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Neutrophils in the tumor microenvironment: trying to heal the wound that cannot heal

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Summary

Neutrophils are the first responders to infection and injury and are critical for antimicrobial host defense. Through the generation of reactive oxidants, activation of granular constituents and neutrophil extracellular traps, neutrophils target microbes and prevent their dissemination. While these pathways are beneficial in the context of trauma and infection, their off-target effects in the context of tumor are variable. Tumor-derived factors have been shown to reprogram the marrow, skewing toward the expansion of myelopoiesis. This can result in stimulation of both neutrophilic leukocytosis and the release of immature granulocytic populations that accumulate in circulation and in the tumor microenvironment. While activated neutrophils have been shown to kill tumor cells, there is growing evidence for neutrophil activation driving tumor progression and metastasis through a number of pathways, including stimulation of thrombosis and angiogenesis, stromal remodeling, and impairment of T cell-dependent anti-tumor immunity. There is also growing appreciation of neutrophil heterogeneity in cancer, with distinct neutrophil populations promoting cancer control or progression. In addition to the effects of tumor on neutrophil responses, anti-neoplastic treatment, including surgery, chemotherapy, and growth factors, can influence neutrophil responses. Future directions for research are expected to result in more mechanistic knowledge of neutrophil biology in the tumor microenvironment that may be exploited as prognostic biomarkers and therapeutic targets.

Keywords

cancer; inflammation; injury; neutrophils; wound repair

1 INTRODUCTION

In the late 19th century, Elie Metchnikoff made seminal discoveries pertaining to phagocyte biology. He first discovered the role of phagocytes through his investigation into the

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CONFLICTS OF INTEREST

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inflammatory responses of starfish larvae when impaled with rosebud thorns. He observed that white blood cells from various animals were attracted to bacteria, followed by phagocytosis and killing of pathogens. These studies remain the foundation for our understanding of innate immune responses to infection. There has been an extraordinary expansion in our understanding of neutrophils at the cellular and molecular level. We have learned about bacterial products (e.g. formylated peptides) and host-derived chemoattractant molecules that attract neutrophils to sites of infection and injury. Knowledge has been gained on the molecular mechanisms by which neutrophils sense microbial products and traffic to sites of infection. Key pathways for neutrophil-mediated host defense (e.g. NADPH oxidase and neutrophil granular constituents) have been elucidated. In addition, we have learned about distinct modes of neutrophil death and termination of neutrophilic inflammation.

Neutrophils are the first responders to infection and injury. Pathogen motifs and endogenous products of necrosis (damage-associated molecular patterns; DAMPs) activate similar pathogen recognition receptors and downstream signaling in neutrophils. From a teleological standpoint, recruitment and activation of neutrophils by DAMPs is likely to be of benefit following traumatic injury. Advanced cancer has been likened to a wound that does not heal. Conditions within the tumor microenvironment—hypoxia, nutrient starvation, cellular proliferation, and necrosis—lead to the release of DAMPs that can recruit and activate neutrophils. While beneficial in the context of fighting infection, neutrophilic inflammation in the tumor microenvironment has less predictable and less understood effects. Neutrophils have the capacity to kill tumor cells. However, specific neutrophil responses may promote tumor progression through a number of signaling pathways, including interaction with tumor, inflammatory, and stromal cells in the tumor microenvironment.

A major theme of our review will be to understand neutrophilic inflammation in cancer in the context of a persistent wound. We explore the roles of neutrophils in antimicrobial host defense and how those responses are co-opted in the tumor microenvironment in ways that can promote tumor progression. Tumor-derived factors (e.g. granulocyte-colony stimulating factor; G-CSF) can expand granulopoiesis, leading to an increased accumulation of mature and immature granulocytic populations in circulation, in the primary tumor, and in distant organs where they can create a “premetastatic niche” that facilitates tumor seeding. The interactions between neutrophils and platelets while trafficking to tumor and their potential effects on tumor cell signaling are examined. In addition, we discuss the relationship between activated neutrophils and T cell signaling, including the potential for mature neutrophils to suppress T cell responses in the tumor microenvironment. There is also a growing appreciation for heterogeneity among granulocytic cell populations in the tumor micro-environment. This heterogeneity relates to their maturation status, transcriptome profiles, and functional roles, which can either be pro-tumorigenic or favor tumor control. We consider the effects of cancer therapy on neutrophils and how neutrophils may affect tumor responses. Finally, we propose future directions for research that targets neutrophils as novel strategies to enhance immunotherapy for cancer.

2 IN THE TUMOR MICROENVIRONMENT: THE TWO FACES OF INFLAMMATION

The presence of white cells within tumors was observed in the 19th century by Rudolf Virchow and raised the notion that inflammation may play a role in cancer. It is now recognized that inflammation plays critical roles at multiple stages of cancer: initiation, growth, metastasis, and response to therapy. While genetic damage is required for tumor initiation, the inflammatory responses may contain or eradicate the tumor or be the “fuel that feeds the flames”.¹ Nuclear factor kappa B (NF- κ B), a critical driver of inflammation, can promote inflammation-associated cancer,² but acts in a cell type-specific manner by influencing survival genes in the tumor and pro-inflammatory responses in the tumor microenvironment.³ Importantly, immune subsets that promote tumor progression possess plasticity and can be therapeutically depleted or re-programmed.⁴

It is now well-established that cytotoxic T lymphocytes (CTL) are critical effectors of anti-tumor immunity. We observed extraordinary advances in cancer immunotherapy based on strategies that augment anti-tumor CTL function, including the use of checkpoint inhibitors, genetically modified T cells, and vaccine-based approaches. In 2013, *Science* selected cancer immunotherapy as the “Breakthrough of the Year.” Harnessing natural killer cells and antibody-dependent anti-tumor immunity are additional examples of promising research. With these advances, it becomes even more important to understand immune responses that can enhance or obstruct durable anti-tumor immunity.

