Remote control: the cancer cell-intrinsic mechanisms that dictate systemic inflammation and anti-tumor immunity
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Neutrophils in cancer: neutral no more

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Abstract

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.

Key points

- In solid cancer patients, neutrophils expand both in the tumor microenvironment and systemically and are generally associated with 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 anti-cancer therapy efficacy 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 anti-cancer therapeutics.

Introduction

The name neutrophil – given to polymorphonuclear, granulocytic cells by Paul Ehrlich in the late 19th century – is based on their inability to retain acidic or basic dyes and for their preferential uptake of pH neutral dyes[1]. Although 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. Their behavior is 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 last 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 incredibly influential, 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 their potential as clinical biomarkers and therapeutic targets.

Neutrophil origins and life cycle: homeostasis versus cancer

In humans, neutrophils make up the most abundant immune cell population, representing 50-70% of all leukocytic cellularity. The production rate of these cells may reach upwards of 10^{11} cells per day[2], and tumors can increase this number 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 amazingly 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 a hematopoietic stem cell (**Fig. 6.1**). LMPPs further differentiate into a granulocyte/monocyte myeloid progenitor (GMP) and many transcription factors required for this process are known (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 MMPs, that

protect against opportunistic infections and mediate the resolution of inflammation (reviewed in [12,13]). If large quantities of neutrophils are used up during infection or cancer, a process called emergency granulopoiesis overtakes steady state granulopoiesis to rapidly increase the demand of 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].

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 signaling governs neutrophil formation[20]. The transcription factor 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 GM-CSF, IL6 and KIT ligand (KITL) – can play a redundant, but lesser role[22-24]. Tumors in many cancer models upregulate these cytokines, causing overactive granulopoiesis and neutrophilia[25-31].

Retention and release from bone marrow

One of the salient features of granulocytes that sets them apart from every other immune cell is their release from the bone marrow as a terminally differentiated, mature cell. 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 receptors, CXCR4 and CXCR2. Constitutive CXCL12 expression from osteoblasts and other bone marrow stromal cells tether CXCR4+ neutrophils in the bone marrow, while CXCL1 and CXCL2 from endothelial cells and megakaryocytes encourage their release into the circulation via CXCR2 signaling[33-38] (**Fig. 6.1**). Several adhesion molecules, such as ITGA4 and 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 regulate 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 curbing the secretion of IL-23 [46-49] – a cytokine that controls IL-17 expression by $\alpha\beta$ T cells, $\gamma\delta$ T cells, innate lymphoid cells and other lymphocytes[50,51]. Since 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].
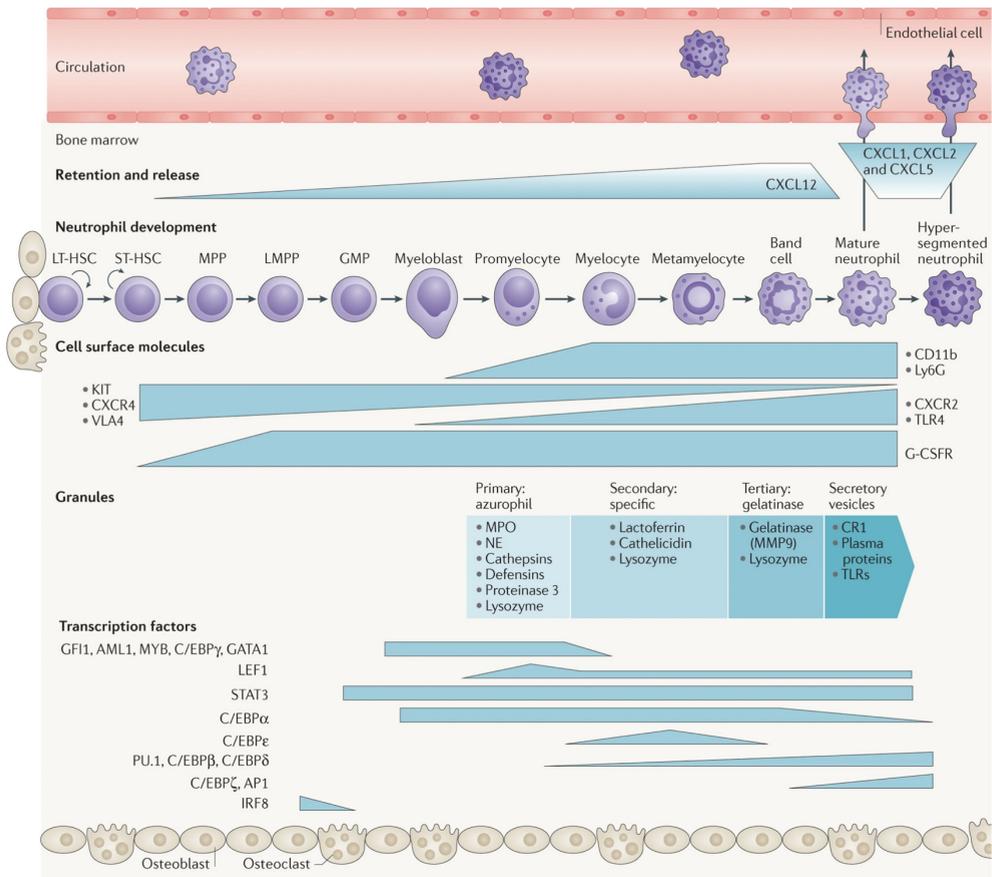
Figure 6.1: Granulopoiesis during homeostasis. Neutrophil development in the bone marrow starts in the stem cell niche. A self-renewing long-term hematopoietic stem cell (LT-HSC) differentiates into a short-term hematopoietic stem cell (ST-HSC) and subsequently a multipotent progenitor (MPP) that has lost its self-renewing capacity. MPPs give rise to lymphoid/myeloid-primed progenitors (LMPP). LMPPs differentiate into granulocyte/monocyte progenitors (GMP), which in turn give rise to granulocytes[5,6,19]. When GMPs commit to neutrophil generation under the direction of G-CSF or GM-CSF, myeloblasts differentiate from a promyelocyte, a myelocyte and a metamyelocyte into a band cell, and finally 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 down-regulate expression of various receptors, including cKIT, VLA4 and CXCR4, while up regulating CXCR2 and TLR4. Under steady state conditions, ligands for cKIT, VLA4 and CXCR4 (such as KITL, VCAM-1 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 and their expression levels during maturation are shown here. Under homeostatic conditions, only fully differentiated neutrophils exit the bone marrow into the circulation.

