculated as ([Absorbance of the treatment - Absorbance of the control group]/ average of the control group).

Co-Culture Assay

T3M4 and CD18/HPAF cell lines were added to a 96-well plate (5000 per well) and incubated overnight in serum-containing media. The next day, the media was changed, and MPRO, HL60 differentiated, and HL60 undifferentiated cells were added to the tumor cells' wells. After 24-hour incubation, the neutrophils were collected and transferred to new wells while the media of the tumor cells was replaced again with serum-containing media. W.S.T. (Sigma Aldrich, Milwaukee, WI, U.S.A.) was added to each well as described above, and the plate was measured for the absorbance at a wavelength of 450 nm using an ELx800 (Bio-Tek, Winooski, VT, U.S.A.) plate reader. Percent growth was calculated as ([Absorbance of the treatment- Absorbance of the control group]/ average of the control group).

Immunofluorescence

Murine pancreatic cancer tissue slides from animal models (KC: K-rasLSL.$^{G12D/+;}$ Pdx-1-Cre) and (KCC: K-rasLSL.$^{G12D/+}$; Pdx-1-Cre; Cxcr2$^{+/-}$) were obtained through Dr. Surinder Batra's lab at the University of Nebraska, Medical Center. These tissue sections were deparaffinized in xylene followed by rehydration in 70% ethyl alcohol. The tissue region was marked with a P.A.P. pen and was subsequently washed three times with phosphate-buffered saline (PBS). The slides were blocked using blocking buffer (10% goat serum) for one hour and then stained with primary antibodies **(Table 2.1)** overnight at 4°C. The slides were washed with PBS the next day, and a secondary immunofluorescence antibody **(Table 2.1)** was added. After a one-hour incubation at room temperature, the slides were rewashed with PBS, VectaFluor™ Duet Immunofluorescence Double Labeling Kit (Vector Laboratories, Burlingame, CA, was

added, and a coverslip was added on the tissue section. The slides were observed under a Nikon Eclipse E800 microscope (Nikon, Melville, NY, U.S.A.) and NIS-Elements BR 5.11.00 software (Nikon). The numbers of neutrophils and NETs were calculated per high power frame. The list of antibodies used can be found in **Table 2.1**.

Gene Expression Analysis

RNA Isolation

Total RNA from neutrophil cell lines was isolated by spinning the cells down, washing them with PBS, subsequently followed by lysing the pellet in 1 ml TRIzol reagent (Invitrogen, Carlsbad, CA). Then, 0.2 ml chloroform was added to the sample. These samples were then centrifuged at 12000g for 15 minutes at 4°C and the clear aqueous phase was transferred to separate tubes. An equal volume of isopropanol was added to each tube to precipitate the RNA and was incubated at room temperature for 10 minutes. Subsequently, these tubes were centrifuged at 12000g for 10 minutes at 4°C. The supernatant was discarded, and the pellet was washed in 1 ml of 75% ethanol. The sample was centrifuged once more at 7500g for 5 minutes at 4°C. The supernatant was discarded, and the remaining pellet was dissolved in 20 μl diethyl pyrocarbonate water. We measured the concentration of RNA using a Thermo Scientific NanoDrop 2000 Spectrophotometer (Thermo Scientific, Carlsbad, CA).

Quantitative RT-PCR

Five μg of total RNA was used to prepare complementary DNA using SuperScript™ Reverse Transcriptase (Invitrogen) and oligo (dT) primers. The complementary DNA (cDNA) was stored at -20°C until further use.

We quantified human CXCL1, CXCL2, CXCL8, IL23, INOS, TGFB, TNFA, MMP9, along with RPL13A (to normalize gene expression). These experiments were performed using PowerUp SYBR Green Master Mix (Applied Biosystems, Thermo Fisher Scientific) and primers **(Table 2.2)**. The cDNA was diluted to a 1:5 ratio. The Quant Studio 3 Real-Time PCR Detection System (Thermo Fisher Scientific) and the Quant Studio design and analysis software were used to run and analyze the experiments. ($-\Delta C_t$) was calculated by the difference between cycle time ($C_{.T.}$) housekeeping gene RPL13A and target specific $C_{.T}$. Relative expression was calculated as ($2^{(-\Delta Ct)}$). Subsequently, we calculated fold change ($2^{(-\Delta\Delta Ct)}$) by comparing mRNA expression of target gene($2^{(-\Delta Ct)}$) vs. *RPL13A*($2^{(-\Delta Ct)}$) and normalizing it to serum-free HL60 ($2^{(-\Delta\Delta Ct)}$) and differentiated HL60 ($2^{(-\Delta\Delta Ct)}$) expression. The list of gene-specific primers used can be found in **Table 2.2**.

Neutrophil Extracellular Trap Quantification Assay

5 x 10^4 undifferentiated HL60 cells were plated in a 96-well plate and treated with supernatants derived from CD18/HPAF supernatants and serum-free media for 2 hours. Phorbol 12-myristate 13-acetate (Sigma) was added to the positive control for 2 hours at 20 nM prior to reading the plate. 10 nM of Sytox Green (Invitrogen) was added to each sample 15 minutes prior to measuring the plate via an EVOS FL auto microscope. The number of green fluorescent NETs was counted per HPF. Representative pictures were also taken through this microscope.

Table 2.1

Antibody	Source	Dilution
Murine Anti-Histone H3 (citrulline R2 + R8 + R17) antibody	Abcam, MA, U.S.A, ab5103	1:200 IF
Murine Anti-Myeloperoxidase	Abcam, MA, U.S.A.	1:100 IF
Murine Anti-Cathepsin G	Santa Cruz, SC 6514 Biotechnology, Europe	1:100 IF
Biotinylated Goat Anti-Rabbit IgG Antibody	Vector Laboratories, CA, U.S.A.	1:500 IF

Table 2.2 List of Primers

TNF-A	Forward 5'-GAGCTGAGAGATAACCAGCTGGTG-3' Reverse 3- GAGATAGATGGGCTCATACCAGGG
GRO1	Forward 5'-ATTCACCCCAAGAACATCCC-3' Reverse 5'-CACCAGTGAGCTTCCTCCT-3'
GRO2	Forward 5'-GCAGGGAATTCACCTCAAG-3' Reverse 5'-AGCTTCCTCCTTCCTTCTG-3'
GRO3	Forward 5'-GCAGGGAATTCACCTCAAG-3' Reverse 5'-GGTGCTCCCCTTGTTCAGT-3'
TGFB2	Forward 5'-CAGCACACTCGATATGGACCA-3' Reverse 5'-CCTCGGGGCTCAGGATAGTCT-3'
MMP9	Forward 5'-CATCGTCATCCAGTTTGGTG-3' Reverse 5'-AGGGACCACAACTCGTCATC-3'
CXCL8	Forward 5'-ACATACTCCAAACCTTTTCCACCC-3' Reverse 5'-CAACCCTCTGCACCCAGTTTTC-3'
Il23	Forward 5'-TGCAAAGGATCCACCAGGGTCTGA-3' Reverse 5'-TAGGTGCCATCCTTGAGCTGCTGC-3'
IL17	Forward 5'-AGATTACTACAACCGATCCACCT-3' Reverse 5'-GGGGACAGAGTTCATGTGGTA-3'
INOS	Forward 5'-TCCAAGACACACTTCACC-3' Reverse 5'-TTCCTGTTGTTTCTATCTCC-3'
RPL13A	Forward 5'-CCTGGAGGAGAACAGGAAAGAGA-3' Reverse 5-TTGAGGACCTCTGTGTATTTGTCAA-3'

RESULTS

Neutrophil Infiltration increased as PDAC progressed and is dependent on the CXCR2 receptor

A systemic increase of neutrophils in circulation is often observed in PDAC, evident through the high NLR ratios measured in patients with non-resectable PDAC[65]. Large numbers of $CXCR2^{+}$ neutrophils are also recruited in the TME and are often facilitated by various chemokines such as CXCL1, CXCL2, and CXCL8 released by the tumor.

We investigated whether our pancreatic mouse model increased neutrophil infiltration in tumors from KC animal models (KC: K-rasLSL.$^{G12D/+;}$ Pdx-1-Cre) at different time points by immunostaining the tissue sections for MPO and cathepsin-G **(FIG 2.1).** We observed an increase in neutrophil infiltration as the tumor progressed from 10 weeks to 50 weeks.

Based on previous studies in our lab[76], we hypothesized that the CXCR2 receptor on the neutrophils was responsible for the increased recruitment. We performed similar MPO and cathepsin-G immunostaining on tumors from CXCR2 knocked out KC mice (KCC: K-rasLSL;$^{G12D/+}$; Pdx-1-Cre; $Cxcr2^{+/-}$) and observed a significant decrease in neutrophil infiltration when compared with our wild type KC murine model **(FIG 2.2).** These results suggest that neutrophil recruitment in the TME becomes more intense as PDAC progresses and is linked with the host CXCR2 receptor.

The PC cell-free supernatant enhanced neutrophil survival

Although neutrophils are universally considered short-lived cells, there has been mounting evidence suggesting that neutrophils in the TME survive for extended periods of time[77]. To investigate whether neutrophils exhibit a similar effect in the PDAC TME, we treated undifferentiated and differentiated human HL60 and murine MPRO cells for 24

hours with supernatants derived from CD18, gemcitabine resistant CD18 (CD18GR), T3M4, gemcitabine resistant T3M4 (T3M4GR), BXPC-3 and AXPC1 tumor cell lines **(FIG 2.3**). We found that the HL60 cells exhibited higher survival in a concentrations dependent-manner (v/v) when treated with tumor cell supernatants than control-treated with serum-free (SF) media. The differentiated HL60 cells also produced a similar result. Murine neutrophils also displayed higher survival when treated with CD18 and CD18GR cell supernatants. Meanwhile, we also attempted to treat MPRO cells with tumor cell supernatant for 72 hours; however, we observed a significant change in neutrophil survival at the higher concentrations (75% v/v). These results suggest that undifferentiated and differentiated neutrophils can survive in the PDAC TME for more extended periods.

Co-culture assay between tumor cells and neutrophils increased tumor cell survival and decreased neutrophil survival

After examining the effect of tumor cell supernatant on the survival of neutrophils, we proceeded to investigate how the direct interaction between tumor cells and neutrophils affected their survivability. We co-cultured MPRO cells and undifferentiated and differentiated HL60 cells with CD18, CD18GR, T3M4, and T3M4GR tumor cells for 24 hours. We then separated the neutrophils and tumor cells and measured their survival compared to controls treated with SF media. We observed a significant increase in the survival of most tumor cell lines when treated with neutrophils **(FIG 2.4).** Surprisingly enough, we did not observe an increase in CD18 tumor cell survival when co-cultured with undifferentiated HL60 neutrophils.

Moreover, our co-culture assay displayed a significant decrease in MPRO and differentiated HL60 neutrophil survival (**FIG 2.5)**. Undifferentiated HL60 survival remained unchanged during this experiment. These results indicate that the interaction between neutrophils and tumor cells in the TME of PDAC can reasonably increase the survival of tumor cells in PDAC and correspondingly result in a decrease in neutrophil survival.

