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REVIEW

The interplay between neutrophils and microbiota in cancer

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Abstract

The role of the microbiota in many diseases including cancer has gained increasing attention. Paired with this is our expanding appreciation for the heterogeneity of the neutrophil compartment regarding surface marker expression and functionality. In this review, we will discuss the influence of the microbiota on granulopoiesis and consequent activity of neutrophils in cancer. As evidence for this microbiota-neutrophil-cancer axis builds, it exposes new therapeutic targets to improve a cancer patient's outcome.

KEYWORDS

cancer, granulopoiesis, microbiota, neutrophils, TAN

1 | INTRODUCTION

The reported half-life of circulating neutrophils ranges from 10 hours to 5.4 days in humans,[1–4] and, more consistently, 8–12.5 hours in mice.[2,5,6] As their lifespan is much shorter than other blood leukocytes, some have questioned the immunomodulatory role of neutrophils in a chronic disease such as cancer. Despite their short time in circulation, neutrophils are swift first responders to inflammation and can bring about a potent, complex milieu of either proapoptotic, prosurvival, mutagenic, or wound healing signals, making their role in cancer pathogenesis a complex one.[7,8] High blood neutrophil-to-lymphocyte ratios are associated with larger tumor size, less differentiated tumors, increased tumor vascularization, poor overall survival, and recurrence-free survival rates in numerous cancers.[9–11] However, other studies link neutrophil markers to better survival rates.[12–17] Rather than their sheer number, the types of tumor-infiltrating neutrophils and their state of differentiation/activation, as we will describe here, may play a significant role in cancer. Their ability to leave the bone marrow as a functional and mature, or attenuated and immature granulocyte can be heavily influenced by the microbiota. Therefore, the three concepts merge into a microbiota-neutrophil-cancer axis that is a burgeoning area of research.

Abbreviations: ABX, antibiotics; ARG1, arginase 1; BMN, bone marrow neutrophil; DAMP, damage-associated molecular patterns; DSS, dextran sulfate sodium; FLT3L, FMS-like tyrosine kinase 3 ligand; fMLF, formyl Met-Leu-Phe; FPR, formyl Met-Leu-Phe receptor; FXR, farnesoid X receptor; GF, germfree; GFI-1, growth factor independent 1; gMDSC, granulocytic myeloid-derived suppressor cell; HDN, high-density neutrophil; HGF, hepatocyte growth factor; HIF, hypoxia-inducible Factor; ILC3, type 3 innate lymphoid cell; LDN, low-density neutrophil; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; MAMP, microbial-associated molecular patterns; MDSC, myeloid-derived suppressor cell; MMP, matrix metalloproteinase; MPO, myeloperoxidase; NET, neutrophil extracellular trap; NOD1, nucleotide-binding oligomerization domain-containing protein 1; PBN, peripheral blood neutrophil; PRR, pattern recognition receptor; RNS, reactive nitrogen species; ROS, reactive oxygen species; SCF, stem cell factor/ c-kit ligand; SCFA, short-chain fatty acid; SFB, segmented filamentous bacteria; SPF, specific-pathogen-free; TAN, tumor-associated neutrophil; TBI, total body irradiation; TCM, tumor-conditioned medium; VEGF, vascular endothelial growth factor

2 | NEUTROPHIL DIFFERENTIATION

Neutrophil development begins in the embryonic yolk sac, then liver, spleen, and later in the bone marrow.[18] Newborn blood neutrophil counts more than double over the first 30 hours postdelivery, but reduce to levels approximate to adults after 72 hours.[19,20] Compared to adults, neutrophils collected from newborns shortly after birth exhibit significantly less phagocytosis until 3 days postbirth,[21] impaired chemotaxis until 1 week to 1 month postbirth,[22–24] and dampened rolling and adherence[25] until 5–24 months postbirth.[22] This impaired functionality may be due to the high presence of immature neutrophils in newborns during the first 30 hours[19,20] as these nascent neutrophils failed to complete the controlled, stepwise process of development called granulopoiesis.

Granulopoiesis is stimulated by the release of G-CSF, stem cell factor (SCF, or c-kit ligand), GM-CSF, IL-3, FMS-like tyrosine kinase 3 ligand (FLT3L) and IL-6 from either hematopoietic cells or non-hematopoietic, stromal cells (Fig. 1).[26] Reactive oxygen species (ROS) production within the bone marrow also appears important for neutrophil development.[27] The ability of the neutrophil to leave the bone marrow is dictated by an exchange in the expression of chemokine receptor CXCR4 for CXCR2.[28,29] This change decreases the neutrophil's association with CXCL12-expressing (a CXCR4 ligand) osteoclasts, endothelial cells, and spindle-shaped stromal cells and

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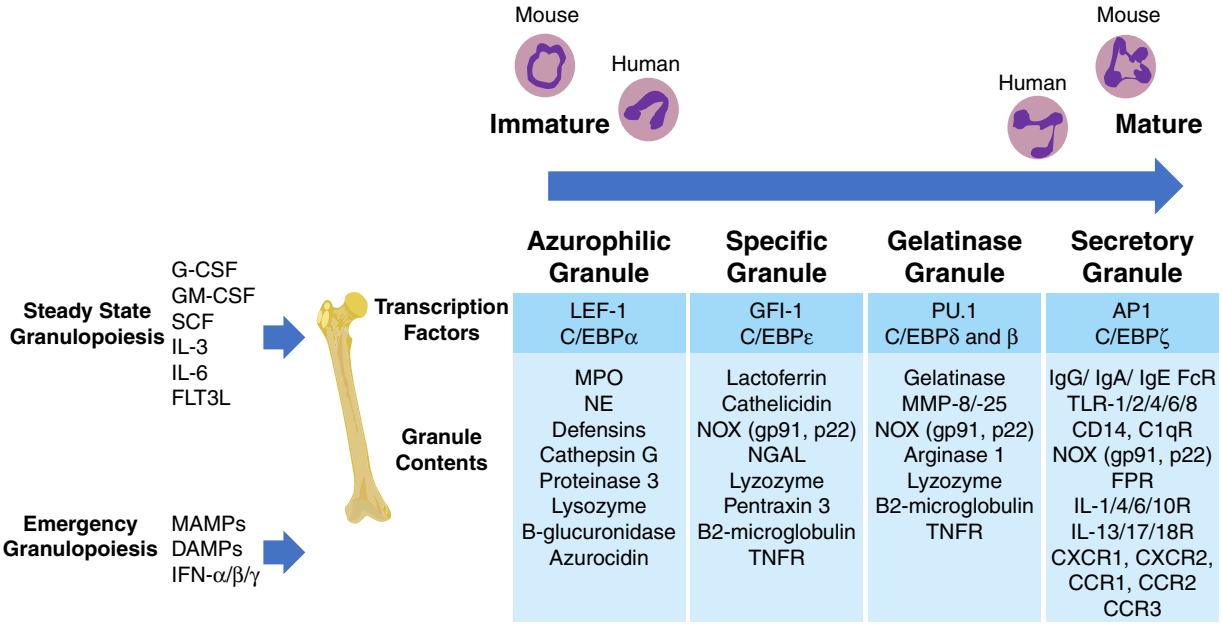