There are numerous obstacles to anti-tumor immunity, including low immunogenicity of cancer-specific antigens, poor trafficking of effector T cells to the tumor microenvironment, and induction of immunosuppressive pathways. Tumor-associated macrophages (TAMs) possess a similar phenotype to the alternatively activated (M2) macrophages involved in wound healing, and also suppress T cells.^{5–8} B7-H4-expressing macrophages constitute a specific TAM population that suppress tumor-specific T cell immunity, and are a target for immunotherapy.^{5,9} Regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and immune checkpoint molecules (e.g. CTLA-4 and PD-1) are additional examples of immunosuppressive pathways that accumulate in the tumor microenvironment and impede T cell immunity. Seen in this light, both mature neutrophils and immature granulocytic cells are important components of the tumor microenvironment that can influence the balance between tumor control and escape. Neutrophilic recruitment and activation can have broad effects on tumor cells and the microenvironment, which include direct cellular injury from release of oxidants and granular constituents, remodeling of the extracellular matrix, release of pro-angiogenic products, and cross-signaling to other inflammatory cells and tumor stromal constituents. Granulocytic cells can also affect tumor progression independently of T cell immunity. Pekarek et al.¹⁰ showed that granulocyte depletion inhibited the growth of tumor cells in nude mice, which are deficient in cellular immunity. However, one of the most translationally important effects of tumor-associated neutrophils (TANs) relates to their effect on CTL responses. To understand the mechanisms by which neutrophils influence the tumor microenvironment, it is of value to understand neutrophil biology in the context of antimicrobial host defense and wound repair.

3 THE GOOD AND BAD OF WOUND REPAIR RESPONSES

Acute neutrophilic inflammation and wound repair responses evolved to act in a concerted and stepwise manner to target infections and to promote repair of damaged tissue (Fig. 1A). The innate immune system is rapidly activated by injury, such as trauma or bacterial infection. In the first stage, neutrophils and platelets are activated and recruited to the site of injury. Following elimination of the acute insult (e.g. spontaneous drainage of an abscess or removal of a foreign body), the next stage of wound healing involves a transition to a less injurious monocytic inflammatory response. Macrophages help to clear neutrophilic inflammation through efferocytosis (removal of dying cells by phagocytosis). In addition, efferocytosis of apoptotic neutrophils by macrophages can limit neutrophilic inflammation by suppression of IL-23/IL-17-dependent granulopoiesis.¹¹ The next stage involves resolution of the wound, characterized by repair, regeneration, remodeling of tissue, and fibrosis. M2 macrophages are thought to facilitate wound healing by suppressing inflammatory responses and producing pro-angiogenic factors. In contrast, pathological wound healing is characterized by a persistent and disorganized inflammatory response in which neutrophil recruitment and injury are ongoing and regenerative responses are ineffective (Fig. 1B). In this review, we will discuss neutrophil biology in the tumor microenvironment in the context of pathological wound healing.

Thirty years ago, Dvorak¹² referred to cancer as a wound that does not heal, and noted the similarities between tumor stroma generation and wound healing responses. Indeed, the tumor microenvironment is characterized by persistent injury from a number of causes (e.g. ischemia, nutrient starvation, and inflammation) that co-opts the normal wound healing responses in ways that can promote tumor spread. Pathological wound healing responses result in activation of pro-angiogenic and tissue remodeling pathways that may facilitate metastasis. In addition to these shared features with persistent wounds unrelated to cancer (e.g. from ischemia or infection), cancer has its own unique features, including the release of tumor-derived factors that can influence marrow reprogramming and neutrophil recruitment and activation.

4 GRANULOCYTIC EXPANSION DURING CANCER

Advanced cancer is associated with an expansion of the granulocytic compartment, including both mature neutrophils and immature granulocytic cells. Elevations in circulating neutrophils, the neutrophil to lymphocyte ratio, and tumor-infiltrating neutrophils have been associated with poor outcomes in several cancers, including hepatocellular, head and neck, lung, and gastrointestinal malignancies.^{13–20} In patients with advanced ovarian cancer, the pretreatment circulating neutrophil count²¹ and the neutrophil to lymphocyte ratio²² correlated with poor outcomes.

The potential causes for expansion and increased circulation of neutrophils in advanced cancer are multi-factorial. A hallmark of advanced cancer is cellular necrosis, which is associated with the release of DAMPs that may stimulate neutrophilic leukocytosis. In addition, tumor-derived cytokines and growth factors can promote myeloid differentiation. Wu et al.²³ showed that circulating hematopoietic stem and progenitor cells (HSPCs) from

patients with solid tumors had “myeloid-biased differentiation”. Tumor-derived G-CSF, granulocyte-macrophage-colony stimulating factor (GM-CSF), and interleukin-6 (IL-6) promoted myeloid differentiation of HSPCs, and circulating myeloid precursors (granulocyte-macrophage progenitor; GMP-positive) correlated with advanced disease and were enriched in tumor.²³ Casbon et al.²⁴ showed that tumor-derived G-CSF resulted in reprogramming of hematopoiesis in bone marrow that stimulated granulocyte expansion. This reprogramming occurred in early stage disease and resulted in systemic expansion of circulating neutrophils that were T cell suppressive.²⁴ Strauss et al.²⁵ identified the retinoic-acid-related orphan receptor 1 (RORC1) as a key pathway driving tumor-stimulated myelopoiesis in response to colony stimulating factors. This myeloid bias is not unique to cancer. Rather, it is a hallmark of infection, trauma, and other causes of systemic injury. The unique feature of cancer relates to tumor-derived factors that are a persistent stimulus for marrow reprogramming skewed toward myeloid differentiation.

