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ORIGINAL ARTICLE

The Multifaceted Roles Neutrophils Play in the Tumor Microenvironment

Ronit Vogt Sionov · Zvi G. Fridlender · Zvi Granot

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Abstract Neutrophils are myeloid cells that constitute 50–70 % of all white blood cells in the human circulation. Traditionally, neutrophils are viewed as the first line of defense against infections and as a major component of the inflammatory process. In addition, accumulating evidence suggest that neutrophils may also play a key role in multiple aspects of cancer biology. The possible involvement of neutrophils in cancer prevention and promotion was already suggested more than half a century ago, however, despite being the major component of the immune system, their contribution has often been overshadowed by other immune components such as lymphocytes and macrophages. Neutrophils seem to have conflicting functions in cancer and can be classified into anti-tumor (N1) and pro-tumor (N2) sub-populations. The aim of this review is to discuss the varying nature of neutrophil function in the cancer microenvironment with a specific emphasis on the mechanisms that regulate neutrophil mobilization, recruitment and activation.

Keywords Neutrophil function · Tumor Microenvironment · Pre-metastatic niche · Chemokines

Introduction

Within the tumor microenvironment, there is a continuous crosstalk between tumor cells, stromal cells and cells of the immune system [1–3]. Tumor cells secrete a wide range of cytokines and chemokines that not only attract and affect macrophages, neutrophils, dendritic cells, NK-, B- and T-cells, but also regulate other stromal cells, endothelial cells and the tumor cells themselves. Cells of the innate and adaptive immune system in turn mutually affect the activation status of other immune cells and attract more leukocytes into the tumor cell mass. Surprisingly, non-malignant immune cells can make up 90 % of the total tumor mass [2–5]. However, it is the integration of all signals encountered within the tumor microenvironment that affects the composition and activation of the various immune cells within the tumor cell mass and ultimately leads to either anti-tumor or pro-tumor activities.

Signals emanating from the tumor may also affect immune cell activities in the circulation and in distal pre-metastatic organs such as the liver and the lung to either restrain or encourage metastasis. Immune cell inhibition of tumor growth is part of the process termed immune surveillance, where circulating or tissue-associated immune cells eliminate malignancies. Conversely, immune cells secrete factors that can stimulate angiogenesis, remodel the extracellular matrix and promote cell growth thereby facilitating tumor growth and dissemination. The balance between these opposite actions is dictated by multiple factors secreted by the tumor cells, the tumor-associated stromal cells and the immune cells themselves. A future goal is to develop strategies that can modulate the tumor microenvironment in such a way that the anti-tumorigenic activities of the immune cells will prevail.

The goal of this review is to provide insight into the complexity of the tumor-stroma interaction, with a specific emphasis on the role played by neutrophils, which have recently emerged as a central player in the above-described interplay.

R. V. Sionov · Z. Granot (✉)
Department of Developmental Biology and Cancer Research, Institute for Medical Research Israel Canada, Hebrew University Medical School, 91120 Jerusalem, Israel
e-mail: zvikag@ekmd.huji.ac.il

Z. G. Fridlender
Institute of Pulmonary Medicine, Hadassah-Hebrew University Medical Center, 96920 Jerusalem, Israel

The Cancer-Neutrophil Crosstalk

In this review we will explore the influence of the microenvironment or niche on the phenotype and function of neutrophils in the context of tumor initiation, growth and metastatic progression. Significant controversy surrounds the function of neutrophils in the context of cancer. Neutrophils are often recruited to the tumor microenvironment where they have been shown to either promote or inhibit tumor growth [6–11]. There are several lines of evidence suggesting that neutrophils may exert direct cytotoxic activities towards the tumor, or indirectly lead to tumor regression through recruitment and induction of tumor-specific T cell responses [12–18]. For instance, tumor infiltrating neutrophils in tumor-bearing rats secrete chemotactic factors for T lymphocytes and recruit T lymphocytes to the tumor bed [19]. When neutrophils were selectively depleted at the time of in vivo priming with γ-irradiated tumor cells, the growth of subsequent transplanted syngeneic tumors was not inhibited, indicating that neutrophils are important for mounting specific anti-tumor immune responses [19]. However, even in the presence of activated neutrophils, cancer cells may generate an immunosuppressed microenvironment, preventing cytotoxic immune responses, while enhancing the tumor-promoting activities of the immune cells.

Below we discuss the interplay between cancer cells and neutrophils, and their ultimate role in tumor growth and metastatic progression. We propose that in the context of tumor growth and metastatic progression, neutrophils possess both pro- and anti-tumor properties and that their function is determined in a niche-dependent fashion.

Niche-dependent Neutrophil Function

While chronic inflammation may promote tumorigenesis [5, 20], the tumor cells themselves also attract immune cells through the secretion of a wide range of chemokines such as IL-8 (CXCL8 in human/CXCL2 in mouse), CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), CXCL6 (huGCP-2), and KC (CXCL1), and cytokines such as IL-1β, IL-6, TNFα, GM-CSF, and G-CSF, thereby inducing inflammation [21–32]. Cytokines from tumors may regulate tumor growth or modify the anti-tumor immune responses [33–36]. Cytokines and chemokines from tumor cells also modify their normal surrounding non-malignant stroma by modulating the function of epithelial cells, endothelial cells, fibroblasts and inflammatory cells to generate a supportive microenvironment [37–39]. For instance, TNFα, IL-6 and IL-17 were shown to promote tumor growth by modifying the function of stromal cells surrounding the tumor [40–42]. TNFα produced by tumor cells or inflammatory cells in the tumor microenvironment can promote tumor cell survival through the induction of NFκB–dependent anti-apoptotic molecules [43]. TNFα was also shown to promote angiogenesis [44], and induce the expression of VEGF and HIF-1α in tumor cells [45]. IL-6 promotes angiogenesis and the expression of VEGF [46] through JAK2/STAT3 signaling [42] and the tumor-promoting effects of IL-17 are in part mediated through up-regulation of IL-6 [42].

Traditionally, immune cells are viewed as protectors of the host where they take part in immune surveillance, eliminating both microbial infections and potentially cancerous cells. However, in the context of a tumor, the function of these cells is modified and they are "alternatively activated" to act against the host and promote tumor growth and metastasis. It is thought that tumors secrete factors that elicit a wound-repair response by tumor-associated macrophages (TAMs) and tumor-associated neutrophils (TANs) and that this response inadvertently stimulates tumor progression [47].

The phenomenon of alternative activation and the shift from anti-tumor to pro-tumor function of immune cells has been extensively described for macrophages. Macrophages have both pro- and anti-tumor actions depending on the activation signals, and have accordingly been classified into anti-tumor "M1" and pro-tumor "M2" macrophages (Fig. 1) [48, 6]. M1 macrophages show increased production of inflammatory cytokines (e.g., IL-1β, TNFα, IL-6, IL-12, IL-23) and Th1, Th17 and NK cell-attracting chemokines such as CXCL9/Mig and CXCL10/IP-10 [6]. Alternatively activated M2 macrophages have a strikingly different gene expression profile compared with M1 macrophages and express the immunosuppressive cytokine IL-10, tumor growth factors (e.g., EGF, FGF1, TGFβ1), pro-angiogenic factors (e.g., VEGF), matrix remodeling factors (e.g., fibrin and matrix metallopeptidases), and chemokines such as CCL17/TARC, CCL22/MDC and CCL24/Eotaxin-2, that are involved in regulatory T (Treg) cell, Th2 cell, eosinophil and basophil recruitment [6, 49, 50]. M2 macrophages contribute to the formation of an immunosuppressed microenvironment. TAMs often show a M2 phenotype [51] and are characterized by the secretion of VEGF, HIF, TGFβ, IL-10, Arginase I and reactive oxygen species (ROS) [52], as well as various chemokines such as CCL2, CCL5 (RANTES), CXCL9, CXCL10, and CXCL16 [53]. TAMs contribute to tumor growth and progression through extracellular matrix remodeling, promotion of tumor cell invasion and metastasis, angiogenesis, lymphangiogenesis and immune suppression [6]. TAMs may also secrete cytokines IL-1β, IL-6 and IL-23 that trigger the proliferation of IL-17-producing $CD8^+$ Tc17 cells [54], a T cell subset that is often found to be present in various tumors [55]. IL-17 increases the synthesis of C-X-C chemokines from epithelial cells [56] leading to increased neutrophil infiltration into the tumor [57]. IL-17 also induces the production of G-CSF [58], thus creating a G-CSF-IL-17-IL-23 feedback axis that regulate neutrophil homeostasis [59].

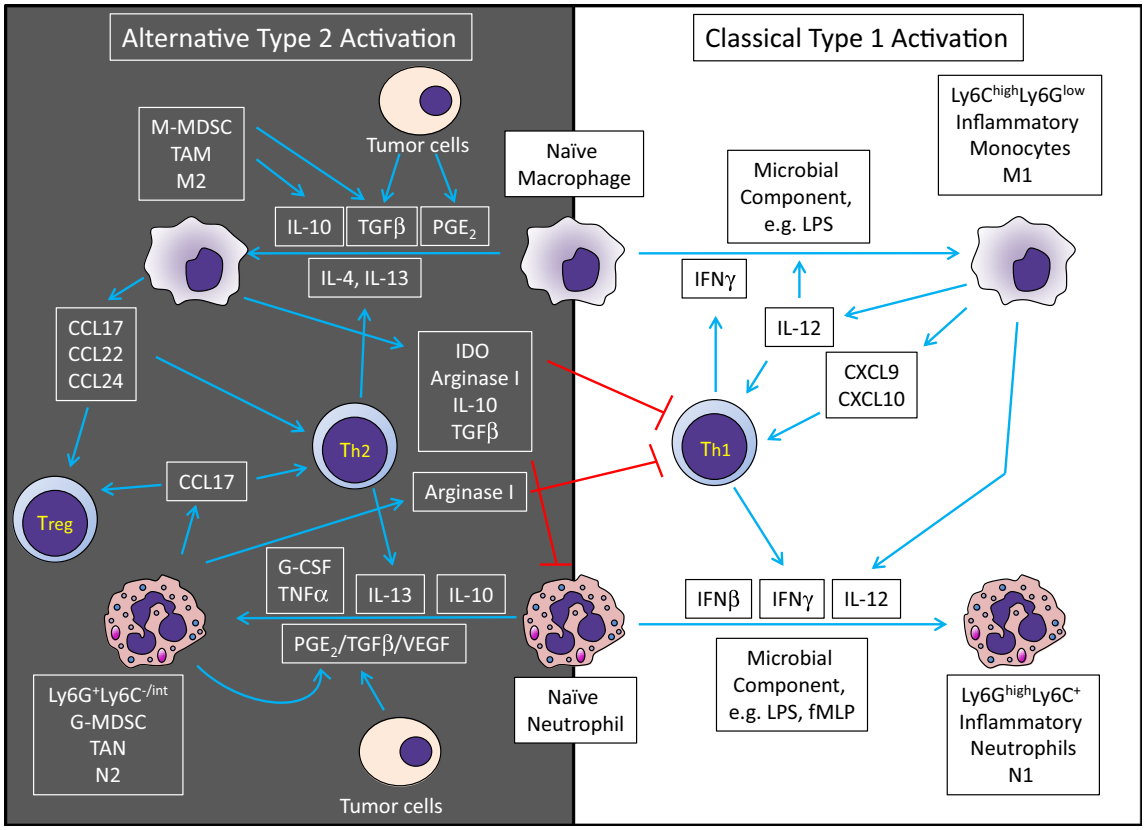

Fig. 1 Polarization of neutrophils and macrophages. Two major activation pathways have been described for macrophages and neutrophils. These immune cells can be activated through the classical pathway involving microbial components and T-cell derived factors such as IL-12 and IFNγ, into Type 1 inflammatory cells that combat acute infection. Recent data suggest that IFNβ is also important for the polarization of neutrophils into N1 cells. In the presence of immunosuppressive components such as IL-10, TGFβ, PGE_2, or excessive Th2 responses characterized by IL-13 and IL-4 production, macrophages and neutrophils undergo an "alternative" activation pathway into Type 2 cells, which exhibit immunosuppressive activities. Also, excessive G-CSF and TNFα production may further promote Type 2 activation of neutrophils. Usually, the Type 1 activation prevails under acute inflammation, while Type 2 activation dominates under chronic inflammation, including the tumor microenvironment. Type 2 activation seems to be a negative feedback mechanism that counteracts excessive immune responses

Tumor-associated neutrophils ("TANs"), like macrophages, may acquire an anti-tumorigenic "N1" or pro-tumorigenic "N2" phenotype and are classified according to their activation state, cytokine repertoire and effects on tumor growth [7, 9–11, 60]. The N1 cells are characterized by cytotoxic activity towards tumor cells and an immunostimulatory profile (i.e., $TNF\alpha^{high}$, $CCL3^{high}$, $ICAM\text{-}1^{high}$, $Arginase^{low}$), whereas N2 neutrophils are characterized by upregulation of the chemokines CCL2, 3, 4, 8, 12, and 17, and CXCL1, 2, 8 and 16 [10]. N1 cells produce more superoxide and hydrogen peroxide and express higher levels of Fas, TNFα, CCL3 and ICAM-1, but lower levels of Arginase, CCL2, CCL5, VEGF, CXCR4 and MMP-9 than N2 cells [10]. Proinflammatory N1 neutrophils have also been shown to promote $CD8^+$ T cells recruitment and activation by producing T cell-attracting chemokines (e.g., CCL3, CXCL9, and CXCL10) and proinflammatory cytokines (e.g., IL-12, TNFα, GM-CSF, and VEGF) [61]. There is a mutual interplay between neutrophils and $CD4^+$ T helper 17 cells (Th17) [62]. While IL-17 and CXCL8 secreted by Th17 cells induce the recruitment of neutrophils, secretion of CCL2 and CCL20 by activated neutrophils attracts Th17 cells [61–63]. Th17 cells may further modulate neutrophil activity through secretion of TNFα, IFNγ and GM-CSF [62].