Neutrophils cultured in the PC supernatant secreted anti-tumor factors

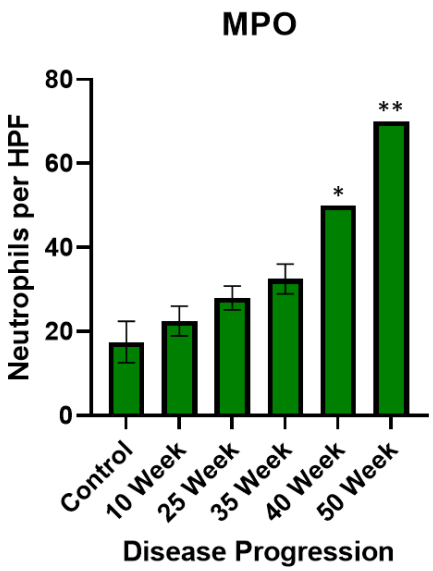
We treated undifferentiated and differentiated HL60 neutrophils with supernatant derived from ASPC1 and BXPC3 tumor cell lines to further investigate the neutrophil-tumor cell interaction. We measured the expression of various secretory factors **(Fig 2.6, 2.7, 2.8, 2.9).** Our undifferentiated HL60 cells displayed a decrease in CXCL1 and TGF-beta expression **(Fig 2.6)**. CXCL2, iNOS, CXCL8, IL23, and TNFA expression remain unchanged. We also observed no significant difference in the expression of any secretory factors in our differentiated HL60 neutrophils **(Fig 2.7)**. Also, our differentiated HL60 cells displayed a significant increase in CXCL2, CXCL8, and IL23 expression and a significant decrease in CXCL1 and iNOS expression compared to undifferentiated HL60 cells in serum-free media **(Fig 2.8)**. Finally, the differentiated neutrophils significantly increased CXCL1, CXCL8, and IL23 expression **(Fig 2.9)**.

NET Infiltration increased as PDAC progressed and is dependent on the CXCR2 receptor

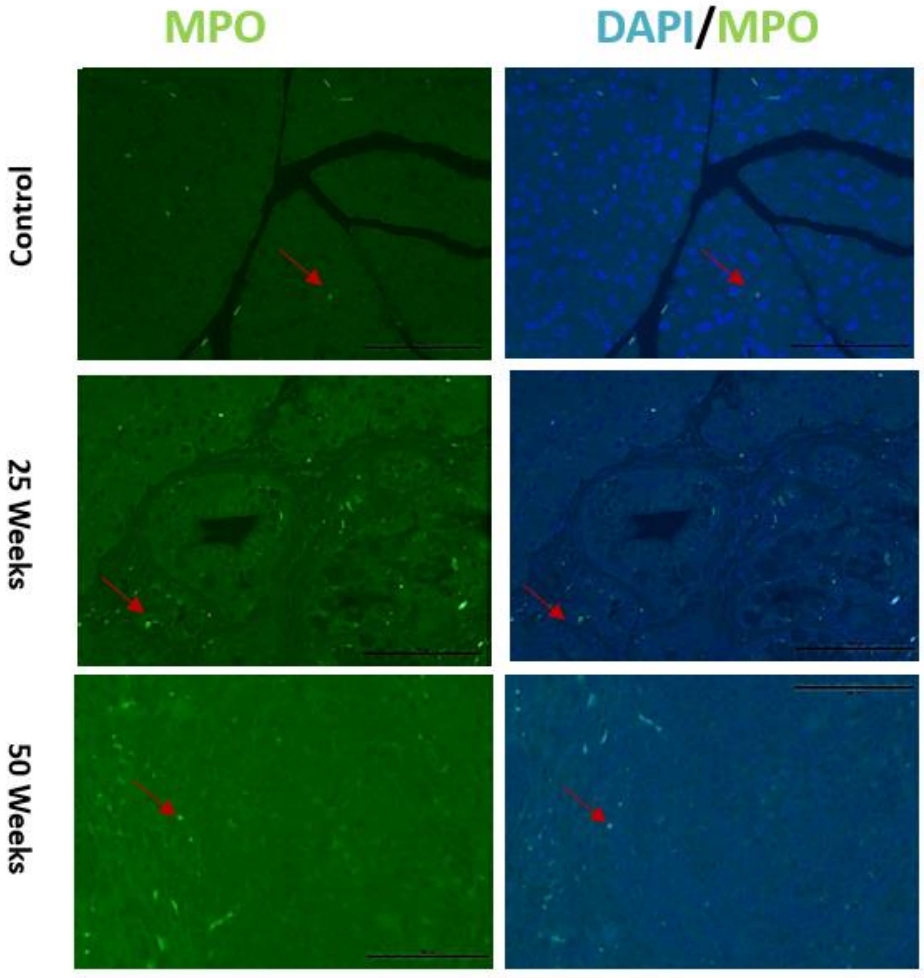
NETs discovered in 2004 by Brinkmann et al. [47] have been widely implicated in cancer progression. They are known to orchestrate circulating tumor cell (CTC) extravasation, impair the immune system, and release proteases such as MMP9[48,53].

We found that our KC pancreatic mouse model also showed an increase in NETs as the disease progressed in its later stages **(Fig 2.10)**. Like our previous studies examining neutrophil infiltration, we also looked at the NET expression in our KC and KCC model and observed a significant decrease in NET counts **(Fig 2.11)**.

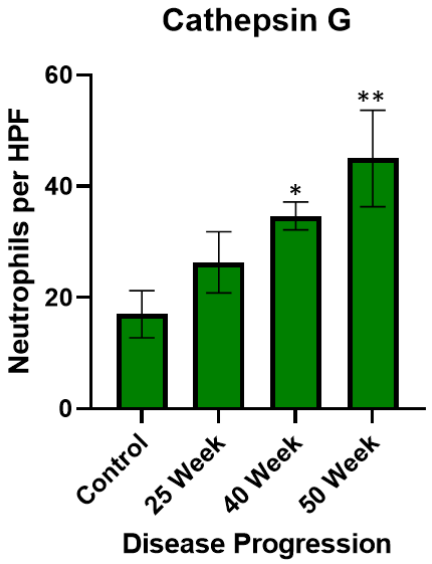
We also discovered that NET formation was favored when neutrophils were treated with tumor cell supernatants derived from L3.3 and L3.6 cell lines **(Fig 2.12)**. These results suggest that neutrophil infiltration and NET formation are closely tied to each other, and the TME in PDAC might stimulate NETosis in these neutrophils.


MPO
Neutrophils per HPF
80
60
40
20
0
**
*
Control
10 Week
25 Week
35 Week
40 Week
50 Week
Disease Progression

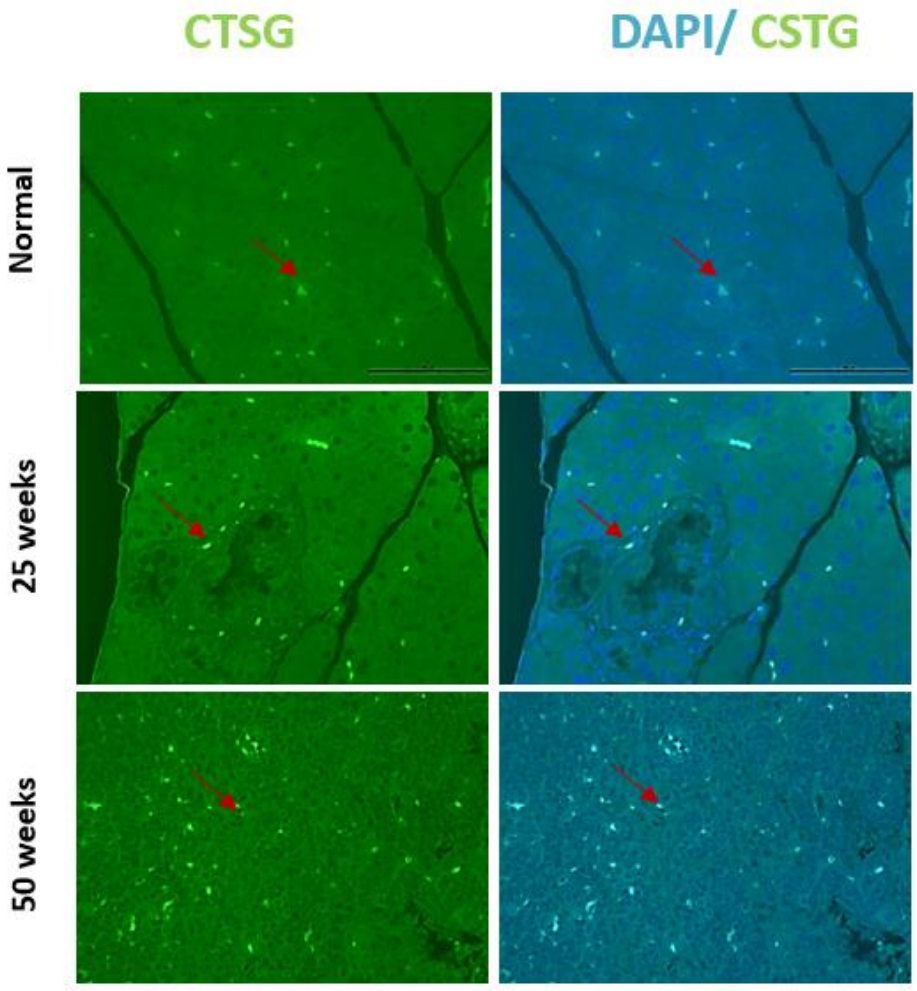
(A)


MPO
DAPI/MPO
Control
25 Weeks
50 Weeks

(B)


Cathepsin G
Neutrophils per HPF
60
40
20
0
*
**
Control
25 Week
40 Week
50 Week
Disease Progression

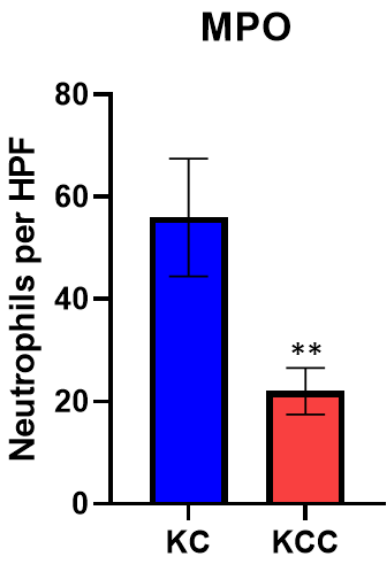
(C)


CTSG
DAPI/ CSTG
Normal
25 weeks
50 weeks

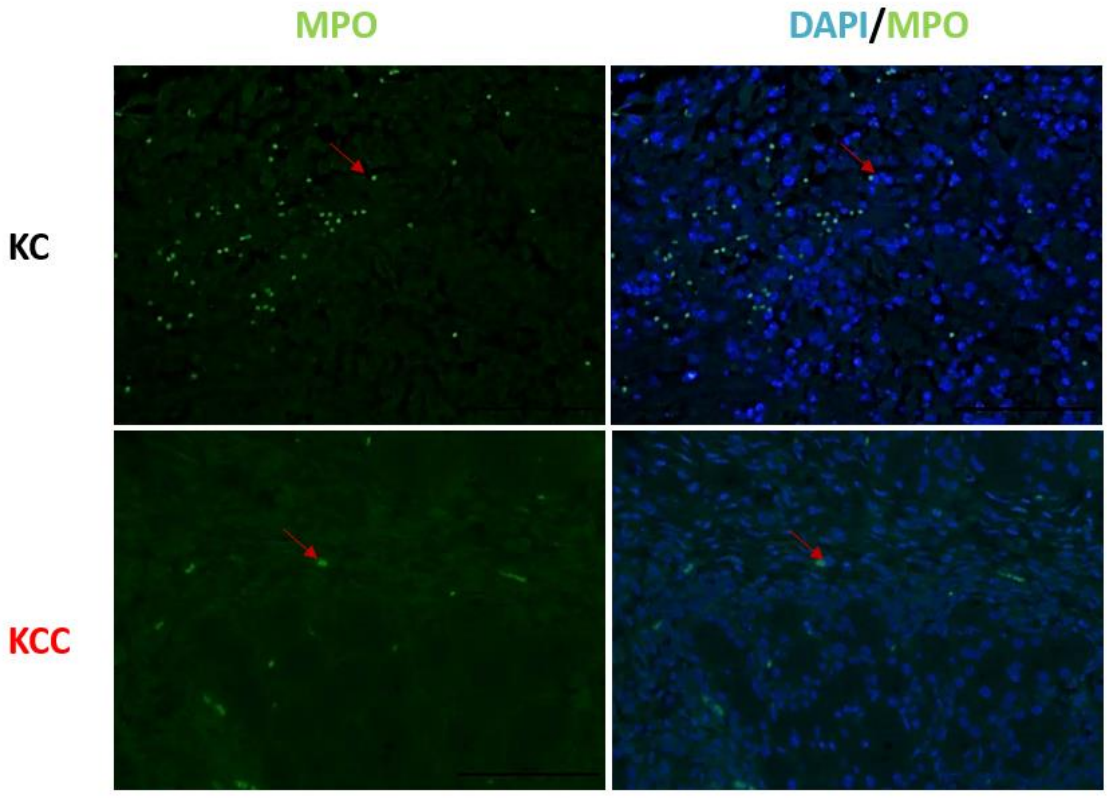
(D)