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 (**Fig. 6.2**). Cancer cells themselves produce these cytokines[27,28,58], but stromal and immune cells can also contribute to their elevated expression in tumor-bearing animals. 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 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 is often so intense in tumor-bearing hosts that undifferentiated cells are sometimes 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 cKIT[26,31], a marker of lymphoid, myeloid and neutrophil progenitor cells[25,67], suggesting these cKIT-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 cKIT – are found in inflammatory models and patients with inflammation[69-73]. These cKIT+ cells differentiate into fully mature neutrophils *in situ* at sites of *S. 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 chemical-induced cancer models 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, since granules are synthesized at specific stages of neutrophil development[12] (**Fig. 6.1**). Recent studies using density gradient purification methods have shown that distinct populations of neutrophils with different *ex vivo* properties 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].
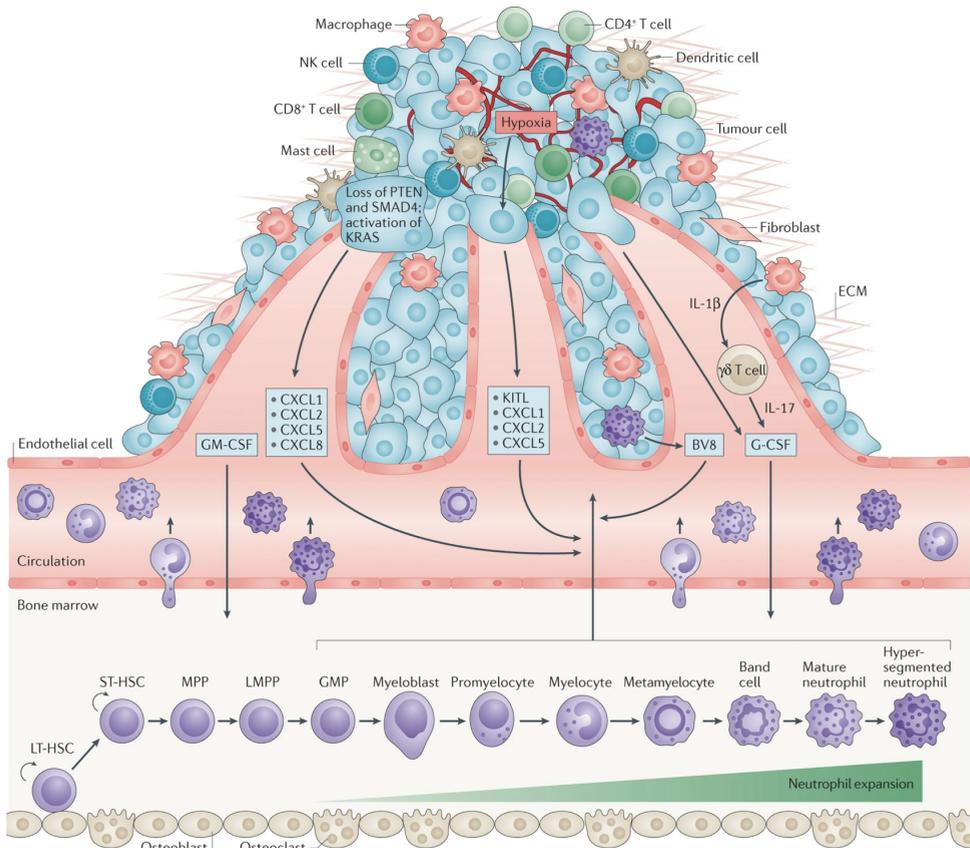
Figure 6.2: Tumor-induced emergency granulopoiesis. Tumors impact bone marrow neutrophils both in their development as well as their release. Tumor-induced increase in G-CSF and GM-CSF levels skews hematopoiesis towards a myeloid cell production, greatly increasing GMP and neutrophil progenitor generation[25-29,58]. In addition, tumors interfere with neutrophil retention in the bone marrow by up-regulation of various cytokines and chemokines. Composition of these mediators depends on tumor type, mutations and oxygen levels in the tumor. Expression of KITL and CXCR2 ligands, CXCL1, 2 and 5, by cancer cells increases in response to hypoxia[31,140]. KRAS signaling in cancer cells increases GM-CSF and several ligands of CXCR2, including CXCL1, 2, 5 and 8 [30,106,109,110]. In addition, cancers cells either directly or indirectly – through IL-1β-producing macrophages and IL-17-producing γδ 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 and mature neutrophils into the circulation.