FIGURE 1 **Granulopoiesis and neutrophil granule development.** During steady-state conditions, stromal cells release G-CSF, GM-CSF, SCF, IL-3, IL-6, and FLT3L in the bone marrow to induce the differentiation of GMPs into neutrophils. Emergency granulopoiesis occurs when support cells and leukocytes detect MAMPs or DAMPs in the periphery and release granulopoietic cytokines in addition to IFN-$\alpha/\beta/\gamma$. A stepwise process of maturation begins with nuclear condensation/segmentation and granule formation. Azurophilic granules are generated with the activation of LEF1 and C/EBPα. These granules contain MPO, defensins, and digestive enzymes. Upon induction of GFI-I and C/EBPε, specific granules form and contain lactoferrin and cathelicidins. PU.1, C/EBPδ and β promote gelatinase granule production, containing MMPs, gelatinase, and arginase 1. Finally, AP1 and C/EBPζ lead to secretory granule formation, which houses membrane proteins necessary for neutrophil activation. Secretory granules are the first to be exocytosed, then gelatinase granules, specific granules, and finally azurophilic granules

increases their attraction to CXCR2 ligands in the periphery: CXCL1, CXCL2, CXCL5, CXCL8 (IL-8).[28,30]

Nuclear segmentation is a marker of neutrophil maturation.[31,32] Murine metamyelocytes show ring-shaped nuclei while a human metamyelocyte nucleus is more kidney-shaped, before both begin to thin and segment into the banded morphology, then finally a fully mature, hyper-segmented nucleus (Fig. 1).[32] Humans and mice also differ both in their abundance of blood neutrophils (10–25% of leukocytes in mice and 50–70% in humans) and expression of proteins such as defensins, IL-10, IL-6, and myeloperoxidase (MPO).[33–35] Under steady-state conditions, neutrophils mature within approximately 5 days from a hematopoietic stem cell to a mature granulocyte.[1] During neutrophil maturation, metamyelocytes begin the stepwise expression of the transcription factors LEF-1 and C/EBPα, GFI-1 and C/EBPε, then PU.1 and C/EBPδ/β as the cell forms three types of neutrophil granules containing different antimicrobial peptides: azurophilic (or primary, MPO$^+$) granules, followed by specific (or secondary, lactoferrin$^+$) granules, then gelatinase$^+$ (or tertiary) granules (full granule contents described in Fig. 1).[36–38] Finally, a fourth structure known as the secretory granule is formed, following AP1 and C/EBPζ activation, that contains most of the neutrophil's activating receptors: TLRs, formyl Met-Leu-Phe (fMLF) receptors (FPR), complement, immunoglobulin, and cytokine receptors (Fig. 1).[37] Each granule type contains a distinctive set of antimicrobial peptides (listed in Fig. 1) and varies in its propensity to be exocytosed based on the concentration of vesicle-associated membrane protein 2 within the granule membrane.[37] In the presence of altered tissue homeostasis due to infections or other sources of tissue damage, neutrophil differentiation is accelerated and immature neutrophils are prematurely released out of the bone marrow in a process known as emergency granulopoiesis. Because granule development is essential for neutrophil functions, immature peripheral neutrophils may not contain a full arsenal with which to fight a challenge.

Emergency granulopoiesis due to systemic infection or myeloablation (the depletion of the bone marrow compartment, usually due to chemotherapy or radiation therapy) can be stimulated by direct binding of microbial- or damage-associated molecular patterns (MAMPs or DAMPs, respectively) to pattern recognition receptors (PRRs) on progenitor cells.[26,39,40] In addition, release of the above-mentioned granulopoietic cytokines as well as type I and II interferon (IFN-$\alpha/\beta/\gamma$)[41,42] from activated leukocytes, epithelial, and stromal cells may indirectly induce emergency granulopoiesis.[26] Upon this emergency signal, immature granulocytes are suddenly released into the periphery and may exhibit altered phenotypes (less antimicrobial peptides, impaired ROS production, phagocytosis, and chemotaxis) and tissue migration properties (reduced migration to liver and spleen).[38,43–45] The increase in the number of immature leukocytes in the blood is termed "left shift" because of the reduced nuclear segmentation of the immature neutrophils. As neutrophils with aberrant or immature morphologies have been noted in the blood and tumors of cancer patients, granulopoiesis may be differentially regulated in this disease.[31,32,38,46,47]

