Neutrophils in cancer: neutral no more

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Summary
Neutrophils are indispensable antagonists of microbial infection and facilitators of wound healing. In the cancer setting, a newfound appreciation for neutrophils has come into view. The traditionally held belief that neutrophils are inert bystanders is being challenged by recent literature. Emerging evidence indicates that tumors manipulate neutrophils, sometimes early in their differentiation process, to create diverse phenotypic and functional polarization states able to alter tumor behavior. In this Review, we discuss the involvement of neutrophils in cancer initiation and progression, and their potential as clinical biomarkers and therapeutic targets.

The name neutrophil – given to polymorphonuclear, granulocytic cells by Paul Ehrlich in the late 19th century – is based on the inability of these cells to retain acidic or basic dyes and for their preferential uptake of pH neutral dyes[1]. Although their neutral staining led to the identification of these cells, neutrophils in the cancer setting are anything but neutral. Neutrophils in tumor-bearing hosts can oppose or potentiate cancer progression. These two types of behavior are controlled by signals emanating from cancer cells or stromal cells within the tumor microenvironment, which educate neutrophils to execute the demise of the tumor or facilitate support networks that lead to its expansive spread. These functions can occur locally in or around the tumor microenvironment, as well as systemically in distant organs.

Until the past few years, other immune cells such as macrophages have overshadowed the role of neutrophils in cancer. But recent studies and the development of new genetic tools have provided the cancer community with new insights into the profound influence of these dynamic cells by uncovering distinct capabilities for neutrophils throughout each step of carcinogenesis: from tumor initiation to primary tumor growth to metastasis. During these processes, neutrophils take on different phenotypes and sometimes opposing functions. Emerging evidence also indicates that these cells are highly influential, and are able to change the behavior of other tumor-associated cell types – primarily other immune cells. In this Review, we focus on how tumors manipulate the generation and release of neutrophils from the bone marrow. We discuss the mechanisms identified in animal models by which neutrophils participate in tumor initiation, growth and metastasis. Finally, we highlight the potential of these cells as clinical biomarkers and therapeutic targets.

Neutrophil origins and life cycle: homeostasis versus cancer [Au: subheading too long, please shorten to <39 characters; possibly 'Homeostasis versus cancer' would work?]

Comment [SC1]: I don't like "Homeostasis versus cancer" The reader has no idea what we are referring to until they read the subheading "Granulopoiesis." What about "Neutrophil origins and life cycle"?

In humans, neutrophils are the most abundant immune cell population, representing 50-70% of all leukocytes. Over 10^{11} neutrophils may be produced per day[2], and tumors can increase this number by even more. Indeed, patients with various cancer types, including but not limited to breast, lung and colorectal cancer, often exhibit increased numbers of circulating neutrophils[3,4]. Recent studies have identified key pathways that tumors exploit to disrupt normal neutrophil homeostasis and these are discussed below.

Granulopoiesis

To accommodate for the notably high production and turnover of neutrophils, the bone marrow devotes about two-thirds of its space to the formation of neutrophils and monocytes

in steady-state conditions[5]. During granulopoiesis, neutrophils arise from lymphoid/myeloid-primed progenitors (LMPPs)[6], which are derived from hematopoietic stem cells (**Figure 1**). LMPPs further differentiate into granulocyte/monocyte myeloid progenitors (GMPs) and many transcription factors required for this process have been identified (reviewed in [5,7,8]). Neutrophil maturation then begins, as GMPs differentiate through the following sequence: myeloblast, promyelocyte, myelocyte, metamyelocyte, banded neutrophil and, finally, a segmented neutrophil (reviewed in [5,9-11]). The transition from myeloblast to promyelocyte is marked by the first appearance of primary granules. Secondary and tertiary granules form sequentially during the myelocyte to metamyelocyte and band cell to segmented cell stage, respectively[5,12]. These granules compartmentalize an arsenal of defensive factors and enzymes, such as myeloperoxidase, elastase, defensins, cathelicidins and matrix metalloproteinases (MMPs), that protect against opportunistic infections and mediate the resolution of inflammation (reviewed in [12,13]). If large numbers of neutrophils are used up during infection or cancer, a process called emergency granulopoiesis overtakes steady state granulopoiesis to rapidly increase neutrophil formation[11]. In tumor-bearing mice and humans with pancreatic or colon cancer (and most likely other tumor types), the spleen is an alternative source of neutrophil production[14].

Granulocyte-colony stimulating factor (G-CSF) is the master regulator of neutrophil generation and differentiation[15-17]. G-CSF acts at the level of myeloid progenitors to induce their proliferation and differentiation. Its receptor, G-CSFR, is expressed throughout the myeloid lineage from early stem and progenitor cells to fully differentiated neutrophils[18,19], and G-CSFR-STAT3 (signal transducer and activator of transcription 3) signaling governs neutrophil formation[20]. The transcription factor RAR-related orphan receptor γ1 (RORC1) is a recently identified regulator of myelopoiesis in tumor-bearing mice and its expression may be induced by G-CSF[21]. However, G-CSF is not absolutely required for granulopoiesis, as other molecules – such as granulocyte-macrophage-colony stimulating factor (GM-CSF), interleukin 6 (IL-6) and KIT ligand (KITL) – can play a redundant, but lesser role[22-24]. Tumors in many mouse models of cancer upregulate these cytokines, causing overactive granulopoiesis and neutrophilia[25-31].

Neutrophil retention and release from bone marrow

One feature of granulocytes that sets them apart from every other immune cell is their release from the bone marrow as terminally differentiated, mature cells. Circulating mature neutrophils account for only 1-2% of all neutrophils throughout the body under homeostatic conditions[32]. Mature cells are retained in the bone morrow by an interplay between two C-X-C chemokine receptors, CXCR4 and CXCR2. Constitutive CXCL12 expression by

osteoblasts and other bone marrow stromal cells tether CXCR4^{+} neutrophils in the bone marrow, whereas secretion of CXCL1 and CXCL2 by endothelial cells and megakaryocytes encourage the release of neutrophils into the circulation via CXCR2 signaling[33-38] (**Figure 1**). Several adhesion molecules, such as integrin subunit α4 (ITGA4) and vascular cell adhesion molecule 1 (VCAM1), as well as some proteases are also important in neutrophil retention[39-41]. In addition to its positive influence on granulopoiesis, G-CSF is a well-known disruptor of neutrophil retention[42]. G-CSF pressures the bone marrow to release neutrophils through thrombopoietin (TPO)-induced upregulation of CXCR2 ligands on megakaryocytes[38], reduction of CXCL12 expression by bone marrow stromal cells[43,44] and downregulation of CXCR4 on neutrophils themselves[45].

Outside the bone marrow, a cascade of other cell types and cytokines, involving IL-23-expressing phagocytes and IL-17-producing lymphocytes, tightly regulates the production of G-CSF so that neutrophil numbers are maintained in the circulation. In this feedback mechanism, macrophages and dendritic cells phagocytose apoptotic neutrophils[47-49], curbing the secretion of IL-23[46] – a cytokine that controls IL-17 expression by $\alpha\beta$ T cells, $\gamma\delta$ T cells, innate lymphoid cells and other lymphocytes[50,51]. Because IL-17 is upstream of G-CSF[52,53], lower levels of IL-17 equate to reduced expression of G-CSF and steady-state release of neutrophils from the bone marrow[46]. Commensal bacteria and enterocyte-derived CXCL5 in the gut also play a role in neutrophil homeostasis by increasing or inhibiting IL-17 production, respectively[54,55]. IL-1β that is released from dying cells or upregulated in response to inflammatory stimuli is another potent inducer of the IL-17-G-CSF axis[56,57].