The immunosuppressive transforming growth factor β (TGFβ) was shown to promote the N2 neutrophil phenotype [13], while interferon β (IFNβ) promotes the N1 phenotype [64] (Fig. 1). Inhibition of TGFβ signaling using the small molecule SM16 increased the mRNA levels for neutrophil chemoattractants (CXCL2 and CXCL5) in macrophages isolated from the tumor and increased the recruitment of $CD11b^+$ neutrophils into the tumor with concomitant reduced tumor growth [13]. Depletion of neutrophils prevented the SM16-induced growth inhibition, suggesting an anti-tumor activity mediated by neutrophils [13].

TANs differ from naïve neutrophils and G-MDSCs (the granulocytic sub-population of myeloid-derived suppressor cells) as they harbor low amounts of the various neutrophil granules and generate low amounts of ROS, while showing an enhanced chemokine secretion profile [10]. TANs

express higher levels of CXCL2, CXCL1 and CCL3 than naïve bone-marrow neutrophils [65], thus generating a positive feedback loop for recruiting more neutrophils as well as other immune cells to the tumor site. While TANs at the primary tumor site seem to acquire an N2 phenotype induced by high TGFβ levels, circulating neutrophils and neutrophils in metastasis-free organs tend to have an N1 anti-tumor-phenotype, due to paracrine exposure to activating tumor-secreted factors such as the chemokines IL-8, CCL2, CCL5 and CXCL5 [32, 66]. Indeed, two studies have clearly demonstrated an anti-tumor function for neutrophils at the pre-metastatic lung [32, 66]. These neutrophils have been coined tumor-entrained neutrophils (TENs), as these have been educated by the tumor to possess anti-tumor properties [32].

Immunosuppression in the Tumor Microenvironment

Ultimately, tumors do develop in the presence of a functional immune system, suggesting that tumor cells acquire properties that help them evade immune surveillance [67]. Indeed, tumors have been shown to escape the anti-tumor immune responses by generating an immunosuppressed tumor microenvironment. Tumors often produce soluble immunosuppressive factors, such as TGFβ [13, 68, 69], VEGF [69, 70], IL-10 [69, 71], iNOS [72, 73], PGE_2 [74, 75], and gangliosides [76, 77], that act on neutrophils and other tumor infiltrating immune cells. Often, progressive immunosuppression is observed at advanced tumor stages, which is partially mediated by tumor-infiltrating immunosuppressive immune cells such as regulatory T (Treg) cells, Th17 cells, regulatory dendritic cells, TAMs, TANs and MDSCs. The mechanisms that mediate the immunosuppressive microenvironment deserve an in-depth exploration, which is beyond the scope of this review. Here we will only describe the effects of some of these immunosuppressive factors that are relevant to neutrophil function.

TGFβ

Transforming growth factor β (TGFβ), which exists in at least three isoforms β1, β2 and β3, has been linked to the regulation of tumor initiation, progression and metastasis [78] and TGFβ1 is frequently upregulated in human cancers [79, 80]. Tumor-secreted TGFβ is usually sequestered to the extracellular matrix as an inactive complex, and becomes activated through enzymes such as neutrophil-derived elastase and matrix metalloproteinase (MMP)-9, or expression of $\alpha_v\beta_6$ integrin [78]. In addition, reactive oxygen free radicals produced by activated neutrophils can activate latent TGFβ [81]. Thus, activated neutrophils, through production of elastase, MMP-9 and ROS, may contribute to TGFβ-mediated immunosuppression, a mechanism that may drive a negative feedback that prevents excessive immune responses. Tumor-associated MDSCs and TAMs are also significant sources of TGFβ production [82, 83]. As MDSC and TAMs phenotypes are affected by TGFβ [84], their initial appearance in the tumor would further contribute to the immunosuppressed microenvironment by enhancing the recruitment of these cells to the tumor bed.

The importance of TGFβ in immunosuppression was demonstrated by introducing the expression of a dominant-negative TGFβ type II receptor in mouse tumor-specific T cells. These ex vivo expanded TGFβ-insensitive $CD8^+$ T cells infiltrated the tumor and mediated apoptosis in tumor cells [68]. Fridlender et al. [13] showed that inhibition of TGFβ signaling using the small molecule inhibitor SM16 conferred anti-tumorigenic activity to neutrophils. Low concentrations of TGFβ1 were further shown to inhibit neutrophil degranulation, as measured by lactoferrin release, in response to lipopolysaccharide (LPS) and formyl peptides [85]. TGFβ was also shown to prevent the production of ROS, reactive nitrogen intermediates and IL-1β by neutrophils [86]. Furthermore, TGFβ has even been shown to be a potent chemoattractant for neutrophils taking a central part in their recruitment to sites of inflammation [87–89]. However, another study showed that TGFβ reduces the expression of the adhesion molecule L-Selectin, resulting in impaired neutrophil recruitment to sites of inflammation [86]. Blockage of TGFβ signaling increased the numbers of neutrophils in tumors, which was associated with increased amount of chemokines and cytokines within the tumor, concomitant with increased ICAM-1 expression on endothelial cells [13, 90]. The increase in intra-tumor neutrophil number observed following anti-TGFβ signaling therapy can be explained by the ability of TGFβ to inhibit endothelial adhesiveness of neutrophils and neutrophil transmigration in vivo [91]. Abrogation of TGFβ signaling in mammary carcinomas also led to increased infiltration of $Gr\text{-}1^+CD11b^+$ MDSCs into the invasive front of tumor tissues facilitating tumor cell invasion and metastasis through a process involving metalloproteinase activity [82, 92]. Interestingly, MDSCs from TGFβ signaling deficient tumor-bearing hosts produced higher levels of VEGF, MMP-2, MMP-13 and MMP-14 than those isolated from normal mice [82].

PGE_2

Prostaglandins (PGs) are small-molecular derivatives of arachidonic acid, produced by cyclooxygenases (COXs; constitutively active cyclooxygenase COX-1 and inducible COX-2) and PG synthases [93]. COX-2 expression is induced by cytokines and growth factors at sites of inflammation and is usually not detected in normal tissues [94]. Increased COX-2 expression and PGE_2 production has been reported in

human colon carcinoma [95], breast cancer [96, 97], renal cell carcinoma [98], and lung carcinoma [99].

Prostaglandin E_2 (PGE_2) directly suppresses various immune cells such as macrophages, neutrophils, Th1, CTL and NK cells, while it promotes Th2, Th17 and regulatory T cell responses as well as the development of tumor-associated suppressive macrophages [93, 100–103]. The Th17-promoting activity of PGE_2 is related to its ability to suppress the production of the Th17-inhibitory IL-12, while enhancing the Th17-supporting IL-23 secretion by dendritic cells [104]. As mentioned above, IL-17 promotes neutrophil recruitment [59], such that increased Th17 activity caused by PGE_2 has indirect effect on neutrophil function. Indeed, PGE_2 has been shown to stimulate IL-23/IL-17-induced neutrophil migration in inflammation by inhibiting IL-12 and IFNγ production, which may be one mechanism for its pro-inflammatory effect [105]. PGE_2 also promotes MDSC recruitment to tumor through the local induction of CXCL12/SDF-1 [100]. In addition, PGE_2 promotes the recruitment of $CD4^+CD25^+$ Tregs to the tumor and has direct positive effects on tumor progression [106]. PGE_2 may also promote tissue influx of neutrophils [107], macrophages [108] and mast cells [109]. One mechanism is through PGE_2-stimulated production of IL-8 by epithelial cells [107] and pulmonary microvascular endothelial cells [110], as well as MCP-1 by mast cells [108]. Furthermore, activated neutrophils have been shown to express COX-2 and secrete PGE_2 [111, 112], which may represent another mechanism for immune response restriction. However, PGE_2 may also contribute to dysregulated inflammatory responses by increasing vascular permeability that facilitates influx of pro-inflammatory polypeptides. While PGE_2 prolongs neutrophil half-life through upregulation of intracellular cAMP levels and inhibition of apoptosis [113], it also attenuates PMA-, fMLP- or GM-CSF-stimulated ROS and LTB_4 production in neutrophils [114–117]. The Th2 cytokine IL-13 was found to increase neutrophil PGE_2 production concomitant with increased expression of complement receptor type 1 (CR1) and type 3 (CR3), and increased neutrophil phagocytosis [118].

In line with the immunosuppressive, tumor promoting function of PGE_2, inhibitors of prostaglandin synthesis, such as indomethacin and aspirin, have been shown to inhibit tumor growth [101] and restore anti-tumor activity by altering the balance between IL-10 and IL-12 [119]. Recently, this issue has reached a renaissance where specific COX2 inhibitors have been proposed to be potential drugs for tumor prevention [120]. Non-steroidal anti-inflammatory drugs (NSAIDs), such as diclofenac, increased neutrophil superoxide production, but also promoted L-Selectin down-regulation [121]. Interestingly, a mutual interplay between TGFβ and PGE_2 seems to exist, where TGFβ stimulation of $CD4^+$ T cells induces production of PGE_2, and PGE_2 contributes to TGFβ-induced suppression of T cells [122].

Other Immunosuppressive Molecules

The expression of indoleamine 2,3-dioxygenase (IDO), a tryptophan catabolizing enzyme, in tumors may lead to dysfunctional T cell response through depletion of tryptophan from the tumor microenvironment [123, 124]. IDO is also produced by MDSCs, regulatory dendritic cells and TAMs [6, 52, 125, 126]. Silencing of IDO within the tumor using *Salmonella typhimurium* as a tumor-homing vector to deliver a short-hairpin RNA targeting IDO, allowed tumor infiltration of activated ROS-producing neutrophils and consequent tumor cell death [127].

Another mechanism leading to suppression of T-cell mediated immune responses is excessive production of adenosine by the cell surface enzyme CD73 (ecto-5′-nucleotidase) [128, 129]. CD73 is usually expressed on endothelial and epithelial cells [130], subsets of leukocytes [131] and $Foxp3^+$ Tregs [128, 129], but also on several cancer types [132, 133]. CD73 acts in concert with CD39 (ecto-apyrase) to produce adenosine in a coordinated two-step enzymatic conversion. Both CD39 and CD73 seem to attenuate neutrophil trafficking into the lungs during LPS-induced injury [134] suggesting that CD73 expression on tumor cells is likely to limit neutrophil infiltration. CD73-deficient mice have increased anti-tumor immunity and are resistant to experimental metastases [135]. Similarly, anti-CD73 antibody therapy was found to inhibit breast tumor growth and metastasis [136].

Regulation of Neutrophil Mobilization, Recruitment and Activation in Cancer

Neutrophil Mobilization in Cancer

Human cancers often induce elevated numbers of circulating neutrophils [6, 7, 9–11, 137–159]. The consequences of cancer-induced neutrophilia in human patients will be further discussed in the section discussing "Prognostic Values of Neutrophils and Other Myeloid Subtypes in Cancer Patients". In tumor bearing mice, a phenomenon similar to what occurs upon inflammation, is observed, namely, the number of circulating neutrophils increase dramatically and are associated with the progression of the disease [32, 160–162]. For example, using the 4T1 mammary tumor model in Balb/c mice, we showed that within 1 week after orthotopic inoculation of the tumor, circulating neutrophil numbers increased from ~17 % to over 30 % [32]. This increase continues with the

progression of the disease, reaching a state of acute neutrophilia with neutrophils making ~90 % of all circulating white blood cells [32]. Similar increase in circulating neutrophil numbers was seen in other mouse models of cancer including Lewis lung carcinoma [163] as well as in spontaneous mouse models such as the MMTV-PyMT and MMTV-Wnt1 transgenic mice [32], where tumor initiation is driven by a transgene, rather than an engrafted tumor. In a rat model of 13762NF mammary adenocarcinoma cells, the number of circulating neutrophils did not increase in poorly metastatic cells, whereas the number rose 50-fold in rats bearing a highly metastatic clone [164]. An intermediate rise in neutrophil number (12–14-fold) was observed in moderately metastatic tumors [164]. The increase in neutrophil number correlated with the ability of the tumor cells to secrete CSF [165]. These tumor cells did not induce a cytotoxic neutrophil response, while *i.v.* co-injections of tumor-elicited neutrophils caused a dose-dependent increase in extrapulmonary metastases that was associated with increased production of heparanase and type IV collagenolytic enzymes by the neutrophils [164]. In contrast to tumor-elicited neutrophils, normal or proteose peptone-elicited neutrophils did not alter the invasive potential [166].

