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Review Article

Tumor-associated neutrophils in pancreatic cancer progression and metastasis

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Abstract: Pancreatic cancer (PC) remains a challenge to modern-day cancer therapeutics, with a dismal five-year survival rate of 12%. 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 often results from infiltrating leukocytes and pro-inflammatory cytokines released into the tumor microenvironment (TME). Neutrophils, one of our body's most prominent immune cells, are essential in sustaining the inflammation observed in the TME. Recent reports demonstrate that neutrophils are complicit in cancer progression and metastasis. Additionally, abundant data suggest that tumor-associated neutrophils (TANs) could be considered as one of the emerging targets for multiple cancer types, including PC. This review will focus on the most recent updates regarding neutrophil recruitments and functions in the cancer microenvironment and the potential development of neutrophils-targeted putative therapeutic strategies in PC.

Keywords: Neutrophils, cancer progression, metastasis, neutrophil extracellular traps, neutrophil polarization

Introduction

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 [1]. 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 leading cause of death in the United States, behind cardiovascular diseases.

Cancer is the uncontrolled growth of host cells, which can invade other body parts. Weinberg and Hanahan eloquently detailed specific characteristics a tumor cell develops during its life cycle in their landmark article in 2000 [2]. 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 [2]. Since then, Weinberg and Hanahan have broadened their horizon and added two additional hallmarks, 'dysregulating cellular metabolism' and 'avoiding immune destruction' [3].

One of the enabling characteristics of these hallmarks is tumor-promoting inflammation [3]. Cancer is often considered the 'wound which never heals' and is a constant source of inflammation. This results in the recruitment of different immune cells into the tumor region, further instigating inflammation and creating a positive

feedback loop. Many articles have highlighted the link between inflammation and tumor development, particularly in pancreatic cancer. Inflammation in pancreatic cancer derives from the cross-talk between immune cells and the tumor, carried out through various pro-inflammatory cytokines [4].

Pancreatic cancer

Although the future looks promising for most types of cancer, the threat of pancreatic cancer still looms. 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 leading cause of cancer-related deaths.

As the name suggests, pancreatic cancer originates from the pancreas, a vital organ in digestive and endocrine functions. Most of these cancers originate from exocrine cells (85%), thus called exocrine cancers. These cancers are challenging to diagnose early, and most patients end up being diagnosed when the cancer has metastasized to various organs, such as the liver. Unfortunately, surgical intervention is no longer viable at this point. The patient must undergo chemotherapy-based regimens, which are ineffective in 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 12% [5].

As in most cancers, pancreatic cancer tumorigenesis stems from oncogenic mutations in the exocrine cells. Almost 90% of these tumors have the KRAS oncogenic mutation involved in downstream signaling pathways enhancing survival [6]. Like most cancers, the p53 tumor suppressor gene is commonly found to be mutated in pancreatic cancer [7]. 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 [8]. It is broadly defined as intrinsic or acquired resistance [9]. Intrinsic resistance in PC 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) [6]. This stroma acts as a physical barrier preventing chemotherapeutic drug entry into the tumor, diminishing the effectiveness of chemotherapy regimens. Multiple pathways attain the acquired resistance or resistance conferred after prolonged treatment with chemotherapeutic drugs. A common pathway involves the efflux of hydrophobic drugs through the cell membrane using ATP-binding cassette transporters [10]. Another cancer cell trait showing an exciting role in chemoresistance is epithelial to mesenchymal transition (EMT), a crucial step toward metastasis in cancer [11]. 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 [12]. 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 the chemotherapeutic drug capecitabine in colorectal cancer patients [13]. 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 (HSCs) and mature in the bone marrow. About 10^{11} neutrophils are generated daily, making up for their short life spans of less than 24 hours [14]. 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 and can rapidly recruit other immune cells, like dendritic cells and T-cells, to the site of infection by releasing cytokines like CCL3 [15] and CXCL12 [16], respectively. These cells are also very potent phago-

cytes and will usually assist in clearing pathogenic invasions. Neutrophil infiltration is intertwined with cancer progression and is known to be involved in tumorigenesis, angiogenesis, immunosuppression, and metastasis [17].