Many of the molecules that control neutrophil release from the bone marrow are frequently upregulated in tumors or systemically as a result of a tumor[25-28,58]. These factors override retention signals in the bone marrow, facilitating neutrophil egress and elevated numbers of circulating neutrophils (**Figure 2**). Cancer cells themselves produce these cytokines[27,28,58], but stromal and immune cells can also contribute to their elevated expression in tumor-bearing mice. For example, tumor-associated macrophages are a well-known source of IL-1β[59]. Recently, we showed that neutrophils expand in mammary tumor-bearing *K14-Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mice because of increased macrophage-derived IL-1β stimulation of the IL-17-G-CSF axis[26]. Ectopic overexpression of IL-1β in tumors derived from cancer cell lines or a genetically engineered gastric cancer model also increases the number of circulating neutrophils[60-63]. As such, aberrant production of cytokines by tumors or stromal cells can offset the balance of neutrophil retention and release from the bone marrow.

The pressure on the bone marrow to release neutrophils can often be so intense in tumor-bearing hosts that undifferentiated cells are set free prematurely. Nuclear staining of circulating neutrophils from mammary and lung tumor models has revealed the existence of

ring-like, banded and segmented nuclei[26,64-66]. We and others recently reported that a proportion of these cells express KIT[26,31], a marker of lymphoid, myeloid and neutrophil progenitor cells[25,67], suggesting that these KIT-expressing cells are most likely meta-myelocytes and/or banded neutrophils[67]. Circulating neutrophils from breast, lung and colorectal cancer patients also show a similar mix of differently shaped nuclei[64,68]. However, the consequence of immature neutrophils in the bloodstream of tumor-bearing hosts is not entirely understood. Interestingly, immature neutrophils and neutrophil progenitor cells – some of which express KIT – are found in mouse models and patients with inflammation[69-73]. These KIT^+ cells differentiate into fully mature neutrophils *in situ* at sites of *Staphylococcus aureus* infection[70,74]. Thus, it is tempting to speculate that differentiation at inflammatory sites or tumors primes immature neutrophils for functions they would not ordinarily perform.

The ectopic appearance of immature neutrophils in the circulation may have profound consequences on tumor progression. An example of this was shown in mice with chemically induced cancer crossed with histamine-deficient mice, where the lack of histamine stalled differentiation of immature neutrophils and increased tumor incidence and growth[75]. These data suggest that immature cells have independent functions from mature neutrophils. Indeed, the phenotype and behavior of mature, aged neutrophils is not the same as young, newly released circulating neutrophils, even in tumor-free mice[76]. One explanation for the difference between immature and mature neutrophil function may be their distinctive composition of granules, because granules are synthesized at specific stages of neutrophil development[12] (**Figure 1**). Recent studies using density gradient purification methods have shown that distinct populations of neutrophils with different *ex vivo* properties can circulate within the same tumor-bearing mouse and individual cancer patients[64]. Whether these populations are truly committed to divergent cell fates or represent cells at assorted stages of maturation remains undetermined.

Neutrophil lifespan

One reason neutrophils have received less attention than other immune cells in the cancer arena is the commonly held belief that neutrophil lifespan is too short to influence cancer progression. The current paradigm is that circulating neutrophils have a half-life of around 7 hours in healthy humans[2,77] and 8-10 hours in mice[78]. However, there are an equal number of reports challenging these kinetics as too short or too long (reviewed in [79]). The discrepancy between these studies lies mainly in limits of the methodology and neutrophil labeling techniques currently available, and therefore the lifespan of neutrophils in tumor-bearing hosts is unclear. Animal experiments in calves and mice have shown that a small pool of non-circulating neutrophils can survive in tissue for several days[80,81]. Neutrophils are

also retained longer in tumors than in the spleen[82], suggesting that the tumor microenvironment encourages their survival both locally and systemically. Indeed, pioneering work from Mantovani and his colleagues in the 1990s showed that many tumor-associated cytokines prolong neutrophil survival in culture[83]. In line with this, there is evidence that the half-life of circulating neutrophils is extended in cancer patients to 17 hours[84], which may be the result of pro-survival signaling by G-CSF[20]. A longer life may give neutrophils more time to synthesize new molecules and perform additional effector functions during tumor progression.

Tumor-induced neutrophil polarization and activation

One major theme that has emerged from the cancer field is that not all neutrophils are equal. Neutrophil polarization leads to divergent phenotypes, depending on specific tumor-derived factors. Transforming growth factor β (TGFβ), G-CSF and interferon β (IFNβ) are the most well-studied molecules in this process. TGFβ and G-CSF activate a tumor- and metastasis-promoting program[25,27,65,85-88], by regulating the transcription factors inhibitor of DNA 1 (ID1), retinoblastoma 1 (RB1) and interferon regulatory factor 8 (IRF8) that control the immunosuppressive functions of neutrophils[25,87,89,90]. IFNβ acts as a negative regulator of the pro-tumorigenic phenotype of neutrophils[91,92]. Cytokine concentration and tumor physiology (such as hypoxia) may also be important for neutrophil polarization, because cytotoxic neutrophils are shaped into cancer-promoting cells as tumors expand and evolve[93]. It is currently unclear at which differentiation step these molecules instruct phenotypic changes in neutrophils. For G-CSF, there is evidence that this cytokine can affect gene expression in stem or progenitor cells and fully differentiated cells as G-CSFR is expressed throughout neutrophil development[18,19]. These data suggest that neutrophil polarization is programmed early in the developmental process in the bone marrow, but when and where individual molecules shape neutrophil polarization needs further attention. Understanding the influence of the cytokines discussed here, as well as others, will provide more insights into how neutrophil activation goes hand in hand with granulopoiesis.

Neutrophil polarization states have been divided into ‘N1’ or ‘N2’ categories to mirror the Th1/Th2 and M1/M2 nomenclature of T cells and macrophages, respectively[65]. The study introducing the N1/N2 nomenclature noted a difference in neutrophil polarization after treating mice bearing subcutaneous mesothelioma tumors with a TGFβ inhibitor. Neutrophils in untreated mice supported tumor growth through inhibition of $CD8^+$ T cells, whereas neutrophils from TGFβ inhibitor-treated mice opposed tumor growth through their cytotoxic ability[65]. However, knowledge surrounding N1- and N2-polarized neutrophils has not progressed much beyond this original study. Their surface markers, cytokine expression

patterns, transcription factor regulators and other hallmarks of activation are largely unknown. In non-cancerous disease models driven by type 1 or type 2 immunity, the role of neutrophils in the disease phenotype is not well understood. It is unclear whether neutrophils respond to type 1-associated cytokines (i.e. IFNγ) or type 2-associated cytokines (i.e. IL-4 and IL-13) or whether neutrophils produce these cytokines to affect disease phenotype. Although some studies addressing these issues are emerging[94,95], the lack of concrete evidence in mice or humans raises the question of whether the N1/N2 terminology can be applied to cancer-associated neutrophils.

The study proposing the N1/N2 terminology characterized N1 neutrophils by a hypersegmented nucleus and N2 neutrophils by banded or ring-like nuclei[65]. Because nuclear morphology is a hallmark of neutrophil differentiation[10], it is unclear whether the so-called N2 neutrophils are just immature cells or represent a distinct polarized state, leaving the relationship between polarization and maturation unresolved. Nevertheless, the binary N1/N2 classification system is most likely an oversimplification of neutrophil polarization for the same arguments that have been given against using 'M1' and 'M2' to describe tumor-associated macrophages[96-98]. Similarly to macrophages, neutrophil polarization probably exists as a spectrum of activation states, rather than only two extremes. We suggest that researchers should follow the recent advances in the macrophage field and apply a combinatorial nomenclature that describes neutrophil activation status[99].

A further complication to the picture of neutrophil subtypes is the ongoing debate on the kinship of neutrophils and myeloid-derived suppressor cells (MDSCs), and it is currently unclear whether these are analogous or separate populations (**Box 1**).