How are the Neutrophils Mobilized? Unlike the situation in infection and inflammation where neutrophil mobilizing factors are secreted by endothelial cells and other stromal cells, in the context of cancer, neutrophil mobilizing factors are often secreted by the tumor cells themselves [22]. The most common neutrophil chemoattractants produced by tumors include IL-8 (CXCL8/CXCL2), MIP-1α (CCL3), huGCP-2 (CXCL6) and KC (CXCL1) [167–171]. G-CSF is ectopically expressed in several human tumors such as leukemia [172], bladder [173], pancreatic [174], cervical [175], ovarian [176], head and neck [177], colorectal [178] and breast carcinoma [179]. Similarly, some human cancers show elevated GM-CSF expression levels [31, 180, 181]. It is therefore not surprising that elevated numbers of circulating neutrophils are seen in a wide variety of human malignancies.

GM-CSF and G-CSF are broadly used therapeutically in cancer patients for their positive effects on bone marrow mobilization and immune functions. They are especially important for overcoming neutropenia caused by various anti-neoplastic treatments. However, accumulating studies show that these factors also promote the expansion of myeloid suppressive components, with undesirable consequences on tumor antigen-specific immune responses [182]. For instance, GM-CSF-based anti-tumor vaccine to human metastatic melanoma patients induced a subset of immunosuppressive MDSCs that involved TGFβ secretion [183]. GM-CSF may increase immune responses when administered at low doses, while causing an opposite effect at high doses [182]. While physiological concentrations of GM-CSF are required for normal myelopoiesis, chronic administration of GM-CSF resulted in the generation of immune suppressive Gr-1^{+}CD11b^{+} cells in mice [184]. Experimental tumors overexpressing GM-CSF induced a systemic increase of immature myeloid cells, which was associated with suppression of T cell immune responses [184–186]. However, irradiated cancer cells engineered to secrete GM-CSF elicited potent anti-tumor immune responses in various animal tumor models [187].

Similarly, tumor-secreted G-CSF that contributes to neutrophil mobilization, activation and stimulation of oxidative metabolism [188–191], is also involved in the polarization towards immunosuppressive MDSCs [192, 193]. The presence of G-MDSCs was shown to be important for promoting tumor growth [193]. Besides stimulating neutrophils, G-CSF may stimulate non-hematopoietic malignant tumor cell growth in an autocrine fashion [173, 194]. G-CSF receptor (G-CSFR) expression has been observed in bladder cancer cells [195], ovarian cancer [196], colorectal cancer [197] and Ewing sarcoma [198]. G-CSF administration increased tumor growth of Erwin sarcoma [198], and must therefore be carefully considered before use for stimulating neutrophil recruitment following chemotherapy.

Neutrophil Recruitment to the Tumor Microenvironment

Neutrophils make up a significant proportion of the non-malignant stroma that contributes to the tumor microenvironment [199]. The increase in circulating neutrophil numbers in the context of cancer (see "Neutrophil Mobilization in Cancer" section) may passively lead to an increase in the absolute number of neutrophils marginating at the tumor microenvironment. However, neutrophils are also actively recruited into the tumor microenvironment in both cancer patients and mouse models of cancer. In humans, intratumoral infiltration of neutrophils has been detected in gastric carcinoma [200–202], bronchioloalveolar carcinoma [170, 203], non-small cell lung carcinoma [204], pancreatic neoplasia [205], pancreatic ductal adenocarcinoma [206], bladder cancer [147], glioma [207], cervical carcinoma [139] and breast carcinoma [4, 208] (Table 1). The clinical consequences of intratumoral and circulating neutrophils in cancer patients will be discussed in "Prognostic Values of Neutrophils and Other Myeloid Subtypes in Cancer Patients" section.

Neutrophils are actively recruited to the tumor microenvironment along a chemotactic gradient of tumor-secreted factors. Production of CXCL8/IL-8 and related chemokines occurs downstream to oncogene activation [209–211]. Bellocq et al. [170] observed a direct correlation between the number of neutrophils and IL-8 levels in broncoalveolar lavage fluids from bronchioloalveolar carcinoma patients, suggesting a role for this cytokine in neutrophil recruitment. Neutrophils were mainly located in the alveolar lumen, while seldom in the alveolar wall [170]. CXCR1 and CXCR2 are the major

Table 1 Tumor-associated neutrophils (TANs) in human cancer

Cancer type	Major findings	Reference
Bladder cancer	• Increase in circulating $CD11b^{+}CD15^{high}CD33^{low}$ granulocytes, while no increase in $CD11b^{+}CD15^{low}CD33^{high}$ monocytes. Both cell types are activated. • Presence of circulating monocyte-macrophage $CD11b^{+}HLA\text{-}DR^{+}$, and granulocytic $CD11b^{+}CD15^{+}HLA\text{-}DR^{-}$ myeloid cells. • Myeloid cells secrete CCL2, CCL3, CCL4, G-CSF, IL-8 and IL-6. • Granulocytes inhibit in vitro T cell proliferation through induction of $CD4^{+}Foxp3^{+}$ T regulatory cells.	[147]
Metastatic adenocarcinomas of the pancreas, colon, and breast	• Unusually large number of circulating granulocytes co-purified with low density PBMCs on a density gradient. • Increased oxidative stress and production of H_2O_2 in circulating granulocytes. • H_2O_2-dependent reduction in T-cell receptor ζ chain expression and IFNγ production in T cells.	[278]
Bronchioloalveolar carcinoma	• Neutrophils are present in the alveolar lumen. The number of neutrophils correlate with IL-8 levels.	[170]
Stage III and IV lung and stomach cancer	• Patients with lung and stomach cancer showed increased blood neutrophil count, but decreased level of leukocyte cationic proteins.	[386]
Recurrent localized cervical cancer	• High density of $CD66b^{+}$ neutrophils and $CD163^{+}$ macrophages in peritumoral compartment.	[139]
Hepatocellular carcinoma (HCC)	• Intratumoral $CD66b^{+}$ neutrophils correlate with $CD8^{+}$ T cells, TGFβ expression, BCLC stage and early recurrence. • Increased intratumoral neutrophil numbers were associated with decreased overall survival, while peritumoral neutrophils were not associated with the outcome.	[387]
Hepatocellular carcinoma (HCC)	• Neutrophils from HCC patients produced CCL2 and CCL3. High CCL2 production was associated with reduced overall survival.	[388]
Hepatocellular carcinoma (HCC)	• Pro-inflammatory IL-17-producing cells recruit neutrophils into the peritumoural stroma of hepatocellular carcinoma (HCC) by epithelium-derived CXC chemokines. • Neutrophils promote angiogenesis at the adjacent tumor-invading edge via MMP-9 signaling. • Selective depletion of neutrophils inhibits tumor progression and growth.	[57]
Resectable non-small cell lung cancer	• Intratumoral $CD66b^{+}$ neutrophils were elevated in 50 % of the patients. • An increase in $CD66b^{+}$ cells was associated with high incidence of relapse and worse overall survival.	[204]
Colorectal cancer	• Increase in MPO^{+} and $CD15^{+}$ cell infiltrate in the mucosa. • While MPO^{+} cells were largely $CD15^{+}CD66b^{+}$, a high percentage of $CD15^{+}CD66^{+}$ cells were MPO^{-}. • Only high density of MPO^{+} cell infiltration was associated with improved survival.	[362]
Colorectal carcinoma	• High intratumoral $CD66^{+}$ cells were associated with a poorer prognosis.	[365]
Melanoma stage I/II	• Presence of $CD66b^{+}$ tumor infiltrating neutrophils was associated with poor prognosis.	[389]
Renal cell carcinoma	• The presence of intratumoral neutrophils was associated with increased tumor size and shorter recurrence-free survival.	[390]
Cervical cancer	• The highest densities of $CD66b^{+}$ neutrophils and $CD163^{+}$ macrophages were observed in the peritumoral environment. • High peritumoral and stromal neutrophils were associated with shorter recurrence-free survival.	[139]
Various cancer samples	• $CD15^{+}$ neutrophils were found in substantial amounts in untreated malignant hepatocellular, cervical, colorectal and gastric carcinoma. • The $CD15^{+}$ cells were more abundant in the peritumoral stroma than in the cancer nest. • The peritumoral stroma $CD15^{+}$ cell density was associated with intrahepatic metastasis in liver cancer and lymph node metastasis in gastric cancer.	[391]

Evidence for the presence of neutrophils within human cancer specimens and its prognostic value

neutrophil receptors that mediate neutrophil chemotaxis to the tumor microenvironment in response to IL-8. The same receptors also mediate neutrophil response to other ligands such as CXCL1/2/3, ENA-78 (epithelial-cell-derived neutrophil attractant-78; CXCL5), GCP-2 (granulocyte chemotactic protein-2; CXCL6) and NAP-2 (neutrophil-activating peptide 2; CXCL7) [212–215]. The neutrophil-sensitive human HT-29 colorectal adenocarcinoma and FaDu pharyngeal squamous-cell carcinoma cells secrete IL-8 and GROα, and induced the adhesion of neutrophils to ICAM-1 on microvascular-endothelial cell monolayers resulting in transmigration through the endothelial cell monolayer [216]. Recruited neutrophils also release cytokines and chemokines, which enhance their own recruitment and activation in addition to inducing the migration of other immune cells [217]. Neutrophil-secreted MMP-9 may process the chemokine

CXCL5 to further promote neutrophil recruitment [214]. Similarly, neutrophil-derived Cathepsin G may increase the chemotactic activity of CXCL8, CXCL5 and CCL15 through N-terminal truncation [218].

$Ly6G^+$ neutrophils are the dominant source of CXCR2 in the blood, and CXCR2 deficiency attenuates neutrophil recruitment to the tumor bed [219]. Depletion of $Ly6G^+$ cells purged CXCR2-dependent tumor-associated leukocytes, suppressed established skin tumor growth and colitis-associated tumorigenesis [219]. CXCR2 is thus a potent pro-tumorigenic chemokine receptor that directs recruitment of tumor-promoting leukocytes into tissues during tumor-inducing and tumor-driven inflammation [219]. This notion correlates well with the important role CXCR2 plays in recruiting neutrophils to inflammatory sites.

The granulocyte chemotactic protein (GCP)-2/CXCL6 was shown to be important for the recruitment of neutrophils to melanoma tumors, and is associated with angiogenesis and tumor growth [171, 220]. CXCL6 specific antibodies reduced the recruitment of neutrophils to the tumor, with concomitant reduction in tumor growth [220]. There are several lines of evidence to suggest that $CD8^+$ T cells may promote the recruitment of neutrophils to the tumor, supposedly through secretion of IFNγ [14] and vice versa, neutrophils contribute to T cell recruitment and activation [14]. Elimination of either neutrophils or $CD8^+$ T cells, or administration of IFNγ neutralizing antibodies prevented tumor regression, suggesting a tight co-operation between neutrophils and $CD8^+$ cells in eliminating the tumor [14]. In contrast, regulatory T cells may limit neutrophil recruitment to the tumor site, which might be related to decreased expression of neutrophil chemoattractants such as CCL3, CXCL1 and CXCL2 [221].

CXCL12 and VEGF are also suggested to act as chemoattractants for myeloid cells [222, 223] and may act in concert for recruiting neutrophil to the tumor microenvironment. Under normal conditions, a tight cooperation between myeloid cells and endothelial progenitor cells is required for proper neovascularization [223]. VEGF induces bone marrow-derived myeloid cell mobilization to the circulation and through VEGF-mediated upregulation of SDF1/CXCL12 in activated perivascular myofibroblasts, the myeloid cells are kept in close proximity to angiogenic vessels [223]. VEGF is upregulated in a wide variety of tumors [69, 224–227], as is CXCL12 [228]. CXCL12, that is also upregulated by hypoxia [229], augments CXCR4 expression on vascular endothelial cells [230] and attracts $CXCR4^+$ cells, including neutrophils, to the tumor [223].

