

# The Complex Role of Neutrophils in Tumor Angiogenesis and Metastasis

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## Abstract

Chronic inflammation fosters cancer development and progression and also modulates tumor responses to anticancer therapies. Neutrophils are key effector cells in innate immunity and are known to play a critical role in various inflammatory disorders. However, the functions of neutrophils in cancer pathogenesis have been largely neglected until recently and still remain poorly characterized compared with other immune cells in the

tumor microenvironment. We highlight recent findings on the mechanisms by which tumor cells, in cooperation with tumor-associated stromal cells, induce expansion, recruitment, and polarization of neutrophils. We also review the multifaceted roles that neutrophils play in different aspects of cancer development and progression, with an emphasis on tumor angiogenesis and metastasis. *Cancer Immunol Res*; 4(2); 83–91. ©2016 AACR.

## Introduction

Neutrophils are the most abundant white blood cells (WBC) in the human circulatory system and constitute an important part of the first-line defense against infection (1). Neutrophils are constantly generated through granulopoiesis in the bone marrow and are mobilized in the peripheral circulation to patrol for invading pathogens (1). Chemokines produced at the infection/inflammatory sites attract neutrophils, where they exert anti-infection/proinflammatory functions, such as phagocytosis of pathogens; release of antimicrobial products, including reactive oxygen species (ROS), antibacterial peptides, enzymes, and neutrophil extracellular traps (NET); and production of cytokines and chemokines to recruit other immune cells (1, 2).

Inflammation has been long recognized as a key aspect of cancer development, which can fuel both primary tumor growth and metastasis (3). Therefore, it is not surprising that neutrophils, a key inflammatory cell type, can be mobilized and recruited to tumors. In fact, aberrant accumulation of neutrophils has been documented in a wide variety of tumors and is often associated with poor clinical outcomes (4–11). Emerging evidence also suggests that neutrophils, in response to signals derived from cancer cells or stromal cells, can alter their phenotypes and migration routes and also release factors that act on tumor cells and other cell types (e.g., endothelial cells and immune cells), which we review in the following sections.

## Deregulation of Neutrophils by the Tumor Microenvironment

The induction of granulopoiesis, a cascade of cellular events that lead to neutrophil production, is mainly regulated by

granulocyte colony-stimulating factor (G-CSF) and its receptor, G-CSFR (12). G-CSF is a ~25 kDa secreted glycoprotein encoded by the *csf3* gene (13). It is produced by endothelial cells, fibroblasts, monocytes, and macrophages in response to Toll-like receptor ligands and various proinflammatory cytokines (13, 14). G-CSF binds to its receptor, expressed in neutrophils and in neutrophil progenitor cells, and activates the downstream Janus kinase (JAK)/signal transducer and activator of the transcription 3 (Stat3) pathway (15), an essential signaling pathway for cancer inflammation (16). Activation of the JAK/Stat3 signaling pathway leads to expression of genes that are required for granulopoiesis (e.g., *MYC*, *CEBPB*; ref. 17). As a result, G-CSF promotes commitment to granulocyte development, neutrophil progenitor cell proliferation, and survival of mature neutrophils (13, 14). In addition, G-CSF facilitates release of neutrophils and hematopoietic progenitor cells from the bone marrow through downregulation of the CXCL12/CXC-chemokine ligand 12 (CXCL12)/CXC-chemokine receptor 4 (CXCR4) axis that mediates retention/homing of neutrophils to the bone marrow (18). G-CSFR is a member of the type-1 cytokine receptor family encoded by the *CSF3R* gene (13). It contains a conserved cytokine receptor homologous (CRH) domain, an Ig-like domain, and three fibronectin type III-like domains in the extracellular region; a single transmembrane region; and an intracellular region without intrinsic catalytic activity (19, 20). Upon G-CSF binding, G-CSFR forms a "cross-over," 2:2 ligand:receptor complex, in which each G-CSF molecule binds to both receptors (19, 21). Expression of G-CSFR has been detected in both hematopoietic and nonhematopoietic cell types, including the placenta, neurons, endothelial cells, cardiomyocytes, and cancer cells (22). In the hematopoietic system, G-CSFR is predominantly expressed in myeloid lineage cells such as myeloid progenitor cells, granulocytes, and monocytes (22).

Mice deficient in G-CSF (23) or G-CSFR (24) manifest severe neutropenia under basal and stressed conditions (e.g., bacterial infection; ref. 25). Administration of exogenous G-CSF remains the most efficient method to induce granulopoiesis and mobilization of hematopoietic progenitor cells in humans and animals (14). Other factors, including GM-CSF, IL6, and

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thrombopoietin, can also contribute to granulopoiesis, although to a lesser extent than G-CSF. Knockout mice for GM-CSF (26), IL6 (27), or thrombopoietin (28) have been examined and, in contrast to G-CSF<sup>-/-</sup> (23) or G-CSFR<sup>-/-</sup> (24) mice, appear to have normal neutrophil numbers in the blood. However, mice lacking both G-CSF (or its receptor) and GM-CSF (26), IL6 (29), or thrombopoietin (ref. 28; double knockout) display more severe neutropenia than mice lacking only G-CSF (or its receptor). It is noteworthy that mice lacking all three myeloid colony-stimulating factors (G-CSF, GM-CSF, and M-CSF) can still generate macrophages and granulocytes, albeit at substantially reduced levels (30).

