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 the recent literature. Emerging evidence indicates that tumours manipulate neutrophils, sometimes early in their differentiation process, to create diverse phenotypic and functional polarization states able to alter tumour behaviour. 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 nineteenth 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 tumour-bearing hosts can oppose or potentiate cancer progression. These two types of behaviour are controlled by signals emanating from cancer cells or stromal cells within the tumour microenvironment, which educate neutrophils to execute the demise of the tumour or facilitate support networks that lead to its expansive spread. These functions can occur locally in or around the tumour 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 tumour initiation to primary tumour 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 behaviour of other tumour-associated cell types — primarily other immune cells. In this Review, we focus on how tumours manipulate the generation and release of neutrophils from the bone marrow. We discuss the mechanisms identified in animal models by which neutrophils participate in tumour initiation, growth and metastasis. Finally, we highlight the potential of these cells as clinical biomarkers and therapeutic targets.

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Neutrophil origins and life cycle

In humans, neutrophils are the most abundant immune cell population, representing 50–70% of all leukocytes. More than 10^{11} neutrophils may be produced per day[2], and tumours can further increase this number. 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 exploited by tumours to disrupt normal neutrophil homeostasis; these are discussed below.

Granulopoiesis. To accommodate the notably high production and turnover of neutrophils, the bone marrow devotes approximately two-thirds of its space to the formation of neutrophils and monocytes in steady-state conditions[5]. During granulopoiesis, neutrophils arise from lymphoid-primed multipotent progenitors (LMPPs)[6], which are derived from haematopoietic stem cells (FIG. 1). LMPPs further differentiate into granulocyte–monocyte myeloid progenitors (GMPs) and many transcription factors required for this process have been identified (reviewed in REFS 5,7,8). Neutrophil maturation then begins, as GMPs differentiate through the following sequence: myeloblast, promyelocyte, myelocyte, metamyelocyte, band neutrophil and, finally, segmented neutrophil (reviewed in REFS 5,9–11). The transition from myeloblast to promyelocyte is marked by the first appearance of primary granules. Secondary granules form during the myelocyte to metamyelocyte transition followed by the formation of tertiary granules during the band cell to segmented cell stage[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