Neutrophils and tumor initiation

Over the past two decades, it has become apparent that mutations in normal cells are required but not sufficient for tumorigenesis. Inflammation plays an essential role in initiating tumorigenesis by damaging specific tissues[100], and neutrophils are a critical component of this process. Inflammation-induced models of cancer initiated by chemical carcinogens, such as the dimethylbenz[a]anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA) skin cancer model and the azoxymethane (AOM)/dextran sodium sulphate (DSS) colitis-associated colon cancer model, have established the importance of neutrophils in tumor initiation (**Figure 3**). In these models, neutrophils are attracted to tumor-prone tissues via the CXCR2 ligands, CXCL1, CXCL2 and CXCL5[101-104]. Application of these carcinogens to CXCR2-deficient mice, which show impaired neutrophil trafficking, prevents papilloma or adenoma formation[102,104]. Similarly, CXCR2 ligands are increased in several genetically engineered mouse models, including the intestinal adenoma *Apc*$^{Min/+}$ model, the invasive

intestinal adenocarcinoma *Ah-CreER;Apc$^{F/+}$;Pten$^{F/F}$* model and the spontaneous oral papilloma *K14-CreER;Kras$^{G12D/+}$* model. In these models, CXCR2 deficiency or inhibition retards tumor formation[102]. However, it should be noted that CXCR2 expression is not exclusive to neutrophils. Depletion of the entire neutrophil population using anti-Ly6G antibodies phenocopies CXCR2 deficiency and hinders tumorigenesis in both chemically induced[101,102] and spontaneous tumor models[102]. In a zebrafish model of *Hras^{G12V}*-driven melanoma, wounding-induced inflammation increases the formation of tumors in a neutrophil-dependent manner[105]. Thus, neutrophils can provide a causal link between inflammation and cancer.

Tumors in various mouse models of KRAS-driven lung cancer – such as *Cc10-Cre;Kras^{G12D}* (also known as *Ccsp-Cre;Kras^{G12D}*), *Adeno-Cre;Kras^{G12D}* and *Kras^{LA1}* models – upregulate neutrophil-related chemokines and display expansion of neutrophils[90,106-109] (**Figure 2**). These phenotypes may be a result of direct upregulation of neutrophil-related cytokines like GM-CSF and CXCL8 by KRAS signaling[29,30,110]. The IL-17-G-CSF axis is responsible for expanding neutrophils in at least some of these KRAS models[108], but whether these cytokines are regulated by KRAS is unknown. As in the chemical-induced colon and skin cancer models, depletion of neutrophils or inhibition of CXCR2 signaling reduces the number of pulmonary tumors in these KRAS models[108,109,111], indicating their dependence on neutrophils. The association between KRAS and neutrophils is even stronger in humans and mice exposed to cigarette smoke. Cigarette carcinogens cause specific activating mutations in KRAS[112,113] as well as inflammation and neutrophil accumulation[114]. These data raise the question of whether every KRAS-driven tumor type requires neutrophils for initiation and whether KRAS orchestrates their polarization.

How neutrophils foster tumorigenesis is not completely understood. Neutrophil-derived elastase and the immunosuppressive ability of neutrophils have both been implicated in tumor initiation[108,111,115], but the exact mechanisms need further elucidation. Neutrophil production of reactive oxygen and reactive nitrogen species (ROS and RNS) and angiogenic factors such as MMP9[116] may also be important for tumor initiation (**Figure 3**). In future work, genetically engineered mouse tumor models will be extremely valuable in this area of cancer-related neutrophil biology, as they allow neutrophils and neutrophil-derived factors to be manipulated as tumors arise *de novo*.

Neutrophils and tumor growth

Early studies on neutrophil function during tumor growth set the stage for the ongoing discussion over when and how neutrophils can be anti-tumorigenic or pro-tumorigenic. More than two decades ago, it was shown that neutrophils can mediate tumor rejection of transplanted G-CSF-producing colon cancer cells into mice[117]. A few years later, an opposing

tumor-promoting role was uncovered when mice bearing transplantable tumors that were depleted of neutrophils via anti-Gr1 antibody showed reduced tumor growth[118,119].

Since then, the literature showing a tumor growth-promoting role for neutrophils *in vivo* has largely outweighed the studies showing an opposite effect. One mechanism neutrophils employ to promote tumor growth is the induction of angiogenesis (**Figure 3**), and neutrophil depletion decreased tumor growth and microvessel density in both transplantable and spontaneous tumor models[65,85,91,120-123]. Blocking CXCR2 signaling or transplanting cancer cell lines into CXCR2-deficient mice recapitulated these effects[58,124,125]. In other studies, co-injection of cancer cell lines with neutrophils isolated from tumor-bearing mice increased tumor growth and angiogenesis[126], underscoring their ability to perpetuate proliferation. Several mitogenic and pro-angiogenic molecules have been implicated in neutrophil-driven tumor growth including elastase, prokineticin 2 (PROK2, also known as BV8) and MMP9[115,120,126-129]. Immunosuppression – through amino acid depletion or specific cytokine release – is another predominant mechanism neutrophils use to facilitate tumor progression[130]. Data from other disease models indicate that neutrophils are important players in directing adaptive immune responses (reviewed in [131]), but apart from their effects on cytotoxic T lymphocytes, many of the underlying mechanisms by which this is achieved are unknown in cancer. More recently, a new pro-tumorigenic function of neutrophils emerged showing that these cells counteract senescence via IL-1RA to promote prostate cancer progression in a PTEN-deficient autochthonous model[132].

Even though the literature on anti-tumorigenic neutrophils is less abundant, there have been some intriguing new data in this area. For example, neutrophils in mice with transplanted *MMTV-PyMT;MMTV-cMyc* mammary tumors hindered tumor growth[133], presumably through their cytotoxic effects mediated by H_2O_2. Neutrophil specific-deletion of MET, the hepatocyte growth factor (HGF) receptor, impaired recruitment of neutrophils to tumors and led to enhanced tumor growth of various transplantable cell lines and in a spontaneous liver cancer model[134]. Expression of MET in neutrophils was upregulated by endothelial cell- and cancer cell-derived tumour necrosis factor (TNF) in this study[134]; whereas others have shown that TNF signaling in $CD4^+$ T cells led to increased IL-17 levels and neutrophil accumulation in ovarian tumor-associated ascites[121]. These data suggest that the control of neutrophil behavior by TNF is context dependent. Notably, there are contradictory results regarding neutrophil function using the same transplantable cell lines. Some studies reported a pro-tumorigenic role of neutrophils, whereas other studies reported no effects in the 4T1 mammary[85,133] and the Lewis lung cancer (LLC)[134,135] models. The timing of neutrophil depletion experiments may be critical for the interpretation of these data, as neutrophil function evolves from anti-tumoral to pro-tumoral in mice bearing transplantable cancer cell lines[93]. Antibody-dependent cellular cytotoxicity (ADCC) is another mechanism

neutrophils can use to kill cancer cells after antibody therapy (reviewed in [136]). It remains to be seen whether ADCC occurs *in vivo* without exogenous antibodies, as cancer-induced endogenous antibodies are known to activate pro-tumoral programs in myeloid cells via Fc receptors[137,138]. Taken together, more research emphasis should be put on determining the context in which neutrophil behavior is modulated.

Several studies demonstrated the importance of neutrophils in tumor progression by blocking neutrophil recruitment to tumors, usually via CXCR2 inhibition. For instance, prostate cancer cells in *Probasin-Cre4;Pten$^{F/F}$;Smad4$^{F/F}$* mice upregulated CXCL5 via the Hippo-YAP1 pathway and blocking YAP1 or CXCR2 decreased immunosuppressive neutrophil recruitment to tumors and blunted tumor proliferation[139]. Less attention has been directed at understanding whether these recruitment factors are also important for neutrophil effector functions. In a *de novo* model of endometrial adenocarcinoma, progesterone receptor (*Pgr*)*-Cre;Pten$^{F/F}$* mice, blockade of neutrophil recruitment by genetic deletion of G-CSFR or CXCR2 increased uterine tumor burden[140]. Hypoxia-induced CXCL1, -2 and -5 recruited neutrophils, and these cells impeded tumor growth by promoting cancer cell detachment from the basement membrane via modulation of integrins. Interestingly, neutrophils deficient in MyD88 signaling maintained their trafficking ability, but lost their anti-tumorigenic functions[140]. These data suggest that CXCR2 ligands regulate neutrophil recruitment, not function. Future work should focus on whether the same is true for every tumor type and whether neutrophil-recruiting molecules can be uncoupled from neutrophil-activating molecules.

