Circulating Tumor Cell-Neutrophil Tango along the Metastatic Process

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Abstract

The crosstalk between cancer cells and the immune system is crucial for disease progression and its therapeutic targeting is providing exciting results, in particular with newly developed immune checkpoint inhibitors. Current approaches primarily focus on cellular interactions occurring between tumor cells and T lymphocytes; however, recent data highlight a crucial role of neutrophils in support of tumor progression and suggest yet unexplored treatment opportunities. In this review, we summarize the current understanding of those interactions that occur between neutrophils and cancer cells, focusing on both protumor and antitumor activities of neutrophils at different stages of cancer progression. These include infiltration of neutrophils into the primary tumor, their interactions with circulating tumor cells (CTC) within the bloodstream, and their involvement in the establishment of a metastatic niche. Additionally, we discuss how further investigation of CTCs and their interacting immune cell partners may point towards novel immune checkpoint inhibition strategies and provide new insights on the efficacy of already existing immunotherapies.

Introduction

Cancer development and progression is a multistep process tightly regulated by interactions between tumor and immune cells (1, 2). Studies describing mechanisms involved in this cellular crosstalk provided the groundwork for successful antitumor immunotherapies for numerous solid cancers, including melanoma, non–small–small cell lung cancer, head and neck squamous cell carcinoma, renal cell carcinoma, urothelial bladder carcinoma, prostate carcinoma, gastrointestinal carcinomas, and triple-negative breast cancer (3). Yet, the majority of patients remain unresponsive to immunomodulation approaches, with the highest response rates being observed in melanoma (up to 58%), non–small–small cell lung cancer (up to 48%), and a subset (MSI-H/dMMR) of colorectal carcinomas (up to 60%; refs. 4, 5). Recent advances have revealed that selective exclusion of immune cells from the primary tumor could explain such limited responsiveness, at least in patients with melanoma (6). Intriguingly, beyond interactions that occur between immune cells and cancer cells at the primary tumor site, multiple lines of evidence also indicate active involvement of immune cells along tumor progression, that is, in the circulation and in sites of distant metastasis (Fig. 1; refs. 7–10).

As neoplasms advance towards systemic dissemination, cancer cells experience direct and indirect interactions with organ-resident and circulating white blood cells (WBC) of virtually all types (1, 9, 11). Both the innate and the antigen-specific branches of the immune system are engaged in turn by tumor cells (12). For instance, concerning antigen-specific cells, expression of tumor-restricted neoantigens may result in a response mediated by lymphocytes, which is evaded by malignant tumors via a plethora of escape strategies (9, 13). Among these, T-cell suppressive checkpoints such as the PD-1/PD-L1 axis or the costimulatory molecules CTLA4 and OX40 are valid therapeutic targets, whose blockade or modulation is currently being assessed in the clinic as the most promising immunotherapy strategy for solid cancers (14, 15). Additionally, there is robust evidence for clinically-relevant immune checkpoints involving innate immune cells, one example being “don’t eat me” molecules (e.g., CD47) able to suppress phagocytic activity of macrophages (16). Along this line, an emerging body of evidence has been highlighting a key role for myeloid cells such as neutrophils in cancer evasion and therapy failure upon T-cell–targeted immunotherapies (17, 18).

Neutrophils are the most common type of WBCs in the circulation and are fundamental in inflammatory responses (19). In cancer, these cells were shown to be crucial for disease progression (20), however their contribution to the metastatic process is controversial, as both protumor (12, 21) and antitumor (22, 23) activities were observed. In fact, similarly to tumor-associated macrophages (TAM), neutrophils can infiltrate tumors and differentiate to execute opposite functions (23–25). Neutrophils respond to recruitment signals of damaged tissue such as cell-free DNA, proteins, and proinflammatory chemokines and cytokines (e.g., IL1β) and enter the tumor microenvironment (26). The presence and the number of tumor-infiltrating neutrophils (TIN) was shown to correlate with more aggressive breast cancer subtypes (e.g., hormone-receptor negative) and to be useful as a prediction tool to determine responsiveness to chemotherapy as well as disease prognosis in different carcinoma types, suggesting a broad involvement for TINs during the course of the disease (27–29). Furthermore, during cancer progression, the number of circulating neutrophils increases owing to
tumor-secreted factors like G-CSF, which can stimulate progenitor cells in the bone marrow to produce more neutrophils (Fig. 1; refs. 30–32). In contrast, it is unclear whether increased neutrophil counts in the blood can occur despite low G-CSF production. An increase in the count of neutrophils in circulation has been associated with worse disease prognosis in several cancer types (33–36), although, in contrast, neutropenia has been correlated with better survival in a meta-analysis of nearly 10,000 patients undergoing chemotherapy (37). Importantly, the neutrophil-to-lymphocyte ratio (NLR) may also serve as a prognostic factor depending on tumor type and stage. For instance, meta-analyses of over 4,000 patients with ovarian cancer and 3,000 patients with resected gastric cancer showed a strong correlation between decreased NLR and longer overall survival (38, 39). Moreover, a study on stage IV patients with pancreatic cancer concluded that NLR scoring can be useful for the identification of patients that will not respond to chemotherapy (40).