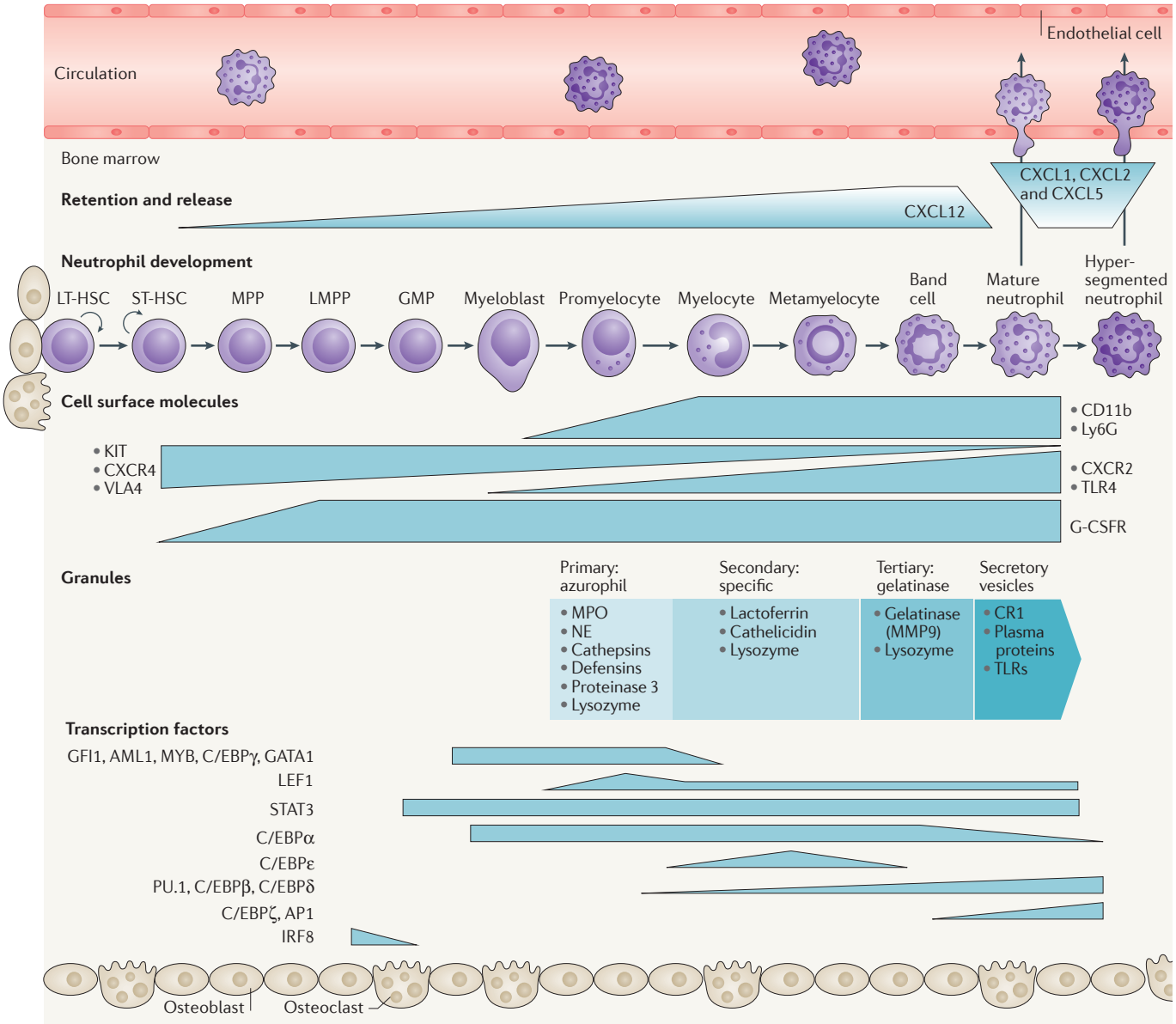

Figure 1 | **Granulopoiesis during homeostasis.** Neutrophil development in the bone marrow starts in the stem cell niche. A self-renewing long-term haematopoietic stem cell (LT-HSC) differentiates into a short-term haematopoietic stem cell (ST-HSC) and subsequently a multipotent progenitor (MPP) that has lost its self-renewing capacity. MPPs give rise to lymphoid-primed multipotent 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-CSF receptor (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 KIT ligand (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, CXCL2, CXCL5 and CXCL8 (in humans only) are expressed outside the bone marrow when neutrophils need to be mobilized[34,37,41]. Neutrophils have three types of granule and other secretory vesicles that contain specific effector proteins — of which a selection is shown here — and these emerge during distinct developmental stages. Primary (also called 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. Various 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. AML1, acute myeloid leukaemia 1; C/EBP, CCAAT/enhancer binding protein; CR1, complement receptor type 1; GATA1, GATA binding protein 1; GFI1, growth factor independent 1; IRF8, interferon regulatory factor 8; LEF1, lymphoid enhancer binding factor 1; MMP9, matrix metalloproteinase 9; MPO, myeloperoxidase; NE, neutrophil elastase; STAT3, signal transducer and activator of transcription 3.

αβ T cells
Most CD4+ and CD8+ T cells are αβ T cells, in which the T cell receptor comprises a heterodimer of an α-chain and a β-chain.

γδ T cells
A small subset of T cells in which the T cell receptor consists of a γ-chain and a δ-chain. These cells behave like innate immune cells and are largely divided into interleukin-17-producing and interferon-γ-producing subsets.

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

the resolution of inflammation (reviewed in REFS 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 tumour-bearing mice and humans with pancreatic or colon cancer (and probably other tumour 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–signal transducer and activator of transcription 3 (STAT3) signalling governs neutrophil formation[20]. The transcription factor RAR-related orphan receptor γ1 (RORC1) is a recently identified regulator of myelopoiesis in tumour-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, also known as KITLG) — may have a redundant, but lesser, role[22–24]. Tumours 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 marrow by an interplay between two C-X-C chemokine receptors, CXCR4 and CXCR2. Constitutive CXCL12 expression by osteoblasts and other bone marrow stromal cells tethers CXCR4+ neutrophils in the bone marrow, whereas secretion of CXCL1 and CXCL2 by endothelial cells and megakaryocytes encourages the release of neutrophils into the circulation via CXCR2 signalling[33–38] (FIG. 1). Several adhesion molecules, for example, integrin subunit α4 (ITGα4) 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 (THPO)-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[46–48], curbing the secretion of IL-23 (REF. 49) — a cytokine that controls IL-17 expression by αβ T cells, γδ 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[48]. Commensal bacteria and enterocyte-derived CXCL5 in the gut also have a role in neutrophil homeostasis by increasing or inhibiting IL-17 production, respectively[54,55]. IL-1β 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 tumours or systemically as a result of a tumour[25–28,58]. These factors override retention signals in the bone marrow, facilitating neutrophil egress and elevated numbers of circulating neutrophils (FIG. 2). Cancer cells themselves produce these cytokines[27,28,58], but stromal and immune cells can also contribute to their elevated expression in tumour-bearing mice. For example, tumour-associated macrophages are a well-known source of IL-1β[59]. Recently, we showed that neutrophils expand in mammary tumour-bearing keratin 14 (*K14)-Cre*;E-cadherin (*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 tumours 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 tumours 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 tumour-bearing hosts that undifferentiated cells are set free prematurely. Nuclear staining of circulating neutrophils from mammary and lung tumour 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 to be metamyelocytes and/or band neutrophils[67]. Circulating neutrophils from patients with breast, lung or colorectal cancer also show a similar mixture of differently shaped nuclei[64,68]. However, the consequence of immature neutrophils in the bloodstream of tumour-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 tumours primes immature neutrophils for functions they would not ordinarily perform.

The ectopic appearance of immature neutrophils in the circulation may have profound consequences on tumour progression. An example of this was shown in mice with chemically induced tumours crossed with histamine-deficient mice, where the lack of histamine stalled differentiation of immature neutrophils and

