Neutrophil dynamics in the tumor microenvironment

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Abstract

The tumor microenvironment profoundly influences the behavior of recruited leukocytes and tissue resident immune cells. These immune cells, which inherently have environmentally-driven plasticity necessary for their roles in tissue homeostasis, dynamically interact with tumor cells and the tumor stroma and play critical roles in determining the course of disease. Among these immune cells, neutrophils were once considered much more static within the tumor microenvironment; however, some of these earlier assumptions were the product of the notorious difficulty in manipulating neutrophils in vitro. Technological advances that allow us to study neutrophils in context are now revealing the true roles of neutrophils in the tumor microenvironment. Here we discuss recent data generated by some of these tools and how it might be synthesized into more elegant ways of targeting these powerful and abundant effector immune cells in the clinic.

Introduction

Recent years have seen a resurgence in neutrophil biology in the context of cancer. Emerging data show that neutrophils are far from the simple homogeneous population they were once thought to be, and depending on context, neutrophil activity can differ in degrees towards pro- or even anti-tumor (1-3). Like other myeloid cells, neutrophils are highly influenced by their environment, therefore, fully understanding the interactions that occur between these cells and their surroundings will enable us to better target them during cancer progression and metastasis. Crucial to our anti-microbial response (4), neutrophils are produced in the tens of millions in the bone marrow and are the largest leukocyte population in the blood of humans. As committed neutrophils are non-proliferative and equipped with an arsenal of proteolytic enzymes and self-destructive effector strategies, they are notoriously hard to purify, manipulate, and study ex vivo. This technical constraint, along with long held, but over-simplistic views of neutrophil biology (i.e. that they are homogenous and inflexible in their response) has meant that neutrophil cancer immunology has lagged behind that of lymphocytes or even the other myeloid cells. Fortunately, recent technological advances allow us to study better than ever how neutrophils contribute to and are influenced by the tumor microenvironment (TME) – both at the primary and secondary sites. Here we review progress in this area and discuss the relative strengths and weaknesses of existing technology and tools to manipulate neutrophils along with examples of how they have benefited knowledge in the field, or in some cases argue why they should be applied to neutrophil biology next considering their contribution to other aspects of in situ cancer immunology.

Neutrophil function at the primary tumor site

The innate immune system co-evolved with infectious microorganisms and its actions are dominated by this primary function (5). Neutrophils contain potent anti-microbial molecules to counter microbial colonization and facilitate tissue repair. This deadly arsenal affords neutrophils the ability to counteract tumor formation and outgrowth (6-14). To recognize and phagocytize cancer cells, neutrophils can use Fc receptors and the immunoglobulins, IgG or IgA, through a process called antibody-dependent cellular toxicity (ADCC). Recent work has shown that blocking the interaction between CD47 – a ligand often expressed on cancer cells that

blocks phagocytosis – and its receptor, signal regulatory protein alpha (SIRPα), on neutrophils enhances ADCC (15). These observations have important implications for cancer immunotherapy, given that inhibitors to the CD47-SIRPα axis are currently being evaluated in cancer patients (16). Neutrophils can also delay tumorigenesis by presenting tumor antigens to killer CD8 T cells and secreting IL-12 to stimulate Type 1 immunity and IFNγ expression from $CD4^{-}CD8^{-}$ unconventional αβ T cells (11, 12, 17). However, many of the effector functions that are important in maintaining host tissue integrity also help tumors initiate and grow, via direct effects on cancer cells (18-21), remodeling the extracellular matrix (ECM) (22, 23), stimulation of angiogenesis (13, 24-35), activation of pro-tumorigenic macrophages (36), inhibition of anti-tumor immunity (35, 37-44), production of reactive oxygen species (ROS) (20, 24, 45, 46), or release of neutrophil extracellular traps (NETs) (42, 47-49) (Figure 1).

Neutrophils arise from bone marrow progenitor cells, and tumors often secrete systemic factors, such as G-CSF, to stimulate granulopoiesis in the bone marrow (50-52). G-CSF is induced by IL-1β and IL-17A in autochthonous and transplantable mouse tumor models of breast and lung cancer (50, 53, 54), indicating that a number of tumor-initiated cell-cell communication events are often required to orchestrate granulopoiesis. In a Kras-driven, p53-deficient cancer model, tumors in the lung activate osteoblastic stromal cells in the bone marrow, which encourage the production of SiglecF-expressing neutrophils that promote cancer progression (55, 56). However, new research indicates that trained immunity (i.e. functional transcriptomic, epigenetic and metabolic reprogramming of innate immune cells evoked by foreign stimuli) can alter granulopoiesis and cancer progression. For example, the fungal-derivative β-glucan can rewire bone marrow progenitor cells through upregulation of Type I interferons to generate anti-tumor neutrophils that can slow the growth of B16 melanoma cells in mice (57). Once released from bone marrow, neutrophils are recruited to tumors by the CXCR2 ligands, CXCL1, CXCL2, CXCL5 and CXCL8 (in humans only) (22, 58-63), that are regulated by KRAS signaling (64), NOTCH signaling (65) and the transcription factor, SNAIL (35). Expression of the CXCL1 chemokine can also be enhanced by obesity in an IL-1β-driven mouse model of esophageal cancer (66), leading to increased neutrophil recruitment to tumors. Tumor growth is slowed in CXCR2-deficient mice or CXCR2 inhibitor-treated mice in mouse models of lung, skin and intestinal cancer (22, 39,