Figure 2.1: Neutrophil Infiltration increased as PDAC progressed in our KC model

(A) Quantitative measurement of MPO positive neutrophils in our KC progressive murine model**. (B)** Representative immunofluorescence imaging of MPO positive neutrophils in our KC progressive murine model. **(C)** Quantitative measurement of cathepsin-G positive neutrophils in our KC progressive murine model**. (D)** Representative immunofluorescence imaging of cathepsin G positive neutrophils in our KC progressive murine model. The values are shown as mean ± SEM, Unpaired t-test; * for $P<0.05$; ** for $P< 0.01$. The pictures were acquired under a magnification of 200X. The scale bars are shown as 10 μm.



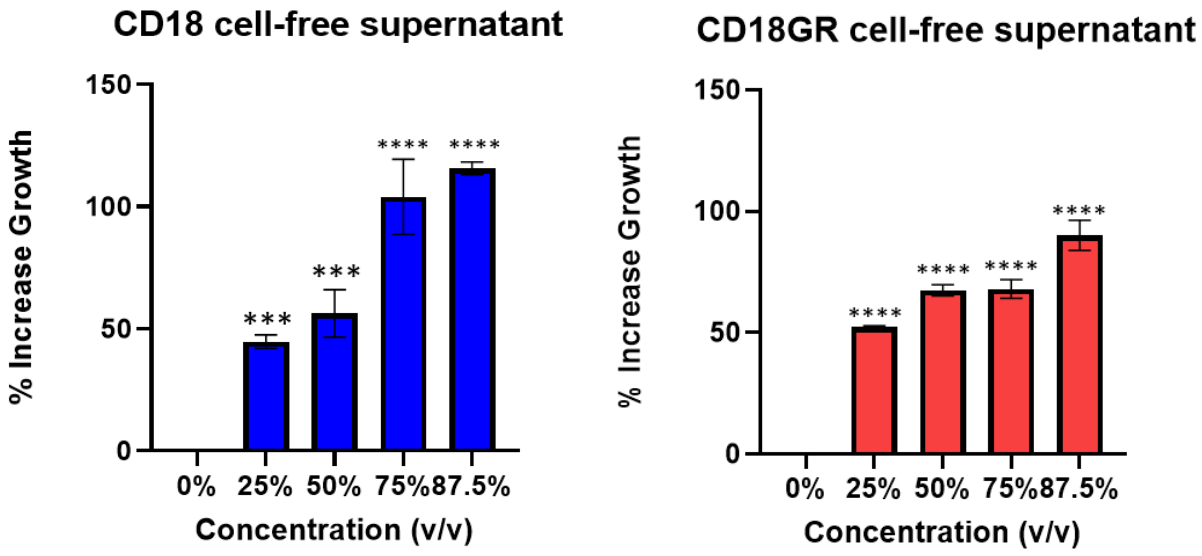
(A)



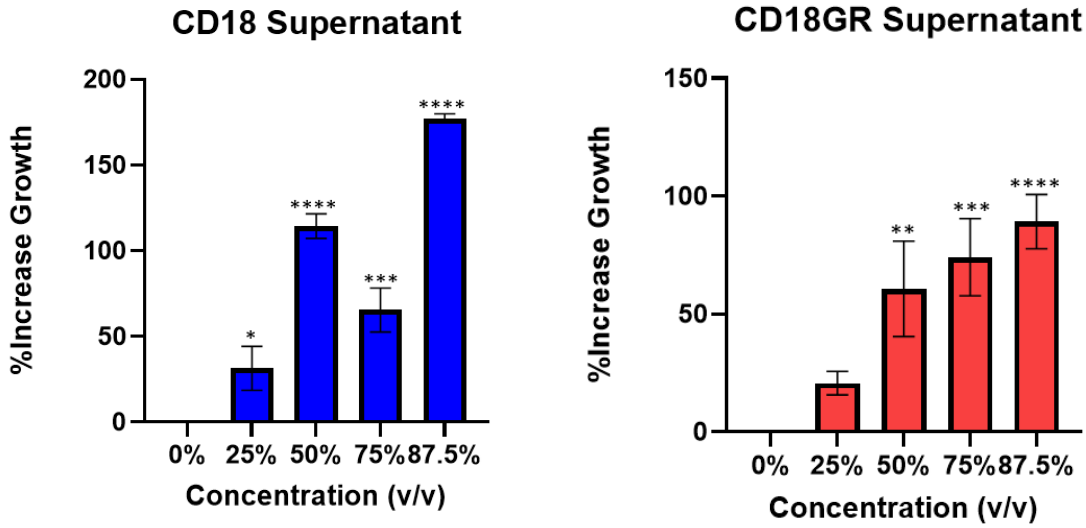
(B)

Figure 2.2: Neutrophil Infiltration decreased in our murine KCC model

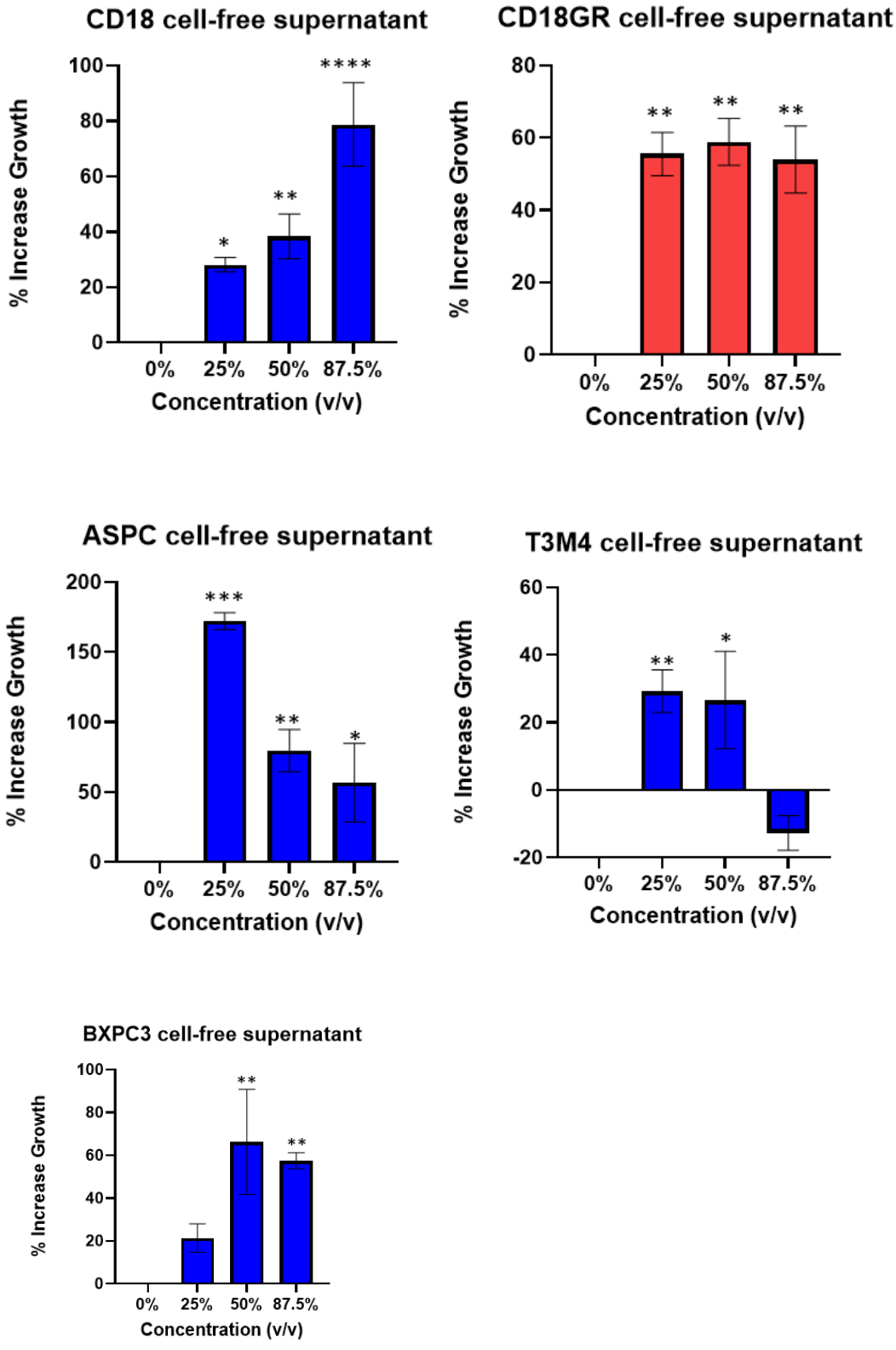
(A) Quantitative measurement of MPO positive neutrophils in our KC and KCC murine model. **(B)** Representative immunofluorescence imaging of MPO positive neutrophils in our KC and KCC murine model. The values are shown as mean ± SEM, Unpaired t-test,** for P< 0.01. The pictures were acquired under a magnification of 200X. The scale bars are shown as 10 μm.