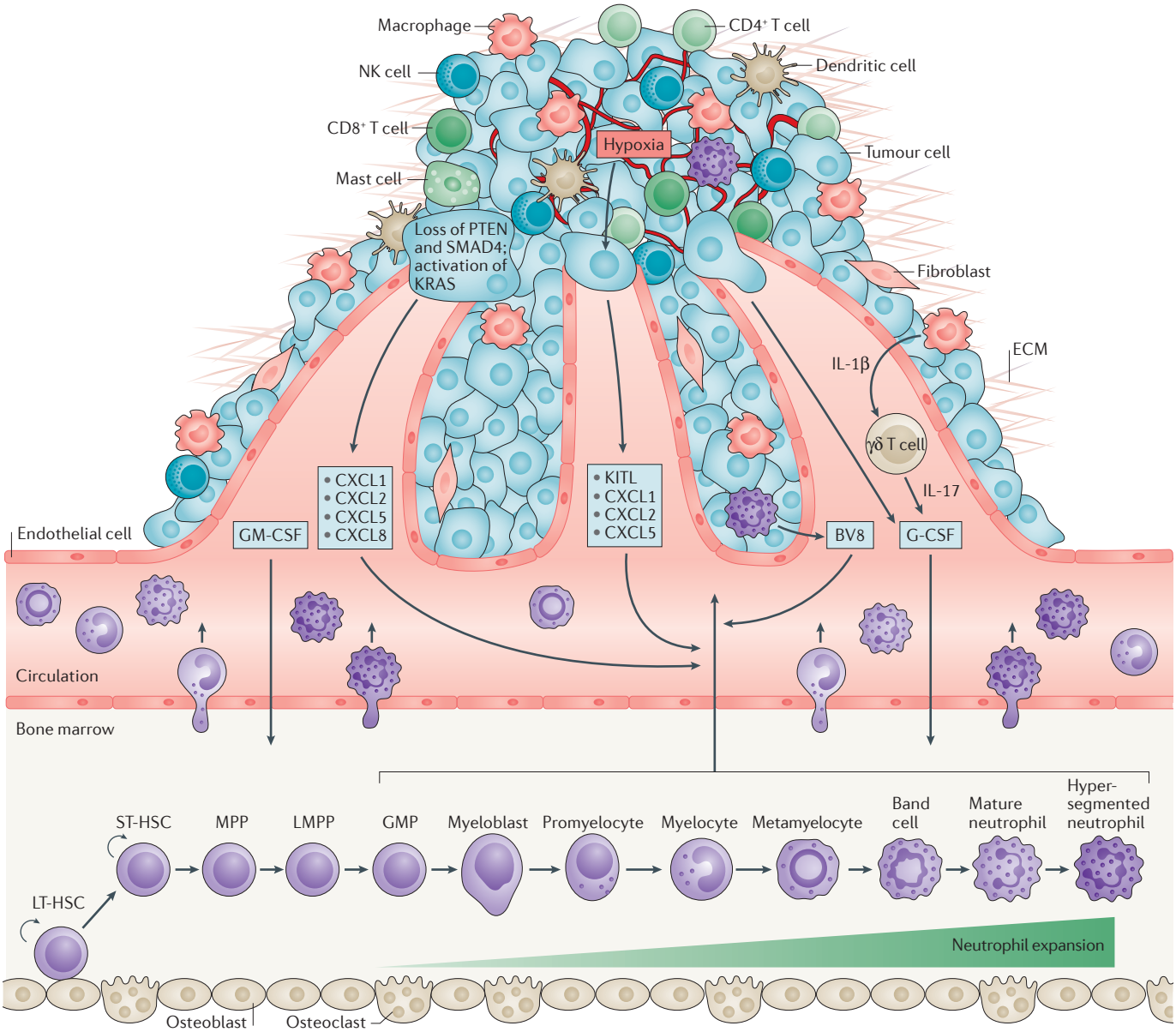

Figure 2 | **Tumour-induced emergency granulopoiesis.** Tumours affect both the development and the release of bone marrow neutrophils. Tumour-induced increases in the levels of granulocyte-colony stimulating factor (G-CSF) and granulocyte–macrophage-colony stimulating factor (GM-CSF) skew haematopoiesis towards production of myeloid cells, greatly increasing the generation of granulocyte–monocyte progenitors (GMPs) and neutrophil progenitors[25–29,58]. In addition, tumours interfere with neutrophil retention in the bone marrow by upregulating various cytokines and chemokines. The composition of these mediators depends on the tumour type, mutations and oxygen levels in the tumour. The expression of KIT ligand (KITL) and the C-X-C chemokine receptor 2 (CXCR2) ligands CXCL1, CXCL2 and CXCL5 by cancer cells increases in response to hypoxia[31,140]. KRAS signalling, as well as loss of PTEN or SMAD4, in cancer cells increases expression of GM-CSF and several ligands of CXCR2, including CXCL1, CXCL2, CXCL5 and CXCL8 (REFS 30,106,109,110,139). In addition, cancer cells either directly or indirectly — through interleukin-1β (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 tumour causes increased generation and release of immature (from GMP to band cell) and mature neutrophils into the circulation[26,64–66]. ECM, extracellular matrix; LMPP, lymphoid-primed multipotent progenitor; LT-HSC, long-term haematopoietic stem cell; MPP, multipotent progenitor; ST-HSC, short-term haematopoietic stem cell.

increased tumour incidence and growth[75]. These data suggest that immature cells have functions different from those of mature neutrophils. Indeed, the phenotype and behaviour of mature, aged neutrophils are not the same as those of young, newly released circulating neutrophils, even in tumour-free mice[76]. One explanation for the difference between the functions of immature and mature neutrophils may be their distinctive

composition of granules, because granules are synthesized at specific stages of neutrophil development[12] (FIG. 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 tumour-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 approximately 7 hours in healthy humans[2,77] and 8–10 hours in mice[78]. However, an equal number of reports challenge these kinetics as too short or too long (reviewed in REF. 79). The discrepancy between these studies is due mainly to limits of the methodology and neutrophil labelling techniques currently available, and therefore the lifespan of neutrophils in tumour-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 tumours than in the spleen[82], suggesting that the tumour microenvironment encourages their survival both locally and systemically. Indeed, pioneering work from Mantovani and his colleagues[83] in the 1990s showed that many tumour-associated cytokines prolong neutrophil survival in culture. 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 signalling by G-CSF[20]. A longer life may give neutrophils more time to synthesize new molecules and perform additional effector functions during tumour progression.

Tumour-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 tumour-derived factors. Transforming growth factor-β (TGFβ), G-CSF and interferon-β (IFNβ) are the best-studied molecules in this process. TGFβ and G-CSF activate a tumour- and metastasis-promoting programme[25,27,65,85–88], by regulating the transcription factors inhibitor of DNA binding 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 tumour physiology (such as hypoxia) may also be important for neutrophil polarization, because cytotoxic neutrophils are shaped into cancer-promoting cells as tumours 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 T_H1/T_H2 and M1/M2 nomenclature of T-helper cells and macrophages, respectively[65]. The study introducing the N1/N2 nomenclature noted a difference in neutrophil polarization after mice bearing subcutaneous mesothelioma tumours were treated with a TGFβ inhibitor. Neutrophils in untreated mice supported tumour growth through inhibition of $CD8^+$ T cells, whereas neutrophils from TGFβ inhibitor-treated mice opposed tumour growth through their cytotoxic ability[65]. However, knowledge surrounding N1-polarized 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 (that is, IFNγ) and/or type 2-associated cytokines (that is, IL-4 and IL-13). It also remains to be elucidated 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 probably an oversimplification of neutrophil polarization for the same reasons that have been given against using M1 and M2 to describe tumour-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).

