



# The complex interplay between neutrophils and cancer

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Received: 28 November 2017 / Accepted: 12 December 2017 / Published online: 9 February 2018  
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## Abstract

Neutrophils are the most abundant type of white blood cell, and are an essential component of the innate immune system. They characteristically arrive rapidly at sites of infection and injury, and release a variety of cytokines and toxic molecules to eliminate pathogens and elicit an acute inflammatory response. Research into the function of neutrophils in cancer suggest they have divergent roles. Indeed, while most studies have found neutrophils to be associated with cancer progression, others have also documented anticancer effects. In this review, we describe the investigations into neutrophil populations that have been implicated in promoting tumor growth and metastasis as well those demonstrating antitumor functions. The collective research suggests a complex role for neutrophils in cancer biology, which raises the prospect of their targeting for the treatment of cancer.

**Keywords** Tumor-associated neutrophils · Cancer biomarker · Neutrophil polarization · Immunosuppression · Metastasis · Neutrophil targeted therapies

## Introduction

Neutrophils are the most plentiful leukocyte in humans, having diverse and critical roles in immunity. They are traditionally known as cells of the innate immune system, and are important first responders to sites of infection and injury (Kolaczowska and Kubes 2013). There, they phagocytose bacteria, produce antimicrobial molecules, and instigate acute inflammatory responses. However, their ability to secrete chemokines and

cytokines, as well as recently described activities such as forming extracellular traps and antigen presentation (Cools-Lartigue et al. 2013; Singhal et al. 2016), has indicated more diversity in neutrophil function than previously appreciated. One role identified in recent years is in the regulation of cancer (Coffelt et al. 2016).

Cancer is a complex disease typically resulting from DNA mutations that impart a set of pathological features onto otherwise normal cells. These include unfettered cell proliferation, resistance to cell death stimuli, genomic instability, abnormal invasion into local tissue, and metastasis to distant organs, among other well-documented characteristics (Hanahan and Weinberg 2000). More recently, inflammation and escape from immune-mediated destruction have been recognized as additional “hallmarks of cancer” (Hanahan and Weinberg 2011). Cancer achieves these using a variety of strategies, several of which are thought to involve neutrophils (Houghton et al. 2010; Bald et al. 2014; Wculek and Malanchi 2015; Kowanetz et al. 2010). Long known to inhabit the tumor microenvironment (TME), tumor-associated neutrophils (TANs) were traditionally considered bystanders. Now, many studies have implicated them in critical aspects of cancer biology. Neutrophils have been shown to promote tumor inflammation (Jamieson et al. 2012), which is a potent enabler of cancer cell proliferation, invasion and angiogenesis within tumors (Hanahan and Weinberg 2011). They have also been described to inhibit the activity of cytotoxic T-cells (Casbon et al. 2015; Rodriguez et al. 2004), both systemically and within the TME, thereby contributing to tumor immune

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evasion. These two activities define an important role for neutrophils in primary tumor growth and secondary metastases (Houghton et al. 2010; Wculek and Malanchi 2015). However, in seeming contradiction, other studies have reported that neutrophils produce cytotoxic compounds that are tumor-suppressing (Fridlender et al. 2009) and can function to promote anticancer T-cell responses (Governina et al. 2017). These activities serve to slow tumor growth and spread. Thus, it appears there is a complex interplay between neutrophils and cancer, one increasingly being recognized as important. Understanding this relationship is a rapidly growing area of cancer research, with significant promise for generating new insights into cancer biology as well as the development of cancer biomarkers and novel therapies.

## Neutrophils as tumor promoters

### Neutrophils associate with poor prognosis

Neutrophils are produced in the bone marrow and spleen in a process called granulopoiesis (Joyce et al. 1979; Spiegel et al. 2016). Once released into circulation, they travel throughout the body to sites of infection and injury where they perform important roles in host defense and tissue repair (Kolaczowska and Kubes 2013). In a normal adult human,  $1\text{--}2 \times 10^{11}$  neutrophils are generated per day in the steady state (Borregaard et al. 2010). Their production is regulated by their rate of apoptosis and consumption in tissue, and increases in response to infection or injury (Navegantes et al. 2017; Borregaard et al. 2010; Kolaczowska and Kubes 2013).

The similarities between tumors and sterile injury (Harold and Dvorak 1986) have prompted investigations into neutrophils as a cancer biomarker and the role they may play in tumor development and progression. It was reported as early as the 1970s that an increase in peripheral circulating neutrophil count correlates with poor survival in cancer patients (Riesco 1970). Indeed, an increased circulating neutrophil to lymphocyte ratio (NLR), similar to circulating C-reactive protein and albumin, has since become a clinically useful biomarker of cancer-related inflammation, and is being studied as a prognostic indicator for many cancer types (Walsh et al. 2005; Shimada et al. 2010; Cho et al. 2009; Koh et al. 2016; Sarraf et al. 2009; Azab et al. 2012; Sharaiha et al. 2011). Today, tumor-associated neutrophilia is an adverse prognostic feature in renal cell carcinoma, melanoma, colorectal cancer, hepatocellular carcinoma, cholangiocarcinoma, glioblastoma, gastric, esophageal, lung, ovarian and head and neck cancer (as reviewed in Donskov 2013). For example, in gastric cancer patients undergoing radical resection combined with cytokine-induced killer-based immunotherapy and postoperative chemotherapy, a preoperative NLR  $<2.995$  was an

independent prognostic factor for disease-free survival (Li et al. 2017). Similarly, in patients with metastatic castration-resistant prostate cancer treated with second-line chemotherapy, a NLR  $<3$  was associated with improved survival (Lorente et al. 2015). Qualitatively similar data have been reported in patients with many other cancer types (Asaoka et al. 2016; Orditura et al. 2016; Zhang et al. 2015; Cedrés et al. 2012). Although NLR cut-off values in these studies vary, it is consistently noted that higher levels of circulating neutrophils are associated with poorer outcomes. Fewer studies have explored the relationship between TANs and cancer prognosis, but those that have found TAN infiltration to be an independent prognostic factor for poor survival in cancers such as clear cell renal cell carcinoma, head and neck squamous cell carcinoma, and pancreatic tumors (Fridlender and Albelda 2012).