Elevated G-CSF, as well as GM-CSF or IL6, have been documented in human and mouse cancers and are often associated with paraneoplastic "leukemoid reactions" (PLR), characterized by high WBC counts of more than 50,000/ $\mu$ L (in humans), with neutrophils being the predominant cell type (16, 31–36). PLR can occur in 10% to 15% of cancer patients and are strongly predictive of poor clinical outcomes (33, 34). We have previously reported that activation of the oncogenic RAS/MEK/ERK pathway induces expression of G-CSF from tumor cells through the Ets transcription factor (37). Blockade of G-CSF release through MEK inhibition, antibody-mediated G-CSF neutralization, or targeted inactivation of the G-CSF receptor in mice, resulted in suppression of aberrant neutrophil accumulation and inhibition of tumor angiogenesis and metastasis (35, 37–39). The significance of tumor-derived GM-CSF or IL6 on neutrophil homeostasis in malignancies has been less extensively characterized. However, it has been shown that augmented expression levels of GM-CSF and IL6 in tumor-bearing mice are associated with increased myeloid-derived suppressor cells (MDSC), a heterogeneous population of monocytic and granulocytic myeloid cells that promotes cancer progression through various mechanisms (40, 41).

Mechanisms of neutrophil recruitment to the inflamed tissues have been reviewed in detail elsewhere (1, 2) and are not discussed here. Of interest, tumors do share some basic chemotaxis mechanisms that regulate recruitment of neutrophil to inflammatory sites. For example, tumor cells and stromal cells in the tumor microenvironment secrete chemokines such as CXCL1, CXCL2, CXCL5, and CXCL8 to attract neutrophils (reviewed in refs. 42, 43). G-CSF induces expression of Bv8/prokineticin-2, a protein that induces angiogenesis, and also facilitates recruitment of CD11b<sup>+</sup>Ly6G<sup>hi</sup> neutrophils in both primary tumors and the metastatic sites (35, 38, 39). Once neutrophils arrive at tumor sites, they can be instructed by tumor-derived factors to "tune up" their tumor-supporting functions. One characteristic feature of advanced solid tumors is hypoxia. In fact, hypoxia promotes HIF-1 $\alpha$ -dependent neutrophil survival (44, 45) but impairs the respiratory burst activity in human neutrophils (46). In addition, it has been reported that blockade of TGF $\beta$  in the tumor microenvironment drives neutrophil polarization from a protumoral "N2" phenotype to an antitumoral "N1" phenotype characterized by enhanced cytotoxic and immunostimulatory activities (47).

## Tumor-Supporting Functions of Neutrophils

Growing evidence supports a protumoral role of neutrophils. In this section, we review the protumoral functions of neutrophils

on tumor cells and tumor-associated stromal cells at primary tumors and metastatic sites.