58, 61-63, 67, 68), providing opportunities for therapeutic intervention. Indeed, CXCR2 inhibitors are being trialed in cancer patients (NCT04477343, NCT03161431, NCT03177187, PRIMUS003).

Although the molecules regulating neutrophil expansion and recruitment to tumors are shared across the entire population, neutrophils can exhibit striking functional differences, and information on their diversity continues to emerge. The mechanisms by which neutrophils are polarized towards pro- or anti-tumor states primarily occurs through cytokines, such as TGFβ, IFNβ, IFNγ, G-CSF and GM-CSF (10-13, 26, 50, 69). Tumor hypoxia is another important regulator of neutrophil phenotype and polarization, since counteracting hypoxia in an autochthonous mouse model of PTEN-driven uterine cancer decreases neutrophil-mediated cancer progression (70). The importance of neutrophil polarization and diversity in cancer has been recently reviewed elsewhere (1-3, 71, 72). However, it is important to mention that specific nomenclature describing neutrophil polarization states have led to confusion when comparing data in the field. These terms include N1/N2 neutrophils, which were coined to mirror T helper cell (Th)-1/2 immunity and M1/M2 macrophages; granulocytic or polymorphonuclear myeloid-derived suppressor cells (G/PMN-MDSCs), which are T cell-inhibiting neutrophils; as well as low-density neutrophils (LDNs) and high-density neutrophils (HDNs), whose name stems from the location of neutrophils in density gradients. There are many biological arguments for and against the continued use of these terms (1, 2, 73, 74), but overall, we argue that to more accurately describe emerging data in the field they should be avoided. The terms above are either too narrow or too simplistic in their ability to capture the inherent plasticity of neutrophils, or they perpetuate the incorrect notion that N1, N2, MDSCs, LDNs, HDNs are cell populations distinct from neutrophils. These terms describe pathological activation or maturation states of neutrophils, rather than separate cell types (75).

Neutrophil participation in metastasis

The importance of neutrophils in cancer spread was established in the 1980s (76, 77), but not until recently have studies started to uncover the mechanisms of neutrophil function during the evolution of metastatic disease. Neutrophils can either help or hinder metastasis formation, independent of any action on primary tumor growth. To counteract metastasis, neutrophils can secrete H_2O_2 to kill cancer cells (7, 78) or

thrombospondin 1 (TSP1) to create an anti-metastatic environment in distant organs (79, 80). These cells can clear antibody-opsonized cancer cells in experimental liver metastasis models by ingesting plasma membrane fragments in a process called trogoptosis (81). However, most studies on this topic report on the ability of neutrophils to encourage metastasis.

Neutrophils can promote metastasis from the vantage point of the primary tumor site by promoting escape of cancer cells into the vasculature (82), in the circulation where they provide mitogenic cues (83), or at the secondary site where these cells accumulate in a variety of models (50, 51, 65, 84-90). In visceral organs, neutrophils can direct disseminated cancer cells to specific locations (89, 91), promote vascular leakiness for easy extravasation (31, 32, 85) or suppress anti-tumor immunity by CD8 T cells and NK cells (50, 51, 65, 69, 84, 86, 90-92). Recent data have provided new evidence of metabolic crosstalk between neutrophils and cancer cells, where neutrophils take up lipids from mesenchymal cells in the lung of mammary tumor-bearing mice and provide them to disseminated cancer cells as an additional energy source to fuel metastasis (93). Another pro-metastatic function of neutrophils is their ability to expel protein-covered nucleic acids, known as neutrophil extracellular traps (NETs), that catch circulating cancer cells and stimulate their adhesion to endothelial cells, invasion and proliferation at secondary sites (23, 94-100). NETs are triggered from neutrophils by inflammatory agents such as lipopolysaccharide (LPS) or Cathepsin C, a cancer cell-secreted protease, in the lungs of mammary tumor-bearing mice to stimulate dormant, non-cycling cancer cells into proliferating or to capture disseminated cancer cells from blood (100, 101). The complement molecule, C3a, also induces NETs and primary tumor progression in an *Apc*-mutated bowel cancer model (48). NETs activate a receptor on breast cancer cells, called coiled-coil domain containing protein 25 (CCDC25), that stimulates intracellular signaling via the ILK–β-parvin–RAC1–CDC42 pathway to promote metastasis formation (102). Whether CCDC25 is expressed by cancer cells across multiple tumor types or whether the interaction between NETs and cancer cells occurs through other receptors is unknown. Furthermore, neutrophil cooperation with platelets and platelet attachment to NETs can contribute to thrombosis. This poses a problem not only for the establishment of metastasis, but also for organ dysfunction at non-metastatic sites in cancer patients (103).