Tumor metastasis

Most neutrophil-centered studies published in the cancer field over recent years pertain specifically to metastasis. Neutrophils actively participate in different steps of the metastatic cascade: cancer cell escape from the primary tumor, intravasation into the blood and/or the lymphatic vascular system, survival in circulation, extravasation into distant organs and outgrowth of metastases (**Figure 4**). As early as the late 1980s – before the importance of neutrophils in primary tumor growth was established[117-119] – co-injection of cancer cells and neutrophils from tumor-bearing rodents intravenously was shown to increase experimental lung metastases[141,142]. Although these studies substantiated the pro-metastatic ability of neutrophils, this research area is surrounded by controversy, as opposing roles for neutrophils exist in the literature and often within the same model system.

The pro-metastatic role of neutrophils

A large body of literature indicates that neutrophils are most important during the early steps of the metastatic cascade. Enhanced retention of human melanoma cells in lungs can be

seen as early as 24 hours after co-injection with neutrophils into nude mice[143]. In experimental lung or liver metastasis models where cancer cell lines are injected into the circulation or spleen, respectively, systemic depletion of neutrophils (via anti-Gr1 antibodies) reduces the formation of metastases[144,145]. Intravital imaging has shown that cancer cells co-localize with endothelial cell-associated neutrophils in a CD11b-dependent manner[144], suggesting that neutrophils guide cancer cells into tissues and/or retain them there rather than supporting the outgrowth of secondary tumors. Neutrophils use neutrophil extracellular traps (NETs) for this purpose to sequester circulating cancer cells in a mesh of nucleic acids, antimicrobial factors and enzymes, and to promote adhesion at distant organ sites[146]. *In vitro*, NETs also stimulate cancer cell migration and invasion[146].

Experimental metastasis models bypass several initial steps of the metastatic cascade, including exit from the primary tumor, intravasation and priming of the pre-metastatic niche. Spontaneous models of metastasis indicate that neutrophils are important for intravasation and formation of the pre-metastatic niche. As mentioned above, neutrophils are potent effectors of angiogenesis[147], providing cancer cells with more routes of escape. Neutrophils can also direct cancer cells towards endothelial cells to promote intravasation into the circulation. For example, melanomas in *Hgf-Cdk4*^{R24C} mice exposed to ultraviolet (UV) light showed cancer cell clustering around blood vessels and increased lung metastasis but no effects on primary tumor growth[148]. In this setting, UV-induced damage to keratinocytes increased the levels of high mobility group box 1 (HMGB1), which recruits TLR4^{+} neutrophils to primary tumors. These neutrophils then facilitate cancer cell angiotropism and metastasis. *In vitro*, neutrophil-derived TNF stimulates the migration of melanoma cells, suggesting that TNF is at least one factor that neutrophils produce *in vivo* to initiate metastasis[148]. The same study found that ulcerated melanomas and the accompanying neutrophilic influx in patients are associated with greater melanoma-endothelial cell interactions and higher metastatic incidence. These data are supported by another study showing a strong correlation between neutrophil infiltration and the extent of ulceration[105]. Taken together, these studies indicate that neutrophils initiate interactions between cancer cells and endothelial cells in the vicinity of the primary tumor microenvironment to expedite metastasis.

An interesting consequence of tumor expansion at the primary site is the accumulation of neutrophils in visceral organs before the arrival of disseminated cancer cells[25,26,28,133,149-152], in what has been termed the pre-metastatic niche[153]. This accumulation of neutrophils in distant organs is highly reminiscent of the swarming behavior of neutrophils that occurs after injury, which is stimulated by neutrophil-derived leukotriene B4 (LTB4), a lipid by-product of the arachidonate 5-lipoxygenase (ALOX5) enzyme[154]. Recent data showed that LTB4 production by neutrophils in the pre-metastatic niche supports LTB4

receptor^{+} metastasis-initiating cells in the *MMTV-PyMT* mouse model, and that inhibition of ALOX5 reduces pulmonary metastasis without affecting primary tumor growth[152]. But why do these neutrophils accumulate in pre-metastatic organs? In tumor-bearing mice, primary tumors release factors that systemically condition distant sites for future metastases. Neutrophil accumulation at distant sites is G-CSF-dependent in some tumor models[25,26,28,152]; however, the original studies characterizing CD11b^{+} myeloid cell recruitment to the pre-metastatic niche implicated vascular endothelial growth factor A (VEGFA), TNF and TGFβ[153,155].

Some or all of these tumor-derived factors may also dictate whether neutrophils promote metastasis at distant locations. Indeed, the genetic loss of TGFβR2 or TGFβ signaling blockade in neutrophils decreased lung metastasis in the 4T1 mammary tumor model[86,88]. Interestingly, the TGFβ-induced immunosuppressive function of neutrophils occurs through an autocrine loop that is activated by regulatory B cells (B_{reg} cells)[88]. G-CSF is another factor that drives a pro-metastatic phenotype in neutrophils, and G-CSF presumably stems directly from cancer cells in the 4T1 model[27,28]. G-CSF induces PROK2/BV8 expression in neutrophils[26,156], which may induce cancer cell migration or vascular leakiness to support metastasis[28,128,129]. We recently identified another mechanism whereby G-CSF modulates neutrophil phenotypes and pro-metastatic functions[26]. In this mechanism, a systemic inflammatory cascade involving the secretion of IL-1β by mammary tumor-associated macrophages leads to IL-17 expression by γδ T cells and subsequently raises systemic G-CSF levels. G-CSF then stimulates neutrophil expansion and converts neutrophils into immunosuppressive cells that block the anti-tumor functions of CD8^{+} T cells, allowing disseminated cancer cells to evade immune detection[26]. Thus, both cancer cells and immune cells can educate the pro-metastatic abilities of neutrophils.

Neutrophil precursors are found ectopically in organs where metastases commonly occur. In the *K14-Cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mouse breast cancer model, we noted that a proportion of neutrophils in various tissues express KIT and display a mixed nuclear morphology[26]. Others have identified KIT-expressing cells in the pre-metastatic niche[28,153,157]. Antagonizing KIT signaling or inhibition of KIT ligand (KITL) expression by cancer cells prevents pulmonary metastasis formation in the 4T1 model[31], suggesting a pro-metastatic role for KIT^{+} neutrophils. In addition, C-C chemokine ligand 9 (CCL9)-CCR1 signaling mediates colon cancer metastasis through recruitment of immature myeloid cells and mature neutrophils[158,159]. These data indicate that the release of neutrophil precursors from the bone marrow supports metastatic progression.

The anti-metastatic role of neutrophils

In stark contrast to the studies above that described a metastasis-promoting role for neutrophils, others have shown that depletion of neutrophils increases metastasis[133,160]. The H_2O_2-mediated cytotoxic behavior of these anti-metastatic neutrophils is controlled by CCL2[133]. However, G-CSF still controls the transcriptional activity and expansion of neutrophils[26-28]. Controversially, these studies used the 4T1 mammary tumor cell line to show an anti-metastatic role[133], whereas other laboratories have used the same cell line to demonstrate a pro-metastatic role of neutrophils[28,88,150]. So, how can different studies of neutrophils produce contradictory results using the same cell line? The timing of neutrophil depletion experiments may be critical, as neutrophils isolated from early-stage tumors exhibit different behavior than neutrophils from late-stage tumors[93,161]. Another possibility may be that the cell lines used by independent labs are not actually the same at all. It is well known that *in vitro* culture places a selection bias on cancer cells, making them more prone to genetic drift[162]. As a result, the 'same' cell lines may diverge in the cytokines they produce. Likewise, the introduction of ectopic transgenes, such as luciferase or green fluorescent protein (GFP), may skew the secretome, immunogenicity or behavior of these cells. Microbiome differences between experimental animal cohorts may also influence neutrophil behavior in conflicting ways. Indeed, neutrophil ageing is controlled by the microbiota in tumor-free mice[76].