### Tumor-promoting mechanisms attributed to neutrophils

The correlation between neutrophils and poor cancer prognosis has prompted an important question: do neutrophils contribute to cancer development, progression and treatment resistance? While the role neutrophils play in cancer biology is far less understood than it is in pathogen infection or sterile injury, a growing body of literature suggests that neutrophils can function to promote tumorigenesis and metastatic spread. Broadly speaking, two mechanisms have been proposed to explain these activities: (1) systemic immunosuppression by circulating neutrophils, and (2) TME modulation by TANs.

#### Systemic immunosuppression by circulating neutrophils

Systemic immunosuppression is one way by which cancer cells can escape from immune-mediated destruction (Mizoguchi et al. 1992; Gunji et al. 1994). The mechanisms responsible are multifaceted, yet have been reported to involve circulating neutrophils by way of engendering T-cell dysfunction (Waldron et al. 2013; Schmielau and Finn 2001). Neutrophils are major producers of reactive oxygen species (ROS) and arginase I (ARG1) (Galli et al. 2011). ROS, which include superoxide anions ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radicals ( $HO^\bullet$ ), are highly reactive oxygen-containing molecules that cause oxidative stress in T-cells (Belikov et al. 2015). ARG1 is an enzyme that metabolizes arginine, an amino acid required for T-cell CD3zeta( $\zeta$ ) chain expression (Galli et al. 2011). Arginine depletion, therefore, results in T-cells that inadequately transmit signals required for their full activation (Waldron et al. 2013). Low arginine levels also inhibit cell cycle regulatory proteins, cyclin D3 and cyclin-dependent kinase 4, thus blocking T-cell proliferation (Waldron et al. 2013). It has been reported that circulating neutrophils negatively correlate with activated, interferon (IFN) gamma ( $\gamma$ )-producing T-cells in cancer patients

(Schmielau and Finn 2001). Those authors also found that  $H_2O_2$ , a significant neutrophil effector molecule, was responsible for suppressing IFN- $\gamma$  as well as other cytokines involved in T-cell activation, such as tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin (IL)-2, and IL-4. Similarly, others have reported that peripheral blood neutrophils isolated from patients with glioblastoma impair CD8+ T-cell function in a cell-based assay (Sippel et al. 2011). Neutrophils also exert immunosuppression in inflammatory contexts besides cancer (Pillay et al. 2013). A subset of neutrophils that express integrin Mac-1 (CD11b/CD18) were found to secrete  $H_2O_2$  into T-cell synapses, thereby suppressing T-cell proliferation in vitro (Pillay et al. 2012). Collectively, these studies suggest that circulating neutrophils help establish systemic immunosuppression within a cancer patient, which promotes immune tolerance toward their tumors.

### TME modulation by TANs

A second tumor-promoting mechanism attributed to neutrophils lies in their capacity to modulate the TME. TANs make up a significant proportion of leukocytes that infiltrate most TMEs (Fridlender and Albelda 2012). Similar to other neutrophils, TANs originate in the bone marrow or spleen and are released into circulation (Cortez-Retamozo et al. 2012). They are recruited to tumors by cancer-secreted factors, wherein their function becomes influenced by the cancer environment (Casbon et al. 2015). The mechanisms governing neutrophil recruitment to tumors are complex, yet are beginning to be unraveled. One commonly used mechanism is through hypersecretion of granulocyte-colony-stimulating factor (G-CSF). G-CSF is normally produced by damaged or infected tissue and is key regulator of granulopoiesis and neutrophil homeostasis in the steady state (Semerad et al. 2002; Kruger et al. 2015). Many cancers have acquired the ability to hijack the G-CSF signaling axis to promote neutrophil recruitment to tumors (Asano et al. 1997; Chakraborty and Guha 2007; Joshita et al. 2009; Kyo et al. 2000; Savarese et al. 2001; Tsukuda et al. 1993). Other mechanisms have also been reported, such as tumor-associated mesenchymal cells producing C-X-C motif chemokine receptor 1 (CXCR1) or CXCR2 agonists (Yu et al. 2017). Cancer-secreted TGF- $\beta$  has also been found to recruit neutrophils to the TME (Fridlender et al. 2009).

Once in the tumor, TANs produce cytokines, chemokines, and proteases that modulate tumor cell proliferation, angiogenesis and metastasis. Antimicrobial factors produced by neutrophils such as proteases, ROS, and myeloperoxidase (MPO) also have wide-ranging biological effects on tumors. For example, neutrophil elastase, a protease that destroys bacteria and aids in tissue remodeling, can promote tumor growth by altering intracellular signaling pathways within cancer cells. It has been reported that neutrophil elastase can degrade insulin receptor substrate 1 (IRS-1), which increases the

interaction between phosphatidylinositol 3-kinase (PI3K) and platelet-derived growth factor receptor (PDGFR), thereby skewing cancer cells toward proliferation (Houghton et al. 2010). MPO production by TANs plays a significant role in tissue remodeling and signal transduction, and has been implicated in the regulation of tumor growth and cancer cell apoptosis (Kessenbrock et al. 2010). Neutrophils have also been reported to stimulate angiogenesis through production of a VEGF-like cytokine called Bv8 (Shojaei et al. 2007).

Neutrophils also promote cancer progression by exerting immunosuppression within the TME. A recent study reported that TANs within gastric cancers inhibit anticancer T-cell activity through strong expression of the immune checkpoint programmed death ligand-1 (PD-L1) (Wang et al. 2017). Tumor cell-derived GM-CSF was found to induce the expression of PD-L1 on neutrophils through the activation of the Janus kinase/signal transducer and activator of transcription 3 (JAK/STAT3) signaling. Importantly, CD3+ T-cells were relieved of the immunosuppression exerted by co-cultured tumor-conditioned neutrophils when treated with anti-PD-L1 (Wang et al. 2017).