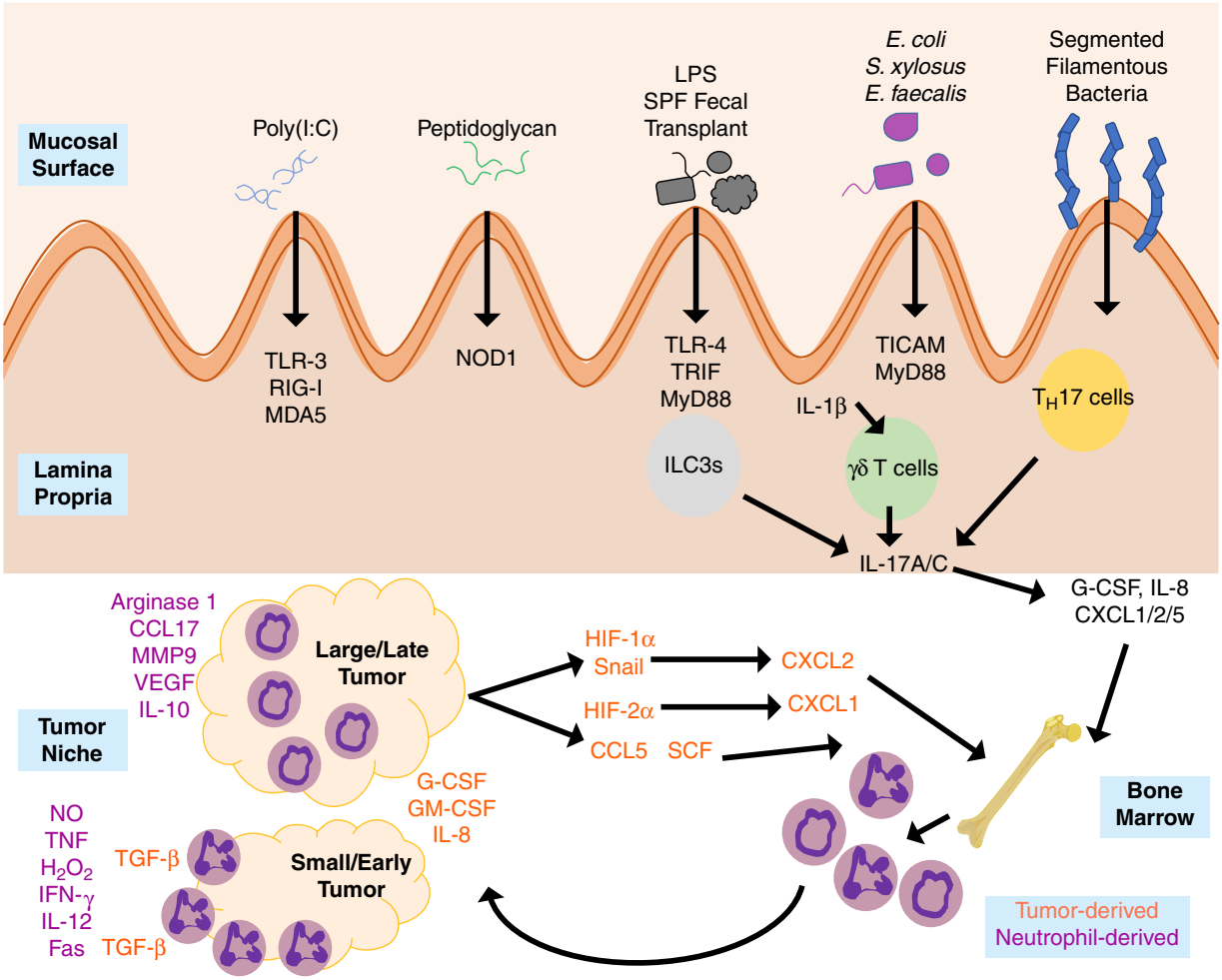

FIGURE 2 **Microbiota, granulopoiesis, and cancer cross-talk.** MAMPs heavily influence steady-state granulopoiesis. GF mice gavaged with poly(I:C), peptidoglycans, LPS and viable bacteria show improved bone marrow and peripheral neutrophil counts. Many groups link this to IL-17 production from ILC3s, T_H17, and $\gamma\delta$ T cells, which further stimulates granulopoietic cytokine release. Since neutrophils leave the bone marrow expressing CXCR2, they are attracted to tissues expressing IL-8 and CXCL1/2/5. Tumors can release these same cytokines and chemokines to recruit neutrophils and modify their function. TGF-β release prevents neutrophils from migrating into the tumor. Small/early stage tumor TANs release more cytotoxic factors (NO, TNF, IFN-γ), while large/late stage tumor TANs exhibit more protumor activity (Arginase 1, CCL17, IL-10)

3 | MODULATION OF NEUTROPHIL DIFFERENTIATION BY THE MICROBIOTA

As mentioned earlier, newborn blood neutrophil counts more than double over the first 30 hours postdelivery[19,20] as the child is exposed a world of microbes, indicating an important connection between microbiota and granulopoiesis. In the late 1980s, studies found that antibiotics (ABX)-treated or germfree (GF) rats exhibit fewer peripheral neutrophils, impaired granulopoiesis, and reduced ROS production.[48,49] Only very recently have the specific mechanisms by which commensal microbiota influence granulopoiesis in vivo started to be characterized.[39,40,50–52]

When murine pups are raised, starting 5 days before birth, on broad-spectrum ABX they demonstrate fewer bone marrow neutrophils than control pups.[50] Both granulocyte-monocyte progenitors (GMPs: c-kit$^+$, Sca-1$^-$, CD127$^-$) and LSK progenitors (Lineage$^-$, Sca-1$^+$, c-kit$^+$) are lower in the bone marrow of GF and ABX-treated specific-pathogen-free (SPF) mice compared to conventional SPF mice.[39] GMPs from GF mice also exhibit less proliferative capacity versus SPF GMPs.[53] Fecal transplants from SPF mice into GF mice,[50,53] colonization of GF mice with a mixture of *E. coli*, *S. xylosus*, and *E. faecalis*[39] or challenging GF mice with heat killed *E. coli*[53] restores bone marrow granulocyte and blood neutrophil counts (Fig. 2).[50] Additionally, GF granulocytes enter the blood much more slowly,[39] possibly because plasma G-CSF[50] and inflammatory cytokine levels (IL-6, CCL2, TNF)[39] are lower in GF mice. Many of these studies link this difference to a MyD88-related pathway, mostly from TLR-4 signaling. Data from Deshmukh et al. further suggests that the microbiota/TLR-4 signaling pathway induces type 3 innate lymphoid cells (ILC3s) to produce IL-17, which ultimately drives granulopoiesis as detailed below.[50]

Other PRRs are purportedly involved in granulopoiesis as well. Treatment with poly(I:C) increases ROS production, CD11b expression and overall survival of neutrophils in the bone marrow.[57] Although this effect was initially linked to TLR-3 expression on mesenchymal stem cells, it should be kept in mind that poly(I:C) also signals through retinoic-acid-inducible protein 1 and melanoma

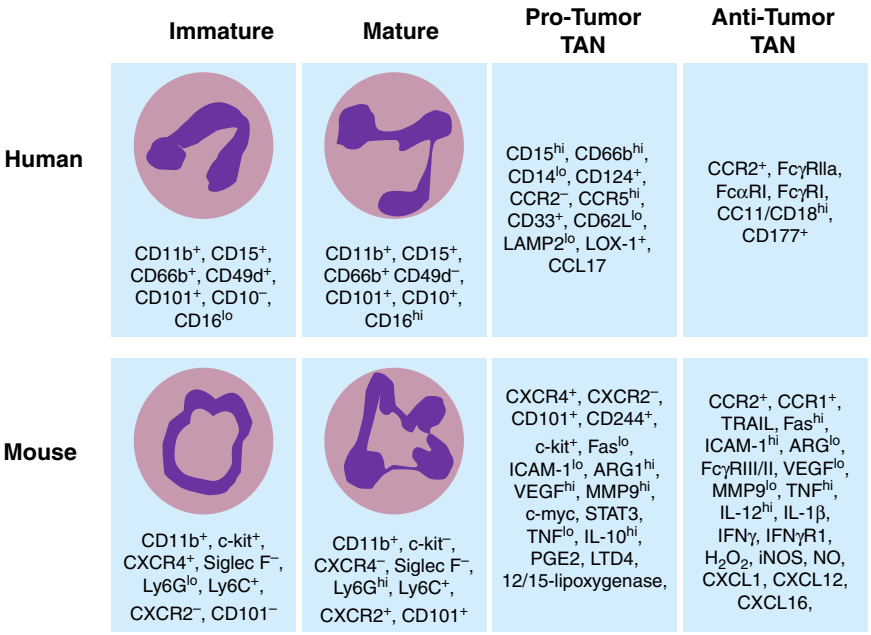

FIGURE 3 **Human and mouse neutrophil markers.** Murine and human neutrophils change their nuclear morphology and surface marker expression during maturation. A few key elements that distinguish pro- and anti-tumor TAN activity are listed here. Many of these markers are described in terms of degree of expression (hi/lo) rather than absolute presence/absence

differentiation-associated protein 5 cytoplasmic receptors (Fig. 2). Microbial peptidoglycans regulate granulopoiesis and neutrophil survival via nucleotide-binding oligomerization domain-containing 1 (NOD1), which also induces intestinal lymphocyte IL-17 production (Fig. 2).[54,55] Gavaging GF mice with a NOD1 ligand restores myelopoietic cytokine production (*Il3*, *Il6*, and *Scf*) and myeloid precursor levels.[56]