The mechanisms by which tumors manipulate neutrophils provide opportunities for therapeutic intervention in cancer patients with metastatic disease. Crosstalk with other immune cells is critical in this process. For example, in autochthonous breast cancer mouse models, macrophages expressing IL-1β in primary tumors stimulate IL-17-producing γδ T cells that control the expansion and phenotype of immunosuppressive neutrophils (50, 84). NK cells also regulate neutrophil behavior, as pro-metastatic neutrophils are converted to anti-metastatic neutrophils in NK cell-deficient mice (92); although, the mechanism by which this occurs is not clear. As mentioned above, TGFβ is an important molecule for neutrophil polarization. Neutrophil-specific deletion of TGFβ receptors decreases metastasis in breast and colorectal cancer models by reverting their suppression of anti-tumor immunity (65, 69). The atypical chemokine receptor, ACKR2, functions in a similar manner as TGFβ in controlling the phenotype and activity of neutrophils. Whereas ACKR2-proficient neutrophils are pro-metastatic, ACKR2-deficient neutrophils are anti-metastatic (104).

Another emerging indicator of neutrophil-driven metastasis is mutational status of tumors. An in-depth comparison of 16 different autochthonous mouse models of breast cancer recently showed that neutrophil-mediated metastasis is dependent on p53 status in primary tumors. p53 null cancer cells increase expression of WNT ligands to activate IL-1β from tumor-associated macrophages, which in turn drive IL-17A production by γδ T cells and neutrophil accumulation, while p53-proficient cancer cells do not (84). The upregulation of WNT ligands stemmed from the inability of p53 to suppress microRNA-34a expression, which subsequently suppresses WNT ligand expression. Using p53-deficient breast cancer models, inhibition of WNT ligands prevents both circulating and lung-infiltrating neutrophils and reduces pulmonary metastasis (84). Interestingly, loss of p53 in models of metastatic colorectal cancer fail to fit within this paradigm; instead, NOTCH1 signaling is the determining factor of neutrophil-mediated metastasis. Gut tumors driven by loss of p53 and KRAS hyperactivation do not metastasize to the liver, but when NOTCH1 signaling is added to this mutational combination, neutrophils are abundant and liver metastasis occurs (65). Moreover, epigenetic changes in renal cell carcinoma results in overexpression of CXCR2 ligands, neutrophilia and neutrophil-mediated lung metastasis that can be blocked with a bromodomain and extra-terminal motif inhibitor (BETi) (105). Breast cancer cells naturally producing Dickkopf-1 (DKK1), a regulator of the WNT

pathway that desensitizes cells to canonical WNT signaling, are inefficient at seeding the lung in part because DKK1 represses neutrophil recruitment to pulmonary tumors (106); although, it is unclear how the genetic makeup of these breast cancer cells results in overexpression of DKK1. These types of analyses should be extended to other tumor types to determine how tumor genotype dictates neutrophil responses.

Implications for the clinic

Because neutrophilia is a common feature in many cancer patients, blood neutrophil-to-lymphocyte ratio (NLR) is a useful and easily attainable biomarker to predict patient outcome, response to chemotherapy, and response to immunotherapy. A high NLR is generally associated with poor prognosis across multiple cancer types (107). NLR may be further refined by incorporating recent discoveries in neutrophil heterogeneity, using surface markers or nuclear morphology. Neutrophil subpopulations may be more pronounced at specific stages of cancer progression than others, so quantifying and using these subsets as biomarkers may be better prognostic indicators of disease severity. Indeed, the frequencies of neutrophil subsets as identified by mass cytometry (CyTOF) change as cancer progresses in melanoma patients (108). With this type of analysis, it will be important to determine optimal low, medium, and high thresholds of neutrophils subsets in order to parse confounding data from cancer patients with infections or other inflammatory diseases (a common side-effect of current immunotherapies), whose neutrophils will dynamically respond.

In addition to circulating neutrophils, the density of neutrophils in primary tumors is often associated with poor outcome (2, 3) and frequently correlates inversely with T cell infiltration (109). CD66b and myeloperoxidase (MPO) are the most common markers used to identify neutrophils by immunohistochemistry; however, these markers are not exclusively specific to neutrophils and can be expressed by other myeloid cell populations. Using gene expression datasets, neutrophil-related gene signatures can also be used as prognostic indicators of outcome. In fact, using the computational method CIBERSORT to quantify cell populations from TCGA data, neutrophils are the greatest indicator of poor outcome among multiple immune cell populations across 39 different cancer types (110).