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

T_H1/T_H2
Two major activation states of $CD4^+$ T-helper cells expressing distinct cytokines and exerting different functions. In general, T_H1 cells provide immunity against intracellular pathogens, whereas T_H2 cells mediate immune responses against extracellular parasites.

M1/M2
Term for macrophage polarization states, in which M1 and M2 represent opposing ends of the macrophage activation spectrum. Historically, M1 represents an antitumour activation state, whereas M2 macrophages are pro-tumoural, although this restrictive nomenclature fails to represent tumour-associated macrophage biology.

N1/N2
Proposed binary classification to distinguish tumour-inhibiting (N1) from tumour-promoting (N2) neutrophils in the cancer setting. However, further evidence to define these polarization states and their relationship to type 1 or type 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.

Neutrophils and tumour initiation

During the past two decades, it has become apparent that mutations in normal cells are required but not sufficient for tumorigenesis. Inflammation plays an essential part in initiating tumorigenesis by damaging specific tissues[100], and neutrophils are a crucial component of this process. Inflammation-induced models of cancer initiated by chemical carcinogens, such as the DMBA–TPA skin cancer model and the azoxymethane (AOM)–dextran sodium sulfate (DSS) colitis-associated colon cancer model, have established the importance of neutrophils in tumour initiation (FIG. 3). In these models, neutrophils are attracted to tumour-prone tissues via the CXCR2 ligands, CXCL1, CXCL2 and CXCL5 (REFS 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 adenomatous polyposis coli (*Apc*)$^{Min/+}$ intestinal adenoma model, the *Ah* (also known as *Cyp1a1*) promoter-driven Cre–oestrogen receptor fusion (*Ah-CreER*);*Apc*$^{F/+}$;*Pten*$^{F/F}$ invasive intestinal adenocarcinoma model and the spontaneous oral papilloma *K14-CreER;Kras*$^{G12D/+}$ model. In these models, CXCR2 deficiency or inhibition retards tumour formation[102]. However, it should be noted that CXCR2 expression is not exclusive to neutrophils. Depletion of the entire neutrophil population using anti-Ly6G (lymphocyte antigen 6 complex, locus G) antibodies phenocopies CXCR2 deficiency and hinders tumorigenesis in both chemically induced[101,102] and spontaneous[102] tumour models. In a zebrafish model of *Hras*^{G12V}-driven melanoma, wounding-induced inflammation increases the formation of tumours in a neutrophil-dependent manner[105]. Thus, neutrophils can provide a causal link between inflammation and cancer.

Tumours in various mouse models of KRAS-driven lung cancer — such as *Cc10-Cre;Kras*^{G12D} (also known as *Ccsp-Cre;Kras*^{G12D}), adenovirus-Cre treated *LSL-Kras*^{G12D} and *Kras*^{LA1} models — upregulate neutrophil-related chemokines and display expansion of neutrophils[90,106–109] (FIG. 2). These phenotypes may be a result of direct upregulation of neutrophil-related cytokines such as GM-CSF and CXCL8 by KRAS signalling[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 signalling reduces the number of pulmonary tumours 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 tumour 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 tumour initiation[108,111,115], but the exact mechanisms need further elucidation. Neutrophil production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and angiogenic factors such as MMP9 (REF. 116) may also be important for tumour initiation (FIG. 3). In future work, genetically engineered mouse tumour models will be extremely valuable in this area of cancer-related neutrophil biology, as they enable neutrophils and neutrophil-derived factors to be manipulated as tumours arise *de novo*.

Neutrophils and tumour growth

Early studies on neutrophil function during tumour growth set the stage for the ongoing discussion over when and how neutrophils can be antitumorigenic or pro-tumorigenic. More than two decades ago, it was shown that neutrophils can mediate tumour rejection of G-CSF-producing colon cancer cells transplanted into mice[117]. A few years later, an opposing tumour-promoting role was uncovered when mice bearing transplantable tumours that were depleted of neutrophils via anti-Gr1 antibody showed reduced tumour growth[118,119].

Box 1 | Neutrophils and myeloid-derived suppressor cells

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 REFS 130,207). The appearance of MDSCs is a consequence of a pathological condition, such as cancer, infection or 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 nonspecific nature of the Gr1 antibody used to identify them (clone RB6-8C5). Gr1 binds to two antigens, lymphocyte antigen 6 complex, locus C (Ly6C) and Ly6G, which identify two major cellular subsets in tumour-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 but also identify 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 distinguish 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 tumour-bearing mice are MDSCs should be avoided because not all CD11b$^+$Gr1$^+$ cells are immunosuppressive in tumour-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 exist predominantly for one purpose or cannot perform 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 re-evaluated, and until convincing evidence is generated that distinguishes neutrophils from G/PMN-MDSCs, we consider G/PMN-MDSCs to be neutrophils with immunosuppressive capabilities.