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 the methodology and neutrophil labeling techniques. Due to these technological limitations, the lifespan of neutrophils in tumor-bearing hosts is unclear. Animal experiments 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 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. Neutrophils are polarized into diverging phenotypes, depending on specific tumor-derived factors. TGFβ, G-CSF and 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 ID1, Rb and IRF8 transcription factors 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, since cytotoxic neutrophils are shaped into cancer-promoting cells as tumors expand and evolve[93]. What is unclear at this point is the differentiation step at which these molecules instruct phenotypic changes. In the case of G-CSF, there is evidence that this cytokine can affect gene expression of both stem/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 these and other cytokines 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 support tumor growth through inhibition of CD8+ T cells, whereas neutrophils from TGFβ inhibitor-treated mice oppose tumor growth through their cytotoxic ability[65]. However, knowledge surrounding N1- and N2-polarized neutrophils has not progressed beyond the original study. Their surface markers, cytokine expression patterns, transcription factor regulators and other hallmarks of activation are largely unknown. In non-cancerous disease models where Type 1 and Type 2 immunity are defined, neutrophil involvement is underdeveloped. Information about neutrophil response to the Type 1/2-associated cytokines (i.e. IFNγ, IL-4, IL-13, etc.) or production of these cytokines to skew immune responses is lacking. Although some studies addressing this issue 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 with a 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 given against using 'M1' and 'M2' to describe tumor-associated

macrophages[96-98]. Similar to macrophages, neutrophil polarization probably exists as a spectrum of activation states, rather than only two extremes. Researchers should follow the recent advances in the macrophage field and apply a combinatorial nomenclature that describes neutrophil activation status[99].

Further complicating this picture is the ongoing debate on the kinship of neutrophils and myeloid-derived suppressor cells (MDSCs) and whether these are analogous or separate populations (**Box 1**).