In addition to their production of H_2O_2[133,160], neutrophils can also limit the formation of metastases through their expression of thrombospondin 1 (TSP1)[163] and MET[134] in experimental metastasis models. However, pro-metastatic neutrophils deactivate TSP1 by elastase- and cathepsin G-mediated degradation after degranulation in lung tissue, and inactivation of TSP1 contributes to metastasis formation[164]. Interestingly, TSP1 can be induced in neutrophils by a peptide derived from prosaposin, a precursor of sphingolipid activator proteins, and treatment of MDA-231-LM2 mammary tumor-bearing mice with this peptide reduced spontaneous formation of pulmonary metastases without affecting primary tumor growth[163]. These data provide proof of principle that the pro-metastatic behavior of neutrophils can be switched *in vivo*, and could open up possible avenues of therapeutic intervention.

Clinical implications

Neutrophils as biomarkers in cancer patients

Although experimental studies have highlighted multifaceted and sometimes opposing roles of neutrophils in cancer, the bulk of clinical evidence assessing neutrophil to lymphocyte ratios (NLRs) mostly supports the notion that neutrophils promote, rather than inhibit, cancer progression[3]. The NLR has thus been proposed to be an attractive biomarker for risk stratification of patients with cancer and to guide treatment decisions. NLRs can easily and

cost effectively be determined using standard blood analyses. That said, at the level of individual patients, it might be challenging to translate a given NLR into a personalized prognosis or treatment plan due to the large variability in neutrophil levels between healthy individuals[165]. In addition, the variation in the reported NLR cutoff points used to allocate patients to high or low risk cohorts complicates the use of a single NLR determination for patient diagnostics and treatment.

To maximize the clinical utility of systemic neutrophil scores, it may be more informative to perform longitudinal measurements of NLR in individual patients. A rise in neutrophil counts and/or NLR over time may indicate disease recurrence or progression, and a drop in these values after initiation of therapy may indicate a good response. Thus far, a limited number of studies have attempted this approach. For example, in colorectal cancer patients, surgical removal of the primary tumor reduces the NLR in a proportion of patients, and a post-surgical low NLR is associated with improved survival[166]. Patients who have metastatic renal cell carcinoma with a low pre-treatment NLR that is maintained during treatment with tyrosine kinase or mTOR inhibitors experience a more favorable outcome[167]. It will be interesting to assess whether parallel scoring of patient serum levels of neutrophil-activating and polarizing soluble mediators, including IL-1β, IL-17, G-CSF, GM-CSF and/or TGFβ, increases the prognostic or predictive power of NLR measures.

In comparison to NLR, the prognostic and predictive power of intratumoral neutrophils is murkier and more variable, and positive (gastric[168]), negative (renal[169], melanoma[170]) or no (lung[171]) correlation with patient outcome has been observed in different studies. Colorectal cancer is one example where controversy surrounds the potential role of intratumoral neutrophils[172,173]. The markers used to identify tumor-associated neutrophils (such as CD66b, myeloperoxidase and cell morphology by haematoxylin and eosin staining) may explain these discrepancies, as expression of these markers in neutrophils may vary in different tumor microenvironments. NLR is more reliable in this way because blood neutrophils are easily separated from other immune cells by flow cytometry. Employing combinatorial markers in tumor sections based on neutrophil polarization may provide some clarity. In fact, combinatorial approaches involving assessment of the expression of multiple neutrophil-related genes have been recently applied to data sets from thousands of patients with cancer. Two independent studies found that the enrichment of neutrophil-associated genes correlates with poor prognosis when encompassing all solid tumor types[4,140]. Thus, moving beyond single markers may be necessary to accurately determine whether the numbers of intratumoral neutrophils has prognostic or predictive power.

Neutrophils as therapeutic targets in cancer patients

Neutrophils and their associated soluble mediators not only serve as prognostic and/or predictive biomarkers in cancer patients, but the versatile functions of neutrophils in cancer biology may also represent therapeutic targets. A relatively straightforward approach to target neutrophils in cancer types in which they are detrimental is via inhibition of their trafficking or activation. Importantly, the cancer field can take advantage of neutrophil-targeting agents that are being developed for the treatment of inflammatory and autoimmune diseases. For example, ongoing clinical trials with a CXCR2 antagonist in patients with chronic obstructive pulmonary disease have shown that treatment results in decreased absolute neutrophil counts, reduced inflammatory biomarkers and reduced disease symptoms[174]. The first clinical trials with reparixin, a CXCR1 and 2 inhibitor[175], are ongoing in cancer patients[176,177]. Importantly, characterization of neutrophil polarization in different tumor types as well as at early and late stages is urgently needed in order to maximize the utility of therapeutic modalities. In tumors in which neutrophils are beneficial, such as early stage lung cancer[161], strategies to magnify their anti-tumor abilities should be explored.

Another neutrophil-associated pathway under intense investigation is the IL-23-IL-17 axis (reviewed in [51]). The US Food and Drug Administration (FDA) approved antagonists targeting IL-12p40 (a subunit of IL-23) in 2009 and IL-17 in 2015 for the treatment of psoriasis, and these agents substantially improve quality of life in people with this disease. It would be interesting to investigate whether these already existing drugs are efficacious in cancer patients because pre-clinical models and clinical samples indicate that this pathway is important for cancer progression[26,68]. Therapeutic strategies aimed at re-polarizing tumor-induced neutrophils or interfering with their downstream pro-tumorigenic effects could offer additional opportunities for intervention[65,152].

Combining neutrophil targeting with other anti-cancer therapies

Successful implementation of neutrophil-targeting approaches in the clinic will require a critical assessment of the most optimal combination therapy strategies. In this regard, we can learn from the growing number of mechanistic studies performed in clinically relevant mouse tumor models that have addressed the impact of neutrophils on the efficacy of anti-cancer therapies. As mentioned above, neutrophils are important mediators of angiogenesis, so perhaps it is no surprise that neutrophils induce refractoriness of experimental tumors to anti-VEGFA therapy in an IL-17- and G-CSF-dependent fashion[178-180]. These data suggest that simultaneous inhibition of neutrophils and anti-angiogenic therapy might be an effective anti-cancer strategy. Indeed, therapeutic synergy is observed when anti-VEGFA therapy is combined with depletion of neutrophils via anti-Gr1 or anti-G-CSF antibodies[179,181].

Chemotherapy is another combination partner for neutrophil-targeting therapeutics; however, many types of chemotherapy negatively affect neutrophil production themselves.

Interestingly, chemotherapy-induced neutropenia is associated with improved survival in patients with non-small cell lung, breast, gastric or colorectal cancer[182-185]. This beneficial association may be explained by two reasons, one of which is neutrophil-independent and the other neutrophil-dependent. Because neutropenia is a surrogate marker of chemotherapy efficacy, lack of neutropenia in patients may indicate insufficient dosing and inadequate tumor killing. Alternatively, the patient survival benefit of chemotherapy-induced neutropenia may arise from reducing the neutrophils that counteract the efficacy of chemotherapy. A growing number of experimental studies have attempted to design strategic combination therapies, and some studies reported a beneficial role for neutrophils in chemotherapy responses, whereas others indicated that neutrophils counteract the anti-cancer efficacy of chemotherapy (recently reviewed in [186]). For example, depletion of $Gr1^+$ myeloid cells or $Ly6G^+$ neutrophils reduced the anti-cancer efficacy of cyclophosphamide and doxorubicin in tumor inoculation models[187,188]. These data contrast to the improved tumor inhibition achieved by combining CXCR2 blockade with doxorubicin, cyclophosphamide or docetaxel in xenograft and *de novo* tumorigenesis mouse models[58,132]. Moreover, some chemotherapeutics, such as gemcitabine and 5-fluorouracil, directly reduce the viability and/or change the functionality of myeloid cells, which then influences the anti-cancer efficacy of these drugs. These drugs trigger IL-1β secretion from immunosuppressive monocytes and neutrophils, setting off a chain of inflammatory events that resulted in a reduced efficacy of chemotherapy on subcutaneous EL4 thymomas in mice[189].

