Title: Mouse versus human neutrophils in cancer—a major knowledge gap

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Abstract

Many types of cancer recruit neutrophils that could have pro- or anti-tumor effects on tumor development. Numerous findings in murine models suggest a predominantly pro-tumoral role for neutrophils in cancer development. However, there are fundamental differences between mouse versus human tumors in the evolution of tumors, genetic diversity, immune response, and also in the intrinsic biology of neutrophils that might have a profound impact on tumor development and the function of these cells. A crucial difference is that the majority of mouse tumor models lack the prolonged initial phases of multistage tumor evolution present in humans when anti-tumoral mechanisms are activated. Here, we discuss the challenges specific to cross-species extrapolation of neutrophil function during mouse versus human tumor development.

Complexities in Understanding the Granulocytes in Human Cancer and Mouse Tumor Models

Neutrophils accumulate in many types of human and murine tumors and represent a significant portion of tumor-infiltrating myeloid cells [1-4]. There is growing evidence indicating that neutrophils have both pro-tumor and anti-tumor effects on cancer development and this dual role has been comprehensively described in many recent reviews [5-9]. Importantly, however, our understanding of the role of neutrophils in tumor development has largely been based on observations made in murine models of cancer. Studies relying on these murine experimental systems have led investigators to postulate a pro-tumoral and immunosuppressive role for neutrophils in cancer development, and this concept is the dominant view in the field. However, this perspective has been derived primarily from murine transplantable tumor models, which are associated with high tumor burden, minimal matrix, and rapid tumor growth. Importantly, these transplanted mouse tumor models use tumor cell lines that, by definition, grow well in mice and have therefore already undergone substantial cancer immunoediting and “Darwinian” selection [10]. Thus, these models bypass the initial phases of tumor evolution that would be expected to occur during tumor development in humans. Given that, data obtained from these mouse models largely reflects the immune response as it occurs during the “escape phase”, where pro-tumoral mechanisms already prevail. All these features are quite dissimilar to the initiation and slow evolution of human cancers which occur over a much longer time period and therefore might have a profound impact on neutrophil phenotype and function in mouse tumors versus human cancers.

For these reasons, an unresolved issue in cancer biology is the nature and function of immune cells, including neutrophils, in human tumor tissues. In this review, we will point out the major obstacles and challenges that exist in the investigation of neutrophils in mouse tumor models and highlight the potential danger in extrapolating these findings to humans.

In murine studies, it appears that tumor-associated neutrophils (TANs) exhibit both pro-tumor and anti-tumor effects [5,11]. Multiple studies have shown that neutrophils promote tumor progression via matrix degradation, the secretion of pro-tumor factors, direct stimulation of tumor cell proliferation, increased metastasis, and enhanced angiogenesis [7,12,13]. On the other hand, anti-tumoral functions have also been reported [12,14]. Specifically, neutrophils have been reported to (1) induce tumor cell death via their potent antimicrobial killing machinery [15], (2) enhance antibody-dependent cell-mediated toxicity (ADCC) [16], (3) produce factors to recruit and activate cells of the innate and adaptive immune system [17], and (4) boost T cell responses following photodynamic and Bacillus Calmette–Guérin (BCG) immunotherapy of cancer [18,19]. Given these varying effects of mouse TANs on tumor growth, the paradigm of anti-tumoral “N1 neutrophils” versus pro-tumoral “N2 neutrophils” was proposed [11] and rapidly adopted.

Although the concept of neutrophil diversity and plasticity has begun to emerge in murine tumor models, uncertainty regarding the phenotypes, functional roles, and relationships between different granulocytic cell populations during tumor progression persists. For instance, N1 neutrophils have a hyper-segmented nucleus characteristic of mature neutrophils, while N2 neutrophil populations consist of cells with a banded or

ring-like nuclei, as seen in immature mouse neutrophils [11]. Given this fact, it remains possible that N1 and N2 neutrophils represent different stages of maturation rather than truly bona fide neutrophil phenotypic subtypes. Alternatively, it is very possible that, similar to our current understanding of the M1/M2 macrophage paradigm [20], TANs could represent a continuum of activation states, rather than two extremes.

Ongoing discussion continues regarding the relationship between neutrophils, TANs, and polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) during tumor progression, because PMN-MDSCs and neutrophils share a common set of markers and exhibit a degree of immunosuppressive capacity similar to N2 neutrophils [8,11,21,22]. Although there have been several efforts to determine the similarities and differences between murine neutrophils and PMN-MDSCs [23-26], further studies are still needed to accurately determine whether TANs, particular N2 neutrophils and PMN-MDSCs, represent two distinct or identical granulocytic cell populations. The recent work performed by Contamine et al. might shed light on this issue. The authors have identified a marker, LOX1, that may distinguish PMN-MDSCs from neutrophils in the blood of patients with a variety of cancers [27]. It should be emphasized that the assumption that all CD11b⁺Gr1⁺ cells in tumor-bearing mice and CD66b⁺CD15⁺ cells in humans are MDSCs should be avoided unless all required functional tests confirm that these cells are indeed suppressive [21].