Neutrophil structure

Neutrophils are relatively small cells with a diameter of around 15 micrometers. Neutrophils are identified by their unique multi-lobular nuclei, 3 to 5 lobes expressed during their maturation, interconnected by chromatin through histone proteins. As is typical of granulocytic cells, their cytoplasm contains a multitude of different granules, containing proteins such as Myeloperoxidase (MPO) and Neutrophil Elastase (NE), each able to release potent anti-microbial and inflammatory enzymes upon activation.

Neutrophil development and life span

Neutrophils originate from HSCs in the bone marrow. After subsequent downstream signaling, these HSCs eventually develop into granulocyte-monocyte progenitors (GMPs). Granulocyte colony-stimulating factor (G-CSF), a type of growth factor/glycoprotein produced in the bone marrow, and other transcription factors are essential in further differentiating GMPs into myeloblasts, which eventually develop into mature neutrophils through stimulation of factors such as CEBP/A, CEBP/E, and GFI1 [18].

The fully matured neutrophils are released in a highly regulated manner to preserve self-tolerance and homeostasis. Many chemotactic factors such as CXCL1 and 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 (PRRs) 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 cross-talk between neutrophils and cancer [19].

Neutrophil methods of activation

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

Phagocytosis

Phagocytosis is the process by which cells engulf other cells or materials through various receptor-mediated signaling processes. Neutrophils are often called '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 and eventually matures into a vesicle with a more anti-microbial composition, killing the pathogen trapped inside [20].

In the context of cancer, neutrophils readily phagocytose opsonized tumor cells through a process known as antibody-dependent cellular phagocytosis (ADCC) [8]. However, tumor cells are usually larger than neutrophils, posing a challenge to the complete phagocytosis of neutrophils [21].

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. There are four types of granules expressed by the neutrophil. Azurophilic granules contain bactericidal enzymes like MPO, hydrolases, cathepsin-G (CG), neutrophil elastase (NE), and defensins [22]. Secondary granules comprise lactoferrin, an integral part of the mucosa, and neutrophil gelatinase-associated lipocalin [22]. Tertiary granules contain

matrix metalloproteinase 9 (MMP9), often linked with ECM remodeling [22]. Last, secretory granules contain various pathogen recognition and complement receptors [22]. The degranulation process is tightly regulated through intracellular molecules β-arrestins and soluble NSF attachment protein (SNAP) [23].

Several enzymes released by neutrophils are linked to cancer progression. CG is a serine protease that resides in neutrophil primary granules. CG is pre-synthesized in promyelocytes in bone marrow, then stored in neutrophils' primary granules as active proteases. The high isoelectric points for CG (12) cause them to be easily caught in negatively charged traps, for instance, neutrophil extracellular traps (NETs) [24]. NE is also known as a serine protease, contributed mainly by neutrophils. Similar to CG, NE is pre-synthesized in promyelocytes and stored in neutrophil granules in active form. The high isoelectric points for NE (larger than 9) also cause them to be easily trapped in negatively charged NET [24]. NE is found to initiate and upregulate cancer-related signaling, for instance, EGFR/MEK/ERK signaling [25] and phosphatidylinositol 3-kinase (PI3K) signaling [26]. Interactions between NE and signaling result in higher levels of pro-cancer factors, such as transforming growth factor-beta TGF-β [27]. NE significantly promotes cancer cell proliferation, metastasis, and therapy resistance [28, 29]. Various studies showed that inhibition of NE reduces tumor progression in multiple cancer types [29, 30].

NE and CG promote lung metastasis by degrading the anti-cancer protein Thrombospondin 1 (Tsp1) [31]. CG and NE are also involved in ECM remodeling in the TME [32]. Moreover, ECM remodeling is very crucial for cancer metastasis. Other than NE and CG, neutrophil-released matrix metalloproteases (MMP) such as MMP-8 and MMP-9, are also found to be involved in ECM remodeling to facilitate cancer progression [32, 33].