While tumor cells are capable of secreting neutrophil chemoattractants, other cells in the tumor microenvironment may also contribute to neutrophil recruitment. For example, tumor-infiltrating T helper type 17 (Th17) cells and IL-17 induce the expression of G-CSF, leading to immature myeloid-cell mobilization and recruitment into the tumor microenvironment [231]. IL-17 may also induce the expression of C-X-C chemokines, notably CXCL8/IL-8, in epithelial cells that in turn recruit neutrophils to the tumor [57]. The presence of Th17 cells within the tumor microenvironment could antagonize and counter the tumor-suppressive IFNγ-producing $CD4^+$ Th1 cells, and are thereby likely involved in the promotion of tumor growth [83]. However, in a lung melanoma mouse cancer model, adoptive transfer of Th17 cells promoted tumor-specific $CD8^+$ T cell activation [232], suggesting for a dual role for Th17 cells in tumor biology. The Th17 cells promoted dendritic cell recruitment into the tumor tissues and stimulated CCL20 chemokine production by the tumor [232].

Recently, another tumor-derived cytokine, IL-35, was shown to promote tumor growth by enhancing myeloid cell accumulation and angiogenesis [233]. IL-35 does not directly inhibit tumor-associated $CD8^+$ T cell activation, differentiation, or effector functions, but IL-35-treated cancer cells showed increased expression of gp130 and reduced sensitivity to CTL destruction [233]. Altogether, these data suggest that tumor cells induce a chronic inflammatory response.

Neutrophil Activation in Cancer

Neutrophils are traditionally perceived as the first line of defense against microbial infections and as mediators of inflammation, which possess favorable properties that protect the host. In the context of cancer, neutrophils were shown to exert both pro- and anti-tumor activities suggesting for a dual mode of activation which is the basis for the distinction between N1 and N2 neutrophils, as described in "Niche-dependent Neutrophil Function" section. Interestingly, several tumor-derived cytokines can activate neutrophil cytotoxic activities, including CCL2, CCL5, CCL3, CXCL1, SDF1/CXCL12 and CXCL16 [32, 66]. However, these chemokines are usually associated with pro- rather than anti-tumor activities. For example, CCL2 (MCP-1), which is overexpressed in a wide range of cancers [234], is associated with poor prognosis in breast, colorectal, cervical and thyroid cancers [235–239]. Tumor-derived CCL2 plays a pro-tumor role by recruiting inflammatory monocytes to pulmonary metastases [240] and MDSCs to the tumor microenvironment [241], as well as promoting angiogenesis [242], tumor cell proliferation [243] and migration [244]. On the other hand, CCL2 prevents apoptosis of neutrophils [245] and activates neutrophils in the pre-metastatic lung towards an anti-tumor phenotype where they produce H_2O_2 to kill disseminated tumor cells [32, 66], suggesting a dual role of this chemokine. Also, CXCL1 was shown to promote tumor cell proliferation [246], tumor angiogenesis [247], invasion and migration [248], in addition to its ability to recruit and activate neutrophils [249]. CXCL1 was shown to be involved in a paracrine

network mediating both metastatic progression and chemoresistance [250]. IL-8 is another chemokine expressed in variety of tumors and is associated with neutrophil recruitment and activation [251]. For example, IL-8 secreted by human fibrosarcoma and prostate carcinoma cells promoted the infiltration of MMP-9^{+} neutrophils [252] and melanoma secreted IL-8 was shown to increase CD11b/CD18 expression on neutrophils, an indication for their activation [253, 254].

A dual role has also been observed for G-CSF. On the one hand, G-CSF is an essential cytokine for mobilization of neutrophils, and under certain conditions, activates them. On the other hand, G-CSF may polarize granulocytes to promote tumor growth and metastasis [26]. These seemingly conflicting roles played by both neutrophils and the tumor-derived chemokines, put forth the question of what determines the overall contribution of neutrophils to cancer? It is likely that the pro- and anti-tumor activities of neutrophils are determined by the overall cytokine and chemokine milieu provided by the tumor and the tumor infiltrating cells rather than the expression level of a specific chemokine. This notion is supported by the fact that TGFβ was shown to be a potent repressor of neutrophil cytotoxicity both in vivo and in vitro [13, 32]. This is best exemplified by the differences in the function of neutrophils at the primary tumor site versus their activity at the pre-metastatic niche. High TGFβ activity in the tumor microenvironment generates immune suppressive conditions, attenuating the potential cytotoxicity of activated neutrophils recruited to the tumor bed [13]. At the same time, the full extent of activated neutrophil anti-tumor cytotoxicity may be manifested in distant organs, i.e. the pre-metastatic niche, where TGFβ levels are low [32, 66].

Tumor-Associated Neutrophils (TANs): Pro- and Anti-Tumor Mechanisms

Neutrophils in Tumor Initiation - Inflammation-Associated Tumorigenesis

Chronic inflammation has been associated with increased susceptibility for cancer [20]. This is well demonstrated in chronic Hepatitis B virus infection [255], where the persisting insult and chronic inflammation ultimately leads to hepatocellular carcinoma. Another well-studied example is the correlation between inflammatory bowel (IBD) disease and colorectal cancer (CRC) with up to 30 % of IBD patients developing CRC [256]. The inflammatory process involves a wide range of immune cells of which neutrophils make a significant fraction. It is therefore reasonable to assume that neutrophils also play a part in inflammation-driven tumorigenesis. Indeed, neutrophils were shown to directly promote tumorigenesis by causing genomic instability [257]. Neutrophil-induced genotoxicity has been related to induction of oxidative DNA damage through release of ROS and myeloperoxidase-related metabolites [258–260]. In addition, neutrophils were shown to promote experimental chronic colitis-associated carcinogenesis in mice [261]. Neutrophil recruitment to the inflamed submucosa was mediated by the chemokine CXCL2 through the neutrophil receptor CXCR2, resulting in increased neutrophil secretion of MMP-9 and neutrophil elastase, and consequent excessive angiogenesis and cell proliferation [261]. Elastase degrades barrier-forming proteins on epithelial cells [262] and neutrophil-derived elastase was found to degrade the adhesion molecule E-Cadherin on pancreatic ductal adenocarcinoma, leading to tumor cell dyshesion and increased migratory capacity [206]. Neutrophil infiltration of pancreatic ductal adenocarcinoma cells was also associated with epithelial-to-mesenchymal transition (EMT). In vitro cultivation of tumor cells with neutrophils led to enhanced expression of the transcription factor TWIST, translocation of β-Catenin to the nucleus, appearance of ZEB1 in the nucleus and downregulation of keratins, a sign of (EMT) [263].

Under certain circumstances, administration of neutrophil-neutralizing antibodies reduced the number and size of tumors [261, 264, 265]. The tumorigenic effect of MMP-9, predominantly provided by neutrophils and mast cells, was also reported in a mouse model of skin cancer where MMP-9 knockout mice showed decreased incidence of invasive tumors [266]. While there is a large body of evidence to support the pro-tumorigenic function of neutrophils, several studies show that neutrophils also possess anti-tumorigenic functions. For example, neutrophils were shown to participate in immune surveillance and eliminate potentially malignant cells and neutrophil MMP-8 was shown to provide protection against carcinogen-induced skin tumors [267].

Neutrophils at the Primary Tumor Site

Neutrophils are frequently found in solid tumors [268] (see "Neutrophil Recruitment to The Tumor Microenvironment" section), and together with macrophages and NK cells make up the vast majority of tumor infiltrating cells. The fact that neutrophils are frequent residents of solid tumors is not surprising as many of these tumors express and secrete high levels of neutrophil mobilizing factors and neutrophil chemoattractants. For example, IL-8/CXCL8, a potent neutrophil chemotactic factor and activator, was found to be expressed in a wide variety of human cancers [253, 269–272]. In most cases, IL-8 is secreted by the tumor cells themselves [210], while in others, other stromal cells such as fibroblasts [273] and macrophages [274] secrete IL-8. Some human cancers secrete high levels of G-CSF and GM-CSF

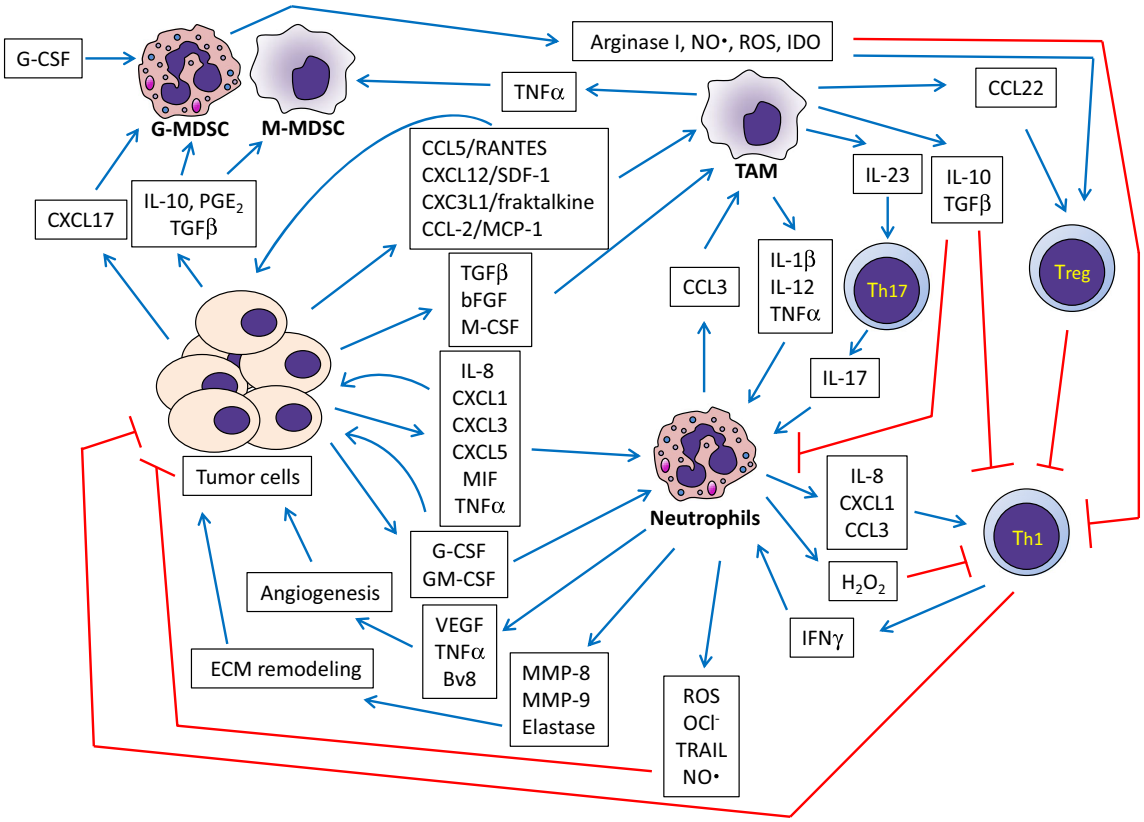

Fig. 2 The crosstalk between cancer cells, neutrophils and other immune cells in the tumor microenvironment. The tumor microenvironment is characterized by a state of chronic inflammation, where tumor cells secrete a range of cytokines and chemokines that recruit and activate neutrophils and other immune cells. Accumulating evidence suggest that neutrophils are essential for mounting an adaptive immune response, as this fails to occur upon depletion of neutrophils. Neutrophils exert both pro-tumor and anti-tumor activities. Through production of ROS, nitric oxide and TRAIL, neutrophils can directly kill the tumor cells. Neutrophils also indirectly prevent tumor growth through eliciting specific anti-tumor $CD8^+$ cytotoxic responses. By virtue of their multifunctional tasks, neutrophils may also stimulate tumor growth through secretion of ECM remodeling enzymes and pro-angiogenic factors. As in chronic inflammation, a negative feedback mechanism involved in restricting immune responses, such as the generation of MDSCs, also occurs within the tumor, generating an immunosuppressed environment that antagonizes the anti-tumorigenic activities of neutrophils. The secretion of immunosuppressive factors by the tumor cells themselves further fortifies this immunosuppressed milieu. (*TANs* Tumor Associated Neutrophils, *TAMs* Tumor Associated Macrophages)

[275, 276], acting as both potent mediators of neutrophil mobilization, recruitment and activation (Fig. 2).