Box 1: Neutrophils and MDSCs

"Myeloid-derived suppressor cell (MDSC)" 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 – they are rarely, if ever, found in homeostatic conditions. The MDSC collection encompasses many immune cells at various stages of differentiation, because of the non-specific nature of the Gr1 antibody (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. At this time, there is no way to single out one 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-MDSC, 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, *i.e.* 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 MDSC 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 capability.

Impact of neutrophils on 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 through damage to 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 (**Fig. 6.3**). 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 – and CXCR2 deficiency or CXCR2 inhibition retards tumor formation in these mice[102]. However, it should be noted that CXCR2 expression is not exclusive to neutrophils. Depletion of the entire neutrophil population via anti-Ly6G
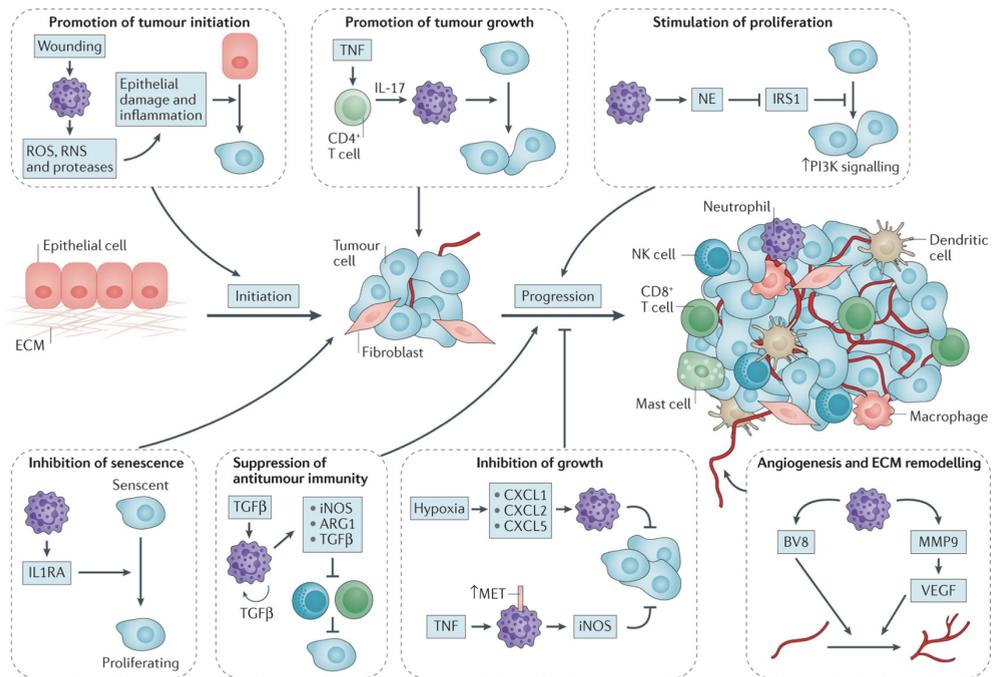
Figure 6.3: Neutrophil function in tumor initiation and growth. There are several mechanisms by which neutrophils either promote or limit tumorigenesis. Transformation of an epithelial cell to a cancer cell can be supported by the production of reactive oxygen or nitrogen species (ROS/RNS) and proteases by neutrophils. These molecules induce epithelial damage and subsequent tumor-promoting inflammation. Epithelial damage by wounding also recruits neutrophils by PGE$_2$ to promote tumor initiation[105]. Promotion of tumor growth can also be mediated by crosstalk between neutrophils that are activated by TNF-induced 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 IRS-1 and activates PI3K signaling[115]. Neutrophils express iNOS or ARG1 to suppress CD8$^+$ T cell-mediated anti-tumor immune responses and promote tumor progression. Immunosuppression can also be accomplished by TGFβ signaling in neutrophils[65,88]. In some contexts neutrophils can also limit tumor growth. Hypoxia in the tumor induces expression of CXCL1, -2 and -5 to recruit anti-tumor neutrophils[140]. Up-regulation of cMET on neutrophils by endothelial-derived TNF causes these cells to produce iNOS, which in this case is cytotoxic to cancer cells[134]. Lastly, neutrophils participate in remodeling of the extracellular matrix (ECM) and induce angiogenesis by Bv8 production and activation of VEGF by MMP9[116,120,126-129].