Segmented filamentous bacteria (SFB) are bacteria that tightly bind the gut epithelium and is made up of several *Candidatus* Savagella (nomenclature yet to be approved) and closely related to the family *Clostridiaceae*.[57,58] SFB also appears to influence neutrophil migration to the gut (Fig. 2). Colonization of Jackson mice lacking SFB with the cecal contents from SFB positive mice significantly increases ileum *Il17a*, *Cxcl1*, and *Cxcl2* mRNA levels and neutrophil counts.[59] SFB-induced neutrophil recruitment is blocked by the administration of anti-IL-17A or anti-CXCR2 depleting antibodies. Both IL-17A and C recruit CXCR2$^+$ neutrophils to a variety of mucosal surfaces, organs and tumors by inducing the expression of CXCL1/2/5.[55,59–65] In addition to ILC3s and T_H17 cells, innate lymphoid $\gamma\delta$ T cells may be a potent source of IL-17 at mucosal surfaces.[50,66,67] Neutrophils, in turn, control SFB levels as shown by the fact that SFB colonization of SFB negative Jackson mice is accelerated by the depletion of neutrophils with anti-Ly6G.[59] Another study confirms the importance of neutrophils in controlling the microbiota makeup. When mice are pretreated with ABX before receiving a cecal challenge with *Entamoeba histolytica*, the mice are dysbiotic and more susceptible to infection and colitis.[68] The dysbiotic mice lack CXCR2$^+$ cecal neutrophils despite high levels of *Cxcl1* and *Cxcl2*.[68] This is because ABX treatment alone decreases neutrophil CXCR2 expression.[68] Neutrophils may control the microbiota by the release of granule antimicrobial peptides[69] and ROS[70] into the gut lumen.

Rare peripheral CXCR4$^+$, CD62L^{lo} neutrophils have been identified as an aged, proinflammatory neutrophil population.[14,29,71] After inflammation, these neutrophils re-gain CXCR4 expression, inducing their migration back to the bone marrow for clearance.[71] This population appears to be regulated by the microbiota as GF mice have fewer aged neutrophils. This can be explained by the fact that GF mice are less exposed to inflammatory challenges. Diet may also heavily influence neutrophil inflammation and recruitment. Short-chain fatty acids (SCFAs) are the product of dietary fiber fermentation by anaerobic intestinal bacteria, listed extensively by Koh et al.[72] SCFAs exert beneficial effects on mammalian metabolism and butyrate, in particular, has anti-inflammatory properties by inhibiting the recruitment and proinflammatory activity (ROS, TNF) of neutrophils.[73,74]

4 | MARKERS FOR NEUTROPHIL SUBSETS IN CANCER

As we begin to discuss the role of neutrophils in cancer, it is important to start by mentioning a few markers and terms for neutrophil subsets as the literature is becoming increasingly complex and confounding in this area. In healthy donors, mature human neutrophils express CD11b, CD10, CD13, CD14, CD15, CD16, CD33, CD66b, and lack CD49d.[75,76] Mature murine neutrophils express CD11b, CD16, CD32, CD101, Ly6G, Ly6C, CXCR2, and lack Siglec F (Fig. 3).[38,75] By flow cytometry, both human and murine neutrophils exhibit an intermediate light side scatter (SSC^{int}) signal, unlike eosinophils that show the highest SSC signal and express CD49d in humans or Siglec F in mice.[75]

Though the presence of nonlymphoid hematopoietic cells in tumors was noted more than 100 years ago, myeloid cells with an ability to inhibit T-cell response were first characterized in the early 1990s and 2000s and referred to as myeloid-derived suppressor cells (MDSCs). These cells were first identified as SSC^{int}, CD11b$^+$, Gr-1$^+$ (Ly6C/Ly6G) in mice and SSC^{int}, CD66b$^+$, CD33^{lo} in humans.[77,78] Unfortunately, these markers are also present on neutrophils in healthy donors and

much of the literature describes the differences in surface marker expression between normal neutrophils and MDSCs in terms of shifts in levels or degrees (low/high) of expression rather than simply positive or negative expression (Fig. 3). Therefore it is important to call a subset "MDSC" only if, compared to the equivalent subsets in tumor-free hosts, the subset acquires the ability to suppress T-cell expansion.[79]

MDSCs are further separated into the granulocytic-MDSC (gMDSCs) and monocytic-MDSC (mMDSCs) subsets. gMDSCs in mice express higher Ly6G and arginase 1 (ARG1), lower Ly6C, and nitric oxide synthase 2 (*Nos2*, which codes for inducible (i)NOS) levels, and absence of F4/80 compared to mMDSCs.[80] In humans, gMDSCs are distinguished from normal mature neutrophils and mMDSCs due to higher CD15 and CD66b expression, lower CD14 levels, and absence of CCR2.[80,81] Fate mapping and cell tracking in a KRAS/p53 lung cancer model revealed that the spleen is a key source of GMPs and gMDSCs that respond to tumor signals (discussed below) and migrate into the cancer cells to become tumor-associated neutrophils (TANs).[82]

Subsets of TANs have been classified using the "type I" and "type II" nomenclature: N1 TANs are Fas$^+$, CD49d$^+$, CD11b$^-$, ICAM-1^{hi} (CD54) and produce proinflammatory factors (TNF, CXCL10, IL-12, CCL3) while N2 TANs are CD49d$^-$, CD11b$^+$, and release repair/anti-inflammatory factors (IL-10, CCL2, CCL5, ARG1).[83,84] These subtypes will be discussed in more detail below. Additional cancer neutrophil makers are gaining attention in their ability to distinguish maturation state (c-kit or CD117, CXCR2, CD101, CCR5), or oxidative and inflammatory activity (lectin-type oxidized LDL receptor 1 (LOX-1), CXCR4, CD177) (Fig. 3).[13,38,66,85–89]