MMP is defined as a cluster of enzymes whose catalytic abilities require the involvement of zinc [34]. MMP-9 is stored in neutrophil tertiary granules [35]. The release of MMP-9 is delicately regulated by various cytokines and growth factors, including the tumor necrosis factor (TNF), TGF-β, and vascular endothelial growth factor (VEGF) [35-37]. After release from the neutrophil granules, MMP-9 plays a pro-tumor role through mechanisms such as remodeling of ECM by degradation of extracellular proteins (type IV collagen, for instance) [34], membrane cleavage [38] or activating pro-tumor factors including TGF-β [39].

NETosis

Neutrophil extracellular traps (NETs) result from 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 been controversial since their discovery in 2004 [40]. 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 [41].

The process of NETosis primarily involves the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which provokes nuclear membrane disintegration and chromatin de-condensation through NE, MPO, and various histones, respectively [42, 43]. Citrullinated histone H3 is commonly used as a NET biomarker. NE and MPO work synergistically by degrading various histones, causing chromatin de-condensation [43]. 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 retains some neutrophil functions such as chemotaxis and phagocytosis and is independent of NADPH oxidase formation [44, 45].

An abundance of various neutrophil activators in the TME, such as CXCL8 and CXCR1/CXCR2 ligands [46, 47], often creates a hospitable environment for NET formation. Like their precursor neutrophils, NETs have been implicated in both pro-tumor and anti-tumorigenic capabilities [41]. They were shown to potentially orchestrate circulating tumor cell extravasation by binding to them [48]. 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 tumor cell degradation [47].

Neutrophil to lymphocyte ratio (NLR) in cancer

Inflammation, one of the ten hallmarks of cancer [3], is a key factor in instigating tumorigenesis in cancer. This systemic inflammation results in invading 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 microenvironment, forming a positive feedback loop.

The NLR ratio is often described as an indicator of 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 (TAN) levels. TANs are often complicit in angiogenesis and tumorigenesis by releasing MMPs and ROS radicals. 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 [49] and PDAC patients after surgical resection [50]. Xiang et al. evaluated the efficacy of the NLR ratio in multiple PDAC patients who had undergone surgical resection. They 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 [51].

Neutrophil recruitment in cancer

As previously explained, HSCs possess the ability to differentiate into neutrophils. Once these neutrophils are fully matured in the bone marrow, they are released into the bloodstream and destined to circulate in 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 inflammation around the tumor microenvironment is often primarily responsible for recruiting neutrophils in the tumor area. A myriad of different chemokines are 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, a critical step toward tumorigenesis [52]. Himmel et al. showed that CXCL8 is produced by regulatory T cells, which could be another potential source for neutrophil recruitment [53].

CXCL1 and CXCL2, highly expressed by tumor cells, are also potent chemokines in neutrophil recruitment through the CXCL1-CXCR1 axis (**Figure 1**). Previous studies have linked G-CSF with this axis and postulate that the combined effect stimulates neutrophil chemotaxis [54]. It is also interesting to note that tumor-associated neutrophils release CXCL1 and CXCL2 and can potentially attract circulating neutrophils into the tumor, thus creating a positive feedback loop.

Neutrophils in chemoresistance

Chemotherapy is one of the most prevalently used tools in the fight against cancer and for patient survival. However, one of the significant challenges when treating cancer is therapeutic resistance. Currently, researchers have found that the tumor microenvironment is closely linked with therapy resistance. The changes in the tumor microenvironment include polarization of immune cells to a pro-tumor type and secretion of cytokines and proteases that promote angiogenesis and metastasis [55]. Neutrophils are a significant component in the tumor microenvironment and are found to play a pivotal role in chemotherapy resistance. NLR is a prognostic indicator of patients' survival and is a valuable marker for resistance to chemotherapy. The higher the NLR, the more likely a patient is to develop resistance when receiving chemotherapy drugs, which indicates lower survival rates for cancer patients [56-58]. Targeting neutrophils in PDAC enhances the thera-