CD18 cell-free supernatant
% Increase Growth
150
100
50
0
***
***
****
****
0% 25% 50% 75% 87.5%
Concentration (v/v)
CD18GR cell-free supernatant
% Increase Growth
150
100
50
0
****
****
****
****
0% 25% 50% 75% 87.5%
Concentration (v/v)

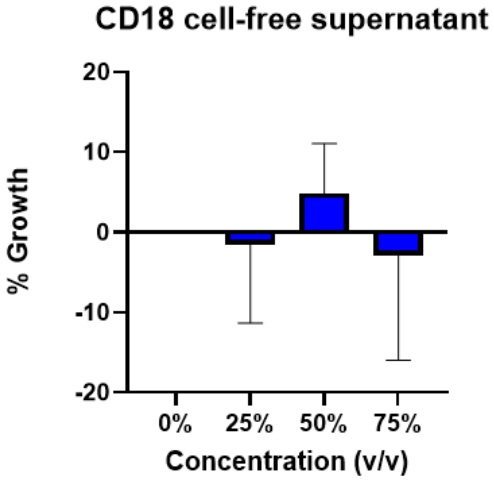
(A)


CD18 Supernatant
%Increase Growth
200
150
100
50
0
*
****
***
****
0% 25% 50% 75% 87.5%
Concentration (v/v)
CD18GR Supernatant
%Increase Growth
150
100
50
0
**
***
****
0% 25% 50% 75% 87.5%
Concentration (v/v)

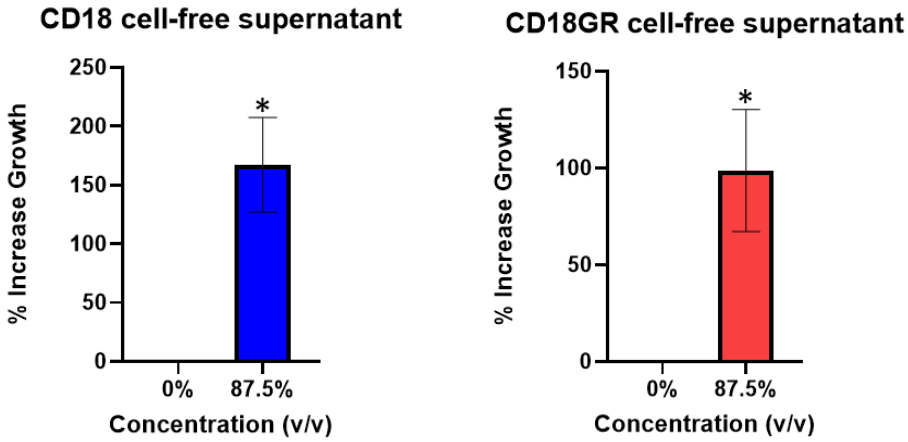
(B)


CD18 cell-free supernatant
% Increase Growth
100
80
60
40
20
0
*
**
****
0%
25%
50%
87.5%
Concentration (v/v)
CD18GR cell-free supernatant
% Increase Growth
80
60
40
20
0
**
**
**
0%
25%
50%
87.5%
Concentration (v/v)
ASPC cell-free supernatant
% Increase Growth
200
150
100
50
0
***
**
*
0%
25%
50%
87.5%
Concentration (v/v)
T3M4 cell-free supernatant
% Increase Growth
60
40
20
0
-20
**
*
0%
25%
50%
87.5%
Concentration (v/v)
BXPC3 cell-free supernatant
% Increase Growth
100
80
60
40
20
0
**
**
0%
25%
50%
87.5%
Concentration (v/v)

(C)


CD18 cell-free supernatant
% Growth
20
10
0
-10
-20
0%
25%
50%
75%
Concentration (v/v)

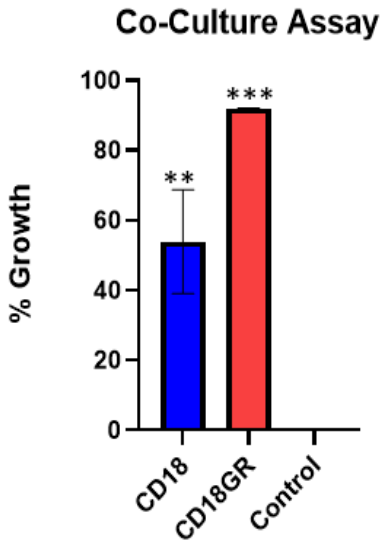
(D)


CD18 cell-free supernatant
% Increase Growth
250
200
150
100
50
0
*
0%
87.5%
Concentration (v/v)
CD18GR cell-free supernatant
% Increase Growth
150
100
50
0
*
0%
87.5%
Concentration (v/v)

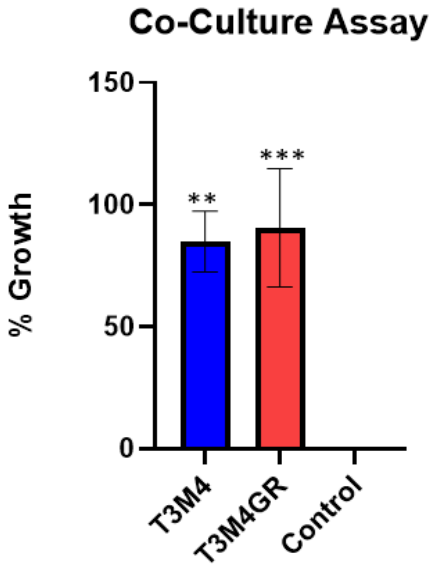
(E)

Figure 2.3: The supernatant of CD18, CD18GR, ASPC, T3M4, and BXPC3 enhanced the survivability of undifferentiated MPRO cells, differentiated and undifferentiated HL60 cells

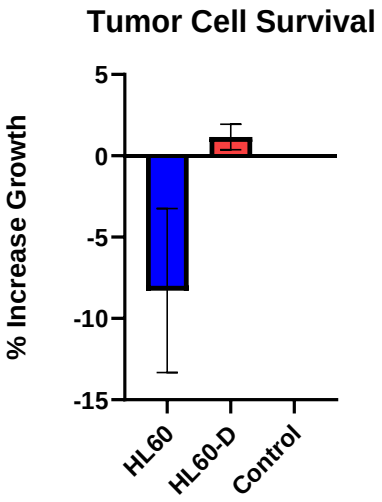
(A) The supernatant of CD18 and CD18GR cells significantly enhanced the survival of undifferentiated HL60 cells. **(B)** The supernatant of CD18 and CD18GR cells significantly enhanced the survival of undifferentiated MPRO cells. **(C)** The supernatant of CD18, CD18GR ASPC, BXPC3, and T3M4 cells significantly enhanced the survival of differentiated HL60 cells. **(D)** The supernatant of CD18 cells had no significant ability to increase the survival of MPRO neutrophils treated for 72 hours. **(E)** The supernatant of CD18 and CD18GR cells significantly enhanced the survival of murine neutrophils. The values are shown as mean ± SEM, Unpaired t-test; * for $P<0.05$; ** for $P< 0.01$; *** for $P \leq 0.001$; **** for $P \leq 0.000$


Co-Culture Assay
% Growth
100
80
60
40
20
0
**
***
CD18
CD18GR
Control

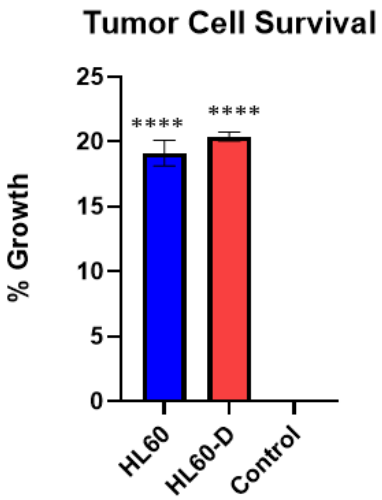
(A)


Co-Culture Assay
% Growth
150
100
50
0
**
***
T3M4
T3M4GR
Control

(B)


Tumor Cell Survival
% Increase Growth
5
0
-5
-10
-15
HL60
HL60-D
Control

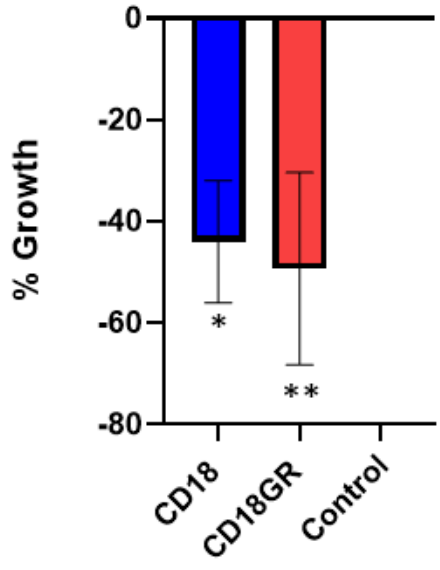
(C)


Tumor Cell Survival
% Growth
25
20
15
10
5
0
****
****
HL60
HL60-D
Control

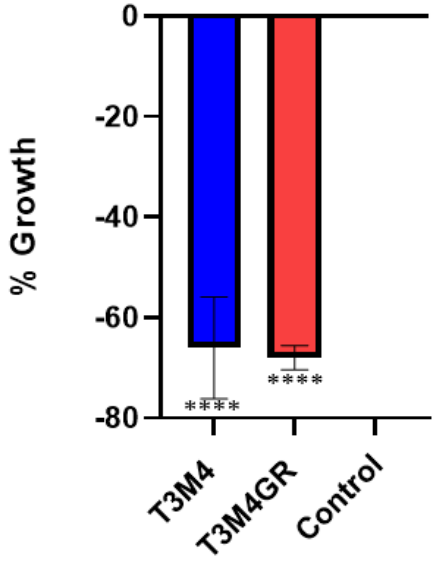
(D)

Figure 2.4: The co-culture assay using CD18, CD18GR, T3M4, T3M4GR cells, and undifferentiated MPRO cells, differentiated and undifferentiated HL60 cells enhanced tumor cell survival

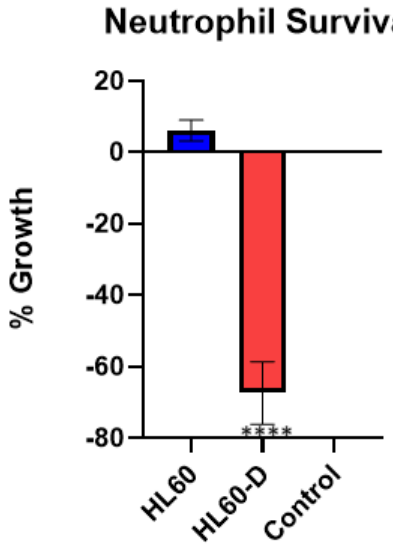
(A) The co-culture assay, including CD18, CD18GR cells, and undifferentiated MPRO cells, enhanced tumor cell survival. **(B)** The co-culture assay involving T3M4, T3M4GR cells, and undifferentiated MPRO cells enhanced tumor cell survival. **(C)** The co-culture assay utilizing CD18 cells and undifferentiated and differentiated HL60 cells did not affect tumor cell survival. **(D)** The co-culture assay that included T3M4 cells and undifferentiated and differentiated HL60 cells enhanced tumor cell survival. The values are shown as mean ± SEM, Unpaired t-test; * for $P<0.05$; ** for $P< 0.01$; *** for $P \leq 0.001$; **** for $P \leq 0.0001$.