5 | RECRUITMENT OF NEUTROPHILS TO THE TUMOR MICROENVIRONMENT

The tumor phenotype likely regulates the type of neutrophil brought within the tumor microenvironment.[90,91] Cancer cells and cancer-supporting fibroblasts can secrete mediators such as IL-8[92,93] and CXCL1/2/5[94,95] that recruit mature CXCR2$^+$ neutrophils; c-kit-ligand (SCF) that recruits immature c-kit$^+$ neutrophils[66,88]; or CCL5 that recruits immature CCR5^{hi} neutrophils.[89] Tumors also release granulopoiesis-inducing factors such as G-CSF, SCF, GM-CSF that support neutrophil production and survival[78,96–98] (Fig. 2). Although more groups are reporting a high proportion of immature neutrophils within tumors,[31,32,38,46,47] most studies do not carefully characterize the TAN nuclear morphology and molecular maturation features (granule contents, ROS production, chemotaxis, surface marker expression). The maturation status matters because, as mentioned before, the ability of the neutrophils to produce ROS, to recognize MAMPs/DAMPS, or to mediate other tumor-promoting or tumor-killing pathways may be dictated by their granule content (Fig. 1), which appear in a stepwise fashion during maturation.[99–101]

Tumor size and level of hypoxia can also establish the extent or type of neutrophil recruitment. Hypoxia-inducible factor (HIF)-2α expression in epithelial cells leads to colon tumor formation and expression of CXCL1.[102] In the KRAS/p53 genetic model of lung carcinogenesis,[103] large tumors contain more neutrophils than small tumors. This ability of larger, more hypoxic tumor to attract neutrophils is due to HIF-1α-induced Snail, which induces *Cxcl2* expression in both the tumors and neutrophils.[103] Snail also promotes epithelial-mesenchymal transition.[103]

The size of a tumor may also dictate where the TANs reside. In transplantable AB12 (mesothelioma model) and Lewis lung carcinoma (LLC) tumors, the number of ICAM-1$^+$ TANs increases over 14 days.[84] The location of the neutrophils transitions from hovering around the periphery of the tumor (early stage, day 7 postimplantation) to full internal invasion of the tumor (late stage, day 14). Neutrophils are a cell subset particularly adept at degrading tight junction proteins to pass through endothelial/epithelial cells, so what prevents neutrophils from invading the inner regions of the tumor during the early stages of tumor growth? So far TGF-β appears to be the main culprit as suggested by the finding that inhibition of TGF-β signaling increases myeloid cell tumor invasion.[47,104]

6 | TAN MECHANISMS IN THE TUMOR

An extensive list of neutrophil anti-tumor mechanisms has been compiled by Sionov et al.,[105] including production of ROS,[47,106,107] IL-12, TNF, cathelicidins, CCL2, CCL3, IL-1β, and TRAIL[16,47,108–111] (Fig. 2). Neutrophils may also mediate antibody-dependent cell-mediated cytotoxicity of tumors,[101,112] become nonprofessional antigen presenters to CD4$^+$ T cells,[113–115] and activate CD8$^+$ T cells,[17,47] B cells,[116] dendritic cells,[117] or NK cells.[118] NK cell-derived IFN-γ promotes the tumor-killing functions of VEGF-expressing neutrophils.[119] As many studies now show that neutrophil markers are linked to better survival rates,[12–17] why do neutrophils have such a bad reputation in the cancer field?

Many neutrophil-mediated tumor-promoting mechanisms have been characterized. Late stage ROS and reactive nitrogen species (RNS) from neutrophils can induce single-stranded DNA breaks and decrease the production and activity of DNA repair enzymes.[120,121] Neutrophil MPO appears to inhibit nucleotide excision repair.[122] Indeed, mice with a myeloid-specific deletion of the antioxidant enzyme glutathione peroxidase 4 and repeatedly exposed to the carcinogen azoxymethane have significantly more epithelial cell mutagenesis and aggressive, invasive tumor formation than littermate control mice.[123] The subset of TANs expressing LOX-1 appear to be particularly oxidative.[87] Besides a possible role of neutrophil factors in mutagenesis and tumor initiation, neutrophils can also promote tumor growth by inhibiting the anti-tumor effects of other immune cells. For example, the high levels of ARG1 in protumor TANs[124] may deplete the tumor microenvironment of L-arginine to metabolically block anti-tumor functions.[124,125] Indeed, L-arginine is a key compound for anti-tumor activity as it is a substrate in the synthesis of cytotoxic nitric oxide, and is an essential factor for T-cell metabolism and anti-tumor activity: IFN-γ production, T-cell receptor expression, expansion, and reduced antigen tolerance.[126–129] Hepatocyte growth factor (HGF) signaling in neutrophils mobilizes them in response to

cancer immunotherapies where they acquire immunosuppressive properties (high *Il10*, *Tgfb2*, *Arg1* expression).[130] Neutrophils also mediate immunosuppression by expressing PD-L1,[130,131] recruiting immunosuppressive T_{regs} via CCL17 release (particularly late stage TANs)[132] and inhibiting NK cell-mediated lytic activity through cleavage of NKp46.[133] Neutrophils can also promote tumors by releasing a multitude of mitogenic cytokines (APRIL, IL-21, IL-17), and factors favoring angiogenesis (MMP9, elastase, VEGF), and tumor progression (HGF, TGF-β) (Fig. 2).[134–136]

Neutrophils may create a framework for tumors to adhere and metastasize. This has been associated with neutrophil selectin adhesion[137] and more recently with neutrophil extracellular trap (NET) formation.[138,139] NETosis is a unique form of cell death induced by chemical or microbial activation that is characterized by the release of decondensed genomic chromatin or mitochondrial DNA and granular contents to the extracellular space.[140–142] NETs then bind pathogens and bombard them with antimicrobial granule peptides to kill them or opsonize them for phagocytosis.[143] Cancer patients show high levels of NET biomarkers, such as citrullinated histone H3, in their blood.[144,145] The tumor microenvironment matrix may also dictate NET formation. Collagen type I binds the inhibitory receptor leukocyte-associated immunoglobulin-like receptor-1 present on mature, activated neutrophils, and prevents NET formation.[146] Tumors also release IL-8 that recruit neutrophils.[92,93] Reports have linked IL-8 to neutrophil survival[147,148] and more controversially[149] to NETosis induction.[140,141] The hypoxic environment of a tumor or the gut may also contribute to NETosis as it increases neutrophil adhesion, degranulation, and toxicity.[150–152] NETs may accelerate tumorigenesis as ROS, RNS, neutrophil elastase (NE), cathepsin G, and matrix metalloproteinases (MMPs) released by NETs may promote DNA mutations, mitogenesis, inhibit T-cell activation, and spur on tumor invasion and metastasis.[144,145,153–158] Toxic NETs damage endothelial and epithelial cells,[159,160] and contribute to kidney dysfunction and thrombosis, namely in autoimmune patients.