### Tumorigenesis, tumor cell proliferation, and survival

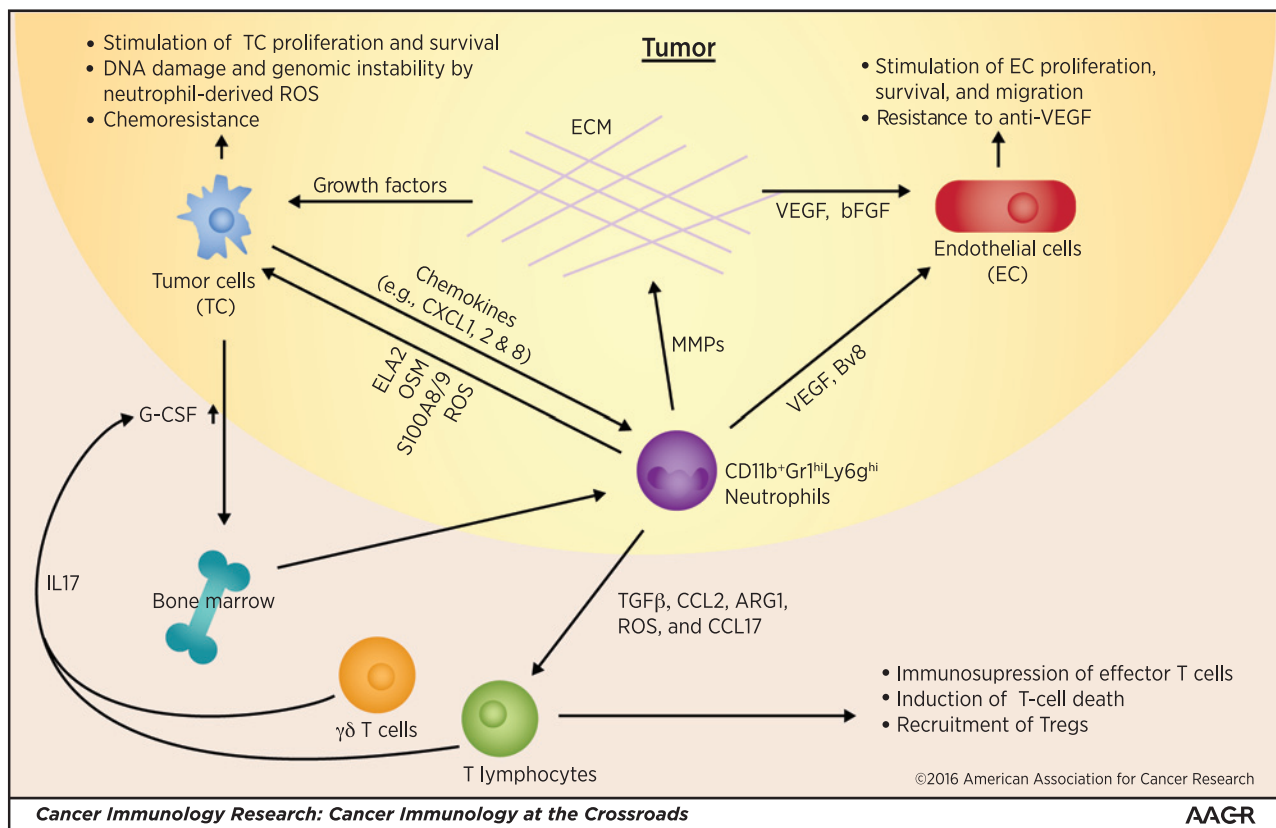
Neutrophils can directly act on premalignant epithelial cells to accelerate tumorigenesis. Work by Coussens and colleagues has shown that matrix metalloproteinase 9 (MMP9), supplied by bone marrow-derived cells, contributes to skin carcinogenesis (48). Among various cell types in the tumor microenvironment, neutrophils are a rich source of MMP9, yet lack expression of tissue inhibitor of metalloproteinases (TIMP), the endogenous inhibitors of MMP9. This renders neutrophil-derived MMP9 more prone to activation and participation in protumoral functions (49). Neutrophils produce ROS (through the action of myeloperoxidase and NADPH oxidase), which is known to cause DNA damage, genome instability, and gene mutation in premalignant epithelial cells and drives oncogenic transformation (50, 51). Together, these observations suggest that neutrophils recruited to chronic inflammation sites may foster tumorigenesis through multiple mechanisms.

During tumor progression, neutrophils release factors that stimulate tumor cell proliferation (Fig. 1). Neutrophil elastase (ELA2) can enter tumor cells, activate phosphoinositide 3-kinase (PI3K) through degradation of its negative regulator insulin receptor substrate-1 (IRS-1), and promote tumor cell proliferation (52). Moreover, breast cancer cells can induce neutrophils to produce oncostatin M (53), a factor known to stimulate tumor cell proliferation through activation of Stat3 (16). A recent study also shows that carcinoma-derived CXCL1/2 facilitates recruitment of S100A8/9-positive granulocytes, which induced tumor cell survival, metastasis, and resistance to chemotherapy (54).

### Angiogenic properties of tumor-associated neutrophils

Angiogenesis is the formation of new blood vessels and involves proliferation, migration, and differentiation of endothelial cells (55). Angiogenesis is a critical step during cancer development, and it has become clear that not only tumor cells but also stromal cells in the tumor microenvironment can supply proangiogenic factors. Tumor-infiltrating neutrophils can mediate the angiogenic switch in a transgenic mouse tumor model (56). Also, tumors grow faster, become highly vascularized, and are more infiltrated by neutrophils in IFN $\beta$ -deficient mice compared with the wild-type mice; depletion of neutrophils eliminates the enhanced tumor growth and angiogenesis in IFN $\beta$ -deficient mice (57). In myxofibrosarcoma patients, elevated numbers of neutrophils positively correlated with tumor microvessel density (58). Moreover, intratumoral infiltration of neutrophils is significantly correlated with tumor grade in glioma patients (11, 59) and with acquired resistance to anti-VEGF therapy in tumor-bearing mice (59).

Vascular endothelial growth factor (VEGF)-A is a potent angiogenic factor and a validated therapeutic target for blocking tumor as well as intraocular angiogenesis (60). Tumor-associated neutrophils contain a large intracellular pool of VEGF that can be rapidly released upon stimulation (61). Moreover, *de novo* synthesis of VEGF mRNA has been reported in tumor-associated neutrophils, in spite of the low gene transcription in mature neutrophils (62). Accordingly, elevated amounts of VEGF are found in neutrophils isolated from the oral cavity