0
-20
-40
-60
-80
% Growth
*
**
CD18
CD18GR
Control

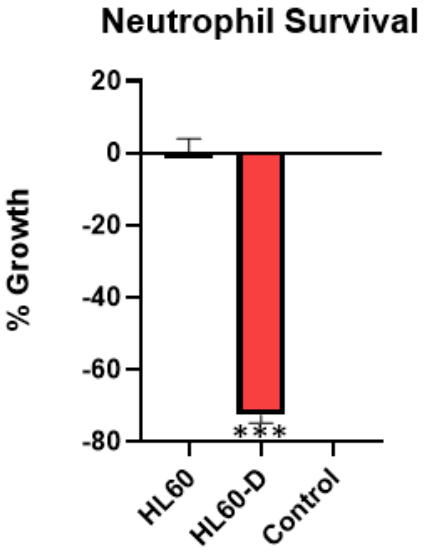
(A)


0
-20
-40
-60
-80
% Growth
****
****
T3M4
T3M4GR
Control

(B)


Neutrophil Survival
20
0
-20
-40
-60
-80
% Growth
****
HL60
HL60-D
Control

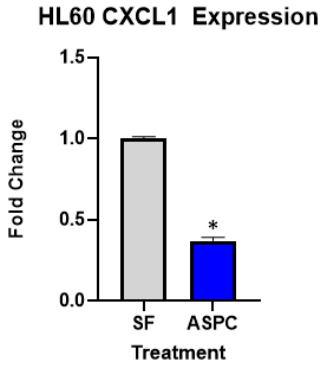
(C)


Neutrophil Survival
20
0
-20
-40
-60
-80
% Growth
***
HL60
HL60-D
Control

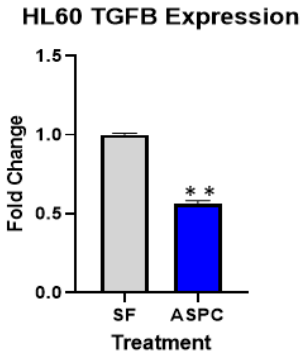
(D)

Figure 2.5: The co-culture assay including CD18, CD18GR, T3M4, T3M4GR cells, and undifferentiated MPRO cells, differentiated and undifferentiated HL60 cells decreased neutrophil cell survival

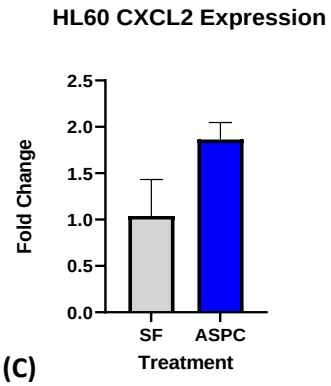
(A) The co-culture assay involving CD18, CD18GR cells, and undifferentiated MPRO cells decreased neutrophil survival. **(B)** The co-culture assay using T3M4, T3M4GR cells, and undifferentiated MPRO cells decreased neutrophil survival. **(C)** The co-culture assay between CD18 cells and undifferentiated and differentiated HL60 cells did not affect neutrophil cell survival. **(D)** The co-culture assay of T3M4 cells and undifferentiated as well as differentiated HL60 cells decreased neutrophil survival. The values are shown as mean ± SEM, Unpaired t-test; * for $P<0.05$; ** for $P< 0.01$; *** for $P \leq 0.001$; **** for $P \leq 0.0001$.


HL60 CXCL1 Expression
Fold Change
1.5
1.0
0.5
0.0
*
SF
ASPC
Treatment

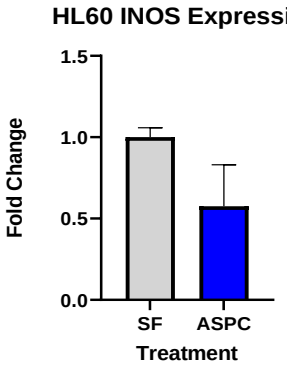
(A)


HL60 TGFB Expression
Fold Change
1.5
1.0
0.5
0.0
* *
SF
ASPC
Treatment

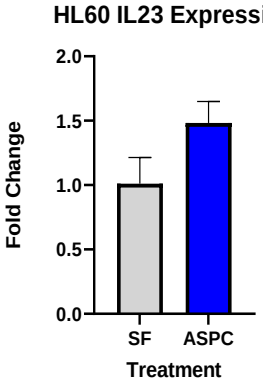
(B)


HL60 CXCL2 Expression
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
SF
ASPC
Treatment

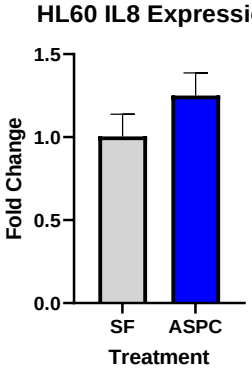
(C)


HL60 INOS Expression
Fold Change
1.5
1.0
0.5
0.0
SF
ASPC
Treatment

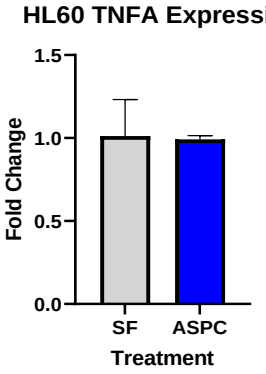
(D)


HL60 IL23 Expression
Fold Change
2.0
1.5
1.0
0.5
0.0
SF
ASPC
Treatment

(E)


HL60 IL8 Expression
Fold Change
1.5
1.0
0.5
0.0
SF
ASPC
Treatment

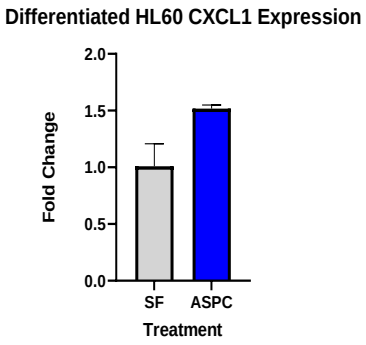
(F)


HL60 TNFA Expression
Fold Change
1.5
1.0
0.5
0.0
SF
ASPC
Treatment

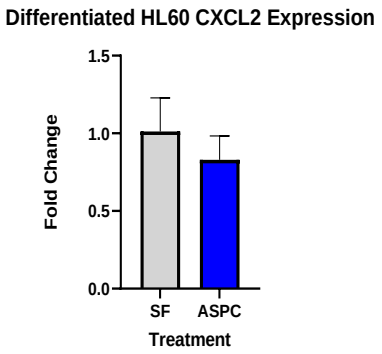
(G)

Figure 2.6: Undifferentiated HL60 cells cultured in tumor cell supernatant downregulated pro-tumor factors CXCL1 and TGFB

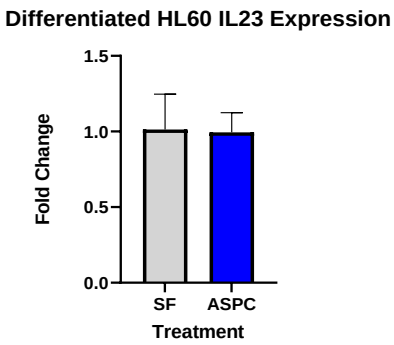
(A) Quantitative RT-PCR for the expression of *CXCL1* in undifferentiated HL60 cells cultured in serum-free media and supernatant derived from ASPC tumor cells. **(B)** Quantitative RT-PCR for the expression of *TGFB* in undifferentiated HL60 cells cultured in serum-free media and supernatant derived from ASPC tumor cells. **(C)** Quantitative RT-PCR for the expression of *CXCL2* in undifferentiated HL60 cells cultured in SF media and supernatant derived from ASPC tumor cells. **(D)** Quantitative RT-PCR for the expression of *iNOS* in undifferentiated HL60 cells cultured in serum-free media and supernatant derived from ASPC tumor cells. **(E)** Quantitative RT-PCR for the expression of *IL23* in undifferentiated HL60 cells cultured in serum-free media and supernatant derived from ASPC tumor cells. **(F)** Quantitative RT-PCR for the expression of *CXCL8* in undifferentiated HL60 cells cultured in serum-free media and supernatant derived from ASPC tumor cells. **(G)** Quantitative RT-PCR for the expression of *TNFA* in undifferentiated HL60 cells cultured in serum-free media and supernatant derived from ASPC tumor cells. The values are mean fold change ± SEM; unpaired t-test, assume both populations have the same SD; **P <0.005; *P<0.0001; *** for P ≤ 0.05.


Differentiated HL60 CXCL1 Expression
Fold Change
2.0
1.5
1.0
0.5
0.0
SF
ASPC
Treatment

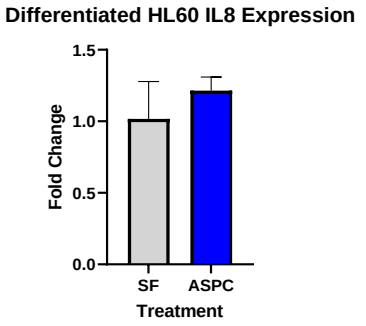
(A)


Differentiated HL60 CXCL2 Expression
Fold Change
1.5
1.0
0.5
0.0
SF
ASPC
Treatment

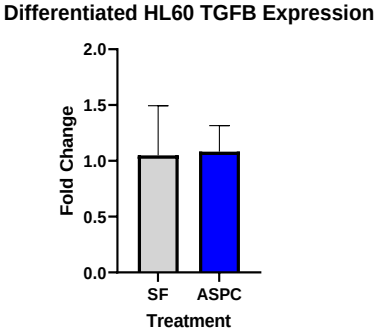
(B)


Differentiated HL60 IL23 Expression
Fold Change
1.5
1.0
0.5
0.0
SF
ASPC
Treatment

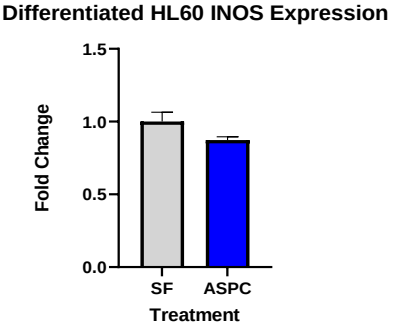
(C)


Differentiated HL60 IL8 Expression
Fold Change
1.5
1.0
0.5
0.0
SF
ASPC
Treatment

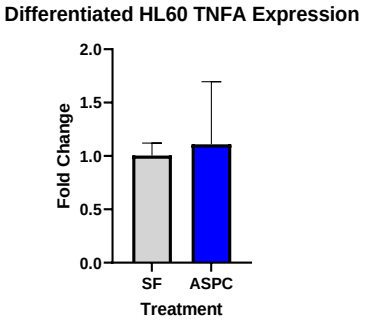
(D)


Differentiated HL60 TGFB Expression
Fold Change
2.0
1.5
1.0
0.5
0.0
SF
ASPC
Treatment

(E)


Differentiated HL60 INOS Expression
Fold Change
1.5
1.0
0.5
0.0
SF
ASPC
Treatment

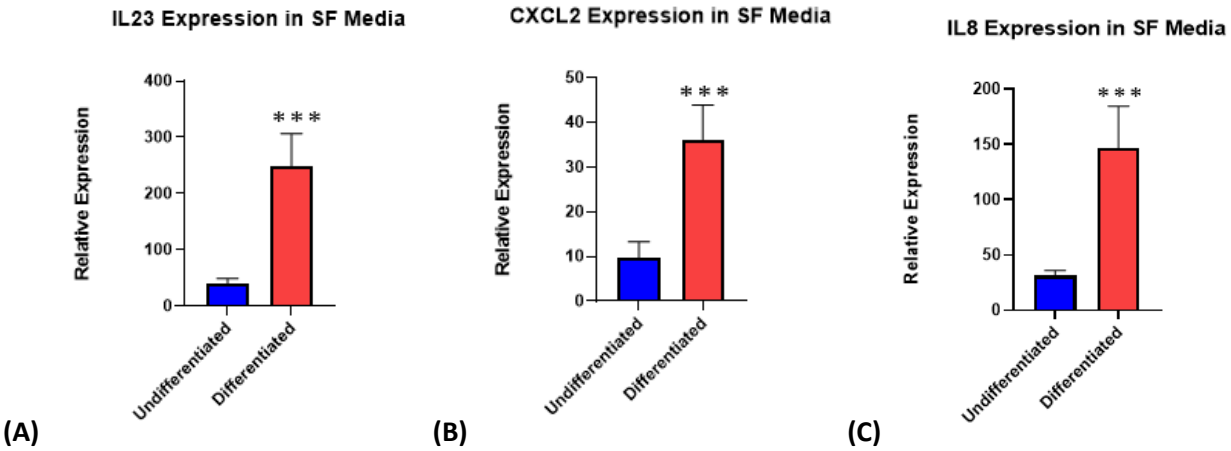
(F)