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 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] (**Fig. 6.2**). This 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. Like 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. Neutrophils production of ROS/RNS and angiogenic factors, such as MMP9 [116], may also be important for tumor initiation (**Fig. 6.3**). In future work, genetically engineered mouse tumor models will be extremely valuable in this area of cancer-related neutrophil biology, since neutrophils or neutrophil-derived factors can be manipulated as tumors arise *de novo*.

Regulation of tumor growth by neutrophils

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 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 transplanted tumor-bearing mice depleted of neutrophils via anti-Gr1 showed reduced tumor growth[118,119].

Since this time, the literature showing a tumor growth-promoting role for neutrophils *in vivo* has largely outweighed the contrasting literature. One mechanism neutrophils employ is the induction of angiogenesis (**Fig. 6.3**), since their depletion decreases 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 recapitulates these effects[58,124,125]. In other studies, coinjection of cancer cell lines with neutrophils isolated from tumor-bearing mice increases 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, Bv8/PROK2 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 these mechanisms 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, some new data exist. For example, neutrophils in mice with transplanted *MMTV-PyMT;MMTV-cMyc* mammary tumors hinder tumor growth[133], presumably through their H_2O_2-mediated cytotoxic ability. Deletion of cMET, the HGF receptor, specifically in neutrophils impairs recruitment of neutrophils to tumors and leads to enhanced tumor growth of various transplantable cell lines and a spontaneous liver cancer model [134]. Expression of cMET in neutrophils is upregulated by endothelial cell- and cancer cell-derived TNF in this study[134]; whereas, others have shown that TNF signaling in CD4+ T cells leads 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. It should also be noted that there are contradictory results regarding neutrophil function using the same transplantable cell lines. Some studies report a pro-tumorigenic role of neutrophils, while other studies report no effect 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, since 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 use to kill cancer cells after antibody therapy (reviewed in [136]). It remains to be seen whether ADCC occurs *in vivo* without exogenous antibodies, when cancer-induced 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 demonstrate 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 upregulate CXCL5 via the HIPPO-YAP1 pathway and blocking any of these molecules decreases CXCR2+ immunosuppressive neutrophil recruitment to tumors and blunts 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 increases uterine tumor burden. Hypoxia-induced CXCL1, -2 and -5 recruit neutrophils, and these cells impede tumor growth by promoting cancer cell detachment from the basement membrane via modulation of integrins. Interestingly, neutrophils deficient in MyD88 signaling maintain their trafficking ability, but lose 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 (**Fig. 6.4**). As early as the late 1980s – before the importance of neutrophils in primary tumor growth was established[117-119] – coinjection 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.
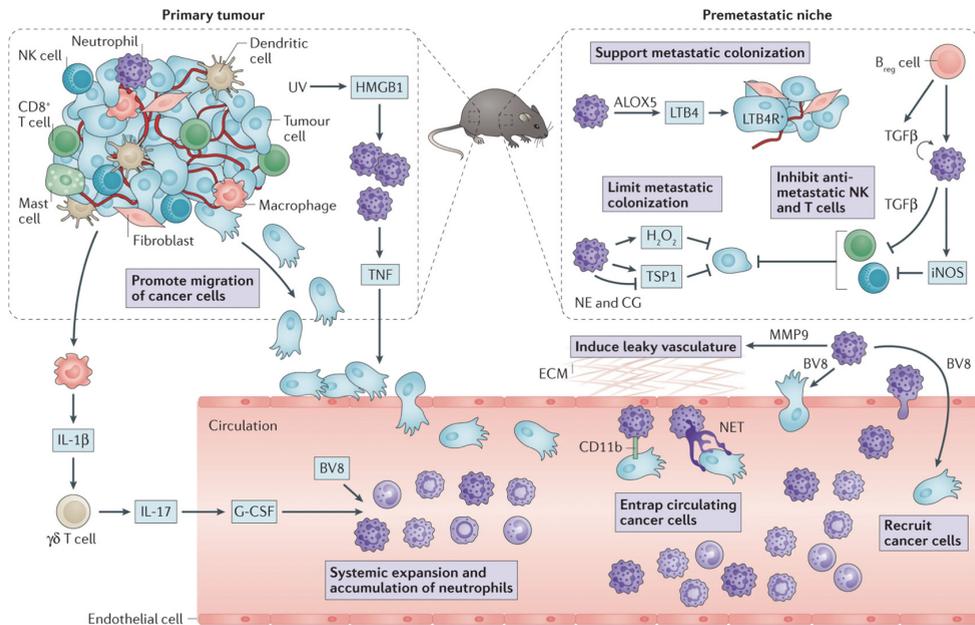
Figure 6.4: Impact of neutrophils on the metastatic cascade. Neutrophils influence several steps of metastasis. In melanoma, UV radiation causes release of HMGB1 from keratinocytes, which recruits neutrophils through TLR4 signaling. These neutrophils induce migration of cancer cells towards endothelial cells by TNF, leading to enhanced metastasis[148]. In mammary tumors, IL-1β-expressing macrophages instigate IL-17-producing γδ T cells, resulting in a G-CSF-dependent systemic expansion of neutrophils. At the metastatic site, these neutrophils limit the anti-tumor CD8+ T cell responses by producing iNOS[26]. Additionally, regulatory B cells instruct neutrophils to limit the T and NK cell response to the metastatic lesion[88]. Neutrophil can support LTB4 receptor-positive metastasis-initiating cancer cells by producing LTB4 at the metastatic site[152]. Neutrophils also capture circulating cancer cells by direct interaction using cell surface molecule CD11b or by releasing neutrophil extracellular traps (NETs), which is associated with increased metastasis formation[144,146]. Neutrophils may also induce leaky vasculature to support extravasation of the disseminated cancer cells by expression of 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 TSP-1, but the latter is degraded by neutrophil elastase (NE) and cathepsin G (CG) during inflammation[133,160,163,164].

