Tumor-associated neutrophils: friend or foe?

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Neutrophils play an established role in host defense and in killing invading microorganisms. Although neutrophils are traditionally considered in the context of their antibacterial functions, it is becoming increasingly clear that tumor-associated neutrophils (TAN) play a major role in cancer biology. Neutrophils make up a significant portion of the inflammatory cell infiltrate in many models of cancer. Like all other leukocytes, they move into tissues under the influence of specific chemokines, cytokines and cell adhesion molecules. The tumor microenvironment has been shown to be responsible for their recruitment in cancer. We have found that TAN are a distinct population of neutrophils, differing markedly in their transcriptomic profile from both naïve neutrophils and the granulocytic fraction of myeloid-derived suppressor cells. Studies have demonstrated specific examples of tumor-mediated signals (such as transforming growth factor-β) that induce the formation of a pro-tumorigenic (N2) phenotype capable of supporting tumor growth and suppressing the antitumor immune response. However, there are also studies showing that TAN can also have an antitumorigenic (N1) phenotype. Herein, we explore the literature on the different mechanisms of TAN recruitment to tumors, the unique characteristics of TAN and what shapes their pro- and/or antitumor effects.

Introduction: neutrophils in cancer

Neutrophils are the predominant circulating leukocyte population in humans, accounting for 50–70% of circulating leukocytes. They have been seen in vivo in close association with tumor cells and within tumor vasculature (1). However, the exact role of neutrophils in the tumor cell microenvironment is the subject of controversy. The interest in the role of neutrophils in cancer increased during the late 80’s and early 90’s, as can be seen from an increase in the percentage of publications on neutrophils out of all cancer-related publications (Figure 1). However, with the rise of interest in T-cell biology, and because no clear roles for neutrophils in cancer were defined, a gradual decrease in the interest in these major cells of the immune system was noted in the following decade, even as new immunotherapy modalities were developing. Interest has increased as recent data suggest more important and significant roles for neutrophils in tumor biology than generally reflected in the literature (2,3).

Neutrophils play a well-established role in host defense, where they extravasate from the circulation and enter tissues (4). There, they phagocytose and kill invading microorganisms (such as bacteria and fungi) by releasing activating cytokines [e.g. tumor necrosis factor (TNF)-α, interleukin (IL)-1, interferons (IFNs), etc.] and defensins, along with toxic substances and reactive oxygen species. Although neutrophils are traditionally considered in the context of their antibacterial functions, it is becoming clear that tumor-associated neutrophils (TAN) and their myeloid precursors [peripheral neutrophils and granulocytic myeloid-derived suppressor cells (G-MDSC)] in the spleen, bone marrow and blood play an important role in cancer biology (2,3,5–8).

In contrast to the well-described ability of inflammatory neutrophils to engulf bacteria, activate the immune system and induce tissue damage in infections (9), it has now become apparent that myeloid cells can also function as immunosuppressive cells in the context of tumors (10). This property has been very well described in recent years for the so-called ‘myeloid-derived suppressor cells (MDSC)’ found in large quantities in the spleens of tumor-bearing animals (10–13) and for tumor-associated macrophages that develop an ‘M2’ or tumor-supportive phenotype (14,15). More recently, we have demonstrated that in untreated tumors, neutrophils can also assume a pro-tumorigenic state, which by analogy to macrophages, we called the ‘N2’ phenotype (6).

The full range of mechanisms responsible for this pro-tumorigenic activity have not yet been elucidated, but neutrophils are known to impact angiogenesis, immune surveillance, as well as to secrete chemokines, cytokines and reactive oxygen species (7). However, we also noted that under certain conditions [e.g. after transforming growth factor-β (TGF-β) blockade], TAN can take on an ‘N1’ phenotype, which is pro-inflammatory and antitumorigenic. It is important to consider which of these two possible phenotypes predominate when interpreting the literature.