Recruited neutrophils were shown to acquire a pro-tumor phenotype similar to that of tumor-associated monocytes, which by analogy to the M2 monocyte phenotype, was referred to as an N2 neutrophil phenotype [13]. TANs were shown to support tumor growth by producing angiogenic factors and matrix-degrading enzymes [8, 57, 64, 252, 264, 277]. Neutrophils were also shown to promote the acquisition of a metastatic phenotype [265] and to suppress anti-tumor immune responses [278]. Neutrophil-derived CXCL2 enhances angiogenesis as well as neutrophil accumulation, ultimately inducing carcinogenesis [247]. Several studies have shown that G-CSF may stimulate angiogenesis and promote tumor growth [279–281]. This effect of G-CSF is mediated through recruitment of $CD11b^+Gr1^+$ MDSCs and increased number of endothelial progenitor cells [279]. The pro-angiogenic effects of MDSCs is in part driven by Bv8 (prokineticin 2/PK2), which is upregulated by G-CSF [282, 283] through a STAT3 dependent mechanism [284]. G-CSF may further contribute to tumor angiogenesis by inducing VEGF-A production in neutrophils [285]. The neutrophil secreted MMP-9 has been functionally implicated in VEGF activation [286], thus further fortifying angiogenesis. A co-operation between cancer cells and neutrophils in promoting angiogenesis was demonstrated by Queen et al. [4], where GM-CSF secreted by breast cancer cells stimulated neutrophils to produce oncostatin M, which in turn stimulated the tumor cells to produce VEGF, thereby augmenting tumor-associated angiogenesis.

IL-17 produced by Th17 cells induces expression of G-CSF, leading to immature myeloid-cell mobilization and recruitment into the tumor microenvironment [231]. These $CD11b^+Gr1^+$ MDSCs produce the pro-angiogenic Bv8 [283], that bypasses VEGF and renders tumors refractory to anti-VEGF therapy [231]. Anti-Bv8 treatment reduced the number of $CD11b^+Gr1^+$

cells in peripheral blood and in tumors along with suppression of angiogenesis [283]. Thus, G-CSF may induce angiogenesis through a Bv8-dependent mechanism.

The important role of neutrophils in the induction of the angiogenic switch in cancer, was further illustrated in a RIP1-Tag2 transgenic mouse model of pancreatic β-cell carcinogenesis [287]. MMP-9-expressing neutrophils were predominantly found in angiogenic islet dysplasias and in tumors, whereas MMP-9-expressing macrophages were localized along the periphery of these lesions [287]. Transient depletion of neutrophils reduced the frequency of initial angiogenic switching in islet dysplasias. Jablonska et al. [64] observed that IFNβ-deficient mice develop faster growing melanoma and fibrosarcoma tumors with better developed blood vessels than did wild-type mice. These tumors displayed enhanced infiltration of $CD11b^{+}Gr1^{+}$ neutrophils that expressed high levels of VEGF, MMP-9 and CXCR4. The transcription factors c-Myc and STAT3 regulating the expression of these proteins, were also elevated in the neutrophils [64]. Thus, endogenous IFNβ seems to inhibit tumor angiogenesis through repression of genes encoding pro-angiogenic and homing factors in tumor-infiltrated neutrophils [64]. Neutrophils in bronchioloalveolar carcinoma produce hepatocyte growth factor (HGF) that stimulates the migration of the tumor cells [203]. Similarly, human cholangiocellular and hepatocellular carcinoma cells induce HGF secretion by neutrophils, which in turn enhances the invasiveness of the cancer cells [288]. TANs can also stimulate tumor growth by releasing growth factors such as epidermal growth factor, TGFβ and platelet-derived growth factor (PDGF) from the extracellular matrix [289]. The important role neutrophils play at the primary tumor site was further demonstrated using neutrophil depletion experiments in mice. In neutrophil depleted mice tumor growth was significantly reduced supporting the notion of pro-tumorigenic neutrophil function at the primary tumor site [287, 264]. While these observations clearly demonstrate a pro-tumor function for neutrophils in the tumor-microenvironment, there are several convincing observations showing the opposite (See "Anti-Tumor Functions of Neutrophils" section). The complexity of the mechanisms that regulate neutrophil function in the primary tumor microenvironment is exemplified in Fig. 2.

Promotion of Cancer Cell Dissemination by Neutrophils

There are several studies showing that neutrophils promote tumor cell motility, migration and invasion [146]. Neutrophils may facilitate invasion through secretion of enzymes degrading the extracellular matrix such as elastase, Cathepsin G, proteinase-3, MMP-8 and MMP-9 [146]. Neutrophils may also indirectly contribute to the degradation of the extracellular matrix through activation of tumor-derived pro-MMP-2 (pro-Gelatinase A), mediated by the neutrophil-derived elastase, cathepsin G and proteinase-3 [290].

IL-8-producing tumor cells have frequently been shown to be more metastatic than the corresponding non-producer cells [291]. IL-8 is a potent chemoattractant for neutrophils and normally recruits neutrophils to the site of wounds. However, in the tumor settings, IL-8 induces the release of specific proteases and heparanases by recruited neutrophils. These proteases and heparanases remodel the extracellular matrix making it easier for tumor cells to intravasate. Neutrophil elastase activates other latent proteases in the tumor microenvironment which cleave and inactivate plasminogen activator inhibitor-1, leading in turn to the release of embedded growth factors such as bFGF, a potent angiogenic factor [291]. An interesting interplay between neutrophil elastase and its inhibitor alpha 1-antitrypsin seems to have a significant impact on cancer development [292]. A deficiency in alpha 1-antitrypsin is associated with increased risk of liver cancer, bladder cancer, gall bladder cancer, malignant lymphoma and lung cancer [292]. Conversely, elevated concentrations of neutrophil elastase might promote the development, invasion, and metastasis of many cancers [292]. Neutrophils also release high levels of MMP-9/Gelatinase B, which further affects extracellular matrix remodelling. HOCl produced by myeloperoxidase of activated neutrophils oxidizes specific sulfur-containing amino acids and activates the MMP pro-enzymes. In parallel, HOCl inactivates TIMP-1, thereby further increasing the proteolytic activity of MMPs [291].

Neutrophils activated by melanoma-secreted IL-8 can facilitate melanoma cell extravasation through an interaction between ICAM-1 (CD54) on the melanoma cells and Mac-1 (CD11b/CD18) on neutrophils [253, 254, 293]. Neutrophils were also shown to be important for entrapping circulating melanoma cells and thus facilitating lung metastasis development [294]. Melanoma-secreted IL-8 not only attracts neutrophils, but also strongly upregulates β2 integrins, which interact with ICAM-1 on melanoma cells, thereby promoting the anchoring of the tumor cells to vascular endothelium [294]. Other studies further show that IL-8 increases neutrophil β2 integrin expression with simultaneous shedding of L-selectin (CD62L), thereby promoting their sequestration in the lung [295]. Similarly, neutrophils were shown to promote breast cancer cell transendothelial migration through an ICAM-1-CD11b/CD18 interaction dependent mechanism [296]. Neutrophils were also shown to interact with colon carcinoma cells, facilitating their dissemination [297]. The interaction was mediated by CD11b, CD11a and L-selectin on neutrophils and CD54 and sialylated, O-linked, protease-sensitive ligands on the tumor cells [297, 298].

Neutrophils in Pre-Metastatic Organs - The "Seed and Soil" Hypothesis

In 1889, Stephen Paget proposed that tumors do not metastasize to random organs and that there are preferable sites of metastasis for specific tumors. This has led to the realization

that successful colonization of a distant organ requires a degree of compatibility between the tumor cell and the future site of metastasis. This realization was the basis of the "Seed and soil" theory. This theory was expanded upon when tumors were shown to directly enhance the seeding of circulating cells in the lungs [299], suggesting that the tumor can modulate the "soil" to make it more compatible with the "seed". Later studies have shown the direct involvement of bone marrow derived VEGFR1$^+$ progenitor cells [300, 301] in generating the pre-metastatic niche that is more receptive toward incoming tumor cells. These cells were identified as of myeloid lineage, and appeared not to differentiate, but maintained their expression of immature surface markers including c-Kit and Sca-1 within the tissue parenchyma [300]. Of note, the formation of the pre-metastatic niche was found to be organ specific. Yan et al. [302] observed that CD11b$^+$Gr-1$^+$ myeloid cells are increased in lung of mice bearing mammary adenocarcinomas before tumor cell arrival. These immature myeloid cells decreased IFNγ production, but increased the production of pro-inflammatory cytokines in the premetastatic lung [302]. In addition, the CD11b$^+$Gr-1$^+$ cells produce large amounts of MMP-9 and promote vascular and ECM remodeling [302]. Deletion of MMP-9 normalized aberrant vasculature in the pre-metastatic lung and diminished lung metastasis [302]. More recently, Gao and colleagues [303] showed that it was the CD11b$^+$Ly6C^{high} monocytic myeloid subpopulation that was promoting metastatic seeding in the lungs rather than the neutrophilic CD11b$^+$Ly6G^{high} subpopulation. Interestingly, the pro-metastatic effect of CD11b$^+$Ly6C^{high} monocytes at the pre-metastatic lung was found to be mediated by secretion of versican which in turn leads to mesenchymal to epithelial transition (MET) and to enhanced proliferation [303]. Still, while these studies do not implicate neutrophils in the process of priming the pre-metastatic niche, the fact the neutrophil chemoattractants such as S100A8 and S100A9 [301] are available in the pre-metastatic lung, together with our own observations [32] showing neutrophils accumulating early in the pre-metastatic lung, support the notion that neutrophils may contribute to the formation of the pre-metastatic niche. Furthermore, Kowanetz et al. [26] showed that depletion of Gr1$^+$ or Ly6G$^+$ cells from the pre-metastatic lung results in reduced metastasis. Similarly, Sceneay et al. [304] also observed increased numbers of granulocytic CD11b$^+$Ly6C^{med}Ly6G$^+$ myeloid cells in the pre-metastastic lungs in mice injected with melanoma or breast carcinoma cells with a concomitant reduction in the cytotoxic activity of NK cells [304]. Erler et al. [305] observed that lysyl oxidase (LOX) secreted by hypoxic breast tumor cells is required for the recruitment of immature myeloid CD11b$^+$F4/80$^-$ cells to the premetastatic lung. Tumor-secreted LOX was found co-localized with fibronectin in the lung, to which CD11b$^+$ cells bound. The CD11b$^+$ cells secreted MMP-2, generating chemoattractive collagen IV peptides, a process leading to enhanced invasion and recruitment of metastasizing tumor cells as well as bone-marrow-derived cells into the lung [305]. Knocking down LOX in the breast tumor cells prevented the recruitment of CD11b$^+$ cells to the premetastatic lung, with concomitant reduction in metastatic growth [305].

A mechanism involving microvascular deposition of neutrophil extracellular traps (NETs) was found to directly facilitate the formation of hepatic micrometastases [306]. Neutrophils were also shown to promote liver metastasis of Lewis lung carcinoma (H-59 subline) cells through Mac1 (CD11b/CD18)-mediated interaction with circulating tumor cells [307]. Neutrophil depletion prior to cancer cell inoculation reduced the number of liver metastases [307]. This effect was reversed when inflamed neutrophils were co-inoculated with tumor cells [307]. H-59 cells showed reduced adhesion to liver sinusoids of CD11b deficient mice [307]. In contrast, our own work, using mouse models of breast cancer, shows that neutrophils are stimulated by tumor-secreted factors and acquire an anti-tumor phenotype [32]. These neutrophils accumulate in the lungs in large numbers during the pre-metastatic phase and provide anti-metastatic protection by eliminating incoming disseminated tumor cells [32]. These tumor-entrained neutrophils (TENs) produced high levels of H_2O_2 and acquire the capacity to kill tumor cells in a contact dependent fashion [32]. Similarly, Lopez-Lago and colleagues [66] showed that tumor-secreted CXCL1 stimulates neutrophils and induces an anti-metastatic response in a model of human renal cell carcinoma. Furthermore, they demonstrated that the metastatic potential of the tumors inversely correlates with the extent of neutrophil mobilization [66]. Finally, we have shown that tumoricidal neutrophils also exist in the circulation of breast cancer patients, but not in healthy individuals, suggesting that anti-tumor neutrophils are generated during the natural course of the disease in patients [32]. These conflicting observations provide the basis for the controversy that surrounds the function of neutrophils at the pre-metastatic niche; do they possess favorable or unfavorable properties? The answer to this question remains obscure. However, it seems that neutrophils may possess both pro- and anti-tumor functions and their actual function in situ is determined by the microenvironment and chemokine milieu (Figs. 1 and 2).