Activated neutrophils can promote metastasis by stimulation of tumor invasion at the primary site. In addition, there is growing recognition of a “premetastatic phase” in which tumor-derived factors stimulate hematopoietic mobilization and tissue-specific responses that enhance metastasis by preparing a distant site for metastatic seeding.^{26–28} Angiogenesis, which is stimulated by injury and activated myeloid cells, plays a critical role in metastasis. Bv8 proteins (named for the frog *Bombina variegata*), also known as prokineticin 1 (Prok1) and prokineticin 2 (Prok2), are upregulated by G-CSF and mobilize myeloid cells from marrow and promote angiogenesis in tumors.²⁹ Anti-Bv8 treatment suppressed circulating and tumor-infiltrating myeloid cells and angiogenesis, and reduced tumor growth in mice.²⁹ The benefit of anti-Bv8 on tumor vascularization and growth occurred at early stages of tumor burden.³⁰ In addition, anti-G-CSF or anti-Bv8 antibodies significantly reduced metastasis in tumor-bearing mice.³¹ These results point to Bv8-expressing granulocytic cells mobilized by G-CSF driving metastasis and as potential therapeutic targets to prevent metastasis at early stages of disease. Wculek et al.³² showed in orthotopic mammary tumor-bearing mice that neutrophils accumulated in the lung before cancer cells and their numbers increased during metastatic progression. Neutrophils in the premetastatic lung augmented the tumorigenic potential of cancer cells in vivo and in vitro, and the pro-metastatic effect of neutrophils was dependent on leukotriene generation.³²

Although a growing body of literature points to activated neutrophils driving tumor progression, this is not a universal finding. Granot et al.³³ showed that neutrophil accumulation in the lung protected mice from mammary tumor metastasis, an effect that was mediated by reactive oxidant generation and tumor-secreted CCL2. Blaisdell et al.³⁴ identified a protective role for neutrophils in a mouse model of PTEN-deficient uterine cancer. In this model, neutrophils were recruited by the hypoxic tumor microenvironment, and their infiltration led to detachment of tumor cells from the basement membrane, and a reduction in tumor growth and metastasis.³⁴ The potential for TANs to be friends or foes in cancer³⁵ is a recurrent theme of this review, and emphasizes the importance of context-dependent factors in the tumor microenvironment and neutrophil heterogeneity that modulate the interactions between neutrophils and cancer.

MDSCs are a subset of heterogeneous myeloid cells, which have been shown to expand in response to tumor-derived factors, and enhance tumor progression by abrogating T cell immunity and stimulating pro-angiogenic and tissue remodeling responses.³⁶ The most important criterion for defining an MDSC population relates to their ability to suppress stimulated T cell activation. However, this hallmark feature is not unique to MDSCs, since mature neutrophils can acquire a suppressive phenotype. In addition, MDSCs are not unique to cancer. Granulocytic populations mimicking MDSCs based on surface marker expression and capable of suppressing T cell responses have been observed in patients with sepsis.^{37,38}

MDSCs have been classified as monocytic and granulocytic based on surface marker expression. These broad categorizations of MDSCs underestimate their heterogeneity regarding tumor-specific factors that promote their expansion or their prognostic significance in circulation and in the tumor microenvironment.³⁹ Granulocytic MDSCs in mice are typically defined based on a combination of myeloid (CD11b⁺) and granulocytic (Ly6G⁺) markers and negative or low expression of the monocytic marker, Ly6C.^{40,41} In humans, there remains a lack of agreement on the optimal markers for MDSCs. A recent interim study to establish consensus criteria for defining MDSCs identified as many as ten putative MDSC populations from peripheral blood mononuclear cells of normal volunteers.⁴² Human granulocytic MDSCs were defined as a population of cells resembling granulocytes that express the granulocytic markers, CD15 and CD66b, and do not express CD14.⁴² This study showed high inter-laboratory variability in putative MDSC populations, especially the granulocytic subsets.⁴²

Another feature commonly associated with MDSCs relates to their immaturity. However, the identification of immunosuppressive mature neutrophils highlights the notion that both the maturational status of granulocytic cells and the inflammatory context can influence their suppressive phenotype. While neutrophils, macrophages, and dendritic cells (DCs) are examples of mature myeloid cells, MDSCs have been proposed to develop from a block in myeloid differentiation. Consistent with this notion, Solito et al.⁴³ identified a human promyelocytic-like population with T cell suppressive activity that was phenotypically similar to MDSCs present in the blood of patients with advanced solid tumors, and correlated with worse prognosis. Very recent evidence points to a key role for HSPCs in promoting tumor expansion through effects on the tumor microenvironment. In tumor-bearing mice, HSPCs are mobilized from the bone marrow to the tumor microenvironment where they differentiate to MDSCs, promoting expansion and metastasis.⁴⁴ In addition, circulating HSPCs in newly diagnosed cancer patients correlated with increased risk of metastasis.⁴⁴

Colony stimulating factors and cytokines promoting MDSCs alter the transcriptional regulation of myelopoiesis. Marigo et al.⁴⁵ noted a key role for activation of C/EBP β transcription factor by G-CSF, GM-CSF, and IL-6 in the expansion of MDSCs that abrogated tumor-specific T cell immunity. In addition, tumor-derived products can activate STAT3 signaling in myeloid cells that can lead to inhibition of DC maturation and stimulation of MDSC development.^{46–49} Waight et al.⁴⁷ identified interferon regulatory factor-8 (IRF-8), a transcriptional regulator of myeloid differentiation and lineage commitment, as a negative regulator of human MDSCs. G-CSF and GM-CSF resulted in

IRF-8 downregulation via STAT3- and STAT5-dependent pathways, while IRF-8 overexpression attenuated MDSC accumulation and enhanced immunotherapeutic efficacy in tumor-bearing mice.⁴⁷ While studies have shown the importance of NOX2 in promoting MDSC accumulation and immunosuppression in tumor-bearing mice,⁵⁰ we found that the presence of NOX2 did not affect MDSC accumulation or tumor progression.⁵¹

Together, studies from patients with cancer and mouse models point to cancers reprogramming the marrow resulting in an expansion of myeloid cells, including granulocytic cells. These granulocytic cells infiltrate tumor and can promote tumor progression by inducing tumor cell proliferation, stimulating angiogenesis and matrix remodeling, and disabling T cell-dependent anti-tumor immunity. In certain contexts, an expansion of a specific myeloid lineage occurs, while other settings result in an expansion of multiple heterogeneous lineages. Gaining more mechanistic knowledge about what drives this “myeloid bias”²³ may lead to new therapeutic approaches and opportunities to enhance existing standard treatment.