## Neutrophils as tumor suppressors

### Neutrophils associate with good prognosis and have tumor suppressor functions

While most studies suggest a tumor-promoting role for neutrophils, there is some evidence that neutrophils may correlate with *improved* survival. For example, studies have indicated that increased TAN infiltration is associated with better outcomes in advanced gastric cancer and colorectal cancer patients (Caruso et al. 2002; Sconocchia et al. 2011). More recently, an increase in CD16<sup>high</sup>CD62L<sup>dim</sup> neutrophils in the circulation of patients with head and neck squamous cell carcinoma was linked to more favorable outcomes (Millrud et al. 2017). The mechanisms by which neutrophils inhibit cancer progression can be broadly categorized into those that promote T-cell-mediated tumor clearance, and those that elicit cancer cell destruction through production of cytotoxic factors.

### T-cell-mediated mechanisms

Several studies have characterized mechanisms by which TANs promote the antitumor activity of T-cells. For example, Singhal et al. (2016) recently reported a role for neutrophils in antigen presentation (Singhal et al. 2016). Using multiparametric flow cytometry, the authors characterized a subset of neutrophils in human lung cancer patients they called “APC-like hybrid TANs”, based on simultaneous expression of APC markers (CD14<sup>+</sup>HLA-DR<sup>+</sup>HLA-

ABC<sup>hi</sup>CCR7<sup>+</sup>CD86<sup>+</sup>CD206<sup>+</sup>) and neutrophil markers (CD11b<sup>+</sup>CD66b<sup>+</sup>CD15<sup>hi</sup>). Functionally, APC-like hybrid TANs were found to produce pro-inflammatory cytokines TNF- $\alpha$  and IL-12, phagocytose, and cross-present tumor antigens for activation of anticancer T-cell responses. Investigations in colorectal cancer (CRC) have also provided evidence that TANs can promote T-cell activity (Governa et al. 2017). The addition of TANs derived from enzyme-digested CRC specimens to CD8<sup>+</sup> lymphocyte cultures increased CD69<sup>+</sup> expression, a marker of early T-cell activation, and enhanced IFN- $\gamma$  release from T-cells. Furthermore, concomitant CRC infiltration of CD66b<sup>+</sup> neutrophils and CD8<sup>+</sup> T-cells correlated with favorable prognosis. TANs were also shown to play a role in promoting antitumor inflammation after radiation therapy (Takeshima et al. 2016). Using syngeneic murine tumor models, a rapid recruitment of neutrophils to irradiated tumors was observed. These so-called “RT-TANs” were capable of producing ROS to induce apoptosis of tumor cells while also activating tumor-specific cytotoxic T-cells.

### Cytotoxic mechanisms

Mechanisms not directly linked to T-cell immunity have also been implicated in the antitumor activity of TANs. Early investigations into neutrophil-tumor biology observed neutrophils exerting direct cytotoxicity toward cultured tumor cells (Ackermann et al. 1989). More recently, neutrophils were found to reduce early-stage tumor growth and metastasis in a mouse model of uterine cancer by promoting basement membrane detachment of viable tumor cells in a process termed “tumor cell sloughing” (Blaisdell et al. 2015). Danger signals released from stressed tumor cells were found to engage toll-like receptors or the IL-1 receptor on neutrophils, which induced Myd88-dependent ROS, neutrophil elastase, matrix metalloproteinase 9 (MMP-9) and protease production to degrade components of the epithelial basement membrane. Another study reported a role for neutrophil extracellular traps (NETs) in neutrophil-mediated cancer suppression. NETs are networks of extracellular fibers primarily composed of DNA (Brinkmann et al. 2004). While they are typically released from neutrophils to bind pathogens as part of an innate immune response, they were recently found to inhibit the migration, proliferation and growth of cancerous cells (Millrud et al. 2017). Finally, a role for MET in antitumor neutrophil recruitment has been identified. MET is a proto-oncogene that codes for a tyrosine kinase receptor of which its only known ligand is hepatocyte growth factor (HGF). MET expression in neutrophils is triggered by inflammatory signals such as TNF- $\alpha$ , and is required for neutrophils to cross an activated endothelium (Finisguerra et al. 2015). In mouse models of HGF-secreting tumors, MET expression by neutrophils was found to be required for neutrophil trafficking to the tumor and

restriction of tumor growth and metastasis through production of nitrogen oxide (Finisguerra et al. 2015).

To summarize: from generating tumor-promoting inflammation and systemic/local-regional immunosuppression, to secreting cancer-killing factors and facilitating anticancer T-cell activation, a growing body of literature suggests multiple, ostensibly divergent roles for neutrophils in cancer biology (Fig. 1).