However, NETs also reportedly exhibit tumoricidal and immunogenic activity.[161–163] Cathelicidins (LL-37 in humans, CRAMP in mice) bound onto NETs are particularly potent activators of monocytes, macrophages, and dendritic cells, leading to type I IFN production, caspase-1 activation, and autoantibody formation.[164–169] Phagocytosed DNA traps may lead to improved anti-tumor activity through cGAS-STING activation.[170] While investigating the biology of NET formation, it is important to note that use of immature bone marrow-derived neutrophils (frequently used in the literature) might not reflect the ability of peripheral neutrophils to form NETs and that each murine strain has different NET forming capabilities, may contain different proteins based on stimuli used and therefore change their immunogenic activity.[171,172] It has also become increasingly evident that leukocytes other than neutrophils and even tumor cells may form DNA traps in response to inflammation.[173,174] The confusion over neutrophil anti- versus protumor effects might be largely due to timing and the stage of tumor development as increasing evidence suggests a switch between these two phenotypes.

7 | EVIDENCE FOR AN ANTI-TUMOR TO PROTUMOR TAN SWITCH

We cited a few identifying markers for neutrophils in the cancer literature, described the terminology of TANs and mentioned mechanisms of neutrophil recruitment into and function within the tumor. Yet, what evidence is there that neutrophils behave differently at various stages of tumor growth? Examination of the AB12 and LLC tumor models at early stages reveal TANs with an anti-tumor N1 phenotype: increased ability to kill tumor cells due to elevated ROS and nitric oxide production, high intracellular TNF levels,[84] and mature nuclear hyper-segmentation, inflammatory gene expression (*Tnf*, *Icam1*, *Il12*, *Ccl3*, *Nos2*) and ability to favor activation of cytotoxic $CD8^+$ T cells.[47] As the tumor grows, it appears that TANs take on more of the tumor-promoting N2 phenotype: immature, banded nuclear morphology with less inflammatory gene expression.[47] For example, neutrophil depletion (using anti-Ly6G) during the early stage of AB12 or LLC tumor implantation in mice (day −1 through day 7) is less effective at inhibiting tumor growth than depletion of neutrophils during the late stage of tumor implantation (day 12 through day 16).[84] If, however, TGF-β signaling is blocked during tumor growth, the N1 TAN phenotype persists and coincides with slower tumor growth that is dependent on the presence of a $Ly6G^+$ population.[47] Similarly, mice with a myeloid-specific deletion of *Tgfbr2* have less LLC and breast cancer (4T1 model) tumor growth due to higher levels of IFN-γ-producing $CD8^+$ T cells and cytotoxic neutrophils than wild type mice.[175] Additionally, SCF from 4T1 tumors may program a metabolic switch in the neutrophils. While neutrophils from tumor-free mice largely rely on glucose for metabolic activity, Christopher Rice in Daniel McVicar's lab (NIH, Center for Cancer Research, in personal communications and presented at Immunology 2018) demonstrates that 4T1-bearing mice expand a $c\text{-}kit^+$ (receptor for SCF) neutrophil population in the periphery that contain more mitochondria (by MitoTracker and complex I, II, III, and V staining) and exhibit significantly more glycolysis-independent mitochondrial metabolism (by Seahorse oxygen consumption rate assay) than $c\text{-}kit^-$ neutrophils. Furthermore, regardless of c-kit absence or presence, the splenic neutrophils from 4T1-bearing mice showed more oxidative activity, induced more T-cell death and blocked T-cell IFN-γ production (in vitro) compared to neutrophils from tumor-free mice. This indicates that the presence of 4T1 tumors "educates" peripheral neutrophils, pushing them toward an altered metabolic phenotype. A similar $CD14^-$, $CD15^+$, $CD10^{lo}$, $CD16^{lo}$, $MitoTracker^{hi}$ neutrophil population with elevated mitochondrial activity is also detectable in ovarian cancer patients, suggesting that therapies targeting this metabolic shift from glycolysis to mitochondrial oxidation in neutrophils may prove to be highly translatable to cancer patients. IFN-β produced from nontumor cells also appears to be significant as *Ifnb1*$^{-/-}$ mice given 4T1 cells have fewer $ICAM\text{-}1^+$ TANs than wild type mice and neutrophils sorted from the blood and tumors of *Ifnb1*$^{-/-}$ mice have less tumor-killing capacity.[176] In vivo treatment with low-dose IFN-β increases the presence of $ICAM\text{-}1^+$ and TNF^+ neutrophils.[176]

Further evidence of varying neutrophil behavior in cancer is obtained by RNA sequencing from each of the neutrophils subsets. Neutrophils obtained from the bone marrow of tumor-free mice (BMN), from the spleen of tumor-bearing mice (termed "gMDSCs" in the article), from the tumors of untreated mice (N2 TANs), and from the tumors of mice treated with TGF-β inhibitor (N1 TANs) show distinct gene expression patterns.[177,178] Key differences include more *Ccl7* (monocyte recruiter) in both TANs over BMN and gMDSCs, more *Cxcl13* (B-cell recruiter), *Ccl6*, *Cxcl10* (monocyte, T and NK-cell recruiter)[177] and higher antigen presentation-related genes in N1s (MHC class-I, TAP1, calreticulin, and tapasin genes),[177] more *Ccl17* (T_{reg} recruiter), *Cxcl14*, *Cxcl1* (neutrophil recruiter) and IL-6 protein in N2s over N1s or BMN,[177,178] and less *Ccl2* (monocyte/macrophage recruiter), *Cxcl4* (neutrophil, fibroblast, and monocyte recruiter), *Cxcl12* in gMDSCs than N2s or BMN.[178] This indicates that each subset is functionally unique.

Similar evidence has also been reported in early stage human lung cancers. These TANs are CD11b^{hi}, CD66b^{hi} CD16^{lo}, CXCR1/2^{lo} compared to peripheral blood neutrophils (PBN) and induce more T-cell proliferation and intracellular IFN-γ levels due to higher expression of costimulatory molecules (OX40L, 4-1BBL, CD86).[14] These patients also possess an antigen presenting cell-like "hybrid" neutrophil population expressing CD66b, CD15^{hi}, CD11b, MPO, NE, CD206, HLA-DR4, CD14, CD86, CCR7, and showing banded nuclei.[14,115] Culturing PBNs or immature BMNs with tumor-conditioned medium (TCM) obtained from digested tumors induces better neutrophil survival and gain of the TAN "hybrid" markers. This is largely due to the GM-CSF and IFN-γ in the TCM.[14,115] Despite a number of transcriptional profiling assays performed on neutrophil subsets, no key transcription factors have been found that distinguishes each subtype. Yet, when PBNs are cultured with this early stage lung TCM or GM-CSF and IFN-γ to induce "hybrid" neutrophils, they lose expression of the transcription factor Ikaros.[115] Loss of Ikaros is also seen in the bone marrow during GMP commitment to monocytes/dendritic cells instead of neutrophils.[179] Other transcription factors that are key to neutrophil development and that may affect TAN polarization include *Irf8* and *Cebpe*, which are higher in immature than mature neutrophils, and *Cebpd* and *Spi1*, which are lower in immature neutrophils.[38]The transcription factor *Egr1* may also prove important as it is present in mature neutrophils, increases with IL-8 or fMLF simuli, and regulates ICAM-1, MMP-9, TGF-β, and IL-1β transcription.[180]