Anti-Tumor Functions of Neutrophils

The recognition that neutrophils have potential anti-tumor functions was first brought forward in the 1970s where neutrophils from patients with bladder cancer were shown to be cytotoxic toward bladder cancer cells [308]. Since then neutrophils were shown to exert their anti-tumor functions via direct cytotoxicity, antibody dependent cell mediated cytotoxicity (ADCC) and through the presentation of specific antigens (Table 2) [7]. Neutrophils need to be activated in order to exert their anti-tumor activities. Various cytokines (e.g., G-CSF, IFNγ, TNFα) and chemokines (e.g., CCL-2, CCL-5,

Table 2 Evidence for anti-tumorigenic effects of neutrophils

Major findings	Reference
Human neutrophils	
• Normal human peripheral blood granulocytes destroy various human cancer cells in vitro, including osteosarcoma, melanoma and lung squamous carcinoma cells, at a higher efficiency than normal fibroblasts.	[392]
• Leukocytes from patients with urinary bladder carcinomas showed cytotoxicity toward human bladder carcinoma cells in vitro.	[308]
• Leukocytes from pregnant women, and patients with toxemia, uterine myoma, ovarian and endometrial carcinoma had cytotoxic effects toward primary ovarian carcinoma cultures.	[393]
• Neutrophils from Stage III and IV lung and stomach cancer patients were more cytotoxic to K562 cells than those from healthy controls.	[386]
• Normal human peripheral blood granulocytes showed cytostatic effect on various tumor cell lines including human K562 myeloid leukemia, human F265 lymphoblastoid, mouse TU-5 kidney cells, mouse RBL-5 lymphoma, human RAJI Burkitt's lymphoma, human W1-38 embryonal lung line, human SV40 transformed fibroblasts.	[394]
• The neutrophils were cytolytic to Chang-A hepatocarcinoma cells, an effect that was enhanced by antibodies.	
• Granulocytes formed clusters among themselves and attached to the target cells.	
• Detachment of adherent cells was observed after 6 h.	
• PMA-stimulated neutrophils mediated cytotoxicity against CEM T-lymphoblast cells.	[314]
• Neutrophil cytotoxicity was mediated by H_2O_2 and myeloperoxidase.	
• Killing of CEM could be inhibited by catalase, the MPO inhibitor cyanide, and the HOCl scavengers tryptophan, methionine and alanine, suggesting a role for the MPO-H_2O_2-Cl- system.	
• The cytotoxic effect was dependent on pH and the effector cell number.	
• Concanavalin A-activated neutrophils can release cytotoxic quantities of H_2O_2 and myeloperoxidase, which in concert with a halide (chloride or iodide) lysed murine LSTRA lymphoma cells.	[315]
• SOD did not affect killing.	
• Unstimulated neutrophils had no effect.	
• Azide, cyanide and catalase prevented the cytotoxicity.	
• Neutrophils were cytotoxic and cytostatic to human tumor cell lines T24 bladder carcinoma, LR melanoma, and SV40-transformed fibroblasts, as well as K562 CML, Raji Burkitt lymphoma and CEM T-ALL cells.	[395]
• The killing of tumor cells was selective, as the neutrophil didn't kill normal human fibroblasts.	
• Protease inhibitors did not inhibit neutrophil cytotoxicity.	
• PMA-activated granulocytes are cytotoxic to CEM T-ALL cells.	[316]
• The myeloperoxidase-hydrogen peroxide-halide system is involved as azide, cyanide and catalase inhibited the killing.	
• The killing was dependent on the presence of a halide.	
• PMA-activated neutrophils are cytotoxic to Raji Burkitt lymphoma cells when incubated at 20:1 E:T ratio.	[317]
• The myeloperoxidase-hydrogen peroxide-halide system is involved as the cytotoxicity can be inhibited by azide, cytochrome C and catalase.	
• Superoxide dismutase enhanced the cytotoxic effect.	
• Neutrophils had a stronger cytotoxic effect on malignant targets (MA-160 prostate cancer, Garr1 colon cancer) than on nonmalignant targets (human embryonic lung and intestinal cells).	[396]
• Neutrophils from colon and breast cancer patients with stage I disease possessed neutrophils which were less effective in killing tumor cells than neutrophils from normal donors.	
• In contrast, neutrophils from colon and breast carcinoma patients with stage IV disease were more effective in killing tumor cells than normal cells.	
• PMA-activated neutrophils kill K562 CML cells. Neutrophil cytotoxicity was inhibited by catalase, while augmented by SOD.	[318]
• Lidocaine enhanced, while verapamil and exogenous adenosine 5′-triphosphate inhibited neutrophil-mediated tumor cytotoxicity.	[397–400]
• Prednisolone inhibited, while chloroquine had no effect on neutrophil-mediated tumor cytotoxicity.	
• TNF-treated neutrophils acquire cytotoxic activity towards tumor cells such as Raji, K562, UCLA-SO-M14 and U937, through production of hydrogen peroxide.	[309, 310]
• Catalase, but not SOD, sodium azide or deferoxamine, prevented the cytotoxic effect.	
• IFNγ enhances the tumor cytostatic effect of neutrophils.	[311]
• Neutrophils induce ADCC of human $GD2^+$ melanoma and neuroblastoma cell lines in the presence of antibodies to the ganglioside GD2.	[336]
• CD11/CD18-deficient neutrophils were defective in inducing cytolysis.	
• Antibodies to CD11b, CD11c and CD18 blocked ADCC by normal neutrophils.	
• Antibodies to FcRII and FcRIII, but not those to FcRI, blocked ADCC.	
• GM-CSF enhances anti-tumor neutrophil ADCC.	
• K652 cells that have been made resistant to H_2O_2 through repeated exposure to increasing amounts of glucose oxidase, expressed elevated levels of Catalase and were resistant to the cytotoxic effect of TNFα-activated neutrophils.	[401]
• Neutrophils from colon carcinoma and breast cancer patients showed elevated cytotoxicity towards Hct-116 colon carcinoma cells, while normal neutrophils or neutrophils from melanoma patients had almost no effect.	[402]
• The cytotoxicity of neutrophils from colon carcinoma could be enhanced by fMLP and prothymosin α1.	
• Prothymosin α1 enhanced the oxidative responses of neutrophils.	
• GM-CSF-primed neutrophils reduced melanoma cell viability.	[325]
• These neutrophils showed increased release of O_2^-.	
• SOD and Catalase when applied separately could not abrogate the neutrophil killing of melanoma cells, while added together partly prevented the killing.	
• Interfering with nitric oxide ($NO^·$) production by N^G-monomethyl-L-arginine significantly protected the melanoma cells against GM-CSF-primed neutrophils.	

Table 2 (continued)

Major findings	Reference
• Neutrophils are cytotoxic to HT-29 colorectal cancer and FaDu pharyngeal squamous-cell carcinoma cells. These tumor cells secrete IL-8 and GROα, leading to the activation of cytotoxic neutrophils.	[216]
• Tumor-entrained neutrophils (TENs) with anti-tumor activities are present in the peripheral blood of breast cancer patients prior to surgical resection, but not in healthy individuals.	[32]
• These TENs showed cytotoxic effect towards MDA-MB-231 breast carcinoma cells.	
• Human neutrophils display a higher cytotoxic activity against poorly metastatic cells compared with highly metastatic renal cell carcinoma cells.	[66]
• Several neutrophil chemokines including CXCL1, CXCL2, CXCL3, CXCL5 and IL-8 were down modulated in the highly metastatic cells.	
• Non-metastatic renal carcinoma cells promote recruitment of neutrophils to the lung of mice. The metastatic activity inversely correlated with the ability of tumor cells to recruit and activate these immune cells.	
Murine neutrophils	
• Neutrophils are attracted by rat Walker Carcinoma 256 cells.	[403]
• Neutrophils interacts with these cells, indent their membranes, leading to a rounding up of the tumor cells. This killing process depends on the presence of other host factors, as in vitro the tumor cells did not induce cytotoxic neutrophils.	
• Cytotoxic effect of rat peritoneal neutrophils against the syngeneic ascites tumor WBP1.	[404]
• Establishment of WBP1 ascites tumor was retarded when the rats were stimulated to produce large amounts of peritoneal neutrophils by intraperitoneal injections of beef heart infusion broth in combination with proteose peptone.	
• Granulocytes from tumor-bearing mice were cytotoxic.	[405]
• PMA-stimulated BCG- or thioglycollate-elicited peritoneal neutrophils kill efficiently P388 and TLX9 lymphoma cells, which correlated with H_2O_2 release.	[406]
• Cell lysis peaked after 4.5 h following co-cultivation with activated neutrophils.	
• Increase in the proportion of myeloperoxidase-positive neutrophils in mice bearing autochthonous M-MuSV (Moloney murine sarcoma virus)-induced tumors.	[407]
• The cytotoxic activity correlated with myeloperoxidase expression.	
• Ha-2 Harvey-MuSV-induced sarcoma and Tu5 Simian virus-40-transformed kidney cells were highly susceptible to the cytotoxic effect of neutrophils (40–60 % killing), while MBA 3-methylcholantrene-induced sarcoma, Ta_3/St 3-methylcholantrene-induced adenocarcinoma, T1699 spontaneous adenocarcinoma and 3 T3 embryonic fibroblasts showed medium sensitivity (20–40 % killing).	
• The cytotoxic activity of neutrophils seems to be non-specific, leading to killing of various tumor cell lines as well as allogeneic, but not syngeneic, fibroblasts. Although the extent of killing of allogeneic mice was much lower (5–20 %) than tumor cells (20–60 %).	
• Total bone marrow derived cells were much more cytotoxic than spleen-derived cells. Cells from the lymph nodes were not cytotoxic.	
• β-1,3-glucan, BCG, Propionibacterium acnes and Zymosan A induced anti-tumor activity of neutrophils toward MM46 mammary carcinoma, MM48 mammary carcinoma, MH134 hepatoma, EL-4 and YAC-1 lymphoma cells.	[408, 409]
• Catalase, but not superoxide dismutase, cyanide or azide, inhibited the killing of tumor cells.	
• Antibody to TNF prevented the cytolytic effect of caseinate-induced inflammatory neutrophils toward MM46 mammary tumor cells.	[332]
• Immature neutrophils obtained from ascites fluid 6 h after caseinate injection showed stronger cytotoxic activity than mature neutrophils obtained 3 h after injection.	
• Myeloperoxidase is expressed 1.6 times more in immature neutrophils.	
• Intraperitoneal injection of *Corynebacterium parvum* 24 h after an intraperitoneal inoculation of a lethal number of mouse ovarian teratocarcinoma cells induced an antitumor response that cured 75 to 95 % of the mice.	[410]
• Isolated peritoneal neutrophils were cytolytic to the tumor cells with cell lysis obtained within 30 min after binding of the neutrophils to the ovarian teratocarcinoma target cells.	
• Overexpression of chemokines such as IL-8, MCP-1 or MIP-1 in CHO tumor cells led to recruitment of neutrophils and concomitant inhibition of tumor growth in nude mice.	[411]
• IFNγ activates rat neutrophils to kill tumor cells by a mechanism dependent on nitric oxide.	[312, 313]
• SOD enhanced the tumor cytotoxic effect.	
• The growth of disialoganglioside (GD2) positive neuroblastoma cells was inhibited by neutrophils in the presence of antibodies towards GD2, an effect that was enhanced by GM-CSF.	[412]
• In the absence of antibodies, neutrophils inhibited growth of one $GD2^+$ cell line, whereas they stimulated the growth of two other $GD2^+$ cell lines as well as the $GD2^-$ cell lines tested.	
• Mouse granulocytes restrict human tumor cell growth in SCID mice.	[413]
• Neutrophils, together with macrophages, mediate antibody-dependent cell cytotoxicity (ADCC) towards tumor cells, which is responsible for the efficacy of monoclonal antibody (mAb)-mediated cancer therapy.	[342]
• Neutrophil-induced ADCC contributes to the anti-tumor activity of the anti-CD20 antibody Rituximab in a non-Hodgkin's lymphoma SCID mouse model.	[341]
• Walker carcinoma W256 activated neutrophils in vitro to produce singlet oxygen.	[326]
• ROS is crucial for neutrophil-mediated tumor cell lysis.	
• Massive granulocyte infiltration at the site of W256 transplants correlates with spontaneous tumor regression.	[414]
• Peripheral blood granulocytes from the tumor-bearing animals are cytotoxic to W256 cells in vitro.	
• Adoptive transfer of granulocytes in the vicinity of W256 carcinoma in rats or Ehrlich ascites tumor in mice reduced tumor cell mass with concomitant increased survival.	[415]
• The presence of MPO in the tumor microenvironment was accompanied by the formation of lipid peroxidase	[416]

Table 2 (continued)