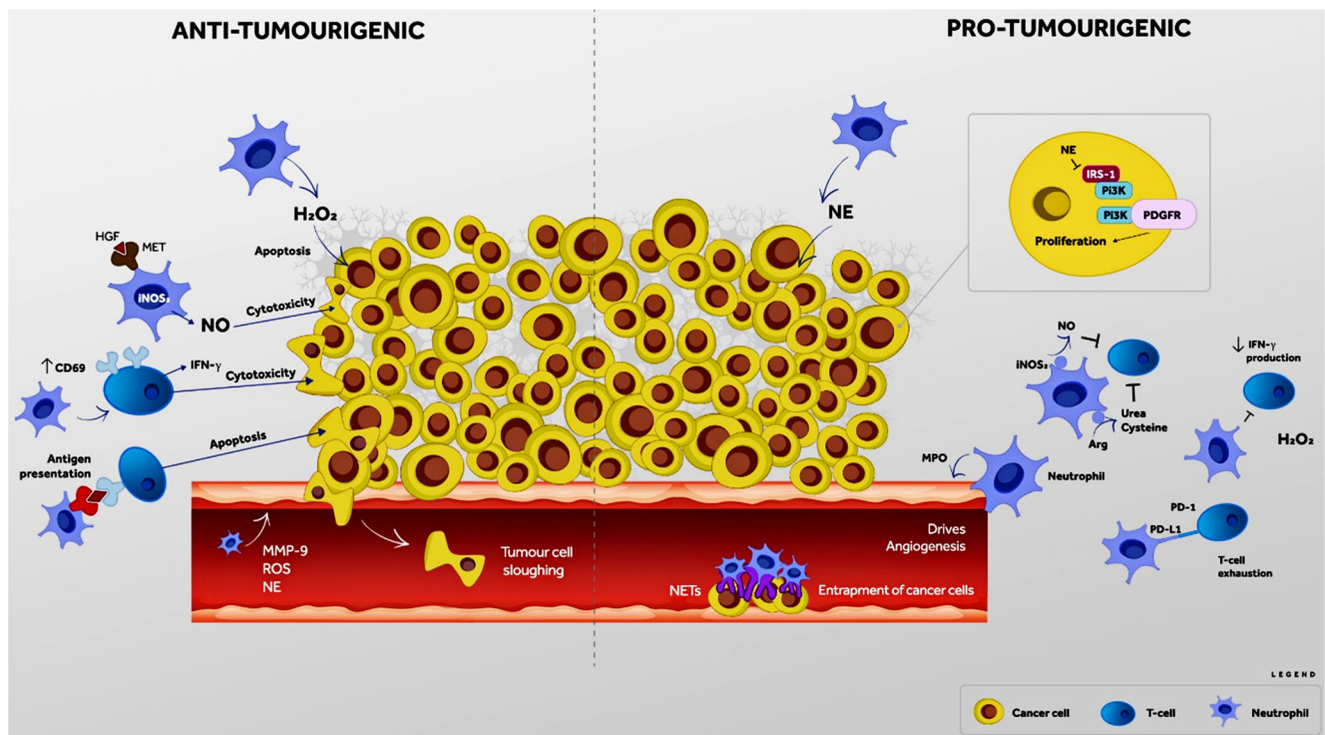
## Why do neutrophils have a dichotomous relationship with cancer?

There are at least two possible explanations for the seemingly conflicting roles of neutrophils in cancer: (1) the heterogeneity and plasticity of neutrophils, and (2) the phase of cancer development represented in the study.

### Neutrophil heterogeneity and plasticity

Based on their distinct morphology and function, it has historically been believed that neutrophils are terminally differentiated cells released from the bone marrow. However, this view has changed in light of observations made in conditions with altered immune states, such as systemic inflammation, autoimmune diseases, and pregnancy, which show that neutrophils are, in fact, a heterogeneous population (Pillay et al. 2013; Scapini et al. 2016; Dumitru et al. 2012; Köstlin et al. 2014). These studies have indicated that neutrophils can possess different morphologies, states of activation, and cell surface markers in different environments (Scapini et al. 2016). In cancer, distinct TAN populations have been identified that differ in terms of cell surface markers and their ability to inhibit or promote tumor growth (Mishalian et al. 2017). As a result, TANs have been categorized into “N1” and “N2” subsets, analogous to the M1 and M2 macrophage and Th1 and Th2 T-cell polarization paradigms (Fridlender et al. 2009; Eruslanov et al. 2014). Broadly speaking, N1 TANs are pro-inflammatory and tumor-suppressing, whereas N2 TANs promote tumor growth and metastasis (Fridlender et al. 2009). Their diverse functions are achieved via differential engagement of tumor-promoting and tumor-suppressing mechanisms. For example, N1 TANs display direct cytotoxic activity against tumor cells (Fridlender et al. 2009; Mishalian et al. 2013), express immune-activating cytokines and chemokines such as TNF- $\alpha$  (Fridlender et al. 2009), and produce low levels of the T-cell suppressive enzyme ARG1 (Fridlender et al. 2009). In contrast, N2 neutrophils recruit regulatory T-cells to the TME via chemokine (C-C motif) ligand 17 (CCL17) (Mishalian et al. 2014), produce large amounts of ARG1, which inhibits T-cell effector functions, and thus support tumor immunosuppression (Fridlender et al. 2009), and induce angiogenesis in the TME through the production of





**Fig. 1** Tumor-promoting and -inhibiting roles of neutrophils in cancer. Neutrophils can support tumor growth using multiple mechanisms (*right side*). The production of nitric oxide (NO), arginase (ARG) and reactive oxygen species (ROS) such as hydrogen peroxide ( $H_2O_2$ ) suppress the activity of T-cells by limiting the production of effector molecules such as IFN- $\gamma$ . Neutrophil elastase (NE) promotes tumor growth by degrading insulin receptor substrate 1 (IRS-1), which increases the interaction between phosphatidylinositol 3-kinase (PI3K) and platelet-derived growth factor receptor (PDGFR), thereby skewing the PI3K axis toward tumor cell proliferation. Through the production of myeloperoxidase (MPO),

neutrophils are involved in tissue remodeling and angiogenesis which supports tumor growth. In contrast, neutrophils may also inhibit tumor growth (*left side*). By releasing factors including NO and  $H_2O_2$ , neutrophils can cause tumor cell cytotoxicity and apoptosis. Neutrophils can also interfere with the growth of tumors through tumor cell sloughing, whereby production of ROS, NE, and matrix metalloproteinase 9 (MMP-9) degrade components of the epithelial basement membrane. Neutrophils can also drive T-cell activation by inducing T-cell CD69+ expression, as well as through antigen presentation to support antitumor T-cell activity and function

various factors such as matrix metalloproteinases and Bv8 (Shojaei et al. 2007; Kessenbrock et al. 2010).