With the growing evidence that neutrophils behave differently at various time points of tumor growth, a large question remaining in this field is whether one wave of N1s resides in the tumor before eventually *becoming* N2s, or whether one wave of N1s is completely *replaced* by another wave of N2s. If the first option is true, this would require a complete reprograming of the neutrophil's transcriptional activity in situ. Some groups believe that neutrophils do not undergo a transcriptional switch so much as a change in "state of activation,"[181] however numerous studies have shown that neutrophils are still transcriptionally active and proliferative after they leave the bone marrow,[38,178,182,183] including immature Ly6G^{lo}, CXCR2^{-}, CD101^{-} murine neutrophils found in tumors.[38] Furthermore, as neutrophils receive multiple prosurvival (IL-8, BCL2, MCL1)[148,184] and granulopoietic (G-CSF, CXCL1, IL-8)[78,96–98] signals from tumors, the early stage cytotoxic N1 TAN could possibly survive long enough within the tumor microenvironment to differentiate in situ into a tumor-promoting late stage N2 TAN.[47,185]

This programing switch may occur systemically, not just in the tumor. When blood is separated by density centrifugation, granulocytes normally settle to be the bottom fraction due to the high density of their granules and are therefore are termed "high density neutrophils" (HDNs).[186] However, under a disease state, granulocytes have been detected in the fraction that also contains "lighter" monocytes, T cells and B cells.[187,188] These have been termed "low density neutrophils/granulocytes" (LDNs). Mice bearing a variety of tumor lines (4T1, AB12, e.g.) show increasing levels of blood LDNs as the tumor grows.[104] Isolated blood LDNs contain a mixture of highly segmented (mature) and banded (immature) nuclear morphologies, express more TRAIL and CCR7, and less Ly6C and CCR5 than HDNs.[104] The LDNs also exhibit less tumor-killing capabilities, ROS production, chemokine production, phagocytosis, and chemotaxis.[104] Therefore, LDNs match a protumor N2 phenotype rather than a tumor-killing N1 phenotype. By labeling and tracking HDNs and LDNs in vivo and ex vivo, it appears that HDNs isolated from tumor-bearing mice exhibit a propensity to become pro-tumor LDNs. HDNs from tumor-free mice do not exhibit this propensity.[104] Culturing HDNs from tumor-bearing mice with TGF-β increased their transition to LDNs, but not so with HDNs from tumor-free mice.[104] This indicates that the presence of tumor cells "educates" neutrophils regardless of their residence in the tumor (TANs) or elsewhere in the periphery (PBN).

What about the hypothesis that one wave of N1s is later replaced by a second, new wave of N2s? To date, any evidence for or against this pathway is largely circumstantial due to the limitations in tracking neutrophils long term. Spleens do appear to be the primary source of the neutrophils that migrate to the tumor[82] and neutrophils are one of the first immune cells to encounter and migrate toward tumor cells.[189–191] Using this logic, if the spleen is removed concurrent to tumor initiation the tumor should have very few TANs. This was observed in a KRAS/p53 mouse model where tumorigenesis is initiated by administration of a Cre-recombinase expressing adenovirus.[82] If only that first wave of neutrophils recruited out of the spleen and into the tumor is the set of neutrophils that matter because this wave of TANs is sufficient to survive and propagate in the tumor, then if the spleen is removed much later posttumor initiation (8 weeks postvirus administration) when the first wave of neutrophils has already migrated to the tumor, then there should be little effect on the number of TANs. Instead, the authors still observe a profound lack of TANs in the tumor when examined at 11 weeks post virus administration.[82] This suggests that multiple waves of neutrophil are continuously recruited from the spleen to replace the TANs in the tumor microenvironment. Furthermore, when mice are examined 11 weeks post virus administration, the spleen of tumor-bearing mice show significantly higher GMP levels than tumor-free mice suggesting that the tumor keeps promoting myelopoiesis. Fluorescently labeled GMPs injected into tumor-bearing mice are found 5 days later predominately in the spleen and tumor.[82] Additionally, when one dose of N1s are transferred by tail vein

injection into 4T1-bearing mice there is significant abrogation of tumor growth; the tumor is completely ablated with multiple injections of N1 neutrophils,[189] again indicating that neutrophils do need to be constantly replaced in the tumor microenvironment rather than one wave of neutrophils being sufficient. We therefore argue that perhaps early TANs begin as N1s, but as the tumor grows it signals changes to the neutrophil transcriptional profile both within the tumor and systemically. The splenic neutrophils are thus affected and as later waves of already N2-polarized neutrophils migrate to the tumor, they replace the previous N1 TANs. It will become easier to confirm this hypothesis as the community develops better methods for tracking neutrophils in vivo such as the Catchup mouse model[192] rather than the toxic quantum dot method, for example.[193] Better tracking methods may establish whether immature, protumor TANs can develop a more mature, antitumor phenotype naturally or with therapeutic intervention as this is currently unknown. Others have also argued that we will not truly get these answers from mouse models that will always mimic advanced stage human cancer because of the high number of injected tumor cells which, because they come from a purified cell line, have bypassed Darwinan selection in order to grow.[194]

8 | THE MICROBIOTA-NEUTROPHIL CROSS-TALK IN CANCER

Though the role of microbiota in influencing neutrophils in cancer progression has only recently been proposed, a few key papers have begun to elucidate this mechanism. The critical steps appear to be how mucosal surface neutrophils respond to a break in the epithelial barrier and microbe translocation, which then primes the body for local and systemic immune activation, which can influence cancer responses. For instance, in a cecal-polyp forming mouse model (*HBUS* mice),[195] precancerous mice systematically lose E-cadherin and Claudin-2 expression as the epithelial barrier degrades allowing neutrophils to migrate nearly to the lumen of the cecum and to release enzymes and antimicrobial peptides (ARG1, COX-2, SERPINE1, REG-3β, and REG-3γ). Soon after, the levels of *Il1a/b*, *Tnf*, *Il17*, *Cxcl2*, and *Ccl17* mRNA rise and promote polyp formation.[195] Fewer tumors were seen in *HBUS* mice treated with anti-Ly6G depleting antibody or vancomycin (targets Gram-positive bacteria), and no tumors were observed in *HBUS* mice on broad-spectrum ABX. When 16S rRNA sequencing was performed on single cecal polyps from *HBUS* mice or normal cecal tissue from ABX-*HBUS* mice or control littermate mice, the bacteria most enriched in the polyps were *Clostridiales*, *Bacteroidales*, and *Desulfovibrionales*.[195]