5 NEUTROPHILS, NEUTROPHIL EXTRACELLULAR TRAPS, AND PLATELETS IN TUMOR PROGRESSION

Neutrophils and platelets are rapidly recruited to sites of injury. This trafficking is mediated by coordinated ligand-receptor interactions involving platelets, neutrophils, and endothelial cells. P-selectin expressed on platelets recognizes and is activated by its ligands, PSGL-1 and CD24, expressed on endothelial cells. Activated platelets induce endothelial expression of ICAM-1, resulting in increased neutrophil adhesion. Consequently, inhibition of neutrophil-platelet interactions protected against acute lung injury in mouse models.^{52,53} Activation of PSGL-1 on neutrophils results in the redistribution of receptors that mediate neutrophil recruitment and trafficking. PSGL-1 is a docking site for activated platelets during inflammation, and inhibition of PSGL-1 was protective in models of organ injury.⁵⁴ Platelets release microparticles during inflammation, which are internalized by activated neutrophils and amplify inflammation in arthritis.^{55,56} In addition, activated platelets release mitochondria into the extracellular environment, which, similar to bacteria, activate and interact with neutrophils in vivo, triggering neutrophil adhesion to the endothelial wall.⁵⁷ Once neutrophils are recruited to the extravascular space, activation of integrins and leukotriene B₄, as well as other chemoattractants, leads to neutrophil clustering in the damaged site and the formation of a wound seal.⁵⁸ Together, co-activation of neutrophils and platelets serves to contain infection. There is precedent for pathogens evading host defense by abrogating neutrophil-platelet interactions⁵⁹; however, there is also growing evidence for these same antimicrobial and wound healing pathways accelerating tumor progression.

Activated neutrophils can elicit anti-tumor functions. They are capable of direct lysis of tumor cells⁶⁰ and killing tumor cells by antibody-dependent cell-mediated cytotoxicity (ADCC).^{61,62} Mittendorf et al.⁶³ showed that breast cancer cells took up neutrophil elastase (NE) released by TANs, which enhanced their susceptibility to CTL lysis. Despite these anti-tumor properties of neutrophils, the overall effect of granulocytic cell expansion and accumulation in the tumor microenvironment is often weighted toward driving tumor growth

and spread. To understand the effect of neutrophils on tumor, it is important to review basic mechanisms of neutrophil-mediated antimicrobial host defense.

Activated neutrophils kill microbes through oxidant-dependent and -independent pathways. The phagocyte NADPH oxidase (NOX2) is a critical enzyme in antimicrobial host defense and in regulating inflammation. Following NOX2 activation, molecular oxygen is converted to superoxide anion and to downstream metabolites, including H_2O_2 and hydroxyl anion. In neutrophils, myeloperoxidase (MPO) converts H_2O_2 to hypohalous acid.^{64–66} In a series of studies by Clark et al.^{67–70}, neutrophil-mediated killing of tumor cells *ex vivo* was dependent on activation of the MPO- H_2O_2 -halide system. In addition, cationic proteins from human neutrophil granules, which are activated by NOX2,⁷¹ were cytotoxic against tumor cells.⁷² However, activation of the neutrophil NOX2/MPO system can also abrogate T cell and natural killer cell function.^{73–75} In murine syngeneic ovarian cancer, tumor progression was similar in wildtype and NOX2-deficient mice, demonstrating a lack of effect of NOX2.⁵¹ Although *ex vivo* studies cannot model the complexity of tumor biology *in vivo*, these studies raise the notion that different tumors and the inflammatory milieu of the tumor microenvironment may influence the sensitivity of tumor cells to neutrophil-generated reactive oxidant attack.

In addition to pathogen killing via oxidant injury from NOX2/MPO activation, NOX2 can augment host defense by intracellular activation of granular proteases⁷¹ and generation of neutrophil extracellular traps (NETs).^{76,77} NETosis is a distinct mode of neutrophil death characterized by the breakdown of membranes and extracellular release of stretches of DNA, histones, and granular constituents.⁷⁷ NETosis can be stimulated by bacterial and fungal infections,^{78,79} as well as non-infectious insults that can mimic infection.^{80–83} It can occur through NOX2-dependent and -independent pathways.⁸⁴ While apoptosis of neutrophils results in non-inflammatory cell death, NETs release products that augment antimicrobial host defense but also cause tissue injury.^{82,83,85,86} In the tumor microenvironment, these same pathways that degrade and remodel extracellular matrix may promote tumor cell migration and invasion.

NETs are pro-thrombogenic *in vivo*, likely due to the released extracellular stretches of chromatin.^{87–92} In addition, NETs release tissue factor, a key component in the coagulation cascade.⁹³ In mouse models, extracellular chromatin and tissue factor contribute to the pathogenesis of deep venous thrombosis, and depletion of NETs results in limiting thrombosis.^{91,92} Peptidylarginine deiminase 4 (PAD4), an enzyme that mediates histone citrullination and NETosis, was required for experimental deep venous thrombosis.⁹⁴ Platelets activated by toll-like receptor 4 signaling can stimulate NETosis during sepsis.⁹⁵ In addition, P-selectin expressed on platelets, through binding to its ligand PSGL-1 on neutrophils, promotes NETosis.⁹⁶ Thus, in the context of inflammation and injury, activated neutrophils and platelets can cross-signal in a positive feedback loop. While in the context of infection, NETosis and activation of thrombosis may limit microbial spread, these same pathways may promote tumor progression.

Cancer-associated thrombosis is a major cause of morbidity and mortality. There is growing evidence for NETs playing a role in cancer-associated venous and arterial thrombosis,^{90,97}

and in cancer progression in tumor-bearing mice.^{98–100} NETs have been identified in a number of cancers in patients, including sarcoma,¹⁰¹ pancreatic cancer,¹⁰² and ovarian cancer (manuscript in preparation). Tumors, as well as their microenvironments, release DAMPs, hypoxia-inducible factors, and pro-inflammatory cytokines, which can attract neutrophils and induce NETosis.^{100,102,103} Demers et al.⁹⁰ showed that in tumor-bearing mouse models, chromatin released by NETs stimulated thrombosis. In addition, systemic infection in tumor-bearing mice resulted in large quantities of chromatin release and worsening of the prothrombotic state.⁹⁰ In small intestinal tumor, lipopolysaccharide induced complement-dependent NETosis resulting in hypercoagulation and metastasis.⁹⁹ Cools-Lartigue et al.⁹⁸ showed in a mouse model of concurrent sepsis and metastatic tumor that NETs trapped circulating tumor cells and promoted metastasis. It is unclear from these results whether targeting tumor-stimulated NETs in the absence of concurrent sepsis would limit metastasis. In patients undergoing curative-intent liver resection for metastatic colorectal cancer, Tohme et al.¹⁰⁰ observed that increased postoperative NET formation was associated with a significantly shorter disease-free survival. Consistent with these results, ischemia reperfusion injury in tumor-bearing mice worsened metastasis, an effect that was abrogated by inhibition of NETosis.¹⁰⁰