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 coinjection 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) reduces the formation of metastases[144,145]. Intravital imaging has shown that cancer cells colocalize with endothelial

cell-associated neutrophils through 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 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. *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, UV exposure of melanomas in *Hgf-Cdk4^{R24C}* mice leads to cancer cell clustering around blood vessels and increased lung metastasis without affecting primary tumor growth[148]. In this setting, UV-induced damage to keratinocytes increases HMGB1 levels, which recruit 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. The same study found that ulcerated melanomas and the accompanying neutrophilic influx in patients is associated with greater melanoma-endothelial cell interactions and higher metastatic incidence[148]. 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 ALOX5 enzyme[154]. Recent data show that LTB4 production by neutrophils in the pre-metastatic niche support LTB4 receptor[+] metastasis-initiating cells in the *MMTV-PyMT* model, and 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 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 decreases 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[88]. G-CSF is another factor driving a pro-metastatic phenotype in neutrophils and G-CSF presumably stems directly from cancer cells in the 4T1 model[27,28]. G-CSF induces Bv8 expression in neutrophils[26,156], which may induce cancer cell migration or vascular leakiness to support metastasis[28,128,129]. We

recently showed another mechanism whereby G-CSF modulates neutrophil phenotype and pro-metastatic function[26]. 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]. Taken together, both cancer cells and immune cells can educate the pro-metastatic abilities of neutrophils.

Neutrophil precursors are found ectopically in metastasis-specific organs. In the *K14-Cre;Cdh1^{F/F};Trp53^{F/F}* breast cancer model, we noted that a proportion of neutrophils in various tissues express cKIT and display a mixed nuclear morphology[26]. Others have shown cKIT-expressing cells in the pre-metastatic niche[28,153,157]. Antagonizing cKIT signaling or inhibition of KIT ligand expression by cancer cells prevents pulmonary metastasis formation in the 4T1 model[31], suggesting a pro-metastatic role for cKIT+ neutrophils. In addition, 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 these studies, 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]; although, G-CSF controls the transcriptional activity and expansion of neutrophils like in other publications[26-28]. The controversial aspect of these observations is that the 4T1 mammary tumor cell line was used 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 produce contradictory results using the same cell line? 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 be divergent in the cytokines they produce. Likewise, the introduction of ectopic transgenes, such as luciferase or 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 microbiota in tumor-free mice[76].

In addition to production of H_2O_2 [133,160], neutrophils can also limit metastasis formation through expression of thrombospondin-1 (TSP-1)[163] and cMET[134] in experimental metastasis models. However, pro-metastatic neutrophils deactivate TSP-1 by elastase- and cathepsin G-mediated degradation after degranulation in lung tissue, and inactivation of TSP-1 contributes to metastasis formation[164]. Interestingly, TSP-1 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 reduces spontaneous pulmonary metastasis formation without affecting primary tumor growth[163]. These data are proof-of-principle that the pro-metastatic behavior of neutrophils can be switched *in vivo*, opening avenues of therapeutic intervention.