Other studies have detected an important role of neutrophil ROS after epithelial barrier breakage. Intestinal damage following total body irradiation (TBI) is absent in mice lacking a functional NADPH oxidase ($gp91^{phox-/-}$) or neutrophil-specific TLR-2/3/4/7/9.[196] On the other hand, in a dextran sodium sulfate (DSS)-colitis model, mice lacking a functional NADPH oxidase ($p47^{phox-/-}$) have more colitis, *Gcsf*, *Cxcl2* expression and neutrophil recruitment than wild type C57BL/6NTac mice.[197] However, transfer of wild type bone marrow into $p47^{phox-/-}$ mice did not reverse the susceptibility to DSS-colitis. This was attributed to the unique fecal microbiota in the $p47^{phox-/-}$ mice compared to wild type mice: *A. muciniphila* was present at high abundance in the knock-out mice before DSS treatment, and bacteria of the genera *Allobaculum* and *Parabacteroides* were more abundant in the knock-out mice after treatment with DSS.[197]

Other microbial species have been linked to neutrophil recruitment and cancer pathogenesis. *Lactobacillus reuteri* has potential applications in cancer therapy as it is associated with larger thymus sizes, decreased circulating neutrophil levels, and decreased cachexia markers.[198,199] Conversely, *Helicobacter hepaticus* increases colonic dysplasia, TNF and iNOS expression,[200,201] and even enhances mammary cancer tumorigenesis in a FVB-Tg(C3-1-TAg)cJeg/JegJ mouse model[202] with a mechanism dependent on neutrophil recruitment. Lung-MAMPs also have a role in neutrophil recruitment and tumor formation. *Haemophilus influenzae* induces IL-17C expression, which promotes release of CXCL1 and 2 from LLC tumor cells to recruit neutrophils.[60] Lipopolysaccharide inhalation promotes neutrophil recruitment, degranulation, and the proteolysis of the anti-angiogenesis factor thrombospondin-1 by elastase and cathepsin G.[136]

The role of diet in affecting the neutrophil/cancer response by modifying the microbiota composition has been explored. The farnesoid X receptor (FXR) is activated by bile acids and, in addition to regulating bile acid synthesis and various aspects of glucose and lipid metabolism, it can signal inflammation. Male $Fxr^{-/-}$ mice maintained on a Western diet with high fat and sugar have worse liver inflammation, tumorigenesis, and neutrophil infiltration than wild type mice.[203] Treatment with broad-spectrum ABX or just polymyxin B, which mostly targets Gram-negative bacteria, improves the liver health of $Fxr^{-/-}$ mice on a Western diet. Similarly, the progeny of noninbred mice (CD-1 Swiss mice) kept on a Western diet display increased lung, liver, and lymphatic neoplasms and a higher number of MPO staining neutrophils in the liver, adipose tissue, and mesenteric arteries.[204]

The microbiota appears to effect cancer chemo- and immunotherapies by modifying the function and maturation of tumor infiltrating myeloid cells. When wild type mice receive 5 gray TBI, the innate immune system activates due to gut epithelia cell damage and gut microbiota translocation to the mesenteric lymph node.[205] Dendritic cells then activate and promote $CD8^+$ T-cell expansion and tumor regression.[205] $Tlr4^{-/-}$ mice or wild type mice treated with ABX before the TBI show less innate immune system activation and impaired tumor shrinkage after radiation.[205] Upon seeing gut bacteria after TBI, ileum neutrophils also migrate to the mesenteric lymph node to activate T cells in a graft-versus-host disease model.[206] Other groups have seen that translocation of gut microbiota due to cecal ligation/puncture leads to the expansion of immunosuppressive TANs, which block T-cell division in vitro and promote tumor growth in vivo.[207] It should be noted that therapy-induced gut barrier permeabilization and microbe translocation may not be necessary to activate myeloid cells in cancer. Some therapies that are not known to cause gut permeabilization, such as intratumoral injection of CpG oligonucleotides, still require the presence of microbiota to promote myeloid-mediated anti-tumor effects.[107,208,209]

Full efficacy of oxaliplatin and cisplatin chemotherapies depends on the presence of the gut microbiota or the ability of the immune system to react to MAMPs.[107] Tumor-bearing GF mice, SPF mice given

broad-spectrum ABX or SPF mice with a genetic deletion of *Myd88* are less responsive to cisplatin and oxaliplatin treatment than control mice. This was associated with decreased ROS production, largely from TANs, in the absence of microbiota. Neutrophils may be a key responder to chemotherapy-induced damage. Cisplatin treatment is linked to mitochondrial disruption and ROS production, both of which could lead to highly immunogenic NET formation.[154,176,210–212] Furthermore, when the mitochondria is disrupted by chemotherapeutics, it can lead to the activation of FPR1 that is highly expressed on neutrophils and leads to ROS production. This is because fMLF is both a MAMP and mitochondrial membrane DAMP, to induce an oxidative burst. Interestingly, *Fpr1*−/− mice are less responsive to the chemotherapeutic anthracycline.[213]

9 | CONCLUDING REMARKS

The subsets and functions of neutrophils infiltrating tumors or infected tissues are more heterogeneous than previously imagined. Each clinical tumor type and the timing of the experimental model likely dictates the tumor's ability to attract neutrophils from a particular tissue reservoir (bone marrow, spleen, or blood), or its ability to influence TAN differentiation (mature or immature) and transcriptionally active (N1 or N2 phenotype). Additionally, it remains to be fully characterized which microbes and which PRR signaling pathways at the mucosal surface dictates the ability of neutrophils to regulate tumor development.

The understanding of the microbiota-neutrophil-cancer axis tumor is rapidly progressing. Future advances in this field will have to take into account the fragile nature of neutrophils. Proper isolation techniques will ensure purity without causing cell activation and is essential for obtaining reliable information.[214,215] Improvement in the quality and reproducibility of cell surface markers to identify the different neutrophil subsets and their differentiation status is greatly needed. In experimental tumor models, the type of tumor and kinetics chosen should be carefully considered. Advances in intravital microscopy and real-time in vitro imaging of neutrophils will also contribute to the study of neutrophil functions in cancer progression and therapy. Already, many neutrophil-targeting therapies are being explored to prevent neutrophil reprograming,[216,217] block neutrophil migration to the tumor[218,219] or take advantage of their migration to deliver anti-cancer drugs.[220,221] The therapeutic gain that could be achieved from a better understanding of the microbiota-neutrophil-cancer axis has the potential to be truly beneficial for a very heterogeneous disease such as cancer.

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DISCLOSURE

The author has no conflicts of interest to disclose.

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