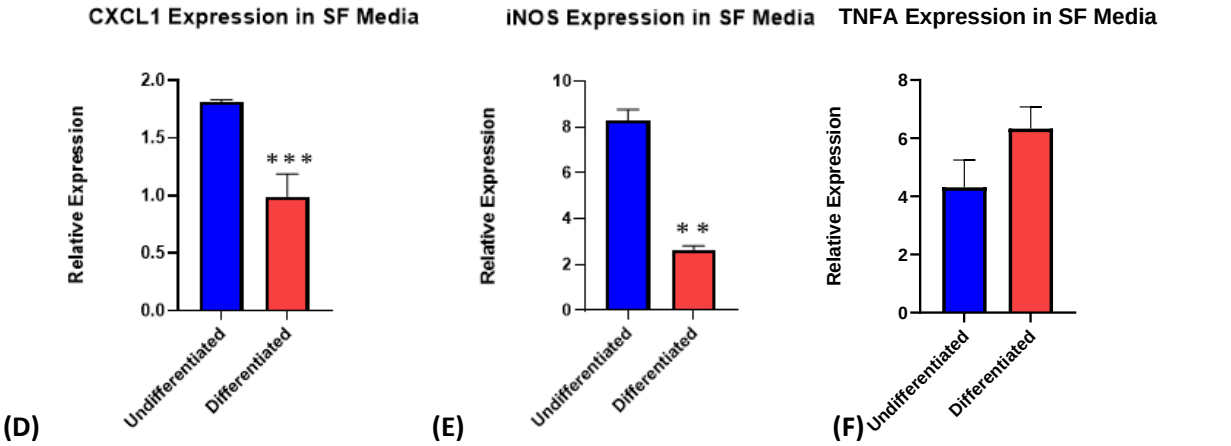

Differentiated HL60 TNFA Expression
Fold Change
2.0
1.5
1.0
0.5
0.0
SF
ASPC
Treatment

(G)

Figure 2.7: Differentiated HL60 cells cultured in tumor cell supernatant displayed no significant changes in secretory factors

(A) Quantitative RT-PCR for the expression of *CXCL1* in differentiated HL60 cells cultured in serum-free media and supernatant derived from ASPC tumor cells. **(B)** Quantitative RT-PCR for the expression of *CXCL2* in differentiated HL60 cells cultured in serum-free media and supernatant derived from ASPC tumor cells. **(C)** Quantitative RT-PCR for the expression of *IL23* in differentiated HL60 cells cultured in serum-free media and supernatant derived from ASPC tumor cells. **(D)** Quantitative RT-PCR for the expression of *CXCL8* in differentiated HL60 cells cultured in serum-free media and supernatant derived from ASPC tumor cells. **(E)** Quantitative RT-PCR for the expression of *TGFB* in differentiated HL60 cells cultured in serum-free media and supernatant derived from ASPC tumor cells. **(F)** Quantitative RT-PCR for the expression of *iNOS* in differentiated HL60 cells cultured in serum-free media and supernatant derived from ASPC tumor cells. **(G)** Quantitative RT-PCR for the expression of *TNFA* in differentiated HL60 cells cultured in serum-free media and supernatant derived from ASPC tumor cells. The values are mean fold change ± SEM; unpaired t-test, assuming both populations have the same SD; **P <0.005; *P<0.0001; *** for P ≤ 0.05.


IL23 Expression in SF Media
CXCL2 Expression in SF Media
IL8 Expression in SF Media
Relative Expression
400
300
200
100
0
50
40
30
20
10
150
***
Undifferentiated
Differentiated
(A)
(B)
(C)


CXCL1 Expression in SF Media
iNOS Expression in SF Media
TNFA Expression in SF Media
Relative Expression
2.0
1.5
1.0
0.5
0.0
10
8
6
4
2
**
Undifferentiated
Differentiated
(D)
(E)
(F)

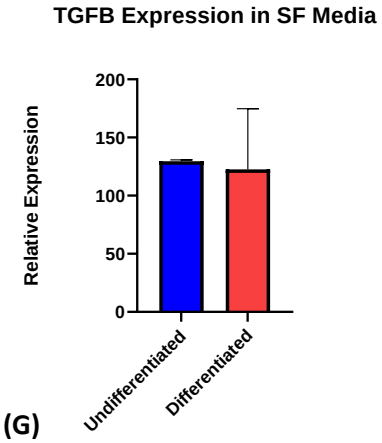
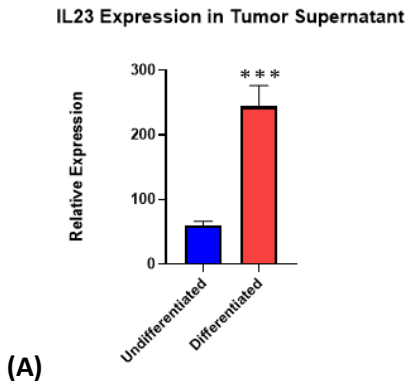
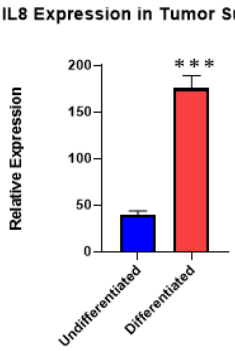

TGFB Expression in SF Media
Relative Expression
200
150
100
50
0
Undifferentiated
Differentiated
(G)

Figure 2.8: Differentiated and undifferentiated HL60 cells cultured in SF media displayed significant changes in secretory factors IL23, CXCL2, CXCL8, CXCL1, and iNOS

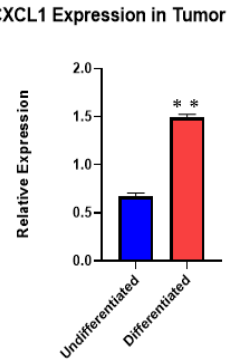
(A) Quantitative RT-PCR for the expression of IL23 in differentiated and undifferentiated HL60 cells cultured in SF media. **(B)** Quantitative RT-PCR for the expression of *CXCL2* in differentiated and undifferentiated HL60 cells cultured in SF media. **(C)** Quantitative RT-PCR for the expression of *CXCL8* in differentiated and undifferentiated HL60 cells cultured in SF media. **(D)** Quantitative RT-PCR for the expression of *CXCL1* in differentiated and undifferentiated HL60 cells cultured in SF media. **(E)** Quantitative RT-PCR for the expression of *iNOS* in differentiated and undifferentiated HL60 cells cultured in SF media. **(F)** Quantitative RT-PCR for the expression of *TNFA* in differentiated and undifferentiated HL60 cells cultured in SF media. **(G)** Quantitative RT-PCR for the expression of *TGFB* in differentiated and undifferentiated HL60 cells cultured in SF media. The values are mean relative expression ± SEM; unpaired t-test, assume both populations have the same SD; **P <0.005; *P<0.0001; *** for P ≤ 0.05.


IL23 Expression in Tumor Supernatant
Relative Expression
300
200
100
0
***
Undifferentiated
Differentiated

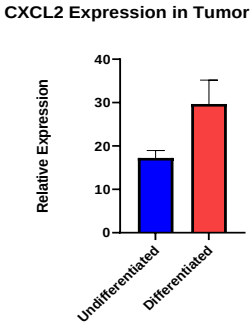
(A)


IL8 Expression in Tumor Supernatant
Relative Expression
200
150
100
50
0
***
Undifferentiated
Differentiated

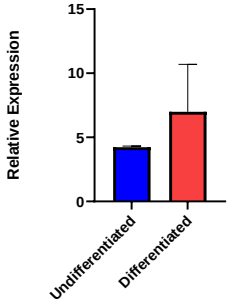
(B)


CXCL1 Expression in Tumor Supernatant
Relative Expression
2.0
1.5
1.0
0.5
0.0
* *
Undifferentiated
Differentiated

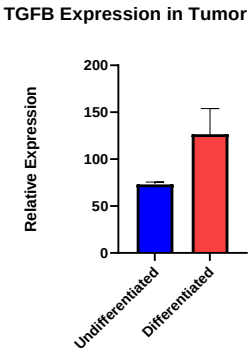
(C)


CXCL2 Expression in Tumor Supernatant
Relative Expression
40
30
20
10
0
Undifferentiated
Differentiated

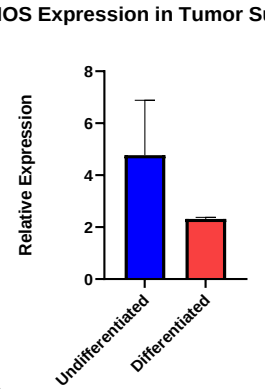
(D)


TNFA Expression in Tumor Supernatant
Relative Expression
15
10
5
0
Undifferentiated
Differentiated

(E)


TGFB Expression in Tumor Supernatant
Relative Expression
200
150
100
50
0
Undifferentiated
Differentiated

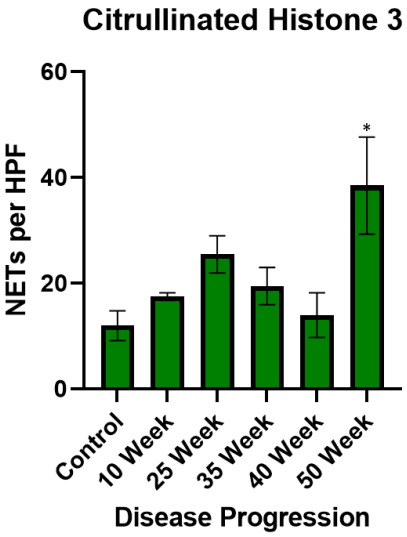
(F)


iNOS Expression in Tumor Supernatant
Relative Expression
8
6
4
2
0
Undifferentiated
Differentiated

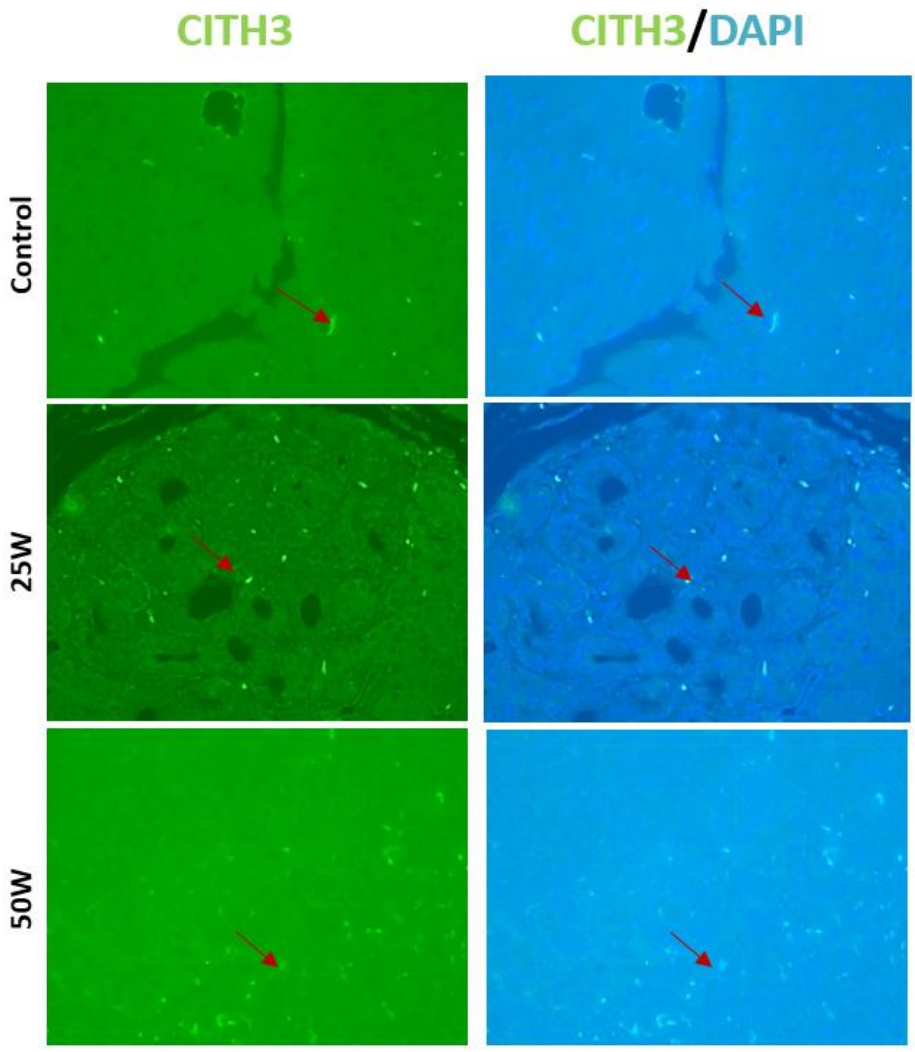
(G)