NETs may facilitate tumor progression through promotion of thrombosis and angiogenesis. Thrombosis can accelerate tumor progression through several pathways, including recruitment of inflammatory cells to the tumor microenvironment, and stimulation of tumor cell migration and adhesion to endothelial cells and of the production of vascular endothelial growth factor (VEGF) and other pro-angiogenic products that increase metastatic potential.¹⁰⁴ In addition, NETs in tumor-bearing mice were associated with vessel damage and organ injury.¹⁰⁵

There is also growing evidence that activated platelets can drive tumor progression. Thrombocytosis has been observed in patients with advanced solid tumors as well as other conditions associated with increased inflammation, such as autoimmune disease. Thrombocytosis at diagnosis of advanced ovarian cancer predicted worse survival.¹⁰⁶ Paraneoplastic thrombocytosis, in addition to being a correlate of advanced cancer, can drive tumor progression through a number of pathways, including stimulation of tumor cell aggregation and proliferation, and the release of growth factors and pro-angiogenic factors.¹⁰⁷ In addition, activated platelets cross-signal to tumor cells to induce epithelial-mesenchymal transition (EMT), which is associated with greater metastatic potential and chemotherapy resistance.^{108–110} Platelet-derived transforming growth factor-beta (TGF- β) plays a key role in EMT, and can act in concert with platelet-derived growth factors and platelet-tumor cell contacts to promote EMT.^{108–110}

TGF- β has multiple signaling properties depending on the inflammatory context. In the context of wound healing, TGF- β plays an essential role in fibroblast activation and scar formation.^{111,112} The effect of TGF- β on T cells varies based on the inflammatory context, and has the potential to stimulate both IL-17-producing lymphocytes and Tregs^{113,114} and to impair CTL activity.¹¹⁵ Within the tumor microenvironment, TGF- β can also induce a population of neutrophils with an immunosuppressive pro-tumorigenic phenotype.^{116,117} Labelle et al.¹¹⁸ showed that platelets stimulate recruitment of neutrophils to tumors that

promote metastasis at early stages of cancer in tumor-bearing mice. These results suggest that during pathologic wound healing in cancer, TGF- β derived from platelets and other cells may have important roles in shaping neutrophil responses and tumor cell biology that promote metastasis. Together, these results support a model in which the tumor cells or their environment stimulate neutrophil and platelet activation through a number of pathways: tumor-derived products, DAMPs, production of pro-inflammatory cytokines and chemokines, and vascular injury and extravasation. These stimuli lead to an inflammatory and thrombogenic cascade that mimic those induced by infection and trauma, and serve to control infection, stop bleeding, and promote wound healing. In the context of cancer where injury is persistent, these antimicrobial and wound repair pathways may promote tumor progression and metastasis through their direct influence on tumor cell biology, and through their influence on inflammation and thrombosis in the tumor microenvironment that create a metastatic niche.

6 EFFECTS OF NEUTROPHILS ON CELL-MEDIATED IMMUNITY

The mechanisms by which neutrophils may alter CTL responses are likely to be the most translationally important, as understanding the roles that neutrophils play in the context of tumor progression and response to immunotherapy may lead to novel strategies to enhance treatment. Following accumulation at sites of infection, neutrophils are able to migrate to draining lymph nodes.^{119,120} This new finding demonstrates the potential for neutrophils to engage with lymphocytes and antigen-presenting cells, at the precise site of inflammation and also at the draining lymph nodes. Neutrophils may interact at these sites either by cell contact or via mediators, such as reactive oxidants, release of granular constituents, and cytokines; and the overall effect may be to prime or dampen antigen-dependent immune responses. During experimental bacterial skin infection, neutrophils migrated from inflamed skin into lymph nodes where they augmented lymphocyte proliferation.¹¹⁹ Lim et al.¹²¹ showed that during influenza infection in mice, the early recruitment of neutrophils to lungs was essential for cell-mediated immune protection. Neutrophils released CXCL12 required for CD8⁺ T cell recruitment and effector functions.¹²¹ In other contexts, activated neutrophils can suppress T cell responses. Immunosuppressive neutrophils are not unique to the tumor microenvironment. During acute systemic inflammation induced by lipopolysaccharide, release of hydrogen peroxide from a subset of activated neutrophils led to suppression of T cell proliferation.¹²² Neutrophils were also shown to limit the spread of T cell responses from draining to distal lymph nodes following protein immunizations.¹²³

TANs exhibit heterogeneity in their capacity to promote or impede T cell immunity. There is growing evidence that neutrophils promote tumor progression through a dual effect of inhibition of CTL responses and promotion of myeloid inflammation and angiogenesis. Langowski et al.¹²⁴ showed that IL-23 promoted tumor incidence and growth in mice. IL-23 stimulated the expansion of IL-17A-producing lymphocytes that, in turn, stimulated myeloid growth factors and pro-inflammatory cytokines that drive neutrophilic inflammation. While important for antimicrobial host defense, the IL-23/IL-17A axis is also associated with autoimmunity. In tumor-bearing mice, IL-23 increased neutrophil and macrophage accumulation in tumors and stimulated angiogenesis and upregulation of matrix metalloproteinase-9 (MMP-9), but reduced CD8⁺ T cell infiltration into the tumor.¹²⁴ MMP-9

is principally expressed in neutrophils, macrophages, and mast cells, and contributes to tumor invasiveness by tissue remodeling and angiogenesis.^{125–127} In tumor-bearing mice, IL-17 stimulated the expression of G-CSF, leading to myeloid cell mobilization and recruitment of Bv8-positive granulocytes to the tumor microenvironment, and promoted resistance to anti-VEGF treatment.¹²⁸ These results point to the IL-23/IL-17 axis as another mechanism to reprogram tumor-associated inflammation from anti-tumor CTL responses towards pro-inflammatory and pro-angiogenic pathways that promote tumor progression.¹²⁹