Neutrophils in human cancer

Relatively little is known about neutrophils in human cancers. Many patients with advanced cancer show high levels of blood neutrophilia (16). The mechanisms by which neutrophilia is induced by tumors is uncertain, although GM-CSF production has been implicated in some tumor systems, such as lung, melanoma, pancreas and breast (17). Several additional cytokines secreted from tumors and stroma cells have been suggested to contribute to neutrophilia and to the induction of suppressive properties of these neutrophils. These include, among others, G-CSF, vascular endothelial growth factor (VEGF), IL-1-β and IL-6 (18). Neutrophilia has been associated with poorer prognosis in many cancers, including bronchoalveolar carcinoma (19), metastatic melanoma (16) and renal carcinoma (20). Interestingly, the neutrophil to lymphocyte ratio has been introduced as a prognostic factor in many tumor types, including colorectal cancer (21) and non-small cell lung cancer (22).

There is surprisingly little data about the presence of neutrophils within human tumors. Intratumoral neutrophils were shown to be a strong, independent prognostic factor for recurrence free, as well as cancer-specific and overall survival in metastatic (23) and in localized (24) clear cell renal cell carcinoma and in head and neck squamous cell carcinoma (25). Infiltration of neutrophils was found to correlate with tumor grade in human gliomas (26) and to be related to more aggressive types of pancreatic tumors (27). This observation is not universal, however. In some tumors (e.g. gastric cancer), a high neutrophil count has been associated with a favorable prognosis (28). We are currently examining a large number of human lung cancers for the presence of neutrophils (myeloperoxidase-positive cells). We are seeing striking heterogeneity, with some tumors heavily infiltrated, some with moderate infiltration and some with no neutrophils. The prognostic implication of neutrophil infiltration in these patients is under study.

Several effects of human tumor cells on neutrophils were demonstrated in vitro. HNSCC tumor-derived factors modulated cellular functions of polymorphonuclear cells and increased their

Abbreviations: CCL, CC chemokine ligand; G-MDSC, granulocytic fraction of myeloid-derived suppressor cells; IFN, interferon; MDSC, myeloid-derived suppressor cells; MMP, matrix metalloproteinase; mRNA, messenger RNA; NE, neutrophil elastase; TAN, tumor-associated neutrophils; TGF-β, transforming growth factor-β; TNF, tumor necrosis factor; IL, interleukin; VEGF, vascular endothelial growth factor.
The percentage of publications that include the MESH terms and MIP2 blood under the influence of specific chemokines (e.g. KC/CXCL1 markers such as F4/80 (33). surface markers CD11b and Ly6G with low expression of macrophage markers such as F4/80 (33).

Neutrophils, like all other leukocytes, move into tissues from the blood under the influence of specific chemokines (e.g. KC/CXCL1 and MIP2/CXCL2 in mice), cytokines (e.g. TNF-α and IFN-γ) and cell adhesion molecules located on their own surface (i.e. CD11b) and on the surface of endothelial cells (i.e. selectins, Intracellular adhesion molecule 1 and Platelet-Endothelial cell adhesion molecule 1) (34). Growth factors may also have a role in the chemotraction of neutrophils into tumors. G-CSF has been shown to indirectly support the extravasation of neutrophils from the blood, mostly by activating the CXCL2–CXCR2 axis (35,36). GM-CSF is well known to have priming and antiapoptotic effects on neutrophils, inducing neutrophilia. More recent studies demonstrated that GM-CSF can also be a strong chemoattractant for neutrophils in vivo (37,38), although this has not yet been shown specifically for their recruitment into tumors. Ueha et al. (39) have recently summarized the dynamics of myeloid cells, including neutrophils, from the bone marrow to the circulation and into mouse tumors. CXCR2 and CXCR4 were shown to cooperatively regulate the release of neutrophils from bone marrow (40). Ueha further suggested that tumor infiltration by neutrophils was at least partly mediated by autocrine CXCL2 production. Indeed, in our recent work using microarrays (41) (see below), we found that the expression of CXCL2 was upregulated by 188-fold in TAN compared with bone marrow neutrophils. Similar results were found for two other known neutrophil chemoattractants—CXCL1 (140-fold compared with bone marrow neutrophils) and CCL-3 (76-fold in TAN compared with bone marrow neutrophils) (41). It seems, therefore, that the neutrophils initiate a positive feedback loop by secreting neutrophil chemoattractants that recruit more neutrophils into the tumor, as described previously in infections (42,43). Another chemokine recently shown to be important in the recruitment of neutrophils to melanoma tumors is GCP-2 (CXCL6). Specific anti-CXCL6 monoclonal antibodies not only reduced recruitment of neutrophils to tumor sites in mice but also caused reduction in tumor growth (44).