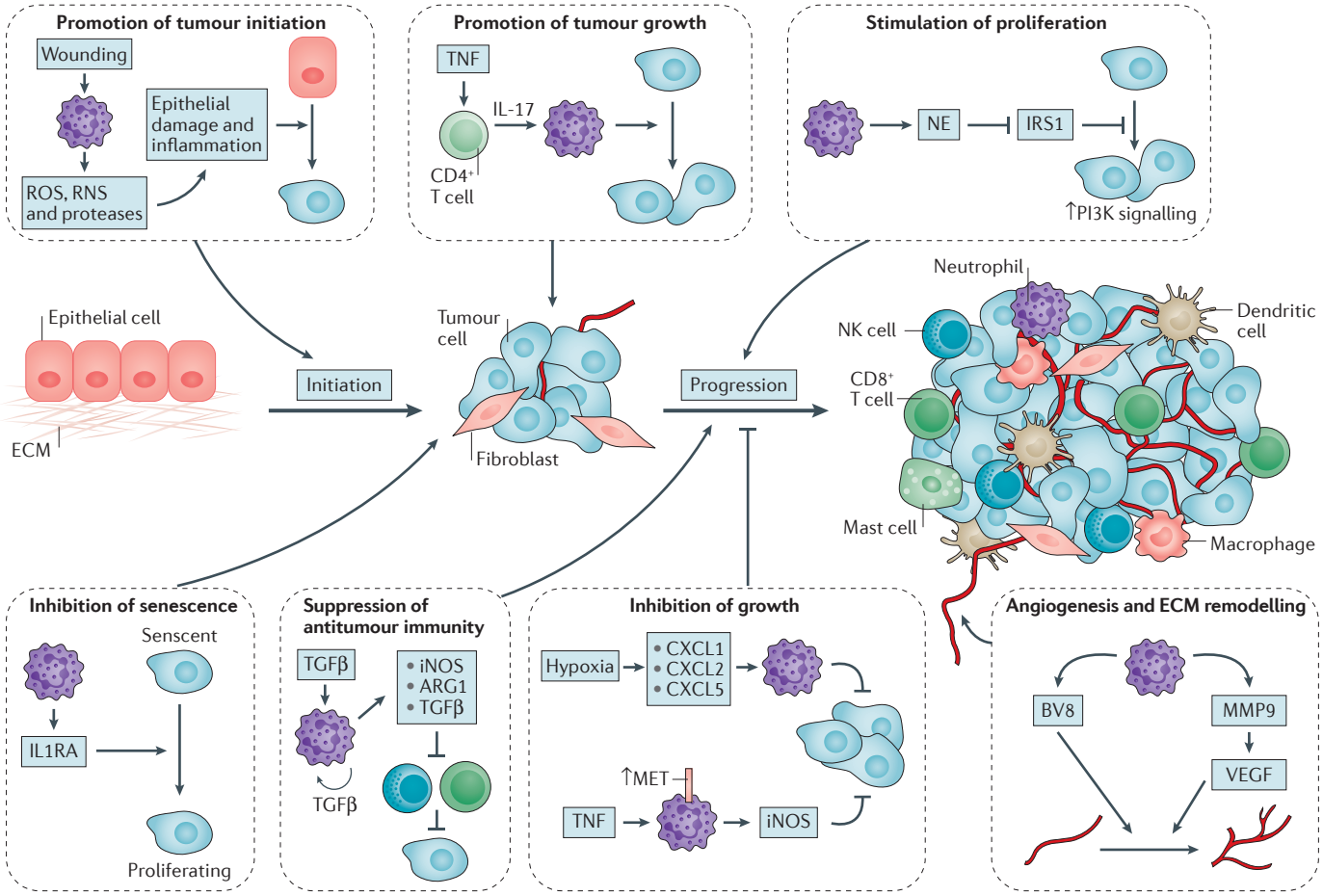

Figure 3 | **Neutrophil function in tumour initiation and growth.** There are several mechanisms by which neutrophils either promote or limit tumorigenesis. Transformation of an epithelial cell to a cancer cell can be supported by the production of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and proteases by neutrophils. These molecules induce epithelial damage and subsequent tumour-promoting inflammation. Epithelial damage by wounding also recruits neutrophils by prostaglandin E2 (PGE_2) to promote tumour initiation[105]. Promotion of tumour 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 tumour initiation, neutrophils promote progression of tumour growth by converting senescent cancer cells into proliferating cancer cells by 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 signalling[115]. Neutrophils express inducible nitric oxide synthase (iNOS, also known as NOS2) or arginase 1 (ARG1) to suppress $CD8^+$ T cell-mediated antitumour immune responses and promote tumour progression. Immunosuppression can also be accomplished by transforming growth factor-β (TGFβ) signalling in neutrophils[65,88]. In some contexts neutrophils can also limit tumour growth. Hypoxia in the tumour induces expression of C-X-C ligand 1 (CXCL1), CXCL2 and CXCL5 to recruit antitumour neutrophils[140]. Upregulation of the hepatocyte growth factor receptor 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 remodelling 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].

Since then, the literature showing a tumour growth-promoting role for neutrophils *in vivo* has largely outweighed the studies showing an opposite effect. One mechanism used by neutrophils to promote tumour growth is the induction of angiogenesis (FIG. 3). Neutrophil depletion decreased tumour growth and microvessel density in both transplantable and spontaneous tumour models[65,85,91,120–123]. Blocking CXCR2 signalling 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 tumour-bearing mice increased tumour growth and angiogenesis[126], underscoring the ability of neutrophils to perpetuate proliferation. Several mitogenic and pro-angiogenic molecules have been implicated in neutrophil-driven tumour growth including elastase, prokineticin 2 (PROK2, also known as BV8) and MMP9 (REFS 115,120,126–129). Immunosuppression — through amino acid depletion or specific cytokine release — is another predominant

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

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

mechanism used by neutrophils to facilitate tumour progression[130]. Data from other disease models indicate that neutrophils are important players in directing adaptive immune responses (reviewed in REF. 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-1 receptor antagonist (IL-1RA) to promote prostate cancer progression in a PTEN-deficient autochthonous model[132].

Even though the literature on antitumorigenic neutrophils is less abundant, there have been some intriguing new data in this area. For example, in mice with transplanted mouse mammary tumour virus promoter-driven polyomavirus middle T antigen (*MMTV-PyMT*);*MMTV-Myc* mammary tumours, neutrophils hindered tumour 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 tumours and led to enhanced tumour growth of various transplantable cell lines and in a spontaneous liver cancer model[134]. Expression of MET in neutrophils was upregulated by endothelial cell-derived and cancer cell-derived tumour necrosis factor (TNF) in this study[134], whereas others have shown that TNF signalling in $CD4^+$ T cells led to increased IL-17 levels and neutrophil accumulation in ovarian tumour-associated ascites[121]. These data suggest that the control of neutrophil behaviour 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[134,135] models. The timing of neutrophil depletion experiments may be crucial for the interpretation of these data, as neutrophil function evolves from antitumoural to pro-tumoural in mice bearing transplantable cancer cell lines[93]. Antibody-dependent cellular cytotoxicity (ADCC) is another mechanism that neutrophils can use to kill cancer cells after antibody therapy (reviewed in REF. 136). It remains to be seen whether ADCC occurs *in vivo* without exogenous antibodies, as cancer-induced endogenous antibodies are known to activate pro-tumoural programmes in myeloid cells via Fc receptors[137,138]. Taken together, more research emphasis should be put on determining the context in which neutrophil behaviour is modulated.