Figure 2.9: Differentiated and undifferentiated HL60 cells cultured in ASPC derived supernatant displayed a significant increase in secretory factors IL23, CXCL8, CXCL1 expression

(A) Quantitative RT-PCR for the expression of *IL23* in differentiated and undifferentiated HL60 cells cultured in ASPC derived supernatant. **(B)** Quantitative RT-PCR for the expression of *CXCL8* in differentiated and undifferentiated HL60 cells cultured in ASPC derived supernatant. **(C)** Quantitative RT-PCR for the expression of *CXCL1* in differentiated and undifferentiated HL60 cells cultured in ASPC derived supernatant. **(D)** Quantitative RT-PCR for the expression of *CXCL2* in differentiated and undifferentiated HL60 cells cultured ASPC derived supernatant. **(E)** Quantitative RT-PCR for the expression of *TNFA* in differentiated and undifferentiated HL60 cells cultured in ASPC derived supernatant. **(F)** Quantitative RT-PCR for the expression of *TGFB* in differentiated and undifferentiated HL60 cells cultured in ASPC derived supernatant. **(G)** Quantitative RT-PCR for the expression of *iNOS* in differentiated and undifferentiated HL60 cells cultured in ASPC-derived supernatant. The values are mean relative expression ± SEM; unpaired t-test, assume both populations have the same SD; **P <0.005; *P<0.0001; *** for $P \leq 0.05$.


Citrullinated Histone 3
NETs per HPF
60
40
20
0
*
Control
10 Week
25 Week
35 Week
40 Week
50 Week
Disease Progression

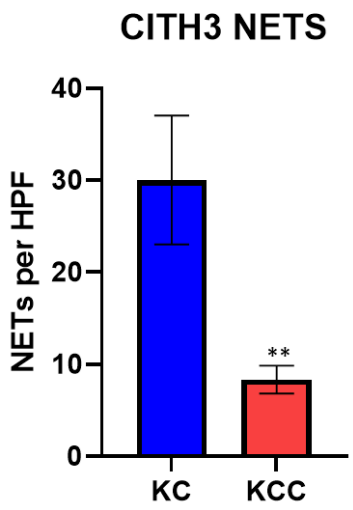
(A)


CITH3
CITH3/DAPI
Control
25W
50W

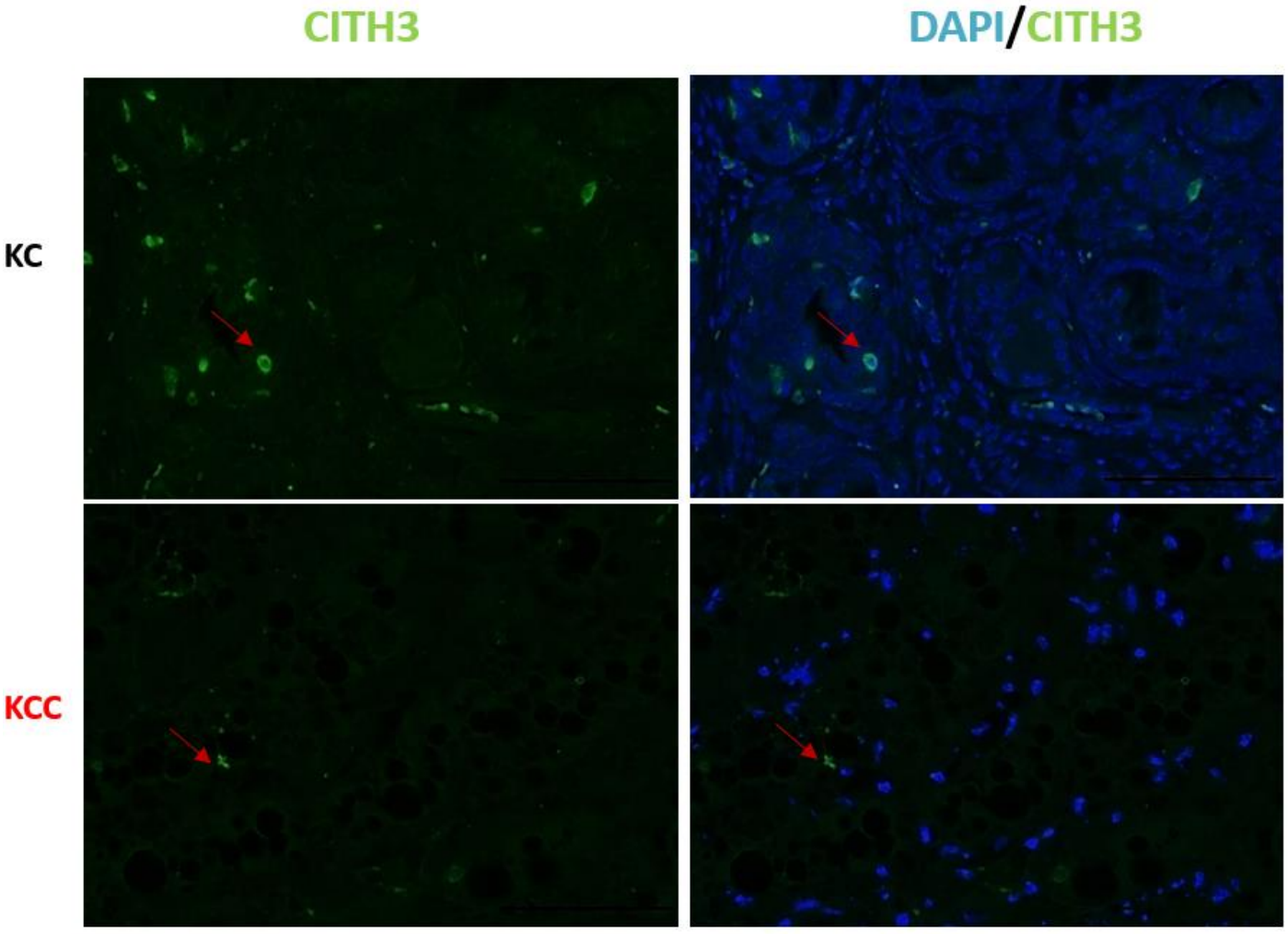
(B)

Figure 2.10: NET Infiltration increased as PDAC progressed in our KC model

(A) Quantitative measurement of citrullinated histone 3 positive NET's in our KC progressive murine model**. (B)** Representative immunofluorescence imaging of citrullinated histone 3 positive NET in our KC progressive murine model. The values are shown as mean ± SEM, Unpaired t-test; * for $P<0.05$.


CITH3 NETS
NETs per HPF
40
30
20
10
0
**
KC
KCC

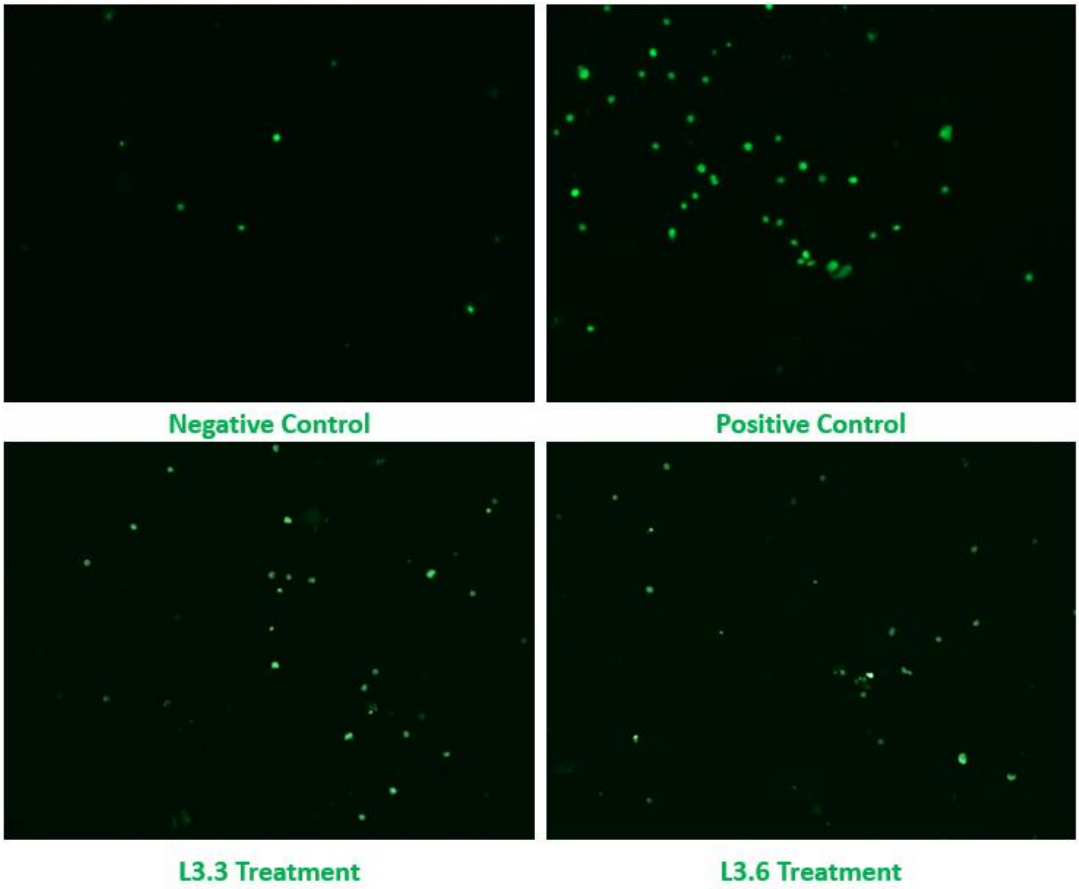
(A)


CITH3
DAPI/CITH3
KC
KCC

(B)

Figure 2.11: NET Infiltration decreased in our murine KCC model

(A) Quantitative measurement of citrullinated histone positive NET's in our KC and KCC murine model. **(B)** Representative immunofluorescence imaging of citrullinated histone 3 positive NET's in our KC and KCC murine model. The values are shown as mean ± SEM, Unpaired t-test,** for P< 0.01. The pictures were acquired under a magnification of 200X. The scale bars are shown as 10 μm.


Negative Control
Positive Control
L3.3 Treatment
L3.6 Treatment

(A) (B)

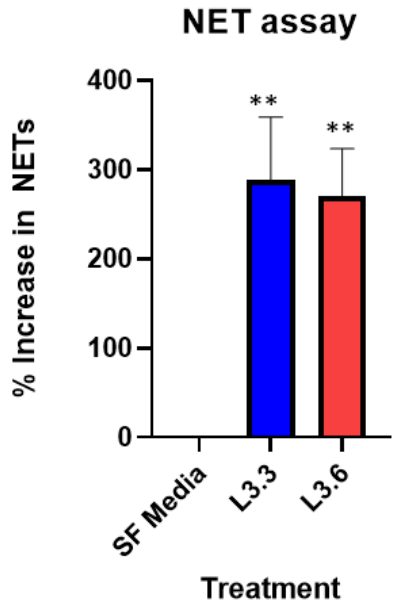

NET assay
% Increase in NETs
400
300
200
100
0
**
**
SF Media
L3.3
L3.6
Treatment

Figure 2.12: NET counts increased when treated with supernatant derived from L3.3 and L3.6 tumor cell-derived supernatant

(A) Representative immunofluorescence imaging of Sytox green positive NETs treated with SF media (negative control), SF media with 20 nM PMA (positive control), and tumor cell supernatant derived from L3.3 and L3.6 tumor cell lines. **(B)** Quantitative measurement of Sytox green positive NETs treated with SF media (negative control) and tumor cell supernatant derived from L3.3 and L3.6 tumor cell lines.