We have shown that TGF-β receptor blockade increases the number of neutrophils in tumors and that this effect occurs through all three parts of the recruitment pathway including increased expression of messenger RNA (mRNA) for CXC chemokines, CC chemokines and activating cytokines within the tumor, as well as upregulating Intracellular adhesion molecule 1 message and protein expression on endothelial cells (6,45). Our preliminary data show that macrophages, as well as endothelial cells, are the important players in neutrophil recruitment, as shown previously in lung inflammation (46). Interestingly, TGF-β appears to inhibit endothelial adhesiveness for neutrophils and neutrophil transmigration through endothelium in vitro (47) and in different inflammatory disease states (48).

Although it has been shown that CD8+ T cell depletion decreases the tissue influx of neutrophils in infectious diseases (42), there are surprisingly few studies examining the effect of CD8+ T cells on the recruitment of neutrophils in cancer. In the only tumor study we were able to identify, a marked decrease in TAN following CD8+ T cell depletion was shown in a model in which CT26 colon carcinoma cells transduced to express G-CSF were placed into mice (49). The mechanisms by which T cells might attract and/or activate neutrophils are not known for certain but include the ability of tumor-stimulated activated T cells to produce GM-CSF (50), KC/CXCL1 and MIP2/CXCL2 (51) or cytokines such as TNF-α and IFN-γ. These cytokines may act to recruit neutrophils by stimulating tumor macrophages or endothelial cells to produce appropriate chemokines and cell adhesion molecules (46,52). Recently Richards et al. (53) demonstrated that T-regulatory cells can play a role in the inhibition of neutrophil recruitment to a site of tumor inoculation. This effect was shown to be mediated by decreased expression of the neutrophil chemoattractants CXCL1 and CXCL2. In contrast, Himmel et al. (54) showed that human T-regulatory cells can actually promote recruitment of neutrophils by secretion of IL-8. A simplified scheme of the factors influencing recruitment of neutrophils into the tumor is shown in Figure 2.

**Unique characteristics of TAN**

‘Imnosculpting’, i.e. the cross talk between immune and tumor cells changing the phenotype of tumor biology, is widely recognized (55). However, until recently, the role of neutrophils in this cross talk has been underestimated. In recent years, several studies have shown specific examples of tumor-mediated signals eliciting pro-tumor responses from neutrophils (56). One interesting illustration of these effects was shown by Queen et al. (56), who demonstrated that cancer cells can stimulate neutrophils to produce oncostatin-M, which in turn increases secretion of VEGF by tumors.

It is worth considering the relationship between TAN and the granulocytic fraction of G-MDSC. MDSC, a heterogeneous population of immune suppressive cells that are produced at high levels in cancer, are defined in mice on the basis of expression of the surface markers CD11b and Gr-1 and by their ability to inhibit T-lymphocyte activation. The CD11b+ /Gr-1+ MDSC population is comprised of at least two subsets—granulocytic (Ly6G+) and monocytic cells (Ly6C+), possibly with different immunosuppressive properties (57).