Given their importance in primary tumor growth and metastasis, neutrophils represent a prime target for immunotherapy in patients with cancer. Three main strategies exist to modulate these cells via

interference with their recruitment, survival, or polarization. As discussed in more detail below, the most well studied method to block neutrophil recruitment is through CXCR2 inhibitors, which are currently being trialed in cancer patients. Neutrophils are very susceptible to various classes of chemotherapy due to their rapid turnover. However, chemotherapy-induced neutropenia may be advantageous in some cases, since this side-effect is associated with improved survival in patients with lung, breast, stomach, and colon cancer (111-114). Neutropenia comes with greater infection risk and must be carefully managed. Conversely, boosting neutrophils may be beneficial when these cells play an anti-tumor role. Increasing neutrophils can be accomplished through administration of G-CSF or GM-CSF. To alter neutrophil polarization and convert pro-tumor neutrophils into anti-tumor neutrophils, targeting cytokines, such as TGFβ or IFNβ, offers a viable approach. These strategies require further exploration with special consideration given to duration of treatment and toxicities. Furthermore, targeting neutrophil recruitment, survival, or polarization may synergize with other cancer immunotherapy modalities, such as checkpoint inhibitors, in patients resistant to these drugs. However, to fully implement neutrophil-related targets in the clinic, a greater understanding of neutrophil biology is required.

Loss- and gain-of-function methods to study cancer-associated neutrophils

Neutrophil depletion / Neutropenia

Neutrophils are rapidly turned over, making depletion studies difficult, especially in long-term cancer models. The Gr1 antibody (RB6-8C5), which binds both Ly6C and Ly6G antigens, as well as the Ly6G antibody (1A8) are used in many studies to specifically target neutrophils (13, 50, 55, 115). However, other cell types can express Ly6C and G including monocytes and eosinophils, respectively, complicating interpretation. In addition, the low levels of Ly6G expressed on immature neutrophils means that these may be inefficiently depleted. Indeed, in a mouse model of head and neck cancer, depletion-resistant neutrophils were present in the tumor and spleen whilst being effectively depleted in the peripheral blood (116). During consistent depletion pressure, neutrophil numbers can rebound, and immature neutrophils can actually increase in tumor-bearing mice compared with controls.

Attempts at refining antibody-mediated depletion of neutrophils using anti-Ly6G together with secondary anti-rat antibody may afford more durable neutrophil depletion (117). Neutrophil trafficking is dependent on CXCR2 signaling; therefore, interference with CXCR2 via genetic deletion or pharmacological inhibitors are useful to block neutrophil ingress into tumors. As mentioned earlier, clinical trials are already underway of CXCR2 inhibitors in cancer patients. However, CXCR2 inhibitors can also affect CXCR2-expressing tumor cells and stromal cells (118, 119). The use of CXCR2 inhibitors may also induce compensatory mechanisms from other myeloid cells, as is observed in pancreatic cancer models (120). A preclinical model known as Genista mice lacks mature neutrophils due to a point mutation in Growth Factor Independence 1 (Gfi1) (121) and has impaired NK cell responsiveness (122) but retains normal T and B cell differentiation. Transplantation of cancer cell lines into Genista mice suggests that neutrophils antagonize cancer progression by blocking the function of IL-17-producing γδ T cells, which are well established promoters of tumor growth and metastasis (123). Neutrophils impede γδ T cells through NOX-2-dependent production of ROS to inhibit their proliferation (124). Interestingly, these mice have a population of Ly6G intermediate cells, which potentially provides a model for studying immature neutrophils. To overcome these blunt approach models, conditional loss of function models have been developed. *Mrp8-Cre* mice crossed with diphtheria toxin receptor mice show 80-95% neutrophil depletion; although, there is minor leakage into the monocyte/macrophage compartment (125).

Neutrophilia

CXCR4 is important for retaining neutrophils in the bone marrow through interaction with its ligand CXCL12 (126), and interference with this molecule can be used to promote neutrophilia. CXCR4-deficient mice die perinatally (127, 128). Therefore, CXCR4 manipulation has mainly relied on pharmacological antagonists, such as Plerixafor (AMD3100), which leads to a rapid release of neutrophils into the circulation. Mice with *LysM-Cre*-driven conditional deletion of *Cxcr4*, which specifically deletes CXCR4 in the entire myeloid compartment, exhibit neutrophilia. Melanoma cells transplanted into these mice have reduced growth and elicit increased NK cell cytotoxic response, indicative of anti-tumor polarized neutrophils (129). Clinical trials targeting CXCR4 to increase trafficking of anti-tumor immune cells in combination with T cell checkpoint

immunotherapy are underway in pancreatic cancer patients (NCT04177810). However, like CXCR2, CXCR4 is expressed by several cell types, suggesting caution is warranted in data interpretation.