**Figure 1.**

Schematic overview of the tumor-supporting functions of neutrophils. Tumor- or stroma-derived G-CSF stimulates neutrophil production in the bone marrow and subsequent release in the circulation. Circulating neutrophils, recruited into the tumor by various chemokines, locally produce factors (e.g., ELA2, OSM, and S100A8/9) that promote tumor cell proliferation, survival, and resistance to chemotherapy. Neutrophil-derived ROS can induce DNA damage in premalignant cells that facilitates oncogenic transformation. Neutrophils then support tumors through stimulation of tumor angiogenesis by releasing proangiogenic factors such as VEGF and Bv8. Neutrophils are a rich source of proteolytic enzymes, including MMPs and serine proteases, which can break down the extracellular matrix (ECM) and release the bioactive forms of growth factors and proangiogenic molecules. Additionally, neutrophils can secrete factors that induce local immunosuppression by impairing T-cell responses, inducing T-cell death, and recruiting regulatory T cells, which permits tumor development and progression. Other immune cells in turn produce factors such as IL17 derived from Th17 and  $\gamma\delta$  T cells that augment G-CSF levels and tumor-induced neutrophilia. ELA2, neutrophil elastase; OSM, oncostatin M.

of cancer patients compared with control subjects, and VEGF amounts in neutrophils are positively associated with disease stages (63).

MMP9 is a proteolytic enzyme that cleaves substrates within, and remodels, the extracellular matrix (ECM), facilitating endothelial cell movement. Moreover, the proteolytic activity of MMP9 releases VEGF and other growth factors that had been sequestered in an inactive form in the ECM (reviewed in refs. 64, 65). When neutrophils secrete TIMP-free MMP9, it liberates bioactive fibroblast growth factor-2 and VEGF from the ECM and induces tumor angiogenesis. Involvement of neutrophils in angiogenic switches has been illustrated in the RIP1-Tag2 transgenic pancreatic neuroendocrine mouse model by counting the number of islets undergoing angiogenesis under different conditions. When neutrophils are depleted by administration of antibodies to Gr1 (a marker on the neutrophil cell surface), the association of VEGF with its receptor is reduced, as is the number of islets undergoing angiogenesis (56). Interestingly, a recent study shows that MMP9-positive neutrophils can compensate for the loss of macrophages in

tumor-bearing CCR2-null mice by supporting tumor angiogenesis and progression (66).

In an effort to identify molecular and cellular mechanisms mediating tumor refractoriness to anti-VEGF therapy, we discovered that tumor infiltration by CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells results in reduced responsiveness to anti-VEGF antibodies, as compared with tumor models with little or no CD11b<sup>+</sup>Gr1<sup>+</sup> cell infiltration (67). Interestingly, a similar tumor responsiveness to anti-VEGF treatment was observed in both immunocompetent and XID mice, indicating that these effects of CD11b<sup>+</sup>Gr1<sup>+</sup> cells do not require B-cell or T-cell function (67). Further studies identified G-CSF produced by tumor or stromal cells as a critical mediator of accumulation of CD11b<sup>+</sup>Gr1<sup>+</sup> cells and consequent tumor refractoriness to anti-VEGF therapy (38, 39).

G-CSF is also induced when tumor-infiltrating T helper type 17 (Th17) cells secrete IL17, which in turn leads to expansion, mobilization, and tumor recruitment of myeloid cells (mostly neutrophils; ref. 68). Upon stimulation with G-CSF, neutrophils upregulate expression of Bv8/prokineticin-2 through activation of the Stat3 pathway (69–71). As noted, Bv8 stimulates

endothelial cell proliferation, survival, and migration (39) and also functions as a chemoattractant for neutrophils and, in some cases, for metastatic tumor cells (35). Blockade of IL17/IL17 receptor, G-CSF/G-CSF receptor, or Bv8—through genetic and pharmacologic approaches—inhibits intratumoral infiltration of neutrophils, tumor angiogenesis, and tumor growth (38, 39, 68). Importantly, Bv8 upregulation in response to G-CSF (and GM-CSF) has been reported in human neutrophils (71). The immunohistochemical localization of Bv8 protein in human tumor-infiltrating neutrophils has also been reported (71). In a complementary fashion, neutrophils can also recruit Th17 cells to the inflammatory sites through secretion of CCL2 and CCL20 (72). Collectively, these results suggest that neutrophils and Th17 cells can cross-talk and work in concert to induce, among other effects, tumor resistance to antiangiogenic therapy.

Besides VEGF, MMP9, and Bv8, recent work in the field has extended the list of neutrophil-derived factors that are known to sustain tumor angiogenesis. These factors include but are not limited to chemokines and cytokines (e.g., CXCL1, CXCL8, IL1 $\beta$ , and IL6), oncostatin M, and urokinase-type plasminogen activator (uPA; reviewed in refs. 73, 74). Nevertheless, whether and how these factors contribute to neutrophil-mediated tumor angiogenesis remains to be determined.