Another unresolved issue is the clinical benefits and risks of using recombinant G-CSF and GM-CSF to counteract chemotherapy-induced neutropenia. Neutropenia predisposes patients to life-threatening infections, therefore recombinant G-CSF or GM-CSF is commonly prescribed to counteract reduced neutrophil numbers brought on by chemotherapy and to lessen therapy-induced mortality[190,191]. However, experimental studies indicate that G-CSF polarizes neutrophils towards a pro-tumorigenic phenotype and promotes metastasis formation[25-28,87]. Two experimental studies examining tumor growth after combining chemotherapy with G-CSF neutralization reported contradictory results[28,192], leaving the debate open. Therefore, it is critical to carefully assess whether the beneficial effect of G-CSF in reducing susceptibility to infections outweighs its potential risk of accelerating disease progression in cancer patients.

Contrasting data also exist about the function of neutrophils in radiotherapy responses. Whereas anti-Ly6G antibody-mediated neutrophil depletion improves the efficacy of radiotherapy in a subcutaneous colon cancer model[193], antibody-mediated depletion of $Gr1^+$ cells does not alter radiotherapy responses of xenografted prostate cancer cells[194]. Taken together, the diverse and sometimes contradictory roles of neutrophils in anti-cancer

therapy responses may reflect differences in tumor type, tumor model, immune status of the host and mechanism of tumor killing by a particular anti-cancer therapy.

A promising therapeutic avenue is the combination of T cell checkpoint inhibitor immunotherapy with neutrophil manipulation[195]. Despite the success of immune checkpoint blockade, disease progression remains unabated in a significant proportion of treated patients[196]. Relieving neutrophil-induced immunosuppression may be one way to improve immunotherapy. Indeed, experimental studies have shown that anti-programmed cell death protein 1 (PD1) or anti-PD1 and anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA4) synergizes with anti-CXCR2 or anti-Ly6G, respectively, to delay tumor growth[197,198]. These studies support the concept that combining cancer immunotherapies with neutrophil suppression may increase therapeutic benefit.

In addition to T cell-based immunotherapies, macrophage inhibitors such as anti-colony stimulating factor 1 receptor (CSF1R) are also gaining traction in the clinic[199]. Data from a genetically engineered skin cancer model and transplantable mammary tumor models indicate that neutrophil infiltration into tumors and their systemic expansion is increased following macrophage blockade via CSF1R or CCR2 signaling[200,201]. Given the tight interplay between neutrophils and macrophages[131], neutrophils may be expected to promote resistance to macrophage-targeting therapies. In fact, neutrophils have been shown to mediate resistance to the anti-angiogenic drug sorafenib after macrophages are blocked in the *RIP1-Tag2* pancreatic and *MMTV-PyMT* mammary tumor mouse models[202]. Thus, targeting one myeloid cell population may require additional targeting of another myeloid cell population to counteract therapeutic resistance.

Conclusion and perspectives

The influential role of neutrophils on cancer biology and their potential as therapeutic targets are now widely recognized. Recent data have shed light on this underappreciated cell type, while at the same time, dispelling the myth of neutrophil neutrality. Currently, the complex roles of neutrophils in cancer not only include their ability to promote or prevent tumor progression, but also encompass various polarization states. Each of these realizations opens up new opportunities for therapeutic intervention. A recurring theme from recent literature that may help in the design of novel neutrophil-targeting, anti-cancer therapies is the crosstalk between neutrophils and other immune cell populations (**Table 1**). Interestingly, several of these communication networks mirror the same pathways in other disease models[94,203], suggesting that neutrophil-related inhibitors designed for specific inflammatory conditions may also be useful in cancer patients.

To gain a better understanding of these pathways and to discover new ones, sophisticated animal models that allow selective neutrophil manipulation are desperately

needed. Neutrophils die quickly during *ex vivo* culture limiting the utility of this technique; therefore, neutrophil biology is best studied *in vivo*. Researchers commonly use two antibodies to deplete neutrophils, anti-Gr1 and anti-Ly6G, but these invaluable tools are far from foolproof. Anti-Gr1 also affects inflammatory monocytes and other Ly6C-expressing cells[204], and neutrophils quickly reappear after antibody depletion in tumor-bearing mice[205]. Recently, a mouse model based on *Ly6g*-driven Cre recombinase was developed, the Catchup mouse, which includes a fluorescent reporter allowing the function of mature neutrophils to be monitored via *in vivo* imaging[206]. One value of this model stems from its ability to specifically delete neutrophil-derived molecules at later stages of these cells' differentiation. We predict that this model and others like it will provide valuable information about the involvement of neutrophils and their molecular products in tumor initiation, growth and metastasis. These models may also generate novel findings in other less-studied areas of neutrophil biology, including the metabolic processes that occur during their tumor-related functions. For the unresolved issues – such as the relationship between neutrophil polarization and maturation, as well as neutrophils versus granulocytic or polymorphonuclear (G/PMN)-MDSCs – single cell sequencing or single cell fate-mapping reporter tools should be coupled with identification of nuclear morphology and surface marker expression to better define the differences between activated neutrophils and immature cells. Together, these new methodologies are destined to provide novel insights into the not-so-neutral behavior of neutrophils in cancer and other diseases.

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Box 1: Neutrophils and MDSCs

Myeloid-derived suppressor cells (MDSCs) is a name assigned to a group of myeloid cells that suppress immune responses and express CD11b and Gr1 (reviewed in [130,207]). The appearance of MDSCs is a consequence of a pathological condition, such as cancer, infection and inflammation, driven by the aberrant expression of cytokines. These cells are rarely, if ever, found in homeostatic conditions. MDSCs encompass many immune cells at various stages of differentiation because of the non-specific nature of the Gr1 antibody used to identify them (clone RB6-8C5). Gr1 binds two antigens, Ly6C and Ly6G, which identify two major cellular subsets in tumor-bearing mice: $CD11b^{+}Gr1^{high}$ cells referred to as granulocytic or polymorphonuclear (G/PMN)-MDSCs and $CD11b^{+}Gr1^{low}$ monocytic (M)-MDSCs. These two populations are more accurately recognized by the use of specific Ly6G (clone 1A8) and Ly6C antibodies: $CD11b^{+}Ly6G^{+}Ly6C^{low}$ neutrophils and $CD11b^{+}Ly6G^{-}Ly6C^{+}$ monocytes. Because G/PMN-MDSCs and neutrophils share a common set of markers and are morphologically identical, there is a great deal of controversy and confusion surrounding the relationship between these cells. There is currently no way to uniquely identify one cell type from the other, so the question of whether neutrophils and G/PMN-MDSCs are distinct populations remains unanswered. Immaturity is often attributed to G/PMN-MDSCs as a feature that distinguishes them from fully differentiated neutrophils[130,207]. However, Gr1 and Ly6G recognize both mature and immature cells, so it is not technically possible to separate neutrophils from their precursors based on these markers. The assumption that all $CD11b^{+}Gr1^{+}$ cells in tumor-bearing mice are MDSCs should be avoided because not all $CD11b^{+}Gr1^{+}$ cells are immunosuppressive in tumor-bearing mice[138,208]. Thus, data in the literature need to be interpreted with caution.