Neutrophil effector functions

Collating the above-mentioned mouse models highlights the complexity and limitations of inducing neutropenia or neutrophilia to study the role of neutrophils in cancer. Knockout or conditional models are used to specifically target key neutrophil-derived molecules. The process of neutrophil extracellular trap production (NETosis) is dependent upon peptidylarginine deiminase 4 (PAD4), so PAD4-deficient mice are used to study NETs in cancer progression (23, 42, 49, 97, 130). Pancreatic tumor-bearing PAD4 knockout mice have even established the potential utility of combining NET inhibitors with T cell checkpoint inhibitors, such as anti-PD1 immunotherapy (42). Neutrophil myeloperoxidase (MPO), another enzyme highly abundant in neutrophils, leads to the generation of reactive oxygen (ROS) and nitrogen species (RNS). MPO knockout mice and inhibitors have been used in mouse models of lung cancer to delay tumor growth with some success (131). However, ROS production by neutrophils can also play a role in cancer cell killing (20, 24, 45, 46), but the context in which ROS is pro- or anti-tumor remains unresolved. Conditional models, such as *Mrp8-Cre* and *LysM-Cre*, are not entirely specific to neutrophils. The *Ly6g-Cre* (Catchup) mouse was generated to increase neutrophil specificity (132), and this mouse has been used to demonstrate the importance of TGFβ-mediated neutrophil polarization in liver metastasis (65) as TGFβ is a major driver of pro-tumorigenic neutrophils in various models (13, 69). These data exemplify the utility of such mouse models. More sophisticated approaches aimed at targeting specific neutrophil effector molecules may shed some light on their role within cancer progression, but ultimately their combination with the more specialized techniques outlined below will likely improve our understanding.

Spatially independent tools to study neutrophils

Flow and Mass Cytometry

As new insights into neutrophil diversity, maturity, and polarization are uncovered (1-3, 71, 72), methods to distinguish these different neutrophil populations become more important. Flow cytometry is an essential

tool in these efforts due to the ability to assess multiple molecules simultaneously. For example, in patients with non-small cell lung cancer (NSCLC), a 27-colour flow cytometry panel has been used to characterize the tumor immune landscape, which revealed neutrophils as the most abundant cell type in NSCLC tumors (109). New markers of neutrophil subsets, including CD10 (133), CD101 (65, 134), CD117/cKIT (50, 135-137), CD177 (14) and SiglecF (55, 56), are easily interrogated by traditional flow cytometry methods. However, as the list of markers grows, data analysis becomes laborious. Automated gating algorithms, such as MegaClust, have aided comprehensive characterization of tumor-associated neutrophils within mouse models (35).

Flow cytometry, though extremely valuable, still has limitations in the number of simultaneous markers possible. Mass cytometry combines flow cytometry and mass spectrometry, using stable isotope labelled antibodies analyzed by mass spectrometry to dramatically increase multiplexing (138). This improvement is imperative for examining precious patient samples with limited total cell numbers. So far, in the context of cancer, neutrophils have mostly been investigated by mass cytometry in the circulation (108, 139). Fluorescence-based cytometry has recently bridged the gap somewhat with mass cytometry, and better optical design and the use of spectrally resolved detectors now allow 30+ markers to be analyzed. Fluorescence-based cytometry removes some of the constraints of mass cytometry, including the need for specialized kits and antibodies for stable isotope labeling and allows the possibility of sorting cells for downstream analysis (whereas mass cytometry destroys the sample). Isolation of neutrophils can be difficult without altering their phenotype/activation status and therefore their functional response in ex vivo assays (140). However, fluorescence activated cell sorting (FACS) of neutrophils for transcriptomic profiling has been important in revealing their role in the TME (141).

RNA sequencing

Mostly due to accessibility, the first studies analyzing neutrophil transcripts in cancer have been performed on blood and bone marrow. RNAseq analysis of circulating neutrophils from *K14-Cre;Cdh1*$^{F/F}$*;Trp53*$^{F/F}$ mammary tumor-bearing mice show an increase in expression of genes encoding the pro-metastatic proteins *Prok2/Bv8*, *S100a8, S100a9* and *Nos2* (which encodes inducible nitric oxide synthase (iNOS)) (50). Transcriptional analysis of sorted neutrophil populations from the blood of mice bearing liver metastases

from 4T1 mammary cancer cells has uncovered differences in expression of transcription factors, where neutrophils produce higher levels of C/EBPε (98). More recently the comparison of neutrophil transcripts from premetastatic lung and peripheral blood revealed the overexpression of lipid droplet-associated genes by pre-metastatic lung neutrophils, allowing the subsequent description of a neutrophil-fueled mechanism of breast cancer metastasis (93).