The results generated in various preclinical models strongly suggest a role for neutrophils in mediating tumor angiogenesis and refractoriness to anti-VEGF therapy. The clinical evidence supporting this notion, although still relatively limited, is growing (58, 63, 71). For example, in a study that investigated the role of inflammatory cells in predicting the clinical outcome in advanced nonsquamous non-small cell lung cancer patients treated with chemotherapy with or without bevacizumab, the authors found that a high number of circulating neutrophils and monocytes and a high neutrophil-to-lymphocyte ratio (NLR) are associated with poor clinical outcome only in patients treated with chemotherapy plus bevacizumab, but not in those treated with chemotherapy alone (75). The same group also reported that a low baseline NLR was associated with the longest progression-free survival (PFS) in colorectal cancer patients receiving bevacizumab as a first-line therapy (76). Also, in patients with metastatic renal cell carcinoma treated with the VEGFR tyrosine kinase inhibitor sunitinib, a low NLR (NLR  $\leq$  3) before treatment was associated with better response rate, longer PFS, and overall survival (OS; ref. 77). Although these findings are consistent with the important roles of neutrophils in mediating tumor angiogenesis and resistance to anti-VEGF therapy (Fig. 1), more studies are clearly needed to address the following concerns: (i) the direct involvement of neutrophils in triggering angiogenesis in human tumors; (ii) the role of neutrophils in predicting the clinical outcomes in patients receiving anti-VEGF therapies; and (iii) whether blockade of the proangiogenic function of neutrophils in cancer patients can reverse resistance to anti-VEGF therapy.

#### Neutrophils as negative regulators of anticancer immunity

Although a comprehensive review of neutrophil immunology is beyond the scope of this article, we want to emphasize that neutrophils can initiate and engage in complex cross-talk with other immune cells throughout their development and activation. Such cross-talk can reciprocally modulate the phenotypes of

neutrophils and other immune cells and thus control inflammation and immune responses under physiologic and pathologic conditions.

It is well established that tumor cells often induce an immunosuppressive microenvironment through impaired antigen presentation, release of immunosuppressive factors, induction of immunologic tolerance and recruitment of immune cells equipped with tumor-supporting machineries (78). In this context, multiple mechanisms have been proposed for the immunosuppressive features of neutrophils and granulocytic MDSCs, a heterogeneous cell population that shares remarkable features including surface markers, cell morphology, and function with tumor-associated neutrophils (79). For instance, tumor-infiltrating neutrophils produce TGF $\beta$  (80), an immunosuppressive cytokine with diverse effects on multiple lineages of immune cells.

Peripheral blood polymorphonuclear leukocytes isolated from patients with hepatocellular carcinoma release significantly more CCL2 compared with healthy subjects. This CCL2 inhibits peripheral blood mononuclear cell (PBMC) production of IFN $\gamma$ , an inhibition that is abolished by antibodies that block CCL2 (81). Neutrophils also exert their immunosuppressive function through production of arginase (ARG1; ref. 82) and ROS. Arginase depletes arginine from the surrounding environment, leading to inhibition of T-cell proliferation and function (83). ROS can suppress T-cell activation and, at high concentrations, induce apoptosis in T cells (84). In addition, tumor-associated neutrophils recruit immunosuppressive regulatory T cells (Treg) into tumors through secretion of CCL17 (85). In summary, the immunosuppressive environment fostered by neutrophils facilitates tumor growth and metastasis (Fig. 1).

#### Neutrophils in premetastatic and metastatic microenvironments

Metastasis remains the leading cause of death for patients with cancer. The multistep process of metastasis involves tumor cell migration, invasion, and escape from primary tumor sites, survival in circulation, extravasation and seeding at secondary sites, overcoming dormancy, and initiation of metastatic outgrowth. Meanwhile, tumor cells need to be protected from attack by the host's immune system throughout the process. Emerging evidence suggests that neutrophils, in response to tumor-derived stimuli, contribute to most if not all of these steps during cancer metastasis. In this section, we highlight recent findings on the input of neutrophils to different phases of cancer metastasis.