Several studies have demonstrated the importance of neutrophils in tumour progression by blocking neutrophil recruitment to tumours, usually by CXCR2 inhibition. For instance, prostate cancer cells in probasin (*Pbsn*)-*Cre4*;*Pten*$^{F/F}$;*Smad4*$^{F/F}$ mice upregulated CXCL5 via the Hippo–YAP1 (Yes-associated protein 1) pathway and blockade of YAP1 or CXCR2 decreased immunosuppressive neutrophil recruitment to tumours and blunted tumour 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 tumour burden[140]. Hypoxia-induced CXCL1, CXCL2 and CXCL5 recruited neutrophils, and these cells impeded tumour growth by promoting cancer cell detachment from the basement membrane via modulation of integrins. Interestingly, neutrophils deficient in MYD88 signalling maintained their trafficking ability, but lost their antitumorigenic 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 tumour type and whether neutrophil-recruiting molecules can be uncoupled from neutrophil-activating molecules.

Tumour metastasis

Most neutrophil-centred studies published in the cancer field in recent years pertain specifically to metastasis. Neutrophils actively participate in various steps of the metastatic cascade: cancer cell escape from the primary tumour, intravasation into the blood and/or the lymphatic vascular system, survival in circulation, extravasation into distant organs and outgrowth of metastases (FIG. 4). As early as the late 1980s — before the importance of neutrophils in primary tumour growth was established[117–119] — intravenous co-injection of cancer cells and neutrophils from tumour-bearing rodents 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 whereby 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 colocalize 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 tumours. 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 tumour, intravasation and priming of the premetastatic niche. Spontaneous models of metastasis indicate that neutrophils are important for intravasation and formation of the premetastatic niche.