In our view, the MDSC nomenclature is self-limiting. Assigning a name to a cell or group of cells based on one function such as immunosuppression implies that G/PMN-MDSCs predominately exist for one purpose or are incapable of performing any other activity. Myeloid cells are extremely dynamic and adaptable cells that carry out many different

functions simultaneously. In fact, neutrophils can be both pro-angiogenic and immunosuppressive[178]. This reality is often overlooked, because individual studies often focus on one particular functional aspect of a cell population while other functions remain untested. Therefore, we suggest that the use of the restrictive term MDSCs be reevaluated, and until convincing evidence is generated that distinguishes neutrophils from G/PMN-MDSCs, we consider G/PMN-MDSCs as neutrophils with immune suppressive capabilities.

Figure legends

Figure 1: Granulopoiesis during homeostasis. Neutrophil development in the bone marrow starts in the stem cell niche. A self-renewing long-term hematopoietic stem cell (LT-HSC) differentiates into a short-term hematopoietic stem cell (ST-HSC) and subsequently a multipotent progenitor (MPP) that has lost its self-renewing capacity. MPPs give rise to lymphoid/myeloid-primed progenitors (LMPPs). LMPPs differentiate into granulocyte/monocyte progenitors (GMPs), which in turn give rise to granulocytes[5,6,19]. When GMPs commit to neutrophil generation under the direction of granulocyte-colony stimulating factor (G-CSF) or granulocyte-macrophage-colony stimulating factor (GM-CSF), myeloblasts differentiate from a promyelocyte, a myelocyte and a metamyelocyte into a band cell, and finally, into a mature, hypersegmented neutrophil[10]. During its differentiation, the developing neutrophil changes its nuclear morphology from a round shape to a banded morphology into a segmented shape. Developing neutrophils express G-CSFR throughout the myeloid lineage[18]. As neutrophils mature, they downregulate expression of various receptors, including KIT, VLA4 (also known as integrin β1) and C-X-C chemokine receptor 4 (CXCR4), while upregulating CXCR2 and Toll-like receptor 4 (TLR4). Under steady state conditions, ligands for KIT, VLA4 and CXCR4 (such as KITL, vascular cell adhesion molecule 1 (VCAM1) and CXCL12, respectively) are produced by the bone marrow stroma to retain the progenitor cells. Ligands for CXCR2, including CXCL1, -2, -5, and -8 (in humans only) are expressed outside the bone marrow when neutrophils need to be mobilized[34,37,41]. Neutrophils have three types of granules and other secretory vesicles that contain specific effector proteins – of which a selection is shown here – and these emerge during distinct developmental stages. Primary (azurophil) granules appear during the myeloblast to promyelocyte stage, secondary (specific) granules appear during the myelocyte to metamyelocyte stage, tertiary (gelatinase) granules appear during the band cell to segmented cell stage of development, and secretory vesicles appear only in mature neutrophils. A variety of transcription factors regulate commitment to the neutrophil lineage and subsequent developmental stages[5,7,8]. A selected list of these transcription factors and

their expression levels during maturation are shown at the bottom of the figure. Under homeostatic conditions, only fully differentiated neutrophils exit the bone marrow into the circulation. CR1, complement receptor type 1; IRF8; interferon regulatory factor 8; MMP9, matrix metalloproteinase 9; MPO, myeloperoxidase; NE, neutrophil elastase; STAT3, signal transducer and activator of transcription 3.

Figure 2: Tumor-induced emergency granulopoiesis. Tumors affect both the development and the release of bone marrow neutrophils. Tumor-induced increases in the levels of granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage-colony stimulating factor (GM-CSF) skew hematopoiesis towards a myeloid cell production, greatly increasing the generation of granulocyte/monocyte progenitors (GMPs) and neutrophil progenitors[25-29,58]. In addition, tumors interfere with neutrophil retention in the bone marrow by upregulating various cytokines and chemokines. The composition of these mediators depends on the tumor type, mutations and oxygen levels in the tumor. The expression of KIT ligand (KITL) and the C-X-C chemokine receptor 2 (CXCR2) ligands CXCL1, 2 and 5 by cancer cells increases in response to hypoxia[31,140]. KRAS signaling, as well as loss of PTEN or SMAD4, in cancer cells increases expression of GM-CSF and several ligands of CXCR2, including CXCL1, 2, 5 and 8[30,106,109,110,139]. In addition, cancer cells either directly or indirectly – through interleukin (IL)-1² -producing macrophages and IL-17-producing ³´ T cells – produce G-CSF[25,26]. Neutrophil-derived BV8 also induces neutrophil expansion[128,129]. This pressure on the bone marrow emanating from the tumor causes increased generation and release of immature (from GMP to banded cells) and mature neutrophils into the circulation[26,64-66]. ECM, extracellular matrix; LMPP, lymphoid/myeloid-primed progenitor; LT-HSC, long-term haematopoietic stem cell; MPP, multipotent progenitor; ST-HSC, short-term haematopoietic stem cell.

Figure 3: Neutrophil function in tumor initiation and growth. There are several mechanisms by which neutrophils either promote or limit tumorigenesis. Transformation of an epithelial cell to a cancer cell can be supported by the production of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and proteases by neutrophils. These molecules induce epithelial damage and subsequent tumor-promoting inflammation. Epithelial damage by wounding also recruits neutrophils by prostaglandin E2 (PGE_2) to promote tumor initiation[105]. Promotion of tumor growth can also be mediated by crosstalk between neutrophils that are activated by tumour necrosis factor (TNF)-induced interleukin (IL)-17-producing $CD4^+$ T cells[121]. In addition to tumor initiation, neutrophils promote progression of tumor growth by converting senescent cancer cells into proliferating cancer cells via IL-1 receptor antagonist (IL-1RA)[132]. Proliferation is directly stimulated by transfer of neutrophil

elastase (NE) to cancer cells, which causes the degradation of insulin receptor substrate 1 (IRS1) and activates PI3K signaling[115]. Neutrophils express inducible nitric oxide synthase (iNOS) or arginase 1 (ARG1) to suppress $CD8^+$ T cell-mediated anti-tumor immune responses and promote tumor progression. Immunosuppression can also be accomplished by transforming growth factor β (TGF²) signaling in neutrophils[65,88]. In some contexts neutrophils can also limit tumor growth. Hypoxia in the tumor induces expression of C-X-C ligands (CXCL)1, -2 and -5 to recruit anti-tumor neutrophils[140]. Upregulation of MET on neutrophils by endothelial-derived TNF causes these cells to produce iNOS, which has cytotoxic effects on cancer cells[134]. Lastly, neutrophils participate in remodeling of the extracellular matrix (ECM) and induce angiogenesis by BV8 production and activation of vascular endothelial growth factor A (VEGFA) by matrix metalloproteinase 9 (MMP9)[116,120,126-129].

Figure 4: Impact of neutrophils on the metastatic cascade. Neutrophils influence several steps of metastasis. In melanoma, ultraviolet (UV) radiation causes release of high mobility group box 1 (HMGB1) from keratinocytes, which recruits neutrophils through Toll-like receptor 4 (TLR4) signaling. These neutrophils induce migration of cancer cells towards endothelial cells by tumour necrosis factor (TNF), leading to enhanced metastasis[148]. In mammary tumors, interleukin (IL)-1β-expressing macrophages instigate IL-17-producing ³´ T cells, resulting in a granulocyte-colony stimulating factor (G-CSF)-dependent systemic expansion of neutrophils. At the metastatic site, these neutrophils limit anti-tumor $CD8^+$ T cell responses by producing inducible nitric oxide synthase (iNOS)[26]. In addition, regulatory B (B_{reg}) cells instruct neutrophils to limit T and NK cell responses to the metastatic lesion[88]. Neutrophils can support leukotriene B4 (LTB4) receptor-positive metastasis-initiating cancer cells by producing LTB4 at the metastatic site[152]. Neutrophils also capture circulating cancer cells by direct interactions using the cell surface molecule CD11b or by releasing neutrophil extracellular traps (NETs), which are associated with increased formation of metastases[144,146]. Neutrophils may also induce leaky vasculature to support extravasation of disseminated cancer cells by expression of matrix metalloproteinase 9 (MMP9) and BV8[128,129]. BV8 is also directly involved in cancer cell migration and the recruitment of neutrophils[28,128,129]. Anti-metastatic functions of neutrophils are mediated by H_2O_2 or thrombospondin 1 (TSP1), but the latter is degraded by neutrophil elastase (NE) and cathepsin G (CG) during inflammation[133,160,163,164]. ALOX5, arachidonate 5-lipoxygenase; ECM, extracellular matrix; TGFβ, transforming growth factor β.