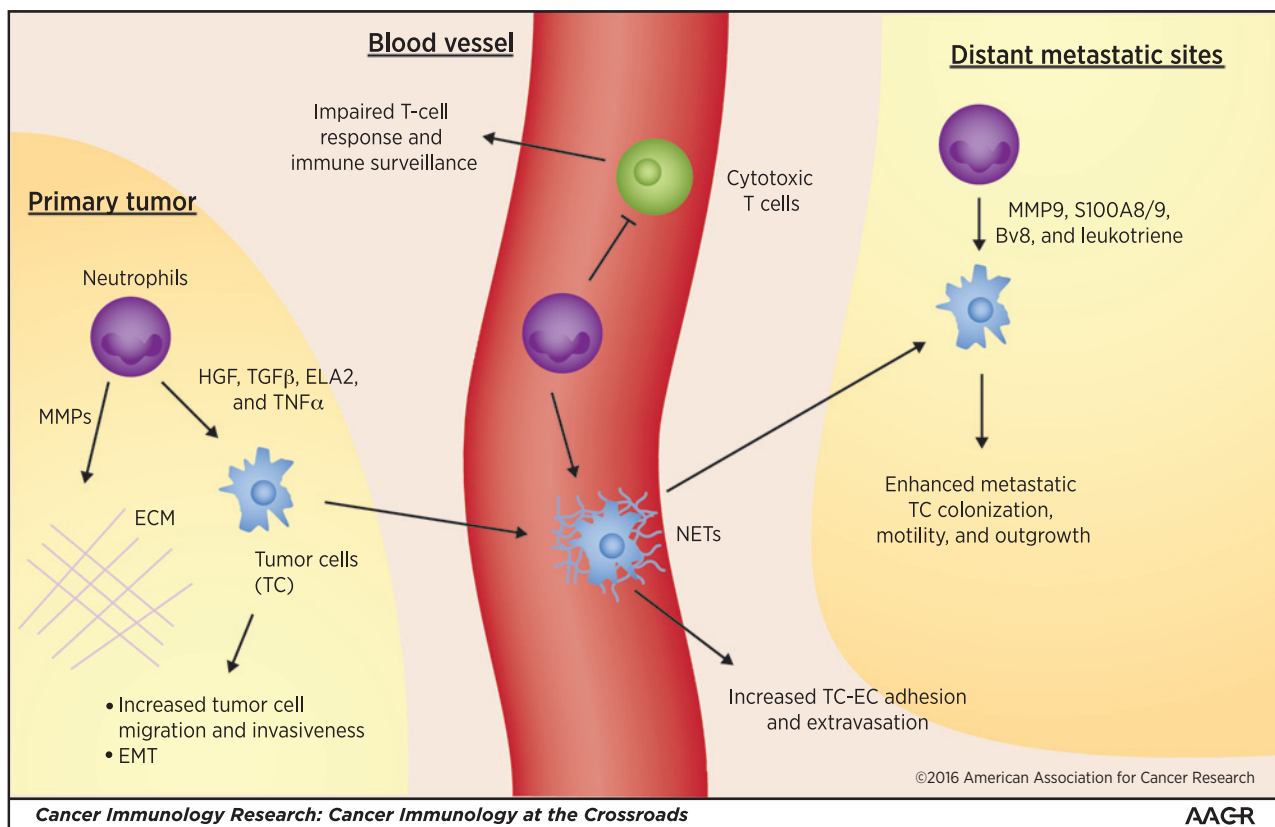
Neutrophils produce a variety of proteins that can stimulate tumor cell migration and invasion. For example, neutrophils maintain a large intracellular pool of serine proteases and MMPs (reviewed in refs. 73, 74) that can be released upon activation, which can facilitate tumor cell migration and invasion through remodeling ECM and increasing the bioavailability of (pro-migration and pro-invasion) signaling molecules. Alveolar neutrophils secrete hepatocyte growth factor (HGF), which induces human lung cancer cell migration (86). Moreover, some neutrophil-derived proteins are known to trigger epithelial-mesenchymal transition (EMT) of tumor cells. EMT is a developmental program that allows stationary epithelial cells to lose tight cell-cell junction and obtain the ability to migrate and invade during development (87). Tumor cells are

known to use this strategy to increase cell motility, invasiveness, and their ability to break/remodel basement membrane and ECM (87). EMT can also prevent circulating tumor cells from dying and facilitate extravasation and seeding at the secondary sites (87). Tumor-infiltrating neutrophils produce among other factors TGF $\beta$  (80), a primary inducer of EMT through upregulation of Snail1/2, Zeb1/2, and Twist1 (87). Neutrophil-derived TGF $\beta$  has been shown to induce EMT in lung adenocarcinoma cells (88), and a recent study suggests that neutrophil elastase can contribute to EMT by degradation of E-cadherin in tumor cells (89). Neutrophils isolated from inflammatory disorders express TNF $\alpha$  upon stimulation (90), which has also been shown to promote EMT (91).

Neutrophils in the peripheral circulation can also facilitate cancer metastasis by inducing cancer cells to adhere to endothelial cells at the extravasation sites. Circulating human melanoma cells secrete IL8, a neutrophil chemoattractant that also induces expression of  $\beta_2$  integrin (Mac-1) on neutrophils, which increases the binding of melanoma cells to neutrophils and endothelial cells, leading to increased metastasis (92). Another

study suggests that neutrophils promote adhesion of lung cancer cells to liver sinusoids and liver metastasis and this effect is partially reversed by Mac-1 or ICAM-1 blockade (93). In addition, Cools-Lartigue and colleagues reported that neutrophil-released neutrophil extracellular traps (NET) can contribute to cancer metastasis (94). NETs are neutrophil-derived structures composed of DNA, chromatin, and granule proteins and represent a host defense mechanism by trapping and killing microorganisms (95). Circulating tumor cells become trapped within NETs, and NET trapping increases formation of liver metastasis (94). Inhibition of NET with DNase or a neutrophil elastase inhibitor impedes metastasis development (94).