There is substantial agreement on the immunosuppressive activity of the monocytic MDSC subset. However, there is still contrasting evidence on the role of the granulocytic fraction. Whereas some have shown that granulocytic MDSC have immunosuppression properties similar to the monocytic fraction (11–13), others have recently demonstrated that they are less immunosuppressive (58,59). It has been shown previously that adoptively transferred MDSC can enter tumors and differentiate to mature tumor associated macrophages or neutrophils (TAN) (59). However, little is known in animals about whether MDSC leave the spleen and circulate. It is thus not clear whether the majority of TAN are actually G-MDSC that were attracted to the tumor or whether they are bone marrow/blood-derived neutrophils that were then converted to N2 TAN by the tumor microenvironment. In order to further evaluate the specific characteristics of TAN in relationship to other populations of neutrophils, we recently used a transcriptomics approach, comparing the phenotype of TAN to naive neutrophils from the bone marrow (NN) and to the G-MDSC (41). In our microarray study, we clearly show that TAN are not ‘tissue-based G-MDSC’ but are a distinct population of neutrophils, differing markedly in their genetic profile from both NN and G-MDSC, with the NN and G-MDSC being more closely related to each other than to TAN.

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**Fig. 1.** The percentage of publications that include the MESH terms neutrophils or granulocytes out of all publications related to cancer for every five years between the years 1971 and 2010.
Fig. 2. A simplified schematic representation of the cells and factors influencing recruitment of neutrophils into the tumor.

Some of the unique characteristics of TAN compared with NN and G-MDSC that were described in this work are described below.

Structural genes. Genes related to cytoskeleton organization and biogenesis, as well as in pathways related to actin binding and polymerization were downregulated in TAN, consistent with their loss of ability to leave the tumor microenvironment after infiltrating the tumor.

Cytotoxic and phagocytic genes. The two cytotoxic pathways of neutrophils, granule proteins production and the respiratory burst (21), are dramatically downregulated in TAN. Interestingly, Shen et al. (60) have shown that TGF-β, of which high levels are found in the tumor microenvironment, can inhibit neutrophils degranulation. An alternative explanation for these findings could be that the mature neutrophils have finished producing granule contents, and the relevant mRNAs are not needed (61). In contrast to these findings, we noted no clear changes in the pathways and genes related to phagocytosis, another major function of neutrophils.

Apoptosis. Despite data suggesting that TAN may be longer lasting cells than circulating neutrophils (62), we found that most genes related to apoptosis were expressed at similar levels. However, we did find that several antiapoptotic members of the nuclear factor-kappa B family were upregulated in TAN. nuclear factor-kappa B may be, therefore, an important regulator of the apoptotic machinery in TAN and it is possible that this pathway is responsible for the notable longevity of TAN compared to other neutrophils.

Immune system. It has become increasingly clear that the contribution of neutrophils to host defense and natural immunity extends well beyond their traditional role as professional phagocytes (9). Neutrophils and their myeloid precursors can be induced to express a number of genes whose products lie at the core of inflammatory and immune responses, suggesting a potential role for these cells in orchestrating the sequential recruitment and activation of distinct leukocyte types to the inflamed tissue (63). Neutrophils, either spontaneously or following appropriate stimulation, have been shown to express and/or produce numerous cytokines, chemokines and angiogenic factors. These include CC- and CXC-chemokines (e.g. CXCL1/2/5/10, CCL-2/4/17), pro- and anti-inflammatory cytokines (e.g. IL1α/β, IL-6, IL-4), immunoregulatory cytokines (e.g. IL-12, IFN-α/γ), colony-stimulating factors, angiogenic factors and members of the TNF superfamily (3). However, until recently, the role of neutrophils in this cross talk between immune and tumor cells has been underestimated. Accumulating data shows that neutrophils can participate in major histocompatibility complex class I and class II restricted antigen presentation, are capable of collecting and cleaving antigens, of forming complexes with major histocompatibility complex-II molecules and of expressing costimulatory molecules (9,64,65). In our work, we also found that TAN show increased expression of gene pathways needed to present antigens that are not expressed in NN, suggesting an enhanced capability of functioning as antigen-presenting cells (41). Indeed, it has been recently shown that mature neutrophils can function as professional antigen-presenting cells capable of priming a T_h1 and T_h17-acquired immune response (65). In inflamed tissues, neutrophils have been shown to engage in complex bidirectional interactions with macrophages, dendritic cells, natural killer cells, lymphocytes and mesenchymal stem cells, affecting proliferation, activation, differentiation and survival (3). This has not been appreciated significantly in the tumor microenvironment.