Major findings	Reference
(LPO)-derived aldehydes such as acrolein, 4-hydroxy-2-nonenal and malondialdehyde. • The presence of acrolein and neutrophil elastase were increased in animals with regressing W256 tumor.	
• Meth A tumor cells induce infiltration of cytotoxic neutrophils, which are responsible for the IFNγ-dependent spontaneous rejection.	[417]
• Inhibition of TGFβ signaling by using SM16 increased neutrophil mediated cytotoxicity towards AB12 mesothelioma cells in a mechanism that depends on ROS.	[13]
• While antibodies to TNFα and N-methylarginine, an inhibitor of iNOS, did not inhibit the cytotoxicity, blockade of superoxide and H_2O_2 by superoxide dismutase (SOD) and catalase, respectively, blocked neutrophil cytotoxicity.	
• $CD11b^+MMP\text{-}9^+$ Neutrophils accumulate in the lungs prior to metastatic seeding of breast carcinoma cells.	[32]
• Tumor-entrained neutrophils (TENs) inhibit metastatic seeding of 4T1 breast carcinoma cells in the lungs by generating H_2O_2.	
• Also, neutrophil depletion in MMTV-PyMT/MMTV-cMyc tumor bearing mice resulted in enhanced metastatic seeding in the lungs.	
• TENs could not prevent the growth of local tumor.	
• Circulating blood neutrophils from 4T1-tumor bearing mice were cytotoxic to the mouse 4T1 and the human MCF7 breast carcinoma cell lines. Similarly, circulating blood neutrophils from B16 melanoma-bearing tumor mice were cytotoxic to both B16 and 4T1 cells.	
• Catalase or TGFβ (at 200pM) abrogated the cytotoxic effects of TENs.	
• Neutrophil-tumor cell contact was required for tumor cytolysis.	
• Tumor-secreted CCL2 mediates the anti-metastatic entrainment of G-CSF-stimulated neutrophils.	
• In vitro CCL2 and CCL5 simulated naïve neutrophils to produce H_2O_2 and to kill 4T1 tumor cells.	
Purified neutrophil products	
• Myeloperoxidase, a product of neutrophils, shows cytotoxic effects against mouse lymphoma cells.	[418, 419]
• Cationic proteins purified from polymorphonuclear leukocytes granules exert a cytotoxic effect on mouse LSTRA lymphoma cells.	[420]
• A ~100kD protein secreted from wheat germ agglutinin or actinomycin D-stimulated neutrophils kills MM46 and MM48 mammary adenocarcinoma cells, MH134 hepatoma cells and L929 fibrosarcoma cells, but not normal splenocytes.	[421]
• No cytotoxic effect of supernatant of unstimulated neutrophils.	
• Human defensins HNP-1, HNP-2 and HNP-3 lysed various human and mouse tumor cell lines.	[335]
• Human defensins in combination with hydrogen peroxide had a synergistic cytotoxic effect on tumor cells.	
• Calprotectin (S100A8/S100A9), a calcium binding protein complex abundantly expressed in neutrophils, induces apoptotic cell death in various tumor cells.	[422]

Evidence for neutrophil mediated tumor cell killing in cancer patients, in animal models of cancer and in using neutrophil derived products

CXCL5 and IL-8) may promote the generation of anti-tumorigenic neutrophils [32, 66, 309–313]. Anti-tumorigenic neutrophils can also be generated artificially by exposing them to the phorbol ester PMA, or to the lectins Concanavalin A or wheat germ agglutinin (WGA) [314–318].

In vivo, the effect of massive infiltration of neutrophils was analyzed by sustained treatment of tumor-bearing mice with GM-CSF, leading to a strong neutrophilia around the tumors. These mice showed a 16-fold lower mortality rate than untreated mice [319]. The same therapeutic strategy was applied in a patient with advanced hepatocarcinoma, who exhibited a complete remission after 4 months of G-CSF treatment. Prolonged administration of G-CSF to squamous head and neck cancer patients led to increased disease-free survival [320]. Other clinical trials using continuous GM-CSF administration in advanced prostate cancer [321] or sustained G-CSF in stage IV melanoma with brain metastases [322] reported better survival, suggesting for an anti-tumor effects of neutrophils.

Direct Cytotoxicity

Neutrophils are armed with a variety of toxic molecules, most of which have anti-microbial properties and are harmless toward eukaryotic cells [323, 324]. Still, several anti-bacterial molecules are also involved in neutrophil cytotoxicity toward tumor cells. Of special importance are the reactive oxygen species H_2O_2 and HOCl, generated during an "oxidative burst" that is mediated by the NADPH oxidase complex and by myeloperoxidase, respectively [325–327]. These molecules are directly involved in the anti-tumor activity [325–327]. Inhibition of myeloperoxidase by azide or cyanide, or addition of catalase that catalyzes the conversion of hydrogen peroxide to water and oxygen, prevents neutrophil-mediated tumor cell killing [13, 32, 309, 314–318]. Neutrophils from patients with myeloperoxidase (MPO) deficiency or defective H_2O_2 production are not cytotoxic to tumor cells [327]. However, addition of superoxide dismutase (SOD) that catalyzes the conversion of superoxide into oxygen and hydrogen peroxide, did not prevent the killing, but sometimes even enhanced it [13, 32, 309, 314–318], possibly by accelerated hydrogen peroxide production, the key effector molecule in neutrophil cytotoxicity. Dissemond and colleagues showed that SOD in combination with catalase could partly prevent tumor cell killing with an involvement of nitric oxide in the killing process [325]. Rat neutrophils stimulated with IFNγ produced nitric oxide that prevented tumor cell growth [312] and induced apoptosis of the tumor cells [313]. However, another study [13] could not relieve the cytotoxic effect with a nitric oxide scavenger. This apparent discrepancy could be due to different neutrophil activation modes and the presence of several neutrophil generated cytotoxic molecules where the inhibition of one is compensated for by the other.

Several studies, including our own, have shown that neutrophil cytotoxicity requires physical contact between neutrophils and tumor cells [4, 32, 328]. Caruso et al. [329] demonstrated intimate contact between neutrophils and tumor cells in samples of early gastric cancer at a stage prior to the appearance of neutrophilia. Following the interaction with neutrophils, tumor cells show varying degrees of damage including disorganization of the intermediate filaments and dilation of the rough endoplasmic reticulum [329]. However, neutrophil cytotoxicity induced by the phorbol ester PMA, a potent agonist of H_2O_2 production and secretion, does not require physical contact [32]. This observation suggests that high levels of neutrophil-generated H_2O_2 are sufficient for cytotoxicity, even when physical contact is restricted. These seemingly conflicting observations were resolved when we realized that rather than secreting H_2O_2 spontaneously, neutrophils secrete H_2O_2 in a contact-triggered fashion [32]. Similarly, Saito et al. [330] demonstrated by visualizing the oxidative process by luminol-dependent chemiluminescence, that ROS accumulate at sites where neutrophils come in contact with tumor cells. Also, the intensity of the hydrogen peroxide-sensitive tracer dichlorofluorescein diacetate preloaded in tumor cells rapidly increased after adding the neutrophils [330]. The interaction between neutrophils and tumor cells results in loss of tumor cell membrane integrity beginning within 15 min of the binding step and completion of the lytic event within 45 min [328]. It is noteworthy to point out that molecules other than H_2O_2, HOCl and NO· may also mediate the tumoricidal effects of neutrophils. These include proteases [331], membrane-perforating agents [331], TNFα [331, 332], TRAIL [333] and defensins [334, 335].

Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)

Another mechanism for neutrophil-mediated tumor cell killing is antibody-dependent cell-mediated cytotoxicity (ADCC) [7, 331] where specific antibodies are used to target malignant cells. Neutrophils express several subtypes of FcRs capable of inducing ADCC, including FcγRI (CD64), FcγRIIa (CD32), FcγRIIIa (CD16a), and FcγRIIIb (CD16b) [336–338], whose surface expression is increased following G-CSF stimulation [338, 339]. FcγRI was shown to mediate neutrophil ADCC activity against glioma, squamous cell and ovarian carcinoma cells [338]. However, binding of IgG to the inhibitory FcγRIIb, might lead to down-regulation of immune responses [340]. The neutrophil-mediated killing of human ganglioside $GD2^+$ melanoma and neuroblastoma cells in the presence of antibodies to GD2, was found to depend on FcγRII and FcγRIII, but not on FcγRI [336]. Neutrophils from a child with leukocyte adhesion deficiency (LAD) devoid of CD11/CD18 adhesion molecules failed to mount any detectable ADCC [336], suggesting the involvement of these adhesion molecules in ADCC. Also, antibodies to CD11b, CD11c and CD18 efficiently blocked ADCC by normal neutrophils, providing further support to this notion [336]. GM-CSF enhanced the anti-tumor neutrophil-dependent ADCC through enhanced expression of CD11/CD18 molecules [336]. Neutrophils were also shown to contribute to the anti-tumor ADCC in Non-Hodgkin's Lymphoma using antibodies to CD20 (Rituximab) [341], in breast cancer using a Tn antigen-specific chimeric mAb [342], and in B-cell lymphoma using a bispecific single-chain fragment variable-specific for HLA class II and FcαRI (CD89) [343]. The bispecific antibody against the myeloid receptor for IgA (FcαR1; CD89) and the B-cell surface marker CD20 induced neutrophil-dependent ADCC toward broad range of B cell lines [344]. Lysis via FcαRI:CD20 bispecific antibodies was enhanced in blood from patients during therapy with G-CSF or GM-CSF [344]. Interestingly, Otten et al. [345] observed that immature neutrophils mobilized from the bone marrow upon G-CSF treatment, efficiently triggered tumor cell lysis via FcαRI (CD89), but were unable to initiate tumor cell killing via FcγR. This may provide a rationale for the disappointing results observed in some earlier clinical trials in which patients were treated with G-CSF and anti-tumor antibody binding to FcγR. An indication for the role neutrophils play in mediating ADCC in human patients was described by Cheung and colleagues [346]. In their study they found that the outcome of treating high risk neuroblastoma patients with anti-GD2 monoclonal antibody strongly correlated with the extent of granulocyte activation [346].

Neutrophils as Tumor-Antigen Presenting Cells

There is accumulating evidence suggesting that neutrophils contribute to the development of an adaptive anti-tumor immune response [12–16, 347]. Elimination of neutrophils prevents the mounting of anti-tumor $CD8^+$ T cytotoxic responses [12–14, 16]. Neutrophils were shown to be required for priming rats with tumor-associated antigens to induce anti-tumor $CD8^+$ effector T cells [348]. This may be related to the ability of neutrophils to attract and activate dendritic cells, macrophages, NK cells and T cells. By virtue of their ability to present antigens, neutrophils may directly activate a T cell response. Neutrophils may also activate dendritic cells and T cells through release of neutrophil extracellular traps (NETs) [349, 350]. On top of secreting T cell chemoattractants, neutrophil-secreted TNFα, Cathepsin G and neutrophil elastase, are able to increase T cell proliferation and cytokine production, which together enhance adaptive immune responses [350]. Jackaman et al. [351] demonstrated the cooperation between tumor-infiltrating neutrophils and $CD8^+$ T

cells in eradicating tumors that received intratumoral injection of IL-2 and anti-CD40 antibodies.

Prognostic Values of Neutrophils and Other Myeloid Subtypes in Cancer Patients

Alterations in circulating leukocyte composition and number are often observed in cancer patients. Cancer-related inflammation and tumor-induced immune suppression are often associated with expansion of myeloid subsets including MDSCs. In parallel, the granulocytes are often activated in cancer patients with a concomitant increase in H_2O_2 production [278]. G-CSF is often secreted by tumor cells [26, 174, 189, 190, 194, 198, 276, 352–359] and is preferentially observed in dedifferentiated or poorly differentiated tumors [360, 361, 354, 190]. Elevated G-CSF blood concentrations in cancer patients have been associated with poor clinical outcome [174, 194, 198, 276, 352, 355, 356].

Recently, a high myeloperoxidase $(MPO)^+$ $CD15^+$ cell infiltration representing neutrophils in colorectal carcinoma was found to be an independent favorable prognostic factor [362, 363]. Also, the increased presence of $CD16^+$ (FcγRIII) myeloid cells of the monocyte/macrophage lineage that were also positive for CD45, CD33, CD11b, and CD11c, but not CD64 or HLA-DR, was found to be associated with improved survival in patients with colorectal carcinoma [364]. This is in contrast to the general concept that high amounts of intratumoral myeloid cells promote tumor progression and hence correlate with poor disease outcome. In particular, colorectal carcinoma infiltration by $CD66b^+$ granulocytes was proposed as a marker of adverse prognosis [365].

Already in 1970, Riesco [366] observed a positive correlation between cancer curability and the total number of lymphocytes, while a negative correlation was found with the total number of peripheral neutrophils (segmented and nonsegmented) when 589 cases of different cancer types were investigated. From then on, a large number of clinical studies have been performed to understand the neutrophil-cancer relationship. Clinical data have often related elevated circulating neutrophil counts or elevated neutrophil-to-lymphocyte ratios (NLRs) as a predictive parameter for poor outcome and formation of distant metastasis in patients with epithelial malignancies [367], including lung [368, 170], gastric [159, 163, 369], renal cell carcinoma [370], ovarian [141], hepatic [143, 148, 371], pancreatic [138], colon cancer [145, 372], and colorectal carcinoma [152] (Table 3). However, Caruso et al. [200] observed that when analyzing the amount of tumor-infiltrated neutrophils in advanced gastric carcinoma, in female but not male, patients with higher TANs had a favorable prognosis.