A recent revisitation of Paget's classic "seed and soil" hypothesis (96) is the "premetastatic niche" (97). Lyden and colleagues reported that VEGFR1<sup>+</sup> hematopoietic progenitor cells are recruited to the premetastatic sites and form cellular clusters before the arrival of tumor cells (97). These VEGFR1<sup>+</sup> cells can then promote adherence and growth of metastatic tumor cells, possibly through production of MMP9 and CXCL12 (97). Our work has suggested that neutrophils, rather than VEGFR1<sup>+</sup> cells,



**Figure 2.**

Neutrophils promote tumor metastasis. At primary tumor sites, neutrophils secrete factors that can induce EMT of tumor cells and support their migration machinery. MMPs produced by neutrophils can break down and remodel the ECM, which in turn facilitates tumor cell migration, invasion, and intravasation. In the peripheral circulation, neutrophils may support adhesion and retention of circulating tumor cells (to the endothelium at the metastatic target sites) through multiple mechanisms. For example, NETs produced by neutrophils can entrap circulating tumor cells and induce extravasation. In addition, neutrophils can facilitate cancer metastasis by suppressing the cytotoxic T-cell-mediated immune surveillance against metastatic tumor cells. At the distant metastatic sites (e.g., lung), neutrophils, along with other bone marrow-derived cells, arrive before tumor cells and constitute the "premetastatic niche" by releasing factors such as MMP9, S100A8/9, Bv8, and leukotrienes that promote colonization, migration, and outgrowth of metastatic tumor cells.

are the major cell type mobilized by signals derived from primary tumors (e.g., G-CSF) and recruited to metastatic sites such as lung and liver, to promote a permissive environment (35). We found that, at the metastatic sites, neutrophils express a spectrum of genes, *Bv8* and *S100A8* being among the most upregulated. As noted, *Bv8* facilitates further recruitment of neutrophils and seeding of metastatic tumor cells (35). Accordingly, treatment with antibodies to G-CSF or *Bv8* reduces aberrant accumulation of neutrophils at premetastatic organs and significantly inhibits lung metastasis (35).

It is noteworthy that several studies have confirmed and further explored the role of neutrophils in fostering metastatic niches and establishing cancer metastasis. The study by Casbon and others confirmed the presence of neutrophils in premetastatic lung tissue and found that prolonged exposure of G-CSF expanded T-cell-suppressive neutrophils, resulting in increased cancer metastasis (98). A recent study by Coffelt and colleagues found that expression of IL17 from  $\gamma\delta$  T cells induced expansion and polarization of neutrophils in mice bearing metastatic tumors (99). Tumor-induced neutrophils facilitated cancer metastasis by suppressing CD8<sup>+</sup> cytotoxic T-cell proliferation and activation (99). This effect was dependent on the IL17/G-CSF axis as neutralization of IL17 or G-CSF prevented neutrophil accumulation, relieved cytotoxic T cells from neutrophil-mediated immunosuppression, and inhibited cancer metastasis (99).

On the other hand, mice deficient in type I IFN signaling (*IFN $\alpha$ R1*<sup>-/-</sup>) have a higher rate of metastasis after tumor implantation compared with wild-type mice (100). This effect is associated with increased G-CSF, neutrophil accumulation, and expression of prometastatic proteins like *Bv8*, *MMP9*, *S100A8*, and *S100A9* at the metastatic sites (100). Type I IFN signaling is negatively correlated with IL17 signaling in T cells (101). It is conceivable that in *IFN $\alpha$ R1*<sup>-/-</sup> mice, IL17 signaling becomes hyperactivated, leading to G-CSF-mediated expansion and mobilization of neutrophils, as observed in this study. As mentioned previously, granulocytic myeloid cells can be recruited to the metastatic sites by tumor-derived CXCL1/2 and secrete *S100A8/9*, supporting metastatic tumor cell survival and chemoresistance (54). Furthermore, blockade of colony-stimulating factor-1 (CSF-1) or its receptor (CSFR1) can lead to increased lung metastasis associated with enhanced serum G-CSF, increased frequency of neutrophils at primary tumors, and metastasis to the (102). Administration of neutralizing antibodies against G-CSF receptor prevents neutrophil accumulation and metastasis promoted by blockade of CSF-1/CSFR1 (102). Additionally, a recent study confirmed the involvement of neutrophils in establishing the premetastatic lung microenvironment and indicated that neutrophil-derived leukotrienes can support colonization of metastatic tumor cells by selectively expanding a sub-pool of cancer cells with high tumorigenic potential (103). These findings are consistent with previous observations that antagonists of the leukotriene generating enzyme (104), or inhibition of leukotriene receptor (105), can suppress tumor metastasis. In summary, these findings are consistent with the hypothesis that tumor-associated neutrophils can promote cancer metastasis through multiple mechanisms that include induction of EMT and tumor cell migration and invasion, assisting extravasation, and creating the metastatic niche and the immunosuppressive microenvironment (Fig. 2).