Importantly, the most broadly used *in vitro* assays to study the suppressive activity of neutrophils or PMN-MDSCs isolated from advanced stages of murine tumors are based on measurements of the proliferation of naïve resting T cells (usually splenocytes) stimulated by anti-CD3/CD28 antibodies. However, the interaction of naïve

T cells with granulocytic cell populations very likely does not reflect what actually transpires in tumors that are infiltrated with effector and memory T cells. In addition, it should be noted that many mouse and human studies use splenic CD11b⁺Ly-6G⁺ cells or circulating low density CD66b⁺CD15⁺ cells as granulocytic MDSCs, respectively. The relevance of these cells to tumor-associated neutrophils and intra-tumoral immune-suppression is not clear. For instance, there are several reports demonstrating that adoptively transferred tumor-specific effector T cells are resistant to intra-tumoral and systemic myeloid cell-induced suppression, and that these tumor-specific T cells can eradicate established tumors *in vivo* [28,29].

Another question to be addressed is the regulation of T cell responses by neutrophils during the early stages of tumor development, where tumor-induced immunosuppressive mechanisms might not be fully developed. Thus, further studies are required to rigorously establish the regulation of anti-tumoral effector T cell responses by tumor-associated neutrophils or PMN-MDSCs throughout all stages of tumor development.

Neutrophils are extremely dynamic and adaptable cells that are able to perform many different functions simultaneously. However, the majority of conclusions presented in the literature are weighted heavily toward one particular functional aspect of the neutrophil, while other functions are given less emphasis or remain untested. Hence, the concept of a predominantly pro-tumoral role of neutrophils and their derived products in tumor biology may be drawn from a small slice of the total physiologic functions of neutrophils, leaving other equally important aspects to be overlooked. For example, the pro-tumoral functions of neutrophil-derived proteases, including neutrophil elastase, have

been attributed to their ability to degrade cytokines, chemokines, and their receptors, remodel the extracellular matrix, increase angiogenesis, and promote metastasis [30-32]. A study by Houghton and colleagues showed neutrophil elastase uptake by lung adenocarcinoma cells, where it localized to endosomes and induced tumor cell proliferation by cleaving insulin receptor substrate-1, inducing hyperactivity of the phosphatidylinositol-3-kinase (PI3K) pathway and uncontrolled proliferation [33]. On the other hand, neutrophil elastase release from TANs is able to potentiate anti-tumor immunity by increasing the susceptibility of tumor cells to tumor-specific cytotoxic T cells (CTLs). For instance, breast cancer cells exhibit a striking uptake of neutrophil elastase from the microenvironment, which yields increased expression of tumor-specific peptides on the cell surface and enhanced susceptibility to peptide-specific CTL lysis [34]. Additionally, neutrophil elastase enhances antigen presentation by stabilizing and reducing membrane recycling of HLA class I molecules expressed on the surface of tumor cells and thus augments tumor-specific CTL [35]. Further confounding its purported pro-tumoral role, neutrophil elastase can also inhibit tumor neoangiogenesis by activating antithrombin [36].

Another example is the multifaceted role of arginine metabolized by Arginase-1 expressed in neutrophils. It is generally assumed that granulocyte-induced arginine deficiency in the tumor microenvironment induces suppression of T cell responses and represents one of the mechanisms of tumor-mediated immune escape [26,37]. However, a recent study convincingly demonstrated that human CD8⁺ T cell antigen-specific cytotoxicity and perforin secretion are completely preserved in the absence of arginine, while antigen-specific proliferation as well as IFN- γ and granzyme B secretion are

severely compromised [38]. Importantly, the production of the immunosuppressive cytokine IL-10 is also suppressed in an arginine-poor environment [39]. In contrast, several studies indicated that arginine depletion might also result in tumor cell death, but only in those tumors that are auxotrophic for arginine, such as renal carcinoma, hepatocellular carcinoma, and melanoma [40,41].

Activated neutrophils can also release neutrophil extracellular traps (NETs) that are extracellular DNA structures composed of chromatin and neutrophil proteases [42]. To date, the bulk of the literature supports the notion that NETs exert strong pro-tumoral effects during tumor progression by degrading the extracellular matrix and promoting cancer cell migration and metastatic colonization [43]. However, some data show potential anti-tumoral activity of NETs. For instance, several components of NETs, including MPO and histones, have been shown to be cytotoxic to tumor and epithelial cells [44,45]. In addition, NETs are able to both activate DCs and directly prime T cells by reducing their activation threshold [46,47].