The most prominent difference that we found between TAN and the other populations of neutrophils was the significant upregulation of cytokines and chemokines, suggesting an important role of tumor neutrophils in the recruitment of immunocytes and in the balance between activation and suppression of the immune system. Among the broad group of chemokines whose mRNAs were upregulated in TAN were the CCL chemokines 2, 3, 4, 8, 12 and 17 and the CXCL chemokines 1, 2, 9 and 16. The upregulation of chemokines in TAN suggests that they have a pivotal role in recruiting other cells of the immune system to the tumor. This is similar to the role that ‘classical neutrophils’ would have in wound healing. At least some of the recruited cells are known to support tumor growth, such as macrophages (by CCL-2 and CCL-7) and T-regulatory cells (by CCL-17) (66).

Table I highlights the major pathways and group genes that we found to be significantly different in one of the three neutrophil populations examined (NN, G-MDSC and TAN) compared with the others (41).

The tumor-supportive roles of TAN
As mentioned previously, we and others have noted that in untreated tumors, TAN appear to develop a pro-tumorigenic phenotype that we have termed ‘N2 TAN’ in analogy to the M2 macrophage phenotype that appears to contribute to tumor growth (5,6,67,68) and suppression of the antitumor immune response (69). Depletion of these ‘pro-tumorigenic’ N2 neutrophils, therefore, inhibits tumor growth (6,67,70) and reduces the level of immunosuppression in the tumor microenvironment, allowing, for example, increased activity of CD8+ cytotoxic T lymphocytes (6). TAN appear to be involved in tumorigenesis and tumor growth through multiple mechanisms:

Initiation, carcinogenesis and tumor growth. Neutrophils have a dual role in the initiation process of tumors, mainly by affecting the
levels following reexposure to IFN-\(\beta\) increased in the absence of IFN-\(\beta\). Extravasation and metastases. Studies have shown indirectly that mainly at the early, rather than late stages of tumor development (41).

The antitumor effects of N1 TAN

Despite the broad literature on the pro-tumor effects of TAN reviewed above, there are several studies reporting antitumor roles for these cells, mostly with engineered tumor cell lines or following specific therapies (7). Interpretation of these studies in the light of the idea of differential neutrophil activation status within tumors is instructive. In contrast to the studies described above, in which depletion of neutrophils inhibited tumor growth, we found that neutrophils can assume a more tumor-cytotoxic N1 phenotype, for example, during TGF-\(\beta\) inhibition (6) or after immunologic or cytokine activation, where they inhibit recruitment and activation of immunocytes (see below), our data demonstrated that N2 neutrophils can inhibit T-cell effector functions; neutrophil depletion of untreated tumor-bearing animals (i.e. removal of N2 TAN) increased the activation status of CD8\(^{+}\) T cells, supporting the idea that N2 TAN can function in an immunosuppressive fashion (6) in the same way that has been proposed for M2 tumor associated macrophages (12,93). A possible suggested mechanism for this suppression of T-cell proliferation and responsiveness to stimulation is by the secretion of stored arginase 1 (ARG1) that degrades extracellular arginine, a factor needed for the proper activity of T cells (94).

Tumor cytotoxicity and inhibition of tumor growth. The direct killing of tumor cells by neutrophils was demonstrated in vivo (99) and in vivo (100) almost three decades ago. Ishihara et al. (101,102) reported that neutrophils from tumor-bearing animals have an enhanced cytotoxicity profile as measured by superoxide anion generation and phagocytosis, inducing a marked decrease in the size and number of metastatic foci in the lung. We and others have demonstrated that
oxidative damage caused by reactive oxygen species secreted from neutrophils are capable of inducing tumor cell lysis (6,103,104). Interestingly, there seems to be a difference between the cytoxicity of neutrophils to primary versus metastatic cells, the latter being less affected (82). Furthermore, it is possible, as recently shown by Granot et al. (105), that tumor-entrained neutrophils can actually inhibit metastatic seeding in the lungs, inducing a neutrophil-mediated inhibitory process at the metastatic site.