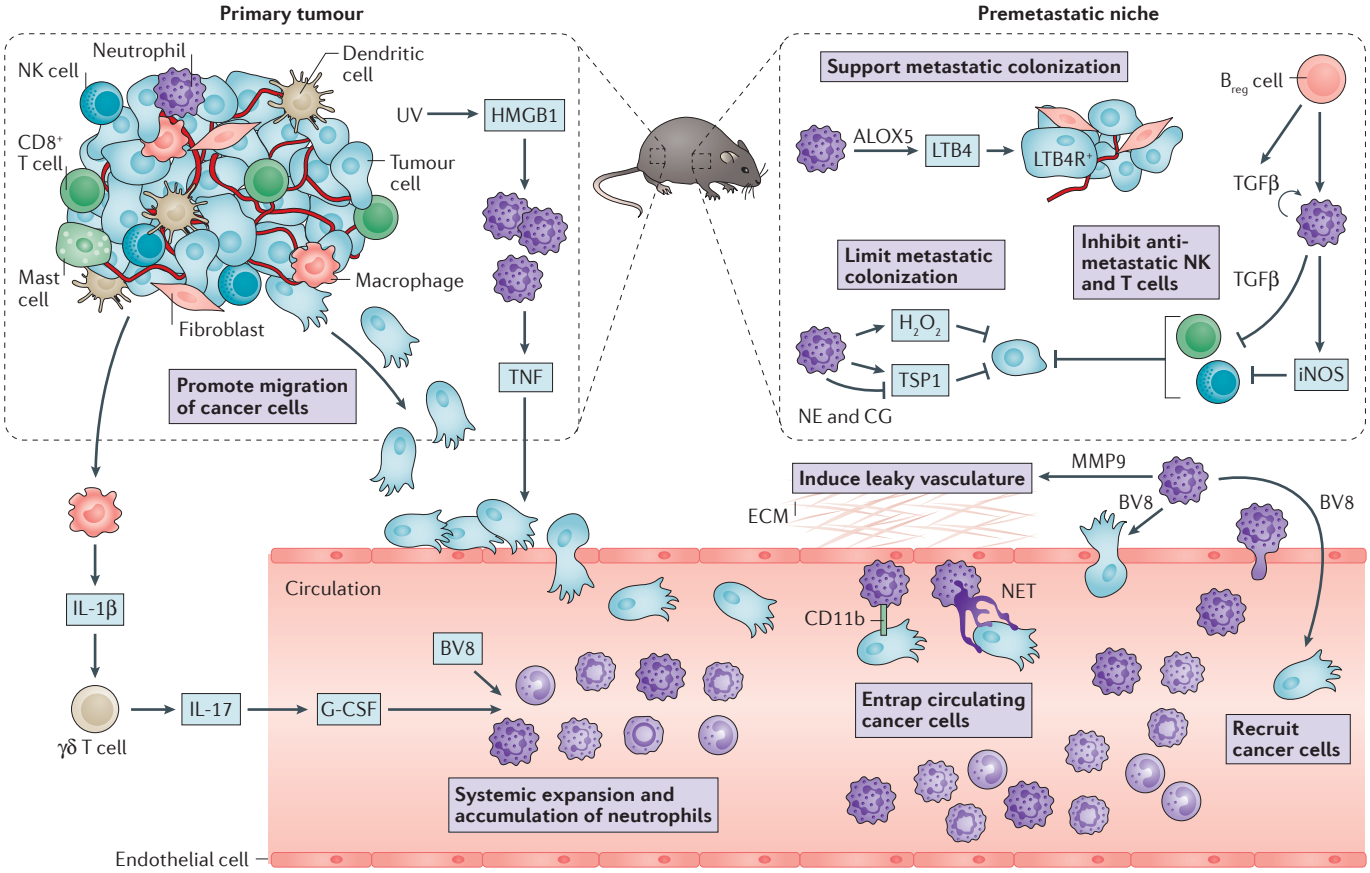

Figure 4 | **Impact of neutrophils on the metastatic cascade.** Neutrophils influence several steps of metastasis. In melanoma, ultraviolet (UV) radiation causes release of high mobility group box 1 (HMGB1) from keratinocytes, which recruits neutrophils through Toll-like receptor 4 (TLR4) signalling. These neutrophils induce migration of cancer cells towards endothelial cells by tumour necrosis factor (TNF), leading to enhanced metastasis[148]. In mammary tumours, interleukin (IL-1β)-expressing macrophages instigate IL-17-producing γδ T cells, resulting in granulocyte-colony stimulating factor (G-CSF)-dependent systemic expansion of neutrophils. At the metastatic site, these neutrophils limit antitumour $CD8^+$ T cell responses by producing inducible nitric oxide synthase (iNOS)[26]. In addition, regulatory B (B_{reg}) cells instruct neutrophils to limit T and natural killer (NK) cell responses to the metastatic lesion[88]. Neutrophils can support leukotriene B4 (LTB4) receptor (LTB4R)-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 (REFS 128,129). BV8 is also directly involved in cancer cell migration and the expansion of neutrophils[28,128,129]. Antimetastatic 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.

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*;cyclin-dependent kinase 4 ($Cdk4$)^{R24C} mice exposed to ultraviolet (UV) light showed cancer cell clustering around blood vessels and increased lung metastasis but no effects on primary tumour growth[148]. In this setting, UV-induced damage to keratinocytes increased the levels of high mobility group box 1 (HMGB1), which recruits Toll-like receptor 4 $(TLR4)^+$ neutrophils to primary tumours. 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 tumour microenvironment to expedite metastasis.

An interesting consequence of tumour expansion at the primary site is the accumulation of neutrophils in visceral organs before the arrival of disseminated

Premetastatic niche
A microenvironment in secondary organs primed by the primary tumour that is populated by non-cancer cells and promotes seeding of metastasizing cancer cells.

cancer cells[25,26,28,133,149–152], in what has been termed the premetastatic niche[153]. This accumulation of neutrophils in distant organs is highly reminiscent of the swarming behaviour 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 premetastatic niche supports LTB4 receptor (LTB4R)$^+$ metastasis-initiating cells in the *MMTV-PyMT* mouse model, and that inhibition of ALOX5 reduces pulmonary metastasis without affecting primary tumour growth[152]. But why do these neutrophils accumulate in premetastatic organs? In tumour-bearing mice, primary tumours release factors that systemically condition distant sites for future metastases. Neutrophil accumulation at distant sites is G-CSF dependent in some tumour models[25,26,28,152]; however, the original studies characterizing CD11b$^+$ myeloid cell recruitment to the premetastatic niche implicated vascular endothelial growth factor A (VEGFA), TNF and TGFβ[153,155].

Some or all of these tumour-derived factors may also dictate whether neutrophils promote metastasis at distant locations. Indeed, the genetic loss of TGFβ receptor 2 (TGFβR2) or TGFβ signalling blockade in neutrophils decreased lung metastasis in the 4T1 mammary tumour 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 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 tumour-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 antitumour functions of CD8$^+$ T cells, enabling 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 premetastatic niche[28,153,157]. Antagonizing KIT signalling or inhibition of 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 signalling 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 antimetastatic role of neutrophils. In stark contrast to the studies above that describe a metastasis-promoting role for neutrophils, others have shown that depletion of neutrophils increases metastasis[133,160]. The H_2O_2-mediated cytotoxic behaviour of these antimetastatic neutrophils is controlled by CCL2 (REF. 133). However, G-CSF still controls the transcriptional activity and expansion of neutrophils[26–28]. Controversially, these studies used the 4T1 mammary tumour cell line to show an antimetastatic 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? The timing of neutrophil depletion experiments may be crucial, as neutrophils isolated from early-stage tumours exhibit behaviour different from that of neutrophils from late-stage tumours[93,161]. Another possibility may be that the cell lines used by different laboratories 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, may skew the secretome, immunogenicity or behaviour of these cells. Microbiome differences between experimental animal cohorts may also influence neutrophil behaviour in conflicting ways. Indeed, neutrophil ageing is controlled by the microbiota in tumour-free mice[76].

In addition to their production of H_2O_2 (REFS 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 tumour-bearing mice with this peptide reduced spontaneous formation of pulmonary metastases without affecting primary tumour growth[163]. These data provide proof of principle that the pro-metastatic behaviour 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 the 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 as 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 owing to

Regulatory B cells
(B_{reg} cells). A subpopulation of immunosuppressive B cells involved in immunological tolerance.

Secretome
The total secreted factors of a cell or tissue.

the large variability in neutrophil levels between healthy individuals[165]. In addition, variation in the reported NLR cut-off points used to allocate patients to high-risk 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 count 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 patients with colorectal cancer, surgical removal of the primary tumour 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 pretreatment NLR that is maintained during treatment with tyrosine kinase or mTOR inhibitors experience a more favourable 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 measurement.

In comparison with NLR, the prognostic and predictive power of intratumoural neutrophils is murkier and more variable, and positive (gastric cancer[168]), negative (renal cancer[169] and melanoma[170]) or no (lung cancer[171]) correlation with patient outcome has been observed in different studies. Colorectal cancer is one example in which controversy surrounds the potential role of intratumoural neutrophils[172,173]. The markers used to identify tumour-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 tumour microenvironments. NLR is more reliable in this regard because blood neutrophils are easily separated from other immune cells by flow cytometry. Using combinatorial markers in tumour sections based on neutrophil polarization may provide some clarity. In fact, combinatorial approaches involving assessment of the expression of multiple neutrophil-related genes have recently been 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 tumour types[4,140]. Thus, moving beyond single markers may be necessary to accurately determine whether intratumoural neutrophils have prognostic or predictive power.