In this review, we explore the current understanding of how immune cell interactions shape the process of cancer metastasis, with a particular focus on neutrophils and their relevance for each fundamental step of tumor progression from primary tumor to metastatic disease.

**Immune Cell Interactions in the Primary Tumor**

Infiltrating immune cells constitute a major component of tumor-associated stroma, where they exert a strong functional influence on tumor development. For instance, targeted depletion experiments have indicated a necessary role for neutrophils and macrophages in promoting primary tumor formation, in contrast to a rate-limiting role for other cell types (e.g., NKs, CD8+ T cells; refs. 41–43). It is well understood that cancer-associated immune cells are often rewired from their physiologic function of antineoplastic immune guards towards an alternative, "tumor-supporting” function.

Defined cellular mechanisms are known to lead to specific profiles of immune escape. The selective exclusion of tumor-reactive T lymphocytes from the primary tumor leads to a first layer of protection, defining the so-called "cold" tumors, that is, tumors impenetrable to immune cells (6). An additional mechanism is constituted by positive recruitment of immune cell subsets that are unable to aggress the tumor and can be later turned into tumor-supportive cells, such as T-regulatory cells, which can be attracted by the tumor or can emerge from the local conversion of infiltrated CD4+ T lymphocytes into suppressors of
Neutrophils become involved in cancer by contributing to a series of proinflammatory activities, which result in a disease-boosting effect under certain conditions (7, 25, 26). Those includes, for instance, production of matrix-degrading metalloproteinases such as MMP9, which pave the way to invasive cancer cells (59, 60). However, besides tumor growth-supportive effects, the powerful inflammatory response brought by circulating neutrophils is also known to result in cancer killing, as demonstrated for certain MEI-expressing neutrophils (21) or in cyto-/genotoxic stress, via production of reactive oxygen species (ROS) that cause cell damage and provoke genetic instability (61), with the likely consequence of fueling tumor evolution. As suggested by these findings, tumor-infiltrated neutrophils are known to be functionally diversified (Fig. 1). Similarly to TAMs, neutrophils can infiltrate tumors to carry opposite functions and undergo polarization into either N1 (antitumor) or N2 (protumor) phenotypes, depending on defined signals. Blockade of TGFβ signaling promotes antitumorogenic neutrophil properties by activating the proinflammatory, N1 phenotype. This may lead to cancer cell aggression via ROS-mediated toxicity, together with a simultaneous curtailment of the immunomodulator activity of the N2 state, normally upheld by TGFβ (23). Intrinsic molecular differences across tumor models might be linked with the proclivity of neutrophils to polarize towards an N1 versus N2 state and could explain the presence of contradictory results, as already proposed for TAMs. Moreover, differences between earlier versus later stages of tumor evolution may provide for further explanation, as shown for lung cancers (62). It follows that neutrophil function in tumor progression is time- and context-dependent, and should be therefore considered as a dynamic process rather than a consolidated scenario.

The cellular mechanisms responsible for the pathologic re-education of neutrophils in cancer are yet largely unexplored. Recently, it was described how a metabolic switch involving FATP2/SLC27A, a regulator of fatty acid processing, can contribute to disease-relevant, functional re-wiring of neutrophils (63). Moreover, another recent work has contributed a first exploration of the metabolic plasticity of different neutrophil subsets, adding evidence for a tendency of low-density neutrophils in facilitating carcinoma metastasis (64). It follows that the integration of signaling events and metabolic cues in patients with cancer may lead to diversification of the behavior of neutrophils within tumor lesions. Speculatively, it is not to be excluded that interpatient environmental factors such as diet, habits, and their combined impact may account for a different propensity of the host's neutrophils to become tumor-supportive. Altogether, the spectrum of protumorigenic activities exerted by neutrophils appears to be vast enough to engage them in multiple aspects of cancer biology, including its progression (65, 66).