Colorectal carcinoma sections were characterized by tumor-infiltrating granulocytes (TIGs) and tumor-associated macrophages (TAMs) and abnormal levels of the cytokines IL-1β, IL-6, IL-8, TNFα, G-CSF and M-CSF [178]. G-CSF was associated with a deeper tumor invasion and a more advanced tumor stage [178]. The granulocyte/lymphocyte ratio was associated with abnormal levels of G-CSF (more than 50 % of the cancer patients) and TIGs were a risk factor for lymph node metastasis in colorectal carcinoma [178]. Similarly, in gastric carcinoma, TIGs were associated with tumor stage and shorter survival time [373].

Studies on neutrophils from patients with head and neck squamous cell carcinoma (HNSCC) showed that these neutrophils differ from their counterparts in healthy donors [374]. The neutrophils from HNSCC showed lower inducible production of ROS, reduced spontaneous apoptosis and increased number of immature neutrophils [374]. The serum concentration of neutrophil related cytokines was higher in HNSCC patients [374]. HNSCC tissue exhibited considerable infiltration by neutrophils, and strong infiltration was associated with poorer survival in advanced diseases [157]. Neutrophil count, neutrophil-to-lymphocyte ratio and serum concentrations of CXCL8 (IL-8), CCL4 (MIP-1β) and CCL5 (RANTES) were significantly higher in the peripheral blood of HNSCC patients than in controls [157]. In vitro, HNSCC-conditioned medium inhibited apoptosis of neutrophils, increased neutrophil chemokinesis and chemotaxis and induced the release of lactoferrin, MMP-9 and CCL4 [157]. Further studies showed that HNSCC activates the p38-MAPK pathway in neutrophils, and stimulates the release of CCL4, CXCL8, and MMP-9 by neutrophils in a CREB-dependent manner [375]. The secretion of CCL4 and MMP-9 by neutrophils was stimulated by macrophage migration inhibitory factor (MIF) produced by the HNSCC cells [376]. MIF was also shown to be produced by other cancer cell types, including breast cancer, esophageal squamous cell carcinoma and hepatocellular carcinoma, where it contributes to angiogenesis [377–380]. Breast cancer patients with positive MIF expression in tumor tissues showed a significantly worse disease-free survival compared with MIF negative patients [377]. Thus, there seem to be a crosstalk between the tumor cells and neutrophils that may affect tumor growth and modify anti-tumor immune responses.

In human hepatocellular carcinoma, TANs accumulate in the peritumoral stroma due to IL-17-dependent release of epithelial cell derived chemokines such as CXCL1/GROα, CXCL2/MIP-2, CXCL3/GROγ and CXCL8/IL-8 [57]. High infiltration of peritumoral neutrophils correlated with tumor progression and predicted poor survival [57]. Neutrophils are the major source of MMP-9 within the hepatocellular carcinoma tissue, which was shown to stimulate the pro-angiogenic activity in hepatoma cells [57]. Selective depletion of neutrophils inhibited tumor angiogenesis and growth in vivo [57]. Also, CXCL5 has been shown to be

Table 3 The correlation between neutrophil blood count and cancer prognosis

Cancer cell type	Effect on prognosis	Reference
Advanced pancreatic adenocarcinoma	Elevated pretreatment NLR>5 was a predictor of shorter survival.	[138]
Recurrent localized cervical cancer	Tumor-associated neutrophil count is an independent factor for short recurrence-free survival	[139]
Epithelial ovarian cancer	Pre-operative NLR in ovarian cancer subjects (mean 6.02) was significantly higher than in benign ovarian tumor subjects (mean 2.57) and healthy controls (mean 1.98). Elevated NLR may predict an adverse outcome.	[141]
Breast cancer	Patients with an NLR>2.5 showed lower disease-specific survival rate than those with an NLR<2.5. This correlation was especially seen in the luminal A subtype.	[423]
Breast cancer	Patients with an NLR>3.3 had a higher mortality rate than those with an NLR<1.8. The high NLR was associated with more advanced stages of cancer.	[424]
Hepatocellular carcinoma (HCC)	The presence of intratumoral $CD66^+$ neutrophils was a poor prognostic factor for HCC after resection	[387]
Hepatocellular carcinoma (HCC)	Pre-operative NLR>5 was an adverse predictor of disease-free and overall survival.	[148]
Hepatocellular carcinoma (HCC)	Patients with an NLR>2.5 showed higher risk for tumor recurrence in HCC patients undergoing liver transplantation.	[371]
Hepatocellular carcinoma (HCC)	High baseline NLR was associated with higher tumor recurrence and worse overall survival in patients after radiofrequency ablation.	[140]
Hepatocellular carcinoma (HCC)	Elevated C-reactive protein together with an NLR>2.3 predicted shorter overall survival.	[425]
Small hepatocellular carcinoma	Elevated post-operative NLR change was a worse prognostic factor.	[143]
Resectable non-small cell lung cancer	An increase in $CD66b^+$ cells was associated with a high cumulative incidence of relapse (CIR) and worse overall survival. An increase in intratumoral neutrophil to $CD8^+$ lymphocyte ratio (iNTR) was associated with high CIR and poor overall survival.	[204]
Advanced non-small-cell lung cancer	A neutrophil count above 4,500/mm^3 was associated with shorter overall and progression-free survival.	[368]
Advanced non-small-cell lung cancer	Patients with normal leukocyte count had a longer median overall survival than those with elevated WBC count (>$10\times10^3/\mu l$).	[426]
Advanced non-small-cell lung cancer	Increased preoperative NLR had a poorer prognosis.	[154]
Bronchioloalveolar carcinoma	The risk of death was increased in patients with a neutrophil percentage of >39 % in the bronchoalveolar lavage fluid.	[170]
Gastric carcinoma	Higher NLR was associated with lymph node metastasis, higher tumor stage, tumor progression and reduced 5-year survival.	[163]
Gastric cancer	Patients with an NLR>2.5 had a poorer prognosis.	[159]
Gastric cancer	NLR was influenced by tumor size. High NLR seems to be a marker for tumor recurrence.	[369]
Advanced gastric carcinoma	Female, but not male, patients with a moderate to extensive amount of tumor-infiltrating neutrophils had a 39 % reduction in their risk of mortality.	[200]
Gastric cancer stage III-IV	Progression-free survival and overall survival was worse for patients with high NLR.	[151]
Colon cancer, stage IIA	Patients with preoperative NLR>4 had a shorter recurrent-free survival.	[145]
Colon carcinoma, stage II	Patients with elevated NLR had a worse overall survival and worse disease-free survival.	[372]
Colorectal cancer	Preoperative NLR>5 correlated with poor pre-operative prognosis.	[158]
Colorectal cancer	The NLR was higher in advanced stages of cancer. The ability to produce ROS at the terminal stage was 33 % lower than in the control group.	[427]
Colorectal cancer	Patients with an NLR>2.5 had a poorer prognosis than those with an NLR<2.5.	[428]
Colorectal cancer	Patients with an NLR>5 showed a worse overall survival.	[429]
Metastatic colorectal cancer	A high NLR was associated with poor overall survival.	[430]
Colorectal liver metastases	The 5-year survival for patients undergoing resection with an NLR>5 was worse than those with normal NLR.	[150]
Non-metastatic renal cell carcinoma	Patients with a pre-operative and post-operative NLR>2.7 had a shorter recurrence-free survival rate.	[153, 431]
Localized renal cell carcinoma	The presence of intratumoral neutrophils was associated with short recurrence-free survival.	[390]
Metastatic renal cell carcinoma	Patients with elevated neutrophil count (>6,500 cells/μl) showed a shorter overall survival.	[432]
Metastatic renal cell carcinoma	Patients with a high blood neutrophil count (>6,000), intratumoral neutrophils and low intratumoral $CD57^+$ NK cells are independent poor prognostic immunological factors.	[370]
Bladder cancer	There was no significant difference between patients with an NLR below or above 2.5 in terms of overall survival.	[144]
Bladder cancer	NLR was an independent prognostic factor in patients treated with radical cystectomy.	[149]
Urothelial carcinoma	An NLR>2.5 was found to be a predictor of invasiveness.	[433]

Table 3 (continued)

Cancer cell type	Effect on prognosis	Reference
Melanoma, stage IV	A high pretreatment neutrophil count (>7,500 cells/μl) predicted shorter overall survival in patients receiving IL-2-based immunotherapy.	[156]
Oral squamous cell carcinoma	Patients with a pretreatment NLR>1.9 showed poorer disease-specific survival.	[434]
Esophageal cancer	Elevated NLR>5 is associated with poor outcomes.	[435]
Esophageal cancer	Patients with a pre-therapeutic NLR>2.2 showed poorer response to chemotherapy.	[436]
Esophageal cancer	No correlation between NLR and overall survival.	[437]
Esophageal cancer	High NLR>5 was associated with poorer overall survival.	[438]
Stomach cancer	Patients with a NLR>7.7 at post-operative day 3 showed a 4.2 times higher cancer recurrence after gastrectomy.	[439]
Metastatic Mesothelioma	An NLR>5 showed poorer overall survival in patients undergoing systemic therapy.	[440]
Metastatic Mesothelioma	An NLR>3 showed poorer prognosis after extrapleural pneumonectomy.	[441]
Metastatic Mesothelioma	A high NLR correlated with sustained neoangiogensis and increased proliferative index.	[442]
Bone metastasis	A high preoperative N/L ratio was associated with poor prognosis after bone metastasis in the surgery group.	[443]

Evidence from clinical studies correlating neutrophil counts and Neutrophil to Lymphocyte Ration (NLR) with cancer prognosis

overexpressed in hepatocellular carcinoma from patients with recurrent disease as well as in highly metastatic hepatocellular carcinoma cell lines [381]. The upregulation of CXCL5 in hepatocellular carcinoma cells correlated with the promotion of tumor growth, lung metastasis and intratumoral neutrophil infiltration [381].

Is it possible that the elevated neutrophil counts in cancer patients are actually the consequence of a more advanced stage, and therefore predict an apparent poorer prognosis? This was acknowledged by Liu et al. [178], who showed that the NLR was significantly associated with a more advanced colorectal tumor stage. Also Ietomi [382] observed that the NLR was higher in Stage IV stomach cancer patients than those having stage I-III stomach cancer. The NLR dropped after surgery, but upon relapse it rose again [382]. Similarly, Sarraf et al. [154] observed higher NLR in more advanced non-small cell lung cancer patients and Fossatti et al. [207] observed a marked and significant correlation between tumor grade of glioma patients and the extent of neutrophil infiltration as determined by the number of $CD15^{+}MPO^{+}$ cells [207]. In low grade tumors 40–50 % show neutrophil infiltration, while in glioblastoma multiforme over 85 % of the samples show neutrophil infiltration [207]. These observations suggest that while neutrophil infiltration is associated with higher-grade tumors this does not mean that poor prognosis is due to increased neutrophil infiltration, rather the tumor is responsible for the elevated neutrophil number.

Concluding Remarks - The Dialogue Between Cancer Cells and Neutrophils

There is a continuous interaction between cancer cells and neutrophils in the tumor microenvironment. Neutrophils are attracted to the primary tumor by tumor-secreted chemokines, which can also induce anti-microbial as well as anti-tumor neutrophil activities. The activation of neutrophils under certain circumstances has been shown to yield prolonged life span in in vivo cancer models. These so-called "Tumor-Associated Neutrophils" (TANs) show enhanced NADPH oxidase activity which leads to the production of reactive oxygen species, especially hydrogen peroxide, that are cytotoxic to tumor cells [32]. After causing tumor cell apoptosis, these cells are then engulfed by neutrophils [383], and processed for antigen-presentation to mount an adaptive $CD8^{+}$ T cytotoxic anti-tumor response [15]. Neutrophils may work together with monocytes to transport tumor cell antigens to secondary lymphoid tissues, where naïve T cells are stimulated [383]. T cells further increase the activity of neutrophils through secretion of IFNγ. The increase in oxygen radicals have additional effects, such as suppression of T cell responses [278, 384], activation of TGFβ [81], and induction of GMP-140 (P-Selectin) expression on the surface of endothelial cells, leading to enhanced neutrophil adherence and activity [385]. The activation of TGFβ suppresses excessive neutrophil function, and may polarize them into N2 neutrophils [13].

Conversely, certain tumor-stimulated neutrophils secrete ECM remodeling enzymes and pro-angiogenic factors that promote, directly or indirectly, the growth of the tumor as well as their detachment and dissemination. When the immunosuppressed environment is induced, there is a positive feedback mechanism to maintain the immunosuppressed condition. Unlike the tumor microenvironment, where immunosuppression prevails, neutrophils in the immunopermissive pre-metastatic organs may show characteristics of anti-tumorigenic cells, providing anti-metastatic protection by eliminating metastatic cells [32]. In light of this, we propose that while neutrophils are largely viewed as a homogenous

cell population, their function in cancer is dictated in a context-dependent fashion, which may seem conflicting. In depth exploration of how neutrophils interpret the orchestra of signals in the different cancer niches will enhance our understanding of how different neutrophil subsets function in the context of cancer.

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