Neutrophils as therapeutic targets in cancer patients. Not only do neutrophils and their associated soluble mediators 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 targeting 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 CXCR2 inhibitor[175], are ongoing in cancer patients[176,177]. Importantly, characterization of neutrophil polarization in different tumour types as well as at early and late stages is urgently needed to maximize the utility of therapeutic modalities. In tumours in which neutrophils are beneficial, such as early-stage lung cancer[161], strategies to magnify their antitumour abilities should be explored.

Another neutrophil-associated pathway under intense investigation is the IL-23–IL-17 axis (reviewed in REF. 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 preclinical models and clinical samples indicate that this pathway is important for cancer progression[26,68]. Therapeutic strategies aimed at repolarizing tumour-induced neutrophils or interfering with their downstream protumorigenic effects could offer additional opportunities for intervention[65,152].

Combining neutrophil targeting with other anticancer 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 tumour models that have addressed the impact of neutrophils on the efficacy of anticancer therapies. As mentioned above, neutrophils are important mediators of angiogenesis, so perhaps it is no surprise that neutrophils induce refractoriness to anti-VEGFA therapy of experimental tumours in an IL-17- and G-CSF-dependent fashion[178–180]. These data suggest that simultaneous inhibition of neutrophils and antiangiogenic therapy might be an effective anticancer 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 themselves negatively affect neutrophil production. 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 have two explanations, 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 tumour killing. Alternatively, the patient survival benefit of chemotherapy-induced neutropenia may arise from reduction of 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 anticancer efficacy of chemotherapy (recently reviewed in REF. 186). For example, depletion of $Gr1^+$ myeloid cells or $Ly6G^+$ neutrophils reduced the anticancer efficacy of cyclophosphamide and doxorubicin in tumour inoculation models[187,188]. These data contrast with the improved tumour inhibition achieved by combining CXCR2 blockade with doxorubicin, cyclophosphamide or docetaxel in xenograft and *de novo* tumorigenesis mouse models[58,132]. Moreover, some chemotherapeutic agents, such as gemcitabine and 5-fluorouracil, directly reduce the viability and/or change the functionality of myeloid cells, which then influences the anticancer efficacy of these drugs. These drugs trigger IL-1β secretion from immunosuppressive monocytes and neutrophils, setting off a chain of inflammatory events that results in reduced efficacy of chemotherapy on subcutaneous EL4 thymomas in mice[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, 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 tumour growth after combining chemotherapy with G-CSF neutralization reported contradictory results[28,192], leaving the debate open. Therefore, it is crucial 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 anticancer therapy responses may reflect differences in tumour type, tumour model, immune status of the host and mechanism of tumour killing by a particular anticancer 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 continues 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; also known as PDCD1) or anti-PD1 and anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA4) synergizes with anti-CXCR2 or anti-Ly6G, respectively, to delay tumour 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 tumour models indicate that neutrophil infiltration into tumours and their systemic expansion are increased following macrophage blockade by CSF1R or CCR2 signalling[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 antiangiogenic drug sorafenib after macrophages are blocked in the *RIP1-Tag2* pancreatic and *MMTV-PyMT* mammary tumour mouse models[202]. Thus, targeting one myeloid cell population may require additional targeting of another myeloid cell population to counteract therapy resistance.

Conclusion and perspectives

The influential role of neutrophils in 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 tumour progression, but also encompass various polarization states. Each of these revelations opens up new opportunities for therapeutic intervention. A recurring theme from the recent literature that may help in the design of novel neutrophil-targeting, anticancer therapies is the crosstalk between neutrophils and other immune cell populations (TABLE 1). Interestingly, several of these communication networks mirror 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 enable 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 tumour-bearing mice[205]. Recently, a mouse model based on *Ly6g*-driven Cre recombinase, the Catchup mouse, was developed, which includes a fluorescent reporter, enabling the function of mature neutrophils to be monitored using *in vivo*

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

Factors	Source	Responder	Outcome	Refs
CXCL1, CXCL2, CXCL5 and CXCL8	• Megakaryocyte • Endothelial cell • Cancer cell	Neutrophil	Neutrophil release from bone marrow in homeostasis and cancer; recruitment to tumours	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 cell	G-CSF-mediated granulopoiesis in homeostasis and cancer	26, 47, 49, 57,121
IL-23	• Macrophage • Dendritic cell	• $CD4^+$ T cell • γδ T cell	IL-17- and G-CSF-mediated granulopoiesis in homeostasis and cancer	49
iNOS and ARG1	• Neutrophil • Monocyte	• T cells • NK cell	Suppression of antitumour immunity	26,130
TGFβ	• Neutrophil • B_{reg} cell	• T cells • NK cell • Neutrophil	Immunosuppression in tumour 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 tumours; MET upregulation in neutrophils	57,121, 134,148
THPO	Unknown	Megakaryocyte	CXCR2 ligand-dependent release of neutrophils from bone marrow in homeostasis	38

ARG1, arginase 1; B_{reg} cell, regulatory B cell; CXCL, C-X-C chemokine ligand; CXCR, C-X-C chemokine receptor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte–macrophage-colony stimulating factor; IL, interleukin; iNOS, inducible nitric oxide synthase; MET, hepatocyte growth factor receptor; NK cell, natural killer cell; TGFβ, transforming growth factor-β; THPO, thrombopoietin; TNF, tumour necrosis factor.

imaging[206]. One value of this model stems from its ability to specifically delete neutrophil-derived molecules at later stages of neutrophil differentiation. We predict that this model and others like it will provide valuable information about the involvement of neutrophils and their molecular products in tumour 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 tumour-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 behaviour of neutrophils in cancer and other diseases.

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Competing interests statement
The authors declare no competing interests.