Single cell (sc)RNAseq allows the detection of heterogeneity in maturation/activation markers in the wider population of neutrophils. Neutrophil heterogeneity in bone marrow, peripheral blood, and spleen has been recently assessed by scRNAseq in homeostasis and bacterial infection (142), but such a comprehensive study is still lacking in cancer. However, an analysis of human tumor biopsies and mouse models of lung cancer showed that neutrophils from humans and mice form a continuum of states with several shared populations amongst species (143). These populations consisted of canonical neutrophils expressing high levels of MMP8/9, S100A8/9 and ADAM8, and several tumor-specific neutrophils which were proposed to promote tumor growth in mice. In these studies, neutrophils exhibit very low transcript counts – a warning that neutrophils can inadvertently excluded using common data filters. Tumor-infiltrating neutrophils only partially overlap blood neutrophil populations, highlighting the influence of microenvironment on neutrophil phenotype (143).

Spatially resolved tools to study neutrophils

Visualizing neutrophils in their anatomical location can help understand how, where and when neutrophils influence tumor cells and other immune cells as well as their role in disease progression and therapy response. Using both routine and more advanced imaging techniques, the spatial context of tumor and stromal cells can be analyzed to investigate local clusters, cell dispersion and interactions in two to four dimensions (Figure 2). For example, immunohistochemistry (IHC) and immunofluorescence (IF) analyses of tumor and metastatic tissue are widely used to characterize neutrophils in tumors. Stratification of human tumors according to the presence of CD66b- or CD15-expressing neutrophils results in different prognostic significance depending on the tumor-type and cellular localization (144, 145). NETs have also been extensively analyzed in fixed tissues (49, 130, 146), usually quantified by co-localized immunofluorescence

staining of extracellular chromatin DNA with granule proteins (e.g. MPO; Neutrophil Elastase, NE; MMP9). NETosis implies chromatin decondensation which usually requires nuclear histone citrullination by PAD4. Therefore citrullinated histones are markers of NETosis but are dispensable in some conditions (147). Highly multiplexed imaging of tissue sections is achievable by multiplexed ion beam imaging (MIBI), which uses metal isotope-tagged antibodies in tissue sections in a similar way to mass cytometry. Using MIBI on triple negative breast cancer biopsies has revealed that neutrophils tend to cluster together and are enriched near the tumor border (148, 149). Furthermore, 3D imaging and tissue clearing techniques that reduce refractive indices and increase imaging depth are being employed to gain a deep understanding of neutrophil location and function throughout entire organs. Imaging neutrophil–T cell interactions in cleared human head and neck tumors has provided direct evidence that T cell activity is decreased when these cells are in close proximity to neutrophils (150). With multiple markers, these techniques could be used to better assess neutrophil heterogeneity (maturation, polarization, etc.) in the TME.

In vivo Imaging

The In Vivo Imaging System **(**IVIS) allows non-invasive, longitudinal fluorescence or bioluminescence imaging of living organisms albeit with limited resolution and sensitivity compared to microscopy. This method can be used to monitor neutrophils in vivo*.* Luminol, a compound that emits luminescence after oxidization, enables the imaging of MPO activity (151). In mice transplanted with 4T1 mammary cancer cells, MPO-expressing neutrophils can be detected at the site of injection only two days after cancer cell transplantation, before tumors are palpable (152). Similarly, a probe to image Neutrophil Elastase Activity (Neutrophil Elastase 680 FAST imaging agent) has shown utility in cancer models (153, 154).

Intravital microscopy (IVM) is a high-resolution technique to gain valuable spatiotemporal information on cells of interest in mice (reviewed in (155-157)), including neutrophils. In transplantable mouse models of head and neck squamous cell carcinoma, IVM revealed that intratumoral neutrophils move slowly, compared with peritumoral neutrophils, which have a higher velocity that increases with cancer progression (158). NETs can also be imaged by IVM to visualize their effects on anti-tumor immune cells (47). Additionally, IVM has uncovered a role for neutrophils in transporting drug nanoparticles to tumors (159,

160). Neutrophil-dependent steps of the metastatic cascade, including neutrophil-mediated cancer cell adhesion to liver endothelium have been visualized by IVM (95, 161). However, some organs are easier to probe by IVM than others, such as the lung, which constantly moves. To overcome these mechanical issues, vacuum-stabilized imaging windows have been developed to visualize neutrophil behavior in the lung following tail vein injection of cancer cell lines (162). Neutrophil activation by cancer cells *in situ* can also be measured with imaging windows (47, 96, 163). Recent advances in permanent lung imaging windows for IVM (164) may allow monitoring of neutrophil behavior during the process of metastasis over time: from development of the pre-metastatic niche to cancer cell seeding to tumor outgrowth.