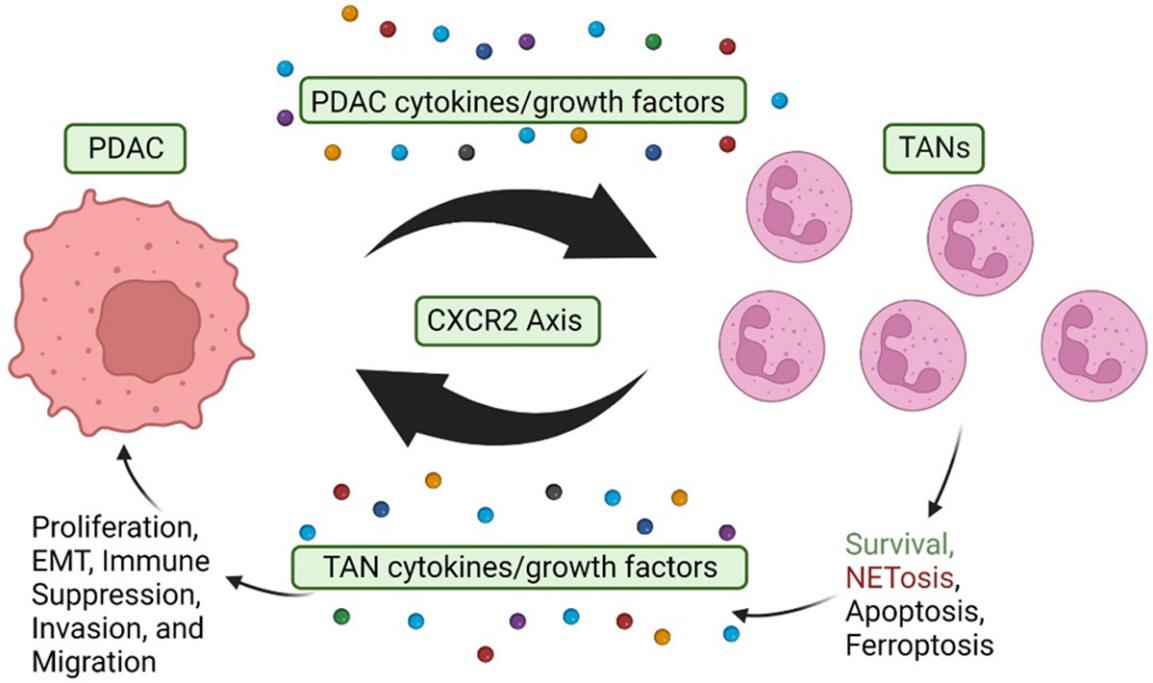

Figure 1. Neutrophil-PDAC interaction in the microenvironment. PDAC and cancer cells interact in the tumor microenvironment (TME) in a manner that still needs to be further elucidated. PDAC cancer cells interacts with neutrophils in the TME via cytokines and growth factors to cause phenotypic change in neutrophil to become tumor associated. Neutrophils then undergo several processes - such as NETosis, Apoptosis, etc. - That causes changes in their cytokine and growth factor production. These changes in turn effect the overall progression of PDAC through various pathways that still need further exploration. Created with BioRender.com.

peutic response to chemotherapy drugs [59]. Neutrophils can release pro-angiogenic factors such as MMP-9, MMP-8, CXCL8, and NE to promote resistance to gemcitabine [60-62]. Gemcitabine is a common chemotherapy drug for multiple cancer types, including pancreatic cancer. Neutrophil-released TGF-β can also be involved in the EMT process, which also promotes tumor cells establishing resistance to gemcitabine [63]. Neutrophils can facilitate cancer cell resistance to chemotherapy drugs and tumor cell resistance to anti-angiogenesis therapy [64]. Tumors gain resistance to VEGF inhibition therapy by neutrophil secretion of IL-17, which positively recruits neutrophils into the tumor microenvironment [65].