Circulating neutrophils are also a heterogeneous population of cells. Indeed, investigations into neutrophil behavior during inflammatory states have revealed distinct populations that can be distinguished by their density. Using a discontinuous density gradient to isolate leukocytes from whole blood, neutrophils from healthy donors sediment above red blood cells in the normal density neutrophil fraction, otherwise known as high density neutrophils (HDNs) (Scapini and Cassatella 2014). In contrast, under inflammatory conditions, low density neutrophils (LDNs) isolate from the mononuclear cell fraction and contain immature neutrophils and activated mature neutrophils which are thought to have de-granulated (Mishalian et al. 2017). Investigations into whether these separate neutrophil populations have a relationship with cancer have revealed that the LDN population increases with tumor growth in mice and cancer patients (Sagiv et al. 2015). Further, these studies suggest that murine LDN and HDN have opposite effects on cancer progression, as LDNs show little of the cytotoxicity toward tumor cells displayed by

HDNs (Sagiv et al. 2015). Which types of neutrophils are represented by the LDN and HDN fractions is not clear. A lack of known cell surface markers clearly distinguishing LDN from HDN subsets leaves it unknown whether, for example, the LDN population consists of immature neutrophils or another neutrophil subtype associated with cancer progression, such as N2 TANs or granulocytic MDSCs (G-MDSC; see below). Traditionally CD16 has been used as a marker for late-stage neutrophils, and recently CD10 has also been suggested to be a marker of mature neutrophils. These markers may be useful in future studies evaluating these neutrophil subsets (Marini et al. 2017).

On the basis of these observations, it is now widely accepted that distinct neutrophil subsets exist, with different roles in cancer progression. However, the degree to which these populations are fixed after neutrophil production and differentiation, or undergo phenotypic change after arriving at the TME, is not well understood (Galli et al. 2011). Some degree of plasticity clearly exists, as neutrophils can be induced to change function with appropriate stimulation (Fridlender et al. 2009). For example, IFN- $\beta$ , a type I interferon, polarizes

neutrophils toward N1 in mice and humans (Andzinski et al. 2016). This study also noted that B16F10 melanoma or MCA205 fibrosarcoma cells grew faster in IFN deficient (*Ifnb1*<sup>-/-</sup> or *Ifnar1*<sup>-/-</sup>) compared to wild-type mice. Indeed, accelerated tumor growth correlated with neutrophil recruitment displaying an N2 phenotype. Others have shown that IL-35, a cytokine that promotes tumor progression and metastasis, indirectly induces N2 polarization by increasing G-CSF and IL-6 production and promoting neutrophil infiltration into the TME (Zou et al. 2017). Additionally, in syngeneic murine models of lung cancer, tumor-derived TGF- $\beta$  was found to enhance tumor growth by driving the accumulation of tumor-promoting N2 neutrophils (Fridlender et al. 2009). Following TGF- $\beta$  receptor inhibition using the inhibitor SM16, levels of N1 TANs and activated CD8<sup>+</sup> T-cells increased, which correlated with decreased tumor growth rates (Fridlender et al. 2009).

### Myeloid-derived suppressor cells (MDSCs)

Another population of “neutrophil-like” cells that have probably contributed to the dichotomous reports of neutrophil function in cancer is MDSCs. MDSCs are a heterogeneous collection of activated immature myeloid cells, comprising a mixture of granulocytic and monocytic subtypes. They have been implicated in various aspects of cancer development including the mechanisms by which cancer evades the immune system (Gabrilovich and Nagaraj 2009). MDSCs lack the expression of cell-surface markers specific to fully differentiated monocytes, macrophages, or dendritic cells (Köstlin et al. 2014), and have historically been defined as CD11b+Gr-1<sup>+</sup> cells in mice, phenotypic markers commonly used to identify neutrophils. Adding further complexity to their identification, the Gr-1 antibody identifies two epitopes: one on the neutrophil-specific receptor, Ly6G, and a second on Ly6C, an antigen expressed by monocytes (Daley et al. 2007). The granulocytic and monocytic subtypes of MDSCs have most clearly been distinguished in mice. G-MDSCs, otherwise known as polymorphonuclear MDSCs (PMN-MDSC), express the cell surface markers CD11b+Ly6G+Ly6C<sup>lo</sup>, while monocytic MDSCs (M-MDSC) express CD11b+Ly6G<sup>-</sup>Ly6C<sup>hi</sup> (Bronte et al. 2016). Because humans lack Gr-1 and Ly6G, human MDSCs have been more difficult to study, but nonetheless have been characterized. In cancer patients, G-MDSCs and M-MDSCs have mainly been described as CD11b+CD14<sup>-</sup>CD15<sup>+</sup> or CD11b + CD14<sup>-</sup>CD66b<sup>+</sup> and CD11b + CD14<sup>+</sup>HLA-DR<sup>-</sup>/loCD15<sup>-</sup>, respectively, although variations on their cell surface markers have been reported (Bronte et al. 2016).

MDSCs are present in most cancer patients and have been shown to inhibit cytotoxic T-cell function and block T-cell enrichment in the tumor (Marvel and Gabrilovich 2015). Indeed, studies have reported associations between MDSCs

and anticancer T-cell function in human patients. The quantity of MDSCs (CD11b+CD33+CD14<sup>-</sup>HLA-DR<sup>-</sup> cells) isolated from the blood of chronic myeloid leukemia patients, for example, was found to negatively correlate with T-cell proliferation (Giallongo et al. 2014). Similarly, CD11b+CD33+HLA-DR<sup>-</sup> MDSCs with increased NO and ROS production isolated from peripheral blood of stage IV melanoma patients was reported to correlate with decreased CD3 $\zeta$  chain expression on T-cells (Sade-Feldman et al. 2016).