Other animal models are extremely useful to study neutrophil dynamics in cancer. Zebrafish larvae are transparent and relatively small, so it is possible to track every neutrophil in the whole organism over extended periods of time (165). In zebrafish implanted with human estrogen receptor positive (ER+) breast cancer cells and neutrophils, neutrophils were observed to promote cancer cell invasion (166).

Conclusion

Recent mechanistic and technological advances have uncovered new aspects of neutrophil biology that offer potential avenues for therapeutic intervention. After years of lagging behind other immune cells, knowledge on neutrophil phenotype and function is finally growing. The community now has spatially independent and spatially resolved methodologies to address critical questions regarding neutrophil behavior. These methodologies should provide details on the context in which neutrophils help or hinder cancer progression. Given the new information on neutrophil diversity, lifespan, and physiological roles (167), these methodologies should be used (in combination) to interrogate neutrophil plasticity more comprehensively. Like other myeloid cells, neutrophils exist in a wide spectrum of phenotypes driven by systemic, tumor-derived signals as well as local, tissue-specific microenvironments (167). However, there is still a serious gap in our knowledge about how the TME and neutrophils influence each other both locally and systemically, and how these mechanisms differ between cancer types. With this information, we can understand the complex roles and responses of these cells during cancer progression and perhaps exploit neutrophils for cancer immunotherapy to benefit cancer patients.