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 pro-inflammatory N1 phenotypes or anti-inflammatory N2 phenotypes. This nomenclature was derived from the M1/M2 classification used for macrophages. Their functional phenotypes define the N1/N2 dichotomy, as no specific cell markers have been discovered. This polarization depends on cytokine signals received in the TME [66], suggesting that the tumor stage (early or established) plays a role in establishing the neutrophil phenotype.

N1 phenotype

Like 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 interferon-beta (IFN)-β expression in the TME [67]. They are pro-inflammatory [18] and are also known to display direct tumor cell cytotoxicity by releasing potent anti-microbial substances like peroxidases and nitric oxide or through ADCC [68]. Because of these reasons, N1 neutrophils are widely regarded as anti-tumorigenic.

N2 phenotype

In contrast, the N2 neutrophil phenotype is comparatively long-lived, has circular nuclei,

and is primarily immuno-suppressive. TGF-β is widely known to induce naïve TANs 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 [69, 70].

ROS and RNS radicals produced by these neutrophils have been shown to cause genetic instability, promoting tumorigenesis in some cancer models [71]. 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.

Although convenient, the N1/N2 classification model remains controversial because of the absence of specific surface receptors and the difficulty of observing these neutrophil subtypes in humans [72]. Moreover, secretory substances released by these neutrophils, such as ROS, possess a dual role in cancer. Depending on their concentrations, they can play anti-tumorigenic or pro-tumorigenic roles [73].

Neutrophil survival in the TME

Neutrophils are universally considered to have a half-life of around 5.5 hours in the bloodstream [74]. However, in the context of cancer, various reports advocate that they persist in the TME for extended periods [75]. It has been suggested that tumor-derived cytokines may attenuate neutrophil apoptosis [19, 76, 77]. TANs and tumors may work in tandem to increase each other's survival, as it was shown that human and neck squamous adenocarcinomas had increased neutrophil survival *in vitro*; in addition, these neutrophils expressed MMP-9, a well-established pro-angiogenic factor [78].

Tumor-associated neutrophil induced immunosuppression

TANs are widely known to have immunosuppressive abilities. These qualities account for the tumor's steady 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 cross-talk between myeloid-derived suppressor cells (MDSCs) and macrophages stimulated M2 polarization [79]. TANs also debilitate NK cell function, which, together with the activity of cytotoxic T-cells, constitute effective tumor cell destruction [80]. Despite sharing the same myeloid precursors, MDSCs are always considered pro-tumorigenic, while neutrophils display pro-tumorigenic and anti-tumorigenic abilities [81]. MDSCs are also usually less dense than neutrophils [81]. Youn et al. showed that tumor-bearing murine MDSC's highly expressed CD115 and CD244 relative to neutrophils might be used as potential surface receptors [82].

Although neutrophils have been shown to impair other innate immune cells, the most significant blow of their immunosuppressive abilities is felt by T-cells, which play a critical role in the adaptive immune system. Arginase-1, upregulated in N2 neutrophils, has been shown to impair T-cell function [83]. Degrading arginine also leads to cell cycle arrest in T-cells, thus preventing replication.

Neutrophils can recruit 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 in MDSCs, interacts with regulatory T-cells and promotes their accumulation [84]. Another study showed that the activation of MDSCs through PGE_2 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 is highly upregulated in MDSCs localized in murine tumors, often suppressing T-cell response [85]. 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 [86], 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 body organs. Tumor cells detach from the primary tumor site, enter the bloodstream, and extravasate into other organs [87]. 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 [88].

Metastatic cascade

The metastatic cascade is a marathon of complex and challenging events that a tumor cell must overcome to progress toward 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 [89]. 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 essential 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 [2, 3].

There is a very significant relationship between angiogenesis and metastasis. Not only does angiogenesis support tumor growth, accelerating its progression toward the advanced metastatic stage, but 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 [90].