Table 1. Bidirectional communication between neutrophils and other immune cells in homeostasis and cancer

Factor(s)	Source	Responder	Outcome	Reference
CXCL1, 2, 5, 8	Megakaryocyte Endothelial cell Cancer cell	Neutrophil	Neutrophil release from bone marrow in homeostasis and cancer; recruitment to tumors	[34,37,38,58,101,102,104,109-111,139,140]
G-CSF	Fibroblast Cancer cell	Neutrophil	Granulopoiesis in homeostasis and cancer; neutrophil polarization and immunosuppression	[15-17,25-28,57,87,133,152,156,178]
GM-CSF	Cancer cell	Neutrophil Monocyte	Granulopoiesis in homeostasis and cancer; neutrophil polarization and immunosuppression	[24,29,30]
IL-1β	Macrophage Dendritic cell	$CD4^+$ T cell γδ T cell	IL-17 and G-CSF-mediated granulopoiesis in homeostasis and cancer	[26,56,57,59-63]
IL-17	$CD4^+$ T cell γδ T cell	Fibroblast Bone marrow stromal cells	G-CSF-mediated granulopoiesis in homeostasis and cancer	[26,46,48,57,121]
IL-23	Macrophage Dendritic cell	$CD4^+$ T cell γδ T cell	IL-17 and G-CSF-mediated granulopoiesis in homeostasis and cancer	[46]
iNOS, ARG1	Neutrophil	T cells	Suppression of anti-	[26,130]

	Monocyte	NK cell	tumor immunity	
TGFβ	Neutrophil B_{reg}	T cells NK cell Neutrophil	Immunosuppression in tumor microenvironment and metastasis	[25,65,85,86,88]
TNF	Endothelial cell Cancer cell	CD4 T cell Neutrophil Endothelial cell	IL-17 and G-CSF-mediated granulopoiesis in homeostasis; neutrophil recruitment to tumors; MET upregulation in neutrophils	[57,121,134,148]
TPO	Unknown	Megakaryocyte	CXCR2 ligand-dependent release of neutrophils from bone marrow in homeostasis	[38]

[ARG1, arginase 1; CXCL, C-X-C ligand; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage-colony stimulating factor; IL, interleukin; iNOS, inducible nitric oxide synthase; TGFβ, transforming growth factor β; TNF, tumour necrosis factor; TPO, thrombopoietin]

Glossary

αβ T cells

Most $CD4^+$ and $CD8^+$ T cells are αβ T cells, in which the T cell receptor (TCR) is composed of a heterodimer of an α and a β chain.

γδ T cells

A small subset of T cells whose TCR consists of a γ and a δ chain. These cells behave like innate immune cells and are largely divided into IL-17- and IFNγ-producing subsets.

Innate lymphoid cells
Innate immune cells that belong to the lymphoid lineage, but lack antigen-specific receptors.

Neutrophil polarization
A state of neutrophil activation in response to specific cues from its environment, which can promote or limit disease progression.

Th1/Th2
Two major activation states of $CD4^+$ T-helper cells expressing distinct cytokines and exerting different functions. In general, Th1 cells provide immunity against intracellular pathogens, whereas Th2 cells mediate immune responses against extracellular parasites.

M1/M2
Term for macrophage polarization states, where M1 and M2 represent opposing ends of the macrophage activation spectrum. Historically, M1 represents an anti-tumor activation state, whereas M2 macrophages are pro-tumoral; although, this restrictive nomenclature fails to represent tumor-associated macrophage biology.

N1/N2
Proposed binary classification to distinguish tumor-inhibiting (N1) from tumor-promoting (N2) neutrophils in the cancer setting. However, further evidence to define these polarization states and their relation to type 1/2 immunity is required before applying this terminology to cancer-associated neutrophils.

Myeloid-derived suppressor cells
A heterogeneous group of immunosuppressive myeloid cells including neutrophils that expand in cancer patients and mouse cancer models.

Autochthonous model
Models of cancer in which tumors arise spontaneously from genetic manipulation or injection of a carcinogen.

Neutrophil extracellular traps
Extracellular neutrophil-derived networks of DNA, fibers and various proteins such as elastase and histones. Release of NETs (NETosis) occurs in response to pathogen infection, sterile inflammation and cancer.

Pre-metastatic niche
A microenvironment in secondary organs primed by the primary tumor that is populated by non-cancer cells that promote seeding of metastasizing cancer cells.

Regulatory B cells
A subpopulation of immunosuppressive B cells involved in immunological tolerance.

Secretome
The total secreted factors of a cell or tissue.

Key points

- In patients with solid cancers, neutrophils expand both in the tumor microenvironment and systemically, and are generally associated with a poor prognosis.
- Genetically engineered mouse models for cancer have been crucial in identifying underlying mechanisms by which neutrophils influence tumor initiation, growth and metastasis.
- Neutrophils exert multifaceted and sometimes opposing roles during cancer initiation, growth and dissemination
- Primary tumors activate granulopoiesis in the bone marrow and actively stimulate the release and recruitment of both mature neutrophils and their progenitors.
- Depending on the spectrum and quantity of soluble mediators produced by cancer cells and cancer-associated cells, neutrophils can be polarized into different activation states by which they elicit various pro- or anti-tumor functions.
- Interactions between neutrophils and other (immune) cells are key in exerting their function, and the interaction networks observed in cancer are often highly reminiscent of those seen in other immunological diseases.
- Neutrophils modulate the efficacy of cancer therapies, and can also serve as biomarkers for progression and therapy response in cancer patients.

- Now that there is a growing understanding of the impact of neutrophils on cancer, the mechanisms by which neutrophils promote cancer progression may be utilized as targets to maximize the efficacy of anti-cancer therapeutics.

Biographies

Max D. Wellenstein

Max D. Wellenstein obtained his M.Sc. degree from Utrecht University in 2014. During his Masters studies, he received several awards to conduct research in Vivek Mittal's lab at Weill Cornell Medical College in New York. Currently, he is a Ph.D. candidate at the Division of Immunology at the Netherlands Cancer Institute in Amsterdam. His work focuses on the impact of neutrophils on breast cancer metastasis.

Seth B. Coffelt

Seth B. Coffelt is a Senior Research Fellow (junior faculty) at the University of Glasgow/Beatson Institute. For the past five years, he was a postdoctoral researcher at the Netherlands Cancer Institute focusing on the metastasis-promoting role of $\gamma\delta$ T cells and neutrophils. Using genetically engineered mouse models, his future studies will center on tumor microenvironmental regulation of metastasis-associated inflammation.

Karin E. de Visser

Karin E. de Visser is Group Leader at the Division of Immunology at the Netherlands Cancer Institute in Amsterdam, the Netherlands. The overall goal of her research is to understand the mechanisms by which the immune system influences breast cancer metastasis and responses to conventional anti-cancer therapies. Using sophisticated mouse models for spontaneous metastatic breast cancer, her lab has recently identified a novel mammary tumor-induced systemic inflammatory cascade involving $\gamma\delta$ T cells and neutrophils that promotes metastasis formation through suppression of anti-tumor T cells. Through these mechanistic insights, her lab aims to contribute to the design of novel immunomodulatory strategies to fight metastatic cancer

Table of contents summary

The traditionally held belief that neutrophils are inert bystanders in cancer has been challenged by recent literature. This Review discusses the involvement of neutrophils in cancer initiation and progression, and their potential as biomarkers and therapeutic targets.