## Antitumor Effects of Neutrophils

While tumor promotion seems to be the predominant outcome of the interaction between neutrophils and tumor/stromal cells, several studies have reported antitumor functions of neutrophils. For instance, it was suggested that neutrophils can directly inhibit tumor cell proliferation and survival through production of TRAIL, a TNF superfamily member that binds to its receptor in tumor cells and induces apoptosis (106). Also, "tumor-entrained" neutrophils have been reported to have antitumor and antimetastatic effects, based on the observation that antibody-mediated depletion of neutrophils in 4T1 and MMTV-PyMT mouse tumor models results in enhancement of metastasis (107). These findings, while intriguing, are contradicted by several reports (see above; refs. 35, 98, 103), showing that, in similar tumor models, tumor-associated neutrophils have clear prometastasis functions. In a different setting, the *cMet* proto-oncogene is expressed in neutrophils and required for chemoattraction and nitric oxide-dependent cytotoxicity of antitumoral neutrophils (108). As a result, *cMet* deletion in neutrophils stimulates tumor growth and metastasis (108).

Neutrophils are essential for development, survival, and activation of other immune cells under basal condition and during induction of immune responses against invading pathogens (reviewed in refs. 1, 2). It has been shown that blockade of TGF $\beta$  polarized the protumoral, immunosuppressive "N2" neutrophils to the antitumor, immunostimulatory "N1" neutrophils (47). It remains unclear, however, whether tumor-associated neutrophils are involved in the development of anticancer immunity without any therapeutic intervention. Previous published results (47, 98, 99) suggest that it is a strong possibility that tumor-associated neutrophils are primarily immunosuppressive, due to chronic exposure to tumor-derived signals, and can be polarized to be immunostimulatory through inhibition of the immunosuppressive signals (e.g., TGF $\beta$ ).

## Potential Strategies to Inhibit Neutrophils

The tumor-promoting actions of neutrophils provide a rationale for the development of therapies that target such cells. However, approaches that eliminate the whole neutrophil population are expected to have major adverse effects because neutrophils constitute a vital defense mechanism against foreign pathogens and depletion of neutrophils may render patients vulnerable to infections. Such approaches might also remove the host-beneficial antitumoral neutrophils. Alternatively, other strategies may be considered: (i) blocking neutrophil mobilization and recruitment to primary tumors and metastatic sites; (ii) polarizing neutrophils from the protumoral phenotype to the antitumoral phenotype; and (iii) specifically targeting neutrophil-derived molecules with tumor-supporting functions. For instance, therapeutics that reduce the expression levels of G-CSF by tumor or stromal cells (e.g., MEK/ERK inhibitor or anti-IL17) or the downstream targets of the G-CSF/G-CSFR axis (inhibitor of the JAK/Stat3 pathway or anti-*Bv8*) can be used to test the first strategy. Antagonists of GM-CSF or other chemokines may also be valuable. Anti-TGF $\beta$  therapy can be used to test the second strategy. To test the third strategy, one might consider antagonists of neutrophil elastase, *S100A8/9*, VEGF, *MMP9*, oncostatin M, neutrophil-derived serine proteases, and scavengers of ROS.

Additionally, blockade of the tumor-supporting functions of neutrophils may be valuable when combined with conventional chemotherapies and other targeted therapies. This is exemplified by the combinational treatment of tumor-bearing mice using antibodies to VEGF and to G-CSF, Bv8, or IL17. Our previous studies indicated that such combination therapies reduce tumor growth in mouse tumor models that are otherwise refractory to anti-VEGF treatment (38, 39, 68).

### Concluding Remarks and Perspectives

Emerging evidence supports an important yet complex role of neutrophils during tumor initiation, growth, angiogenesis, evasion from immunosurveillance and metastasis. Still, much remains unknown, and further characterization of neutrophil functions in the context of cancer and cancer-related inflammation is needed (see Box 1). The knowledge gained by addressing such questions is expected to advance our understanding of neutrophil recruitment, heterogeneity, programming, and functional plasticity, in response to signals derived from tumors and possibly other pathologic conditions. It should also facilitate identification of the most efficient strategies to block the tumor-supporting functions of neutrophils while preserving or even boosting the antitumoral functions.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### Box 1. Outstanding Questions

- Considering the difference between mouse and human neutrophils, to what extent can knowledge generated from mouse tumor models be applied to human cancer patients?
- What are the most efficient and reliable methods to identify and isolate neutrophils from mouse tumor models, and more importantly, human cancer patients?
- What is the developmental status of neutrophils recruited to primary tumors and metastatic sites?
- How do neutrophils interact with other cell types that are recruited to/constitute the metastatic niche? How does this interaction regulate cancer metastasis? What are the relative contributions of immunosuppression and direct stimulation of angiogenesis or tumor cell growth to neutrophil-induced tumor promotion? Are they tumor-type or tumor-stage dependent?
- Are the protumoral and antitumoral neutrophils fundamentally different neutrophil subsets? Or do they simply represent different developmental stages or activation states of neutrophils? Can the protumoral and antitumoral neutrophils coexist in the same tumor tissue? What surface/intracellular markers can be used to distinguish them? Which signaling pathways determine differentiation toward protumoral versus antitumoral phenotypes of neutrophils? How can we develop therapies to target such signaling pathways in order to suppress the protumoral while promoting the antitumoral effects?

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