In a mouse model of colorectal tumor, IL-23 produced by myeloid cells augmented intratumoral IL-17 response and promoted tumor growth and progression.¹³⁰ The authors proposed a model in which barrier erosion by tumor enabled mucosal invasion by bowel flora that triggered IL-23-dependent inflammation and acceleration of tumor growth.¹³⁰ These results are consistent with tumor-infiltrating CD8⁺ and Th1 cells predicting prolonged disease-free survival, and Th17 inflammation predicting poor prognosis in patients with colorectal cancer.¹³¹ Coffelt et al.¹³² showed that IL-1 β stimulated IL-17 expression from $\gamma\delta$ T cells, resulting in G-CSF-dependent expansion of neutrophils in a mouse model of spontaneous mammary tumor development. Tumor-induced neutrophils suppressed CTL responses, and depletion of IL-17 or G-CSF and absence of $\gamma\delta$ T cells prevented neutrophil accumulation within tumors and abrogated the T cell suppressive phenotype of neutrophils. In addition, the absence of $\gamma\delta$ T cells and neutrophils limited tumor metastasis without affecting the size of the primary tumor. Activated neutrophils can suppress T cell responses through a number of mechanisms. TANs, for instance, can stimulate T cell exhaustion. Koyama et al.¹³³ showed that ablating the tumor suppressor genes STK11/LKB1 in a mouse model of KRAS-driven non-small cell lung cancer resulted in the accumulation of neutrophils with T cell suppressive function, and increased expression of exhaustion markers on T cells. TANs were also shown to contribute to an immunosuppressive environment through recruitment of Tregs.¹³⁴

Arginase-1 (Arg-1) is expressed in neutrophil tertiary granules, and released during neutrophil activation.¹³⁵ Arg-1 converts arginine to ornithine, a precursor of proline required for wound healing. Release of Arg-1 by neutrophils can lead to depletion of arginine, resulting in T cell suppression.^{136,137} Activation of neutrophils can stimulate Arg-1 release and induce phenotypic and functional changes similar to granulocytic MDSCs.¹³⁸ As shown in Fig. 2, there can be heterogeneity among neutrophil populations in the tumor microenvironment regarding Arg-1 expression. In mice with spontaneous mammary tumor development, iNOS expression in neutrophils was also associated with a T cell suppressive phenotype.¹³² These results point to modulation of arginine metabolism as a potential mechanism for activated neutrophils to suppress T cell responses.

7 REVISITING THE CONCEPT OF NEUTROPHIL HETEROGENEITY

Modern tools for cellular and transcriptome analyses have led to an appreciation for the extraordinary heterogeneity among macrophages regarding their cellular origin and biological properties.^{139,140} The notion of neutrophil heterogeneity—that neutrophils are made up of biologically distinct populations—has been suggested for decades. Earlier studies largely relied on surface marker expression on neutrophils and differences in

neutrophil responses to ex vivo stimuli to define unique populations.^{141–145} In a prescient paper published more than 30 years ago, Gallin¹⁴⁶ noted the existence of neutrophil heterogeneity, but asked whether it was meaningful. With regard to tumor immunology, the answer is almost certainly yes. There has been a greater appreciation for the potential of neutrophil heterogeneity, including distinct modes of neutrophil death (apoptosis, necrosis, NETosis), reverse transendothelial migration, the ability to present antigen, and acquisition of phenotypes that promote or inhibit adaptive immunity.

One of the major distinctions between neutrophil populations in cancer relates to the N1 versus N2 classification. N1 TANs can promote anti-tumor effector responses both by directly targeting tumor cells and by stimulation of T cell immunity. In contrast, N2 TANs facilitate tumor progression by suppressing T cell responses and upregulating angiogenic factors (e.g. VEGF and MMP-9).^{127,147,148} Sagiv et al.¹¹⁷ recently expanded on these results by characterizing three distinct neutrophil populations in cancer: mature high-density neutrophils, mature low-density neutrophils, and immature low-density neutrophils. The mature high-density neutrophils had an N1-like phenotype and the capacity to kill tumor cells. The low-density population included immature neutrophils (granulocytic MDSCs) and mature neutrophils with T cell suppressive capacity. When treated with TGF- β , the mature high-density neutrophils polarized to become low-density neutrophils, and acquired a suppressive phenotype. Importantly, the immunosuppressive low-density neutrophils increased in relation to tumor burden.

Neutrophils expressing major histocompatibility complex class II and costimulatory molecules have been detected at inflammatory sites, and can mimic properties of professional antigen-presenting cells. Similar to professional antigen-presenting cells, neutrophils recruited T cells via CXCL9 and CXCL10^{116,149} and cross-primed CD8⁺ T cells in vivo and stimulated CTL effector functions.¹⁵⁰ Duffy et al.¹⁵¹ showed that following intradermal injection of a vaccinia virus, neutrophils transported virus from the dermis to the bone marrow and were required for generation of virus-specific CD8⁺ memory T cells. Interestingly, neutrophils were also shown to differentiate into a hybrid population expressing surface markers for both neutrophils and DCs, which were capable of clearing bacteria and generating NETs (characteristics of neutrophils) and presenting antigens to naïve CD4⁺ T cells (characteristic of DCs).^{152,153} It is unknown if these neutrophil-DC hybrids are relevant in the tumor microenvironment or in the context of immunotherapy. However, at a conceptual level, these observations raise the potential for reprogramming neutrophils to a DC phenotype that could be exploitable for tumor antigen presentation and augmentation of cellular immunity.

Neutrophil variability in cancer can result from changes at multiple levels, including epigenetics, metabolic, oxidant generation, post-translational changes, and cross-signaling between cells. We do not know how durable these changes are. As we learn more about the cues that regulate neutrophil diversity, new approaches for their therapeutic modulation are expected to emerge.