DISCUSSION

One of the established hallmarks of cancer is persistent, chronic inflammation in the TME. This inflammation is induced by two pathways[78]. The intrinsic pathway involves genetic mutations, resulting in oncogene activation and diminished tumor suppressor activation. This is commonly observed in PDAC, where the KRAS mutation is often the initiating event of tumorigenesis and is present in 90% of all PDAC tumors[76]. This gene encodes the KRAS protein, commonly associated with cell proliferation and differentiation, and is highly upregulated in PDAC[76]. The other pathway of inflammation involves inflammation generated by the TME[78]. The TME in PDAC is complex and dynamic, consisting of stromal cells, fibroblasts, immune cells, and desmoplasia[103]. Invading leukocytes such as macrophages and neutrophils are highly prone to release ROS and RNS upon activation, often orchestrating downstream inflammatory pathways in the tumor.

Neutrophils, the most abundant white blood cells in our body, play a dubious role in tumor progression. On the one hand, many reports have observed neutrophils carrying out their normal anti-tumor responses, involving inflammation and cytotoxicity[104]. However, an increasing number of studies show that neutrophils play a pro-tumorigenic role in cancer progression by fostering an immunosuppressive environment, driving angiogenesis, and releasing proteinases[104]. This theory is further cemented in PDAC, where a high NLR ratio indicates poor prognosis in patients[13]. This seemingly conflicting nature of these immune cells can, in theory, be interpreted by the presence of different subpopulations of neutrophils in the body[67]. However, this classification is highly controversial, and much work needs to be done in this area. In this study, we investigated the neutrophil-tumor interaction in PDAC.

Our first set of experiments investigated neutrophil invasion in the TME of PDAC. Our KC mouse model Pdx1-cre;LSL-Kras$^{(G12D)}$ displayed increased neutrophil numbers as the disease progressed towards its late stages **(Fig 2.1)**. These results are coincident with other studies indicating that elevated neutrophil infiltration leads to poor prognosis in PDAC[79]. The PDAC TME establishes high expression of the chemotactic cytokines CXCL1, CXCL2, and CXCL5, and neutrophils are attracted to these regions due to their expression of surface receptor CXCR2. The CXCR2 receptor specifically binds to the cytokines mentioned above and is thus involved in chemotaxis. Neutrophils also can release these chemotactic cytokines themselves once activated and possess the ability to create a positive feedback loop in the TME. We observed impaired neutrophil invasion in our CXCR2$^{+/-}$ Cre-LSL-Kras^{G12D} murine model **(Fig 2.2)**. This opens the possibilities of therapeutic strategies involving blocking the CXCR2 receptors, thus decreasing neutrophil invasion leading to potentially improved prognosis.

Once we established an increase in neutrophil numbers as PDAC progressed, we investigated the capability of tumor cells to favor neutrophil survival. Neutrophils were previously ignored in cancer research due to their short lifespan. However, recent reports suggest enhanced neutrophil survival in the TME[58]. Our experiments determined a significant increase in neutrophil survival when treated with supernatants derived from multiple PDAC cell lines **(Fig 2.3)**. These results suggest that neutrophils survive for more extended periods in the PDAC TME. Previous findings suggest that tumors upregulate the expression of the critical neutrophil growth factors G-CSF and GM-CSF, thus creating an environment favoring neutrophil survival in the TME[57].

Moreover, Zhang et al. have recently shown that high expression of G-CSF in human PDAC patients led to an overall poor prognosis[80]. This could be the result of an influx of immunosuppressive neutrophils in the TME. Thus, our studies reaffirm that the TME fosters neutrophil survival in PDAC.

Neutrophils are well established in promoting tumor proliferation in many cancer types. N2 neutrophils behave as anti-inflammatory neutrophils and are involved in immunosuppression and angiogenesis, critical milestones responsible for tumor development and even metastasis. Our co-culture assay **(Fig 2.4)** determined that neutrophil-tumor cell interaction in proximity increased tumor cell survival. Recently, Lianyuan et al. showed similar results where their PDAC cell lines displayed increased survival when co-cultured with neutrophils at low density[81].

However, our co-culture assay also led to a decrease in neutrophil survival **(Fig 2.5)**. This was a very surprising outcome, which leads us to hypothesize that these neutrophils undergo NETosis, resulting in neutrophil cell death and the formation of NET's. NET's have been mainly implicated in facilitating metastasis in cancer by releasing proteases and entrapping circulating tumor cells[58]. To further substantiate these claims, we observed an increase in NET formation in our KC mouse model Pdx1-cre;LSL-$Kras^{(G12D)}$ **(Fig 2.10)** as the disease progressed and also observed a decrease in NET formation in our $CXCR2^{+/-}$ Cre-LSL-$Kras^{G12D}$ murine model **(Fig 2.11)**. Although these results may merely reflect the neutrophil infiltration observed in our previous assays, we also found that neutrophils conditioned with tumor cell supernatant displayed a propensity towards NETosis **(Fig 2.12).** Coincidentally, tumor-expressed G-CSF was determined to be an inducer of NETosis in tumor-associated neutrophils[82]. This could potentially explain

why we observed an increase in NET formation in our experiments. However, the clinical relevance of NETosis in cancer progression is a relatively unexplored area, particularly in the context of tumor proliferation and survival. Still, much work needs to be done in this field.

We also investigated multiple secretory factors released by neutrophils when conditioned with tumor cell supernatant. Surprisingly, our undifferentiated HL-60 neutrophils downregulated CXCL1 and TGFB expression **(Fig 2.6)**. CXCL1 is an important chemokine belonging to the CXC chemokine family and is primarily involved in neutrophil chemotaxis. A mounting pile of evidence suggests that tumor cell-derived TGFB polarizes tumor-associated neutrophils towards the N2 state. Primarily, these studies reported attenuation of tumor development following TGFB blockade in various models[67]. However, the effect of neutrophil-derived TGFB in cancer has been poorly documented. Previous reports suggest that TGFB expression stimulates neutrophil chemotaxis and can thus theoretically explain why we also observe a decrease in CXCL1 expression in tandem with TGFB expression[83]. Moreover, these results indicate the ability of neutrophils to become anti-tumorigenic when exposed to tumor cell supernatant.

Our differentiated HL-60 neutrophils showed no significant changes in any cytokine expression when treated with tumor cell-derived supernatant (**Fig 2.7**). Our differentiated HL-60 neutrophils displayed increased expression of CXCL8, IL23, and CXCL2 when treated with either serum-media or tumor cell supernatant relative to undifferentiated HL-60 neutrophils (Fig 2.8,2.9). Many reports suggest differentiated HL-60 neutrophils possess chemotactic ability compared to undifferentiated HL-60 cells through the upregulation of integrins such as MAC-1 (CD11b-CD18)[84]. These integrins bind to ICAM

molecules on the endothelial cells, permitting neutrophil extravasation in the body. In line with these observations, CXCL2, another member of the CXC chemokine family, widely influences neutrophil chemotaxis in tumors. CXCL8, previously called neutrophil-activating peptide 1, is a neutrophil chemotactic factor and can regulate angiogenesis and metastasis in the TME. Il23 is another pro-inflammatory cytokine involved in T helper 17 cell regulation and function. A major drawback of these assays is that HL-60 cells are not classified as a true neutrophil cell line. They were derived from circulating leukocytes in 36-year-old women with acute promyelocytic leukemia[105]. Thus, they may not accurately represent live neutrophils in the TME of PDAC. However, because of the short lifespan of live neutrophils, the HL-60 cells proliferative ability makes them an ideal neutrophil model in our assays.

Interestingly, it was recently discovered that IL23 contributes towards neutrophil chemotaxis in mice during the bacterial colon infection *Clostridium difficile* colitis[85]. Taken together, upregulation of chemotactic factors CXCL8, IL23, and CXCL2 in our experiments display the propensity of our differentiated HL60 cells towards chemotaxis compared to undifferentiated HL60 cells. To our surprise, we observed a decrease in iNOS expression in our differentiated HL-60 cells. Nitric oxide (NO), produced by inducible nitric oxide synthase (iNOS), is cytotoxic in large concentrations, and INOS is usually downregulated in tumor-associated neutrophils[86]. It is also known that iNOS expression is increased in differentiated neutrophils in an inflammatory environment[87]. However, similar to our result, Kawase et al. [88] discovered that HL-60 cells differentiated via DMSO alone were insufficient to induce iNOS expression in these cells.

CONCLUSION

Taken together, our results examined the intertwined roles of neutrophils and tumor cells in the TME of PDAC. Increased recruitment of neutrophils and their by-product, NETs, were observed as PDAC progressed. This is due to increased expression of CXCL2 ligands and increased neutrophil survival.

Previous reports suggest that neutrophil incidence in the TME becomes widespread as the tumor develops, often leading to poor prognosis in most cancer subtypes. These neutrophils seem to play a distinct role in tumorigenesis and by no means are just by-products of inflammation in the body. Indeed, the neutrophils in our assays were able to stimulate tumor cell survival *in vitro*, hinting that neutrophils might play a pro-tumorigenic role in PDAC.

The increased propensity of neutrophils in our experiments to undergo NETosis highlights the ability of neutrophils to persist in the TME even past death, further inhibiting the body's response against the tumor. However, it still cannot be concluded that neutrophils play a pro-tumorigenic role in cancer. Many studies have reported that various neutrophil subtypes often lead to an anti-tumorigenic attack in the TME[104]. In line with this, our neutrophils showed a decrease in expression of pro-tumorigenic factors when treated with tumor cell supernatant.

We also determined that the differentiation of neutrophils led to an upregulation of multiple chemotactic factors, hinting that neutrophil differentiation in the TME plays a major role in the recruitment of neutrophils from the bone marrow. Overall, our studies attempt to highlight the complexity between neutrophil and tumor interaction in PDAC. They can process both pro and anti-tumorigenic properties. Neutrophil influence in cancer is still a relatively new concept, and extensive work needs to be done in this field.

Nevertheless, we hope neutrophils can become a viable therapeutic target in the future for cancer.

FUTURE DIRECTIONS

There are still many unanswered questions in our work that will be addressed in the future.

Expression of G-CSF and GMCSF in the TME

We theorized that one of the major reasons we observed increased neutrophil survival in our mouse models and *in vitro* assays was the expression of neutrophil growth factors G-CSF and GM-CSF in the TME. Future studies can prove this theory by staining these growth factors on our tissue sections or measuring their concentrations in tumor supernatant via ELISA.

The N1/N2 Polarization

Neutrophil function in the TME is well documented to carry both a pro-tumorigenic (N2) and an anti-tumorigenic (N1) role. It would be interesting to identify what phenotype we come across in our *in vitro* assays. However, the lack of specific surface markers between the N1/N2 phenotype remains to be a challenging aspect in this regard. Influencing this polarization towards the N1 state might lead to an effective therapeutic response. This may be achieved through either TGFB blockade or IFN upregulation in the TME.

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