The mechanistic basis of T-cell suppression by MDSCs is multifaceted. T-cell proliferation and function is impaired by MDSCs mainly through ARG1, inducible nitric oxide synthase-2 (iNOS2) and ROS (Gabrilovich and Nagaraj 2009). MDSCs express ARG1 and iNOS2 in response to specific cytokines, including the Th2 cytokines TGF $\beta$  and IL-10 for ARG1, and the Th1 cytokines IFN- $\gamma$ , IL-1, IFN- $\alpha$ , and TNF- $\alpha$  for iNOS2 (Waldron et al. 2013). Interestingly, it has been suggested that the two MDSCs populations differ in how they regulate T-cells, that G-MDSCs suppress mainly by producing ROS, while M-MDSCs use iNOS2 and ARG1 (Youn and Gabrilovich 2010). Perhaps owing to these differences, studies have shown that, on a per cell basis, M-MDSC are more potent than G-MDSC in suppressing T-cells (Youn and Gabrilovich 2010). It has been shown that MDSCs can also recruit and induce CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> regulatory T-cells, a cell type that functions to inhibit immune responses (Nagaraj et al. 2013). And finally, studies in mice have reported that MDSCs can modulate natural killer cell activity by way of downregulating the production of effector molecules, IFN- $\gamma$  and granzyme B (Zhu et al. 2012). The ability of some cancers to co-opt MDSC function, therefore, seems to provide a number of complementary and non-redundant mechanisms within the TME for promoting tumor growth and survival.

In addition to their effects in the TME, studies have reported that MDSCs can exert systemic immunomodulation. The spleen, for example, has been reported to function as an organ for G-MDSC production, a reservoir of G-MDSC accumulation, and a niche for systemic immunosuppression mediated by G-MDSCs (Youn and Gabrilovich 2010). Numerous studies have reported the occurrence of splenomegaly during cancer development, the accumulation of G-MDSCs in the spleen, and the fundamental importance of the spleen in tumor-induced immune tolerance (Cortez-Retamozo et al. 2012; Spiegel et al. 2016; Jordan et al. 2017). How splenic G-MDSCs exert global immunosuppression is not well understood, although one study suggested that splenic CD11b + Gr-1<sup>int</sup>Ly6C<sup>hi</sup> cells expand in the marginal zone of the spleen, where they cross-present tumor antigens to promote T-cell tolerance (Ugel et al. 2012). In that study, splenectomy restored lymphocyte function and induced tumor regression when coupled with immunotherapy (Ugel et al. 2012).

In summary, these studies illustrate the presence of diverse subsets of neutrophils or neutrophil-like cells with variable

effects on tumor growth. Because most research investigating the relationship between neutrophils and cancer have used identification markers that do not distinguish between the N1, N2 and MDSC phenotypes, the seemingly divergent roles characterized in these studies probably reflects different populations of neutrophils being measured, at least in part. Similarly, the apparently contradicting effect of neutrophil depletion on tumor growth (Fridlender et al. 2009) may likewise be due to the presence of phenotypically distinct neutrophil populations.

### Developmental stage of the cancer

A second explanation for the divergent roles of neutrophils in cancer may reside in the tumor models used and the phase of cancer development they represent (Eruslanov et al. 2014). Most studies in mice employ tumor models established from cell lines previously subjected to immune sculpting *in vivo* and selected on the basis of rapid growth. As such, they mainly mimic late-stage tumor progression (Eruslanov et al. 2017). Several investigations have lent support to the notion that neutrophils are antitumorigenic at primary tumor initiation, but tumor-promoting once the cancer becomes more established. It has been observed that, during the earliest stages of lung cancer, for example, TANs stimulate anticancer immunity by promoting T-cell activation and proliferation (Eruslanov et al. 2014). Additionally, TANs from early tumors are more cytotoxic toward cancer cells and produce higher levels of TNF- $\alpha$ , NO, and H<sub>2</sub>O<sub>2</sub> compared to neutrophils from established tumors (Mantovani et al. 2011; Mishalian et al. 2013). A differential capacity for antigen presentation has also been observed in TANs harvested from tumors at distinct developmental stages. The APC-like hybrid TANs described earlier were found in small tumors, but completely absent from tumors >5–7 cm in diameter (Singhal et al. 2016). Given that larger tumors are characterized by hypoxia, it was proposed that circulating immature neutrophils become polarized toward canonical TANs in large, hypoxic tumors, whereas IFN- $\gamma$  and GM-CSF secreted from smaller tumors promote their polarization toward APC-like hybrid TANs. Thus, it may be that the observed discrepancies in neutrophil–cancer interactions are the consequence of an evolving relationship between neutrophils and the TME throughout disease progression. To this end, the use of genetically engineered mouse models that spontaneously develop tumors more faithfully depicting multistage tumor development may allow for better understanding of this dynamic relationship.