TANs aid in the process of angiogenesis in multiple ways. They are a major source of VEGF, commonly known as a pro-angiogenic factor, which binds to resident epithelial cells, maintaining the 'on' state of the angiogenic switch [91]. They also release MMPs, which are notoriously known for remodeling the ECM, allowing the new blood vessels much-required space to grow [68].

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 [92]. 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 brought upon by various transcription factors such as Snail and Twist [93]. Further downstream signaling results in a dwindling expression of E-cadherin on the cell membrane [94]. Proteins responsible for strengthening the basement membrane and tight junctions and gap junctions around the tumor cells are downregulated because these transcription factors stimulate the transition from epithelial to mesenchymal phenotype [95]. Correspondingly, this transition is characterized by the up-regulation of proteins such as vimentin and N-cadherin [96].

Many studies have linked TANs with the EMT transition observed in tumor cells. It was recently shown that the expression of specific mesenchymal markers was increased when gastric cancer cells were treated with neutrophils *in vitro* [97]. They proposed that neutrophils instigate the JAK2/STAT3 pathway in tumor cells by releasing IL-17, resulting in EMT. In another study, a similar co-culture assay was performed using neutrophils with human epithelial ovarian cancer cells, which decreased E-cadherin expression on the cancer cell [98].

Circulating through the bloodstream

Tumor cells that have successfully escaped from the confines of their primary organ site and entered 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 [99]. Current techniques for measuring CTCs involve using epithelial cell adhesion molecule (EPCAM) as a surface marker, which may underrepresent mesenchymal CTCs. In the PDAC context, neutrophils were clustered around CTCs, potentially behaving as a physical protective barrier against other cytotoxic immune cells like NK and cytotoxic T-cells [100].

Extravasation

Extravasation is another crucial process involving the escape of the CTC into distant tissue. CTCs may undergo a similar approach to leukocyte extravasation, involving selectin-mediated rolling and adhesions with the capillary bed through integrin and cadherin expression [101]. The homing of CTCs to a distant metastatic site is not considered 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].

Recent literature suggests neutrophils help in CTC extravasation by protecting them from natural killer (NK) cells, which are very effective tumor cell killers [103]. Through intravital microscopy, another group of scientists showed that neutrophils might assist in circulating tumor cell adhesion in their murine model for liver metastasis [104]. Neutrophils may also behave as a 'chaperone' to CTCs by interacting with tumor cell-expressed ICAM-1 and various neutrophil receptors [105, 106].

Establishment of metastasis in the secondary site

The final obstacle tumor cells face in the metastatic cascade is establishing 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 the CTC invasion [107]. Paget et al. showed that bone marrow-derived hematopoietic cells were linked with metastasis through the expression of VEGFR1. Like the primary tumor site, a largely immunosuppressive microenvironment persists in pre-metastatic niches. MDSCs, which suppress T-cell activity, are commonly found in these niches [108]. Surprisingly enough, TIMP1, primarily released by tumor cells, was also involved in niche formation in the liver through neutrophils [109]. 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 [110]. Although EMT is a crucial step in metastasis, it is also theorized that MET, mesenchymal-to-epithelial transition, occurs during this colonization phase [111]. 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 proliferation by releasing versican, a large proteoglycan [112].

Conclusion

Although science has significantly improved in fighting pancreatic cancer, a long road is ahead of us. Due to their short lifespans, neutrophils are usually ignored in cancer progression. However, the last decade of research elucidates a 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 can persist in the TME for long periods. They may also undergo NETosis and become NETs, which are linked with cancer progression. Neutrophils have also been complicit in metastasis and assisting tumor cells in each step of the metastatic cascade.

Due to their critical involvement in tumor progression, neutrophils become attractive targets for novel immunotherapeutic strategies. Friedlander et al. have shown that blocking TGF-β in the TME increased the anti-tumorigenic phenotype's neutrophils [69]. 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 [91]. Thus, we can improve cancer therapeutics by steering the polarization of naïve TANs in the TME.

Disclosure of conflict of interest

None.

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