**Immune Cell Interactions in the Circulating Tumor Cell Compartment**

Circulating tumor cells (CTC) are direct precursors of metastasis that travel in the peripheral blood until reaching secondary sites. To successfully form metastasis, CTCs have to overcome multiple obstacles including surviving anoikis, crossing the tissue-blood barrier, overcoming shear stress, and avoiding intravascular immune attack. Surpassing all of these challenges requires skillful manipulation of the environment, for example by bringing along helpful components of
surrounding tissue (67) or taking advantage of the immune system's vulnerabilities (9).

Formation of heterotypic cell clusters between CTCs and WBCs has been previously reported, mostly along the establishment of novel CTC isolation technologies (Fig. 1; refs. 68, 69). Importantly, this interplay has been linked with a worse prognosis, indicating a possible protumor phenotype of associated WBCs (70). A recent study revealed that in the majority of cases these WBCs are neutrophils in both patients and mouse models (65). Although neutrophils are very abundant in circulation, clustering with CTCs appears to occur at the tumor site, as the number of CTC-neutrophil clusters is higher in tumor-draining vessels, that is, right after detachment from the tumor mass, and it does not occur when cancer cells are injected intravenously. The formation of CTC-neutrophil clusters is mediated through cell–cell adhesion and particularly via vascular cell adhesion molecule 1 (VCAM-1), which is known to regulate trans-endothelial migration of WBCs (71–73) and to facilitate both anchoring to the vascular endothelium as well as crossing the tissue–blood barrier during extravasation. Notably, the elimination of VCAM1 prevents productive interactions between neutrophils and cancer cells, by diminishing their proliferative advantage. Such neutralizing cell-dissociation offers therapeutic possibilities, as suggested by a recent study where small drug-mediated dissociation of CTC clusters could dampen the metastatic potential of CTC aggregates (74).

Interactions between neutrophils and CTCs seem to be reciprocal and influence both cell types, as indicated by the polarization of neutrophils into N2-like units and by the increased metastatic potential of CTCs clustered with neutrophils, compared with CTCs traveling alone. These observations are in line with previous reports showing a pro-proliferation effect that neutrophils exert on cancer cells (75, 76). Along these lines, other studies have shown interaction of CTCs with myeloid-derived suppressor cells (MDSC), a less differentiated myeloid cell type that shares some of the functions that are attributed to their full-differentiated counterparts (77).

Neutrophils can also impact CTC biology in an indirect manner. For example, neutrophil extracellular traps (NET), which are formed by DNA and proteolytic enzymes in response to infection, can be induced during cancer-related sepsis. As a result, CTCs can be captured in NETs at the dissemination site and this further promotes metastasis, and in turn, NETs may increase vascular permeability at the metastatic site, enabling CTCs to extravasate (78, 79). Additionally, circulating neutrophils were shown to cooperate with other circulating immune cells able to foster systemic inflammation (e.g., IL17 producing T cells), whereas at the same time suppressing tumor-killing cytotoxic T lymphocytes (12). Finally, neutrophil-induced tumor angiogenesis via MMP9 facilitates extravasation and thus contributes to hematogenous dissemination (59, 60). Altogether, there is overall evidence for a more prevalent tumor-supportive role for neutrophils in their relationship with CTCs, arguing that neutrophil-targeting strategies may provide a new tool to reduce metastatic dissemination.

**Immune Cell Interactions in the Metastatic and Premetastatic Niche**

The arrangement of the metastatic niche is a remotely operated process, resulting from the ability of tumor cells to conduct a systemic conditioning of distant organs. The physical integrity, as well as the normal function of distant organs may be altered during metastatic niche formation (80). For example, in the bone, normal physiologic processes such as bone remodeling by stromal cells and osteoblasts may be jeopardized during metastatic niche formation (81). The notion of "premetastatic niche" implies that host-derived cells may be drawn via distant signals originated from the tumor to initiate the preparation of a metastatic niche ahead of the cancer cells' arrival. The systemic nature of this process implies the involvement of host's cells that are not in the fold of the primary tumor environment.

A robust line of evidence incriminates neutrophils in the preparation of the metastatic niche, mainly as positive actors that enable CTC lodging at the metastatic site. For instance, the co-administration of neutrophils and metastatic cells was shown to help retention of CTCs in lungs by promoting their tethering to the endothelium (72). Intravital microscopy has revealed that liver-metastatic CTCs may adhere directly on top of arrested neutrophils, suggesting that neutrophils may act as a bridge to facilitate interactions between cancer cells and the liver stroma (73). As recurrently observed, bone marrow-derived cells presenting the surface phenotype of both mature and immature neutrophils (Gr1+ CD11b+) can accumulate in the metastatic site before the arrival of cancer cells (22, 82) and their recruitment can be mediated through the recognition of cross-linked collagen, an extracellular matrix component generated by cancer cells via enzymatic secretion (83). The premetastatic niche promoted by these "myeloid "emissaries" may exert its beneficial effects on cancer cells via several mechanisms (84) including in situ vascular remodeling to permissive conditions for the incipient cancer cells (82) as well as production of factors stimulating metastasis initiation (i.e., leukotrienes; ref. 85). Furthermore, there is substantial evidence supporting a role for mature neutrophils as suppressors of the host's in situ immunosurveillance, that is, acting as a subset of metastasis-promoting MDSCs (12, 58).