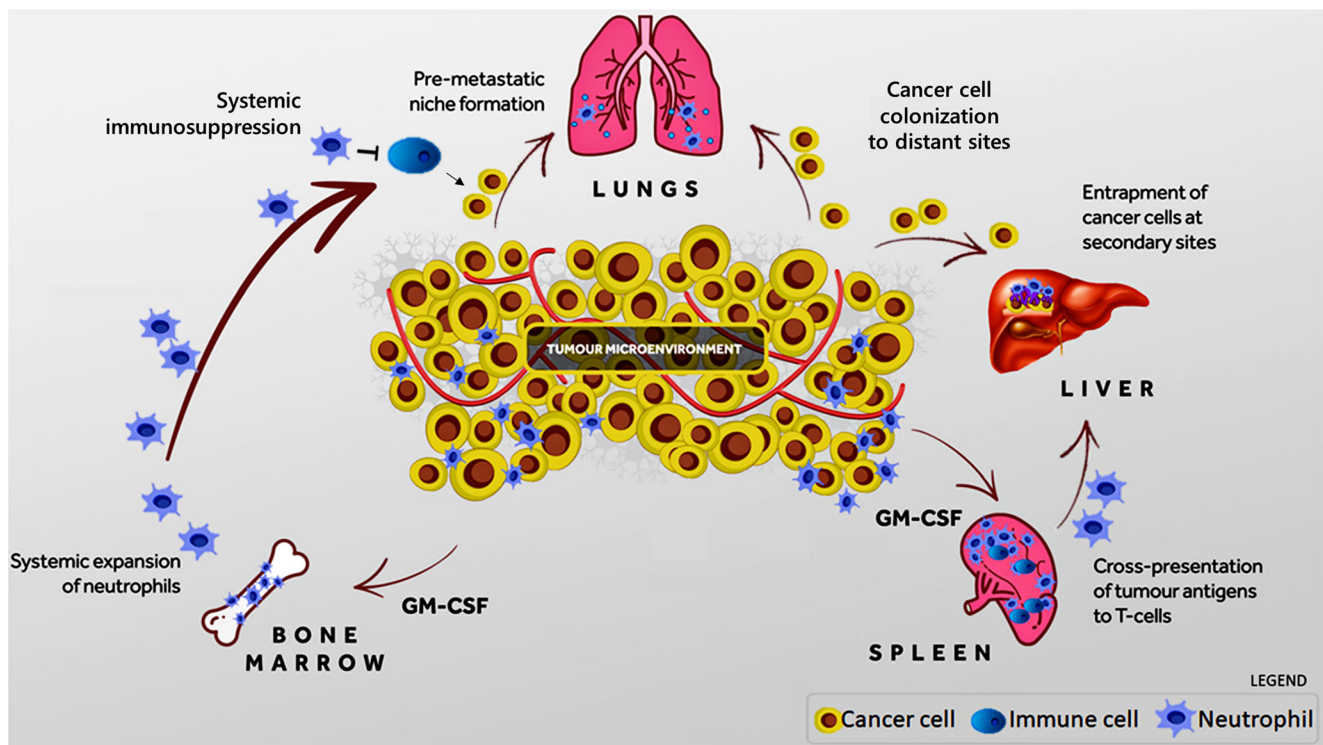
### Neutrophils and metastasis

Investigations have demonstrated a complex relationship between neutrophils and the development and/or regulation of metastasis, a characteristic feature of advanced cancer.

Metastasis is the leading cause of death in cancer patients and occurs when tumor cells from a primary site spread to and grow in distant organs. A growing body of research has demonstrated that neutrophils can play multiple metastasis-promoting roles in cancer. Studies using murine breast cancer models, for example, have reported that tumor implantation leads to an increase in circulating neutrophils, which in turn promote metastatic seeding in the lung (Spiegel et al. 2016; Wculek and Malanchi 2015). Similarly, mice bearing melanoma tumors challenged with circulating neutrophils from tumor-bearing mice developed more metastatic nodules in the lung compared to mice challenged with circulating neutrophils from control hosts (Zhang et al. 2016). Consistent with these studies, depleting neutrophils in breast cancer-bearing mice resulted in a significant reduction in pulmonary metastasis and local invasion into lymph nodes (Coffelt et al. 2015). While studies assessing the role of neutrophils in human metastatic disease are more limited, they have nevertheless revealed a positive correlation between neutrophils and metastases. For example, a high NLR was reported to be associated with the presence of brain metastases in patients with advanced non-small-cell lung cancer patients (Koh et al. 2016). Another study found the development of metastasis in patients with uveal melanoma to be associated with increased numbers of circulating CD11b<sup>+</sup>CD14<sup>-</sup>CD15<sup>+</sup> cells (Achberger et al. 2014).

The mechanisms by which neutrophils promote metastasis are multifaceted and have thus far been attributed mainly to their ability to create a “pre-metastatic niche” and exert systemic immunosuppression (Fig. 2). The concept of the pre-metastatic niche was born out of observations that neutrophils and other cells accumulate in tissues distant from the primary tumor and promote cancer cell recruitment and colonization therein. For example, studies have found that, as breast cancers progress, neutrophils accumulate within the lung and secrete leukotrienes that select for highly proliferative cancer cells through increased extracellular-signal-regulated kinases 1 and 2 activation (Wculek and Malanchi 2015). Depleting neutrophils, by genetically engineering a neutrophil-targeting diphtheria toxin or through the use of an anti-Ly6G blocking antibody, reduced the rate of spontaneous lung metastasis in those models. Other studies have reported that neutrophils are directly involved in cancer cell adherence to blood vessels at non-cancerous sites. In a mouse xenograft model of melanoma, for example, it was observed that ICAM-1 on melanoma cells and  $\beta_2$  integrin on neutrophils interact, which promotes the anchoring of cancer cells to the vascular endothelium, a critical step in cancer cell metastasis (Huh et al. 2010). More recently, a mechanism was elucidated whereby neutrophils support tumor cell adherence to vessels within distant sites via NETs. In one study, neutrophil stimulation caused NET release into liver sinusoids, which resulted in tumor cell anchoring, extravasation and subsequent colonization (Cools-





**Fig. 2** The role of neutrophils in metastasis. Neutrophils influence several steps of metastasis. Tumor-secreted factors such as GM-CSF promote granulopoiesis and release of neutrophils/MDSCs from the bone marrow or spleen. Neutrophils can promote metastasis by creating systemic

immunosuppression to attenuate immune-mediated attack on metastatic cancer cells, or creating a pre-metastatic niche within distant organs such as the lung and liver, which serves to promote the recruitment, entrapment and successful colonization of cancer cells at those sites

Lartigue et al. 2013). Other studies have reported that metastatic cells themselves co-opt NET release to support further colonization. Park et al. (2016) observed that G-CSF secreted by metastatic cancer cells stimulates neutrophil NET formation, and that targeting NETs by DNase I-coated nanoparticles prevented lung metastasis in the 4 T1 breast cancer model (Park et al. 2016). Other cancer cells such as the human cell line AsPC-1 have also been found to directly induce NET production from neutrophils (Abdol Razak et al. 2017).