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Figure Legends

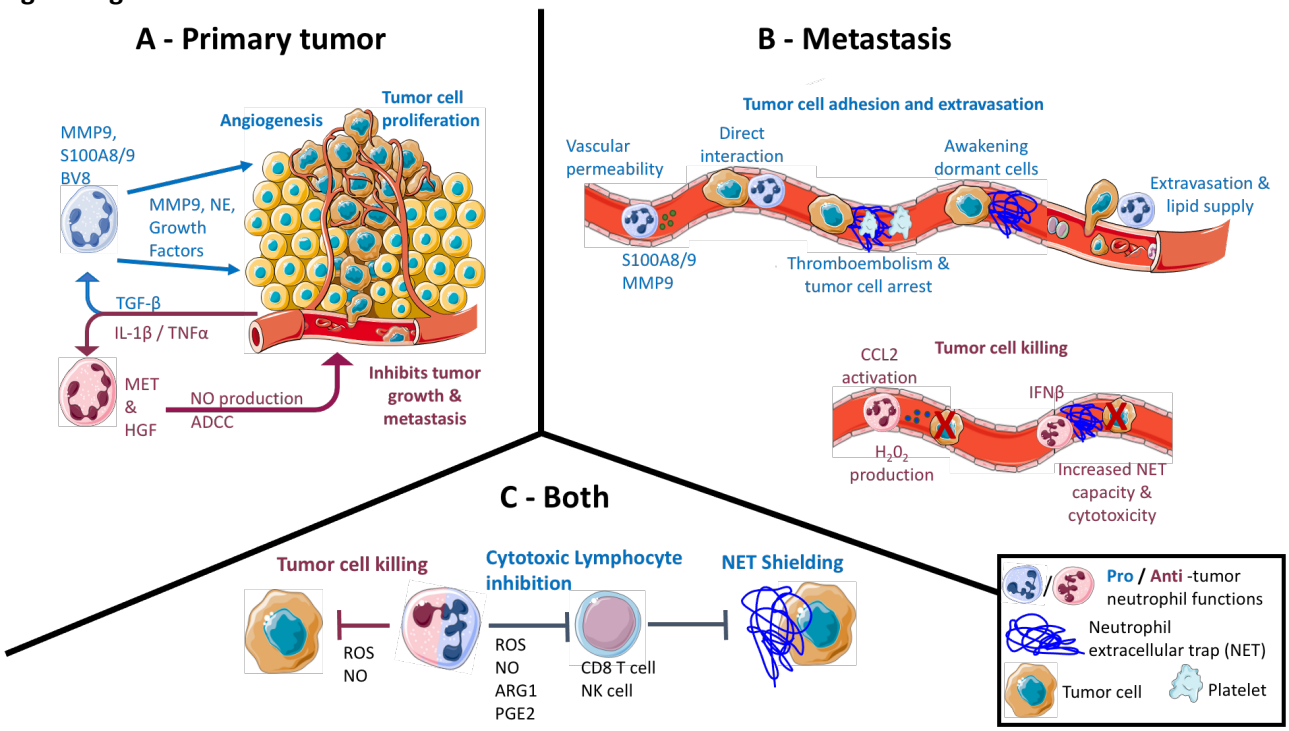

Figure 1. Neutrophil functions during cancer progression. Neutrophils participate in tumor progression by acting both at primary tumors and the (pre-)metastatic niche. (A) In primary tumors, neutrophils can mediate angiogenesis through the release of MMP9, S100A8-A9 and BV8 to activate VEGF. The production of growth factors and laminin degradation by neutrophil-derived proteases NE and MMP9 can assist tumor cell proliferation. Alternatively, inflammatory stimuli (IL-1β and TNF-α) can induce neutrophil MET expression and binding of HGF, leading to NO production and tumor cell killing. Neutrophils also use antibody-dependent cellular cytotoxicity (ADCC) to kill cancer cells. (B) Neutrophils can support metastasis through a number of different factors individually or in combination. Inflammation induced by molecules such as S100A8 increases vascular permeability and therefore extravasation. Direct interactions between cancer cells and neutrophils or NETs can lead to their arrest in the vasculature. In addition, NETs have been suggested to wake dormant tumor cells and neutrophils can feed tumor cells with lipids to aid their survival. Together, these events favor tumor cell extravasation and metastasis. Neutrophils can also aid tumor cell killing. CCL2 produced by the primary tumor can activate neutrophils in the premetastatic niche to produce hydrogen peroxide providing an efficient tumor cell killing mechanism. IFN-β has also been shown to increase neutrophil anti-tumor potential by increasing NET capacity and cytotoxicity towards tumor cells (C) The release of reactive oxygen species (ROS) and nitric oxide (NO) can induce tumor cells

death but conversely, through ROS, NO, ARG, PGE2, or a 'shielding' effect of NETs, neutrophils can suppress cytotoxic immune cell activity.

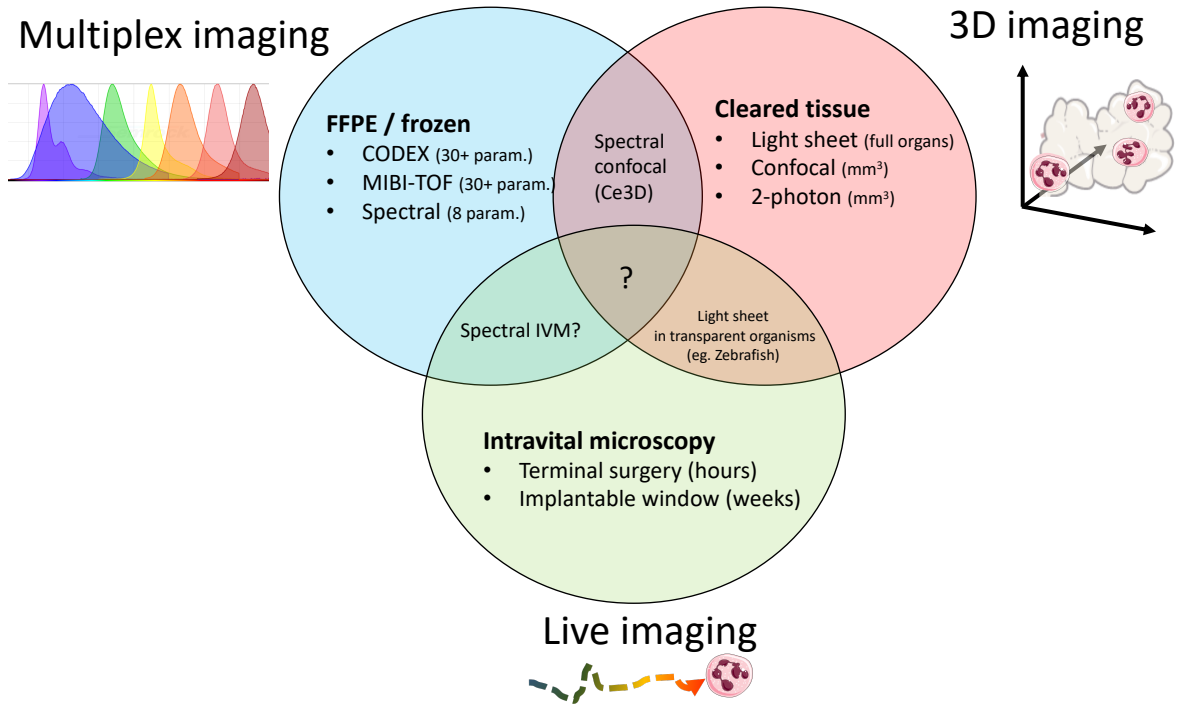

Figure 2. Overlap in state-of-the-art of TME imaging approaches. Current state-of-the-art high-resolution imaging techniques allow highly multiplexed imaging in two dimensions with mass imaging or CODEX and to a lesser extent spectral imaging. It is possible to image large volumes of tissues and even whole organs in three dimensions using tissue clearing techniques in combination with light sheet, confocal or multiphoton microscopy but multiplexing options are currently sparse. To capture cell dynamics in vivo, imaging windows can be implanted in mice to image cells *in situ* in real time. However, tissue penetration and multiplexing options are again currently limited. The use of transparent organisms such as zebrafish embryos and the combination of volumetric imaging/intravital microscopy with spectral imaging could be a way to circumvent some of these limitations.