The role of neutrophils in the metastatic niche is only beginning to unveil its full complexity. Intravital microscopy studies have revealed an immediate immune response taking place in lungs as they are colonized by extravasating CTCs (86). This includes sequential recruitment of myeloid cells in multiple waves, which results in a competition between cells denoted to “patrolling,” that is, antимetastatic protection of the organs (e.g., resident antigen-presenting cells, such as dendritic cells) versus later-appearing phagocytes that support metastatic success. Freshly-recruited neutrophils appear to exert patrolling activity at least during early attempts of metastatic colonization (Fig. 1); only later, the intervention of CCL2-attracted inflammatory monocytes alters the conditions, allowing metastatic niche installation (87). This is in line with other studies showing that metastasis-promoting neutrophils may exert MDSC function only in a defined time window of the metastatic process, and they can emerge by numerical expansion in response to inflammatory cues (e.g., IL17-producing cells; ref. 12). On the contrary, it was observed in other models that the bulk of circulating neutrophils may prevalently exert antimetastatic cytotoxicity, outweighing their role as MDSCs, at least before monocytic involvement (22).

Detailed lineage tracking experiments will be mandatory to clarify exactly when, where and how and tumor-killing neutrophils are taken over by neutrophil variants that serve the (pre-) metastatic niche and by granulocyte-like MDSCs. For instance, it may be inferred from the above-mentioned reports that
neutrophils are not simply going rogue in solitary, but rather cooperate with CCL2-dependent monocytes or macrophages (87). The CCL2–CXCR2 axis has been implicated as a major vulnerability in MDSC-promoted metastasis, as its blockade promotes retention of monocytes in the bone marrow, thus preventing localization in the lungs and local differentiation into functional macrophages (88, 89). However, the blockade of this axis alone is not sufficient to prevent rebound of inflammatory cells able to quickly re-install the metastatic niche (90). Hence, the spark that triggers massive recruitment of inflammatory cells to the (pre-) metastatic niche may not have been fully identified at its core yet, although numerous candidate pathways have emerged. Further exploration of the heterogeneity of metastasis-promoting versus metastasis-restricting neutrophils is thus warranted for the identification of key vulnerabilities. Novel myeloid-restricted "innate" checkpoints may be revealed, whose therapeutic targeting may restore the cytotoxic activity of neutrophils or the ability of phagocytes to remove cancer cells in arrival from the blood.

**Perspectives and Conclusions**

Tumor-promoting neutrophils are directly and indirectly involved in multiple steps of tumor progression (Fig. 1). Besides their numerous protumorigenic inflammatory activities, neutrophils have been long suspected (and in some cases described) as modulators of cancer-restraining immunity, able to team up with myeloid-derived immunosuppressive cells in the management of interactions between cancer cells and the immune system. This major role played by neutrophils in cancer immune evasion entitles them to represent a hurdle for cancer immunotherapy, for instance by promoting T-cell exclusions from cancerous lesions, as also suggested by recent preclinical studies (17, 18, 91). Further, the recent discovery that productive interactions between CTCs and neutrophils can be studied in the circulation opens new avenues to liquid biopsy-based cancer diagnostics (65). We speculate that the association rate of cancer cells with neutrophils and/or the neutrophil-induced proliferative signature may suggest high levels of "conditioning" of neutrophils towards MDSC-like function. This would predict higher rates of T-cell exclusion from cancer foci, and a lower responsiveness to anti-PD-1/PD-L1 targeting. Large cohorts and prospective studies will be needed to understand if CTC-neutrophil clustering appears in those patients that are not eligible for T-cell–targeted strategies, and if these patients may benefit from additional, neutrophil-targeting therapies.

**Disclosure of Potential Conflicts of Interest**

B.M. Szczepanowska has ownership interest (including patents) in the patents filed on CTCs. N. Aceto is a paid consultant for companies interested in liquid biopsy and has ownership interest (including patents) in the patents filed on CTCs. No potential conflicts of interest were disclosed by the other authors.

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Saini et al.


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