6

Clinical implications of neutrophils in cancer

Neutrophils as biomarkers in cancer patients

Although experimental studies highlight a multifaceted and sometimes opposing role of neutrophils, the bulk of clinical evidence assessing neutrophil to lymphocyte ratios (NLR) mostly supports the notion that neutrophils promote, rather than inhibit, cancer progression[3]. NLR has thus been proposed to be an attractive biomarker for risk stratification and to guide treatment decisions. NLR can easily and at low costs be determined from standard blood analysis. That said, at the level of individual patients, it might be more 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 the patients to the high or low risks 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 upon initiation of therapy may be indicative of a good therapy response. Thus far, only 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 post-surgical low NLR is associated with improved survival[166]. Metastatic renal cell carcinoma patients with a low pre-treatment NLR that remain low during treatment with tyrosine kinase or mTOR inhibitors experience a more favorable outcome[167]. It will be interesting to assess whether parallel scoring of 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.

In comparison to NLR, the prognostic and predictive power of intratumoral neutrophils is murkier and more variable: from positive (gastric[168]) to negative (renal[169], melanoma[170]) to no (lung[171]) correlation with patient outcome. Colorectal cancer is one example where controversy surrounds the potential role of intratumoral neutrophils[172,173]. The markers used to identify tumor-associated neutrophils (i.e. CD66b, myeloperoxidase, cell morphology by H&E, etc.) 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 multiple neutrophil-related genes have been recently applied to gene expression data sets containing 1000s of patients. Two independent studies found that intratumoral neutrophil infiltration correlates with poor prognosis when encompassing all solid tumor types[4,140]. Thus, moving beyond single markers may be necessary to accurately determine whether intratumoral neutrophils have 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 where 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 CXCR1/2 inhibitor[175], are ongoing in cancer patients[176,177]. Importantly, characterization of neutrophil polarization in different tumor types as well as early and late stages is urgently needed in order to maximize therapeutic modalities. In tumors where neutrophils are beneficial, like 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 FDA approved antagonist drugs 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. It would be interesting to investigate whether these already existing drugs are efficacious in cancer patients, since pre-clinical models and clinical samples indicate that this pathway is important for cancer progression[26,68]. Therapeutic strategies aimed at re-polarization of tumor-induced neutrophils or interference with their downstream pro-tumorigenic effects represent additional opportunities for intervention[65,152].

Combining neutrophil targeting with other anti-cancer therapies

Successful implementation of neutrophil-targeting compounds in the clinic will require a critical assessment of the most optimal combination therapy strategies. For this purpose, we can learn from the growing number of mechanistic studies performed in clinically relevant mouse tumor models, addressing 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-VEGF 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-VEGF therapy is combined with depletion of neutrophils via anti-Gr1 or -G-CSF[179,181].

Chemotherapy is another combination partner for neutrophil-targeting compounds; although, many types of chemotherapy negatively affect neutrophil production themselves. Interestingly, chemotherapy-induced neutropenia is associated with improved survival in non-small cell lung, breast, gastric and colorectal cancer patients[182-185]. This beneficial association may be explained by two reasons that are either neutrophil-independent or neutrophil-dependent. Since 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 address these questions, and some studies report a beneficial role for neutrophils in chemotherapy response, whereas other studies indicate that neutrophils counteract the anti-cancer efficacy of chemotherapy (recently reviewed in [186]). For example, depletion of Gr1+ myeloid cells or Ly6G+ neutrophils reduces the anti-cancer efficacy of cyclophosphamide and doxorubicin in tumor inoculation models[187,188]. These data stand in contrast to improvement of tumor inhibition by combining CXCR2 blockade with doxorubicin, cyclophosphamide or docetaxel in xenograft and *de novo* tumorigenesis models[58,132]. Moreover, some chemotherapeutics directly reduce the

viability and/or change the functionality of myeloid cells, which influences the anti-cancer efficacy of that drug. Gemcitabine and 5-fluorouracil are two examples. These drugs trigger IL-1β secretion from immunosuppressive monocytes and neutrophils, setting off a chain of inflammatory events that results in reduced chemotherapy efficacy on subcutaneous EL4 thymomas[189].