8 THE OVARIAN CANCER MICROENVIRONMENT: A CASE STUDY ON PERSISTENT INFLAMMATION AND IMMUNOSUPPRESSION

Ovarian cancer is commonly diagnosed at advanced stages. Standard treatment for advanced disease involves cytoreductive surgery and chemotherapy. Although the majority of patients respond to chemotherapy, the median progression-free survival for women with stage III epithelial ovarian cancer was 17 months.¹⁵⁴ There is growing recognition that immune responses in the pretreatment tumor microenvironment are both prognostic biomarkers and potential targets for therapeutic modulation in patients with advanced ovarian cancer. The critical role of immune surveillance in ovarian cancer was demonstrated by correlation of survival with tumor-infiltrating lymphocytes.¹⁵⁵ Intraepithelial CD8⁺ cell accumulation and a high CD8⁺/Treg ratio were associated with favorable prognosis¹⁵⁶ while increased Treg accumulation predicted worse outcome¹⁵⁷ in patients with advanced ovarian cancer.

There is growing evidence that the tumor microenvironment in ovarian cancer is inflammatory, characterized by mature and immature myeloid cell accumulation and expression of pro-inflammatory cytokines and chemokines, as well as immunosuppressive.^{158–164} In pretreatment ascites of patients with ovarian cancer, the myeloid cell population consists of mature macrophages, immature myeloid cells, and granulocytic cells with variable immunosuppressive phenotypes.^{8,165} Accumulation of B7-H4-expressing macrophages in the tumor microenvironment impeded T cell responses and correlated with more rapid tumor progression.^{5,166} Tumor cells and TAMs produce the chemokine CCL22, which mediates trafficking of Tregs to the tumor leading to suppression of tumor-specific T cell immunity.¹⁵⁷

It has been recognized for several years that the ascites in patients with ovarian cancer is thrombogenic, and that coagulation and fibrinolysis could affect tumor invasion.^{167–169} Stone et al.¹⁰⁶ showed that paraneoplastic thrombocytosis in advanced ovarian cancer was driven by IL-6, and correlated with poor prognosis. As previously described, there are several mechanisms by which activated platelets can affect tumor progression, including release of pro-angiogenic factors, cross-signaling with neutrophils, stimulation of EMT, and promotion of a premetastatic niche^{110,118} In addition, platelets increase the proliferation of ovarian cancer cells through TGF- β -dependent signaling.¹⁷⁰ In murine ovarian cancer, platelets were shown to reduce the efficacy of chemotherapy.¹⁷¹

The tumor microenvironment of ovarian cancer is associated with release of products of necrosis that activate innate immune responses. We found that when treated with cell-free ascites, normal donor neutrophils underwent NETosis and suppressed stimulated T cell proliferation in a cell contact-dependent manner (Singel K.L., A.N.H. Khan, T.R. Emmons, P.C. Mayor, K.B. Moysich, K. Odunsi, and B.H. Segal. 2016. Ovarian cancer ascites-activated neutrophils suppress T cell proliferation in a contact-dependent mechanism. *J. Immunol.* 196: 211.16 (Abstr.)). Together these results support a model in which DAMPs in the ovarian cancer tumor microenvironment stimulate innate immune responses and platelet activation that normally function to control infection and promote wound repair. We speculate that these responses may augment tumor cell invasion and metastasis through several pathways (e.g. enhanced tumor cell seeding onto serosa, release of growth factors

and stimulation of angiogenesis, and cross-signaling to tumor cells to promote proliferation and EMT).

9 EFFECTS OF ANTI-NEOPLASTIC TREATMENT ON NEUTROPHILS

In the clinic, myeloid cell responses are not a static process. Cytotoxic chemotherapy results in marrow hypoplasia followed by recovery. Cytotoxic chemotherapy and radiation cause death of tumor cells, but also cause bystander injury to non-tumor cells that can affect the skin, mucosa, and visceral organs. Chemotherapy and radiation are administered in cycles, resulting in repeated tissue injury and wound repair responses. Some of the immune responses to cytotoxic regimens may be beneficial. For example, chemotherapy-and radiation-induced tissue necrosis can result in DAMP release that activates DCs. In addition, specific chemotherapy regimens can target MDSCs, and enhance anti-tumor T cell immunity.^{172,173} However, Bruchard et al.¹⁷⁴ showed that chemotherapy also triggered inflammasome activation in MDSCs that activated IL-1 β and IL-17 responses and inhibited anti-tumor immunity. Treatment with an IL-1 receptor antagonist enhanced the effect of chemotherapy. In addition, myeloid cell recruitment may be an obstacle to radiation therapy. Inhibition of Mac-1 (CD11b/CD18) enhanced tumor response to radiation by reducing myeloid cell recruitment in tumor-bearing mice.¹⁷⁵

Major surgery, including tumor resection, can activate innate immune responses that mimic infection (e.g. fever and leukocytosis). Wound repair entails rapid activation of thrombosis followed by regenerative responses. Particularly in the setting of non-curative intent surgery in which non-resected tumor remains (e.g. primary debulking for metastatic ovarian cancer or nephrectomy for metastatic renal cancer), we do not adequately understand how wound repair responses to surgery affect cancer progression. Resection of tumor is expected to reduce the levels of tumor-derived factors that drive granulocytic responses, including TANs and MDSCs. However, at the same time, wound repair responses activated by surgery may also drive angiogenesis and immunosuppressive responses. Predina et al.¹⁷⁶ showed that incomplete resection of tumor in mice led to the accumulation of immunosuppressive M2 macrophages and Tregs in recurrent tumors that were barriers to anti-tumor vaccines. Surgery can also stimulate NETs that stimulate progression of metastatic tumor.¹⁰⁰

A common side effect for patients undergoing chemotherapeutic treatment for cancer is febrile neutropenia. The National Comprehensive Cancer Network¹⁷⁷ and American Society of Clinical Oncology guidelines¹⁷⁸ recommend a myeloid growth factor as prophylaxis in patients receiving regimens associated with a high risk for neutropenic fever and those with a high risk of infectious complications during neutropenia. The use of recombinant G-CSF to accelerate neutrophil recovery from chemotherapy will, by design, stimulate granulocytic differentiation. We do not fully understand the effects of repeated cycles of chemotherapy and G-CSF on myeloid cell populations within the tumor microenvironment and how they influence tumor cell biology. In murine models, tumor-derived G-CSF can expand MDSCs, worsen tumor progression, and promote resistance to chemotherapy.^{24,47,179–181} These results should not influence clinical practice regarding growth factor support; such decisions should be based on clinical trial data and authoritative guidelines. However, they do underscore the need for additional research regarding the effects of chemotherapy and

growth factor support on the tumor microenvironment with the ultimate goal of improving chemotherapy efficacy and limiting toxicity.