A second mechanism by which neutrophils were shown to be capable of directly inhibiting tumor cells is by mediating Fas-ligand-associated apoptosis (106). This is in line with our observation that an increased percentage of N1 TAN are Fas positive (6). A third mechanism of killing mediated by neutrophils, mostly shown following treatment, is antibody-dependent cellular cytoxicity (2,107), for example, as part of the mechanism of the epidermal growth factor receptor antibodies—panitumumab and zalutumumab (108).

**Activation and proper direction of the adaptive immune system and tumor rejection.** The ability of the adaptive immune system, and specifically the CD8+ cytotoxic T lymphocytes, to reject tumors is a key process for the success of any immunotherapy. As suggested above, N2 neutrophils can be major inhibitors of T-cell effector functions in a similar way previously proposed for M2 tumor associated macrophages (6,12,91–93,109). However, others and we have shown that N1 neutrophils can actually be immunostimulatory, supporting tumor rejection. These proinflammatory N1 neutrophils can promote CD8+ recruitment and activation by producing T-cell attracting chemokines (e.g. CCL-3, CXCL9 and CXCL10) and pro-inflammatory cytokines (e.g. IL-12, TNF-α and GM-CSF) (6,63). Furthermore, neutrophils have been shown to cross-present antigens in vitro, and antigen-pulsed neutrophils promoted the activation of CD8+ T cells (110). Additional examples of this neutrophil-CD8+ lymphocytes interaction include studies showing that proinflammatory therapy-induced CD8+ T-cell induction required the presence of neutrophils (91) and that depletion of neutrophils significantly impaired T-cell trafficking and reduced efficacy of Bacillus Calmette–Guerin immunotherapy of bladder cancer (97).

There is also evidence that TAN can activate dendritic cells via cell–cell contact and through secretion of TNF-α (64), activate CD4+ T cells, promote antitumoral memory (111) and induce IL-12-induced tumor regression (112). Depletion of these N1 TAN thus either augments tumor growth and/or blunts the antitumor effects of immunologic treatments (6,49,91,97). Neutrophils can also be involved in the known ‘bystander’ effect of antitumor treatment with oncolytic viruses. Breitbach et al. (113) demonstrated that in vivo, most of the tumor killing activity of vesicular stomatitis and vaccinia viruses is caused by indirect killing of uninfected tumor cells, mediated by an influx of neutrophils to the tumor, and that depletion of neutrophils inhibited their antitumor effects.

Figure 3 summarizes, in a simplified scheme, the pro-tumor and antitumor effects that have been described in neutrophils, ascribing these effects to N1 versus N2 TANs.

**N1 and N2—polarization or hyperactivation?**

In their recent review on TAN as targets for cancer therapy (7), Gregory and Houghton raised the interesting question whether the differences between N1 and N2 TAN were due to two unique transcriptional programs as suggested in our work (6) or instead represented two states of activation, i.e. that N1 TANs produce the same mediators, but at higher levels. In our unpublished data, comparing the mRNA expression of N1 versus N2 TAN, we found that a vast majority of the changes were indeed upregulation of the same genes and pathways in N1 TAN compared with N2 TAN. However, there were some clear exceptions. For example, we noted that the chemokine CCL-17 (which attracts T regulatory cells) was much more highly expressed in N2 TAN than in N1 TAN. The important question whether TAN can be manipulated to undergo frank irreversible polarization or possibly reversible activation states remains unresolved and should be a matter of further research.

**Summary**

It is becoming increasingly clear that TAN play a major role in cancer biology. TAN are a distinct population of neutrophils, which in their basic unmanipulated state are induced by the tumor microenvironment (by TGF-β and probably other factors) to elicit pro-tumor responses (N2 TAN). However, recent evidence shows that these cells can be altered to assume antitumor roles (N1 TAN). Neutrophils are thus an important underappreciated cell population in cancer biology, and their functions need to be better characterized. A more complete understanding of the way these cells support or fight cancer will be important to develop strategies to direct the immune system against tumors.

**Conflict of Interest Statement:** None declared.

**References**


Received December 21, 2011; revised February 5, 2012; accepted March 10, 2012