It has also been proposed that neutrophils support metastasis by promoting systemic immunosuppression. Investigators have observed that neutrophils contribute to tumor cell survival in circulation by suppressing the activation of peripheral leukocytes (Zhang et al. 2016). Additionally, it has been found that, in a murine breast cancer model, tumor-secreted IL-1 $\beta$  stimulates the release of IL-17 $\alpha$  from  $\gamma\delta$  T-cells, which was responsible for the systemic induction of G-CSF, and an increase in numbers of immunosuppressive neutrophils (Coffelt et al. 2015). Upon neutrophil depletion, an effector CD8<sup>+</sup> T-cell phenotype was enhanced, which was proposed to represent enhanced antitumor immunity (Coffelt et al. 2015). Finally, it has been shown that neutrophils inhibit the functional activation of natural killer cells and thereby the ability of these cells to clear intraluminal tumor cells (Spiegel et al. 2016). Taken together, it appears that cancer has evolved

numerous ways to co-opt neutrophils to aid in its metastatic seeding and growth.

There are some studies, however, that have reported on neutrophils *preventing* metastatic spread. For example, antibody-mediated depletion of neutrophils was found to have accelerated the formation of lung metastasis in a mouse model of kidney cancer (López-Lago et al. 2013). Additionally, Granot et al. (2011) showed that a unique population of cytotoxic neutrophils they called tumor-entrained neutrophils (TENs) can accumulate in the lungs of mice with breast cancer. Activated by tumor-secreted chemokine ligand 2 (CCL2) secretion, TENs were found to inhibit metastatic seeding via H<sub>2</sub>O<sub>2</sub> secretion (Granot et al. 2011). Thus, much like the relationship between neutrophils and primary tumors, the interplay between neutrophils and metastasis can be dichotomous, likely owing to dynamic and context-dependent interactions and heterogeneous populations of cells at play.

## Targeting neutrophils for cancer therapy

The evidence that neutrophils play an important role in primary tumor progression and the development of metastases provides a powerful rationale to target these cells in cancer patients. However, neutrophils are a vital defense mechanism



against infection, and their broad depletion may leave patients immunosuppressed and vulnerable to infection with pathogens. Also, their short life span and high production rate could diminish the efficacy of therapeutic targeting. Further, the evidence for antitumor neutrophils is significant, and neutrophil-targeting therapies lacking specificity may also deplete this function, leading to inadvertent promotion of tumor growth. These challenges have thus far narrowed therapeutic approaches to strategies that target the role of neutrophils in regulating antitumor immunity and in the metastatic cascade.

### Targeting neutrophils to promote anticancer immunity

Therapeutic approaches aimed at relieving neutrophil-mediated immunosuppression have recently been reported. One of these targeted the CCL5–CCR5 axis with CCL5-targeting nanoparticles in combination with Maraviroc, a FDA-approved CCR5 inhibitor (Ban et al. 2017). Myeloid cell CCR5 expression is dependent on autocrine CCL5 signaling in the bone marrow, and CCL5 signaling impedes the maturation of neutrophils, resulting in the generation of immunosuppressive Ly6G<sup>+</sup> myeloid cells. Immunohistochemical analysis of patient tumor samples revealed that immune CCR5 expression inversely correlates with the maturation status of neutrophils in tumors as well as 5-year-survival rates of triple-negative breast cancer patients. Thus, the authors reasoned that blocking CCL5 in bone marrow might attenuate the accumulation of immunosuppressive neutrophils. Indeed, compared with control mice, CCL5-targeting nanoparticle treatment decreased the number of immunosuppressive myeloid cells, as evidenced by arrested M- to G-subset switching, decreased NOS2 expression and enhanced activated CD8<sup>+</sup> T-cell infiltration. Importantly, 4 T1 tumor growth was significantly reduced.

Another approach has been to stimulate the anticancer immune functioning of neutrophils. For example, mice bearing B16 melanomas treated with the synthetic double-stranded RNA analog poly I:C and inactivated Sendai virus demonstrated CD11b<sup>+</sup>Ly6G<sup>+</sup>FAS<sup>+</sup> TAN accumulation in the TME that promoted cytotoxic T-cell activity against the tumor (Yang Chang et al. 2016). This treatment strategy produced similar results in the EL4 murine lymphoma tumor model, with the additional observation that this treatment induced ROS production from TANs, which elicited direct tumor cell death (Shime et al. 2017).

### Targeting neutrophils to prevent metastasis

Other therapeutic approaches have targeted the role of neutrophils in the metastatic cascade. The observation that inflammatory neutrophils associate with circulating tumor cells (CTCs) and pre-metastatic niches has prompted the development of a neutrophil-mimicking nanoparticle (NM-NP) drug

delivery system to deplete CTCs (Kang et al. 2017). NM-NPs were designed by coating surfaces of biodegradable nanoparticles with an inflammatory neutrophil-derived membrane before injection into mice bearing syngeneic breast cancer. Compared with uncoated nanoparticles, NM-NP exhibited enhanced cellular association with 4 T1 cells and improved homing to the pre-metastatic niche. By loading carfilzomib, a second-generation proteasome inhibitor, onto the NM-NP, there was selective depletion of CTCs in the blood, and fewer metastatic nodules and reduced tumor cell proliferation in the lymph nodes of mice. Thus, it appears that mimicking the ability of circulating neutrophils to associate with CTCs can be exploited to inhibit the formation of a metastatic niches and alleviate metastatic burden.

Another therapeutic approach to blocking metastases is based on the observation that neutrophils support metastatic initiation through production of leukotrienes, which select and expand cancer cells with strongly tumorigenic phenotype (Wculek and Malanchi 2015). Blocking neutrophil production of leukotrienes was found to prevent metastasis in a murine breast cancer model, suggesting that this may be a viable approach to inhibiting metastasis and prolonging survival of patients with aggressive cancer types.

### Conclusion and future directions

There is rapidly growing evidence that neutrophils play a complex and essential role in cancer, a discovery that has significantly enhanced our understanding of cancer progression and revealed new therapeutic opportunities. Although recent advances in T-cell-mediated cancer therapies have highlighted the immense power of the adaptive immune response in treating cancer, it is also clear that neutrophils and the innate immune system can likewise be harnessed. Although early approaches show promise, better characterization of the specific mechanisms by which neutrophils exert their influence on primary tumors and the metastatic cascade is needed in order to achieve the full therapeutic potential afforded by this intriguing aspect of cancer biology.

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