10 FUTURE THERAPEUTIC STRATEGIES TARGETING NEUTROPHILS IN THE TUMOR MICROENVIRONMENT

Future therapeutic options that target neutrophils, both mature and immature granulocytic cells, should be based on an understanding of the heterogeneity of these cells and their diverse roles as both promoters of tumor control and promoters of immune evasion and tumor progression. Detailed studies of neutrophil biology in the tumor microenvironment, including modern approaches for transcriptome profiling at the population and potentially single cell level will add insight into neutrophil heterogeneity, and may form the basis for targeted approaches against populations that drive tumor progression. As we learn more about the plasticity of neutrophils,¹⁸² including the N1/N2 axis, we envision that newer approaches will emerge to skew TANs toward an anti-tumor phenotype. As an example, Andzinski et al.¹⁸³ found that type I interferons induced polarization of TANs to an anti-tumor N1 phenotype in tumor-bearing mice, and similar changes in neutrophil activation were observed in melanoma patients receiving type I interferon therapy. Therapeutic approaches to reprogram neutrophils would need to consider the dynamic phenotypes of TANs both in relation to early versus advanced disease and location within the tumor (peripheral versus central) that affect neutrophil biology based on several factors, including oxygen levels, DAMPs, and cytokines.

As previously discussed, there is growing evidence for NETs driving tumor progression. There are currently several experimental approaches that can deplete NETs, including DNase, antibodies against NET constituents, and small molecule inhibitors of signaling pathways required for NETosis.^{82,83,98,184,185} Small molecule inhibitors of peptidylarginine deiminase inhibit NET generation and have been protective in murine models of lupus and atherosclerosis.^{186–188} Prostaglandin E2 was recently shown to inhibit NET generation.¹⁸⁹ Targeting NETs or specific NET constituents merits further investigation as adjunctive therapies for cancer, initially in tumor-bearing mice.

Since neutrophils traffick to sites of injury, including the tumor microenvironment, they are potentially exploitable for delivery of antineoplastic agents. As a proof of principle, Chu et al.¹⁹⁰ observed in murine melanoma that neutrophils enhanced the delivery of nanoparticles to the tumor and augmented the effect of antibody-mediated immunotherapy.

While tumor-derived factors and the tumor microenvironment can reprogram marrow to drive granulopoiesis and generation of neutrophils with distinct phenotypes, other factors may also do so. For example, recent studies point to the microbiome influencing neutrophil lifespan and function, including the propensity to generate NETs.^{191,192} The influence of the microbiome on neutrophil biology may be of particular relevance in patients with cancer where microbiome alterations can result from several factors, including mucosal injury from chemotherapy and radiation and the widespread use of antibiotics. There is tremendous excitement about learning how the bowel microbiome affects cancer risk and response to therapy,¹⁹³ including to checkpoint inhibitors.^{194,195} Seen in this light, the microbiome in

patients with cancer might also influence neutrophil phenotypes that are relevant to control or progression of cancer, and can potentially be therapeutically modified.

Another emerging concept of potential relevance to tumor immunology is innate immune memory or “trained immunity”. Memory characteristics of innate immunity involves a priming event that modifies innate immune cells such that re-exposure to identical or heterologous stimuli results in a heightened response and augmented host defense.¹⁹⁶ The acquisition of memory is associated with epigenetic and metabolic changes.^{197,198} In a chronic parasitic infection model, activated neutrophils can prime a long-lived effector macrophage phenotype that augments host defense.¹⁹⁹ We speculate that as greater knowledge is gained about trained immunity, including in the context of cancer, that newer vaccines that combine adaptive and innate immune memory will be developed.²⁰⁰

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FIGURE 1.

The good and bad of wound repair mechanisms. (A) When injury (e.g. traumatic injury or acute infection) occurs, the inflammatory response follows an ordered transition during normal wound healing. The first stage of acute inflammation involves neutrophil and platelet recruitment and activation, followed by chronic inflammation involving M2 macrophage accumulation and T cell suppression. Finally, the injurious stimulus is alleviated (e.g. clearance of infection) and resolution of inflammation occurs. Resolution includes tissue regeneration, which requires growth factors, angiogenesis, and fibrosis. (B) Pathologic wound healing is associated with a persistent and disordered inflammatory response. Examples include persistent diabetic wounds and cancer. In these scenarios, neutrophil activation is ongoing, which drives the rest of the inflammatory pathways and prevents resolution from occurring. In the context of cancer, pathologic wound healing can drive tumor progression through a number of pathways that are concurrently activated, including thrombosis and angiogenesis, matrix remodeling, release of growth factors, and T cell suppression

FIGURE 2.

Neutrophils within the tumor microenvironment possess heterogeneity in regard to arginase-1 (Arg-1) expression. Flow cytometric analysis of peripheral blood (A) and ascites (B) from a patient with newly diagnosed ovarian cancer prior to therapy. Blood was collected pre-operatively on the day of surgery, and ascites was collected intra-operatively prior to tumor resection. The upper left panel shows the ungated scatter of all cells within the sample, the upper right panel is the proportion of CD45⁺ cells in the ungated population, the lower left is the proportion of CD15⁺ cells in the ungated population, and the lower right panel shows the CD66b and Arg-1 expression of the CD15⁺ cells. In blood there is a single population of CD15⁺ CD66b⁺ Arg-1⁺ cells, while in ascites two distinct populations were observed: CD15⁺ CD66b⁺ Arg-1 low and CD15⁺ CD66b⁺ Arg-1 intermediate. The difference in Arg-1 expression may relate to differences in transcription or Arg-1 release from activated neutrophils. Neutrophils can be tracked throughout the plots by the purple-colored cells