Another unresolved issue is the clinical benefit and risk of using recombinant G-CSF and GM-CSF to counteract chemotherapy-induced neutropenia. Neutropenia predisposes patients to life-threatening infections, so recombinant G-CSF and GM-CSF administration 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 report contradictory results[28,192], leaving the debate open. Therefore, it is critical to carefully assess whether the beneficial effect of G-CSF on reducing susceptibility for infections outweighs the potential risk for accelerating disease progression in cancer patients.

Table 6.1. Bidirectional communication between neutrophils and other immune cells in homeostasis and cancer

Factor(s)	Source	Responder	Outcome	Reference(s)
CXCL1, 2, 5, 8	Megakaryocyte Endothelial cell Cancer cell	Neutrophil	Neutrophil release from bone marrow in homeostasis and cancer; recruitment to tumors	34,37,38,57,58,101,102,104, 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/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	26,46-49,57,121
IL-23	Macrophage Dendritic cell	CD4 T cell γδ T cell	IL-17/G-CSF-mediated granulopoiesis in homeostasis and cancer	46-49
iNOS, ARG1	Neutrophil Monocyte	T cells NK cell	Suppression of anti-tumor immunity	26,130
TGFβ	Neutrophil Breg	T cells NK cell Neutrophil	Immunosuppression in tumor microenvironment and metastasis	25,65,85-88
TNF	Endothelial cell Cancer cell	CD4 T cell Neutrophil Endothelial cell	IL-17/G-CSF-mediated granulopoiesis in homeostasis; neutrophil recruitment to tumors; cMET upregulation in neutrophils	57,121,134,148
TPO	Unknown	Megakaryocyte	CXCR2 ligand-dependent release of neutrophils from bone marrow in homeostasis	38

Contrasting data also exist about the function of neutrophils in radiotherapy response. Whereas anti-Ly6G 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 response of xenografted prostate cancer cells[194]. Taken together, the diverse and sometimes contradictory role of neutrophils in anti-cancer therapy response may reflect the differences in tumor type, tumor model, immune status of the host, or mechanism of tumor killing by a particular anti-cancer therapy.

A promising avenue of potential therapeutic benefit 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-PD1 or anti-PD1/CTLA4 synergizes with anti-CXCR2 or anti-Ly6G, respectively, to delay tumor growth[197,198], supporting the immunotherapy/neutrophil inhibition concept.

In addition to T cell-based immunotherapies, macrophage inhibitors (i.e. anti-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], it may be expected that neutrophils promote resistance to macrophage-targeting therapy. In fact, neutrophils mediate resistance to the anti-angiogenic drug, sorafenib, after blocking macrophages in the *RIP1-Tag2* pancreatic and *MMTV-PyMT* mammary tumor models[202]. Thus, targeting one myeloid cell population may require additional targeting of another myeloid cell population to counteract 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, neutrophil complexity not only includes the ability to promote or prevent tumor progression, but also encompasses 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 6.1**). 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. Because neutrophils die quickly during *ex vivo* culture, the use of the culture dish is severely limited with these cells. Therefore, neutrophil biology is best studied *in vivo*. Researchers commonly use two antibodies to deplete neutrophils, Gr1 and Ly6G, but even 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 to monitor the

function of mature neutrophils via *in vivo* imaging[206]. One value of this model stems from its ability to specifically delete neutrophil-derived molecules at later stages of differentiation. In the coming years, 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 G/PMN-MDSCs – single cell sequencing or single cell fate-mapping reporter tools will need to be coupled with nuclear morphology identification and surface marker expression to better define the differences between neutrophil activation and immature cells. Together, these new methodologies will unravel novel insights into the not so neutral behavior of neutrophils in cancer and other diseases.

Acknowledgements

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.

Th1/Th2 cells
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, while Th2 cells mediate immune responses against extracellular parasites.

M1/M2 macrophages
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, while M2 macrophages are pro-tumoral; although, this restrictive nomenclature fails to represent tumor-associated macrophage biology.
N1/N2 neutrophils
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.

Regulatory B cells (B$_{Reg}$ cells)
A subpopulation of immunosuppressive B cells involved in immunological tolerance.

Secretome
The total of secreted factors of a cell or tissue.

Neutrophil Extracellular Trap (NET)
Extracellular neutrophil-derived network 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.

Myeloid-Derived Suppressor Cells (MDSCs)
Heterogeneous group of immunosuppressive myeloid cells including neutrophils that expand in cancer patients and mouse cancer models.

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.

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

6

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