For all these reasons, the current simplified scheme of classifying neutrophils as anti-tumoral and pro-tumoral, which for many years has served as a satisfactory working hypothesis, might be quite inadequate today, especially for human tumors and TANs.

Caveats in Translating Findings from Mouse Tumor Models to Human Cancers.

The power of mouse models has allowed us to generate many fundamental concepts in tumor immunology. However, it is important to keep an open mind about how closely these findings actually translate to human cancers. It is a sobering fact that the majority of cancer immune therapies that work well in mice fail to provide similar efficacy in humans. The average rate of successful translation from animal models to

clinical cancer trials is less than 8% [48]. Here, we would like to detail some of the species-specific differences in tumor evolution, genetic heterogeneity, immune and inflammatory responses, and intrinsic biology of neutrophils that might have a profound impact on the tumor development and function of neutrophils in mouse tumors versus human cancers (Fig. 1).

It is becoming apparent that human tumor development represents a process of microevolution with a broad range of selective pressures on multiple tumor subclones that harbor different driver and other mutations [49,50]. In addition to many intrinsic and tumor microenvironmental evolutionary pressures, prolonged selective immune pressures result in the development of adaptations that allow a tumor to escape any effective immune responses (Fig. 1) since human neoplastic clones that are susceptible to the immune system will be killed. The remaining clones, which have survived sustained selective pressure by the host's anti-tumor immune response, will be increasingly resistant to adaptive immune system attacks and will gradually become the dominant population within the tumor. These surviving subclones will enter a final stage of tumor microevolution termed the "escape phase," where pro-tumoral mechanisms dominate and help to shape the fitness of these malignant cells (Fig. 1) [10].

In contrast to human tumors, the majority of mouse tumor models use tumor cell lines originally derived from advanced tumors that have already been subjected to immune selection *in vivo*. These cell lines have been selected to grow rapidly *in vivo* and have thus already undergone extensive cancer immunoediting [10]. For this reason, the rapidly growing tumors in murine model systems do not accurately mirror the gradual stages of tumor development and the complex interactions between tumor cells and the

tumor microenvironment that occurs during the slow evolution of human tumors (Fig. 1). Typically, mouse transplant models are characterized by a high tumor burden, minimal matrix, very rapid tumor growth, and the absence of important phases of cancer immunoediting such as elimination and equilibrium. Of note, the tumor-to-normal body ratio in many transplant mouse models can be several thousand times higher than that seen in humans. Furthermore, the fact that the mice are of the same inbred mouse strain means that the model will not reflect the huge human genetic diversity. In addition, most mouse tumors have many fewer mutations (and thus fewer neoepitopes) than seen in many human cancers. All these features are dissimilar to the slow evolution of human cancers, specifically during the early-stage of tumor development, and might have a profound impact on the function of inflammatory tumor-infiltrating cells, including neutrophils, in mouse tumors versus human tumors (Fig. 1).

Genetically engineered mouse models with spontaneous tumor growth more closely reflect the slower multistage tumor development observed in humans. These mouse models are highly useful for evaluating the effects of specific mutation, deletion or gene amplification of one or two genes during murine tumor progression [51]. However, they do not fully reproduce the genetic complexity of human tumors, where genes typically contain multiple point mutations and the specific gain or loss of genes varies enormously from one cell to another even within the same tumor [52]. In humans, premalignant clones with “driver” and “passenger” mutations coexist for prolonged periods of time and only selective pressure with anti-tumoral host responses will determine which carcinogenic mutations are neutral or advantageous. In genetic mouse tumor models, tumorigenesis is induced by engineered, previously characterized driver

mutations that normally result in growing a resistant clone. It is unlikely that tumors initiated by this resistant clone will be subjected to rigorously selective immune pressures and induce sustainable anti-tumor response. Due to a limited number of initiating driver mutations, mouse tumors are typically more homogeneous than human tumors. Therefore, although useful, genetic mouse models likely do not fully recapitulate the evolving interplay between the tumor microenvironment with neutrophils and malignant cells observed in humans. In addition, the slow generation time and insufficient amount of tumor tissue at early stages of development for analysis are other disadvantages of genetic mouse models in the study of TANs.

To date, both transplantable and spontaneous tumor models generate contradictory results regarding the *in vivo* role of neutrophils in tumor development. The majority of studies have shown that systemic depletion of neutrophils results in decreased tumor growth in mouse models, suggesting their pro-tumoral role [11,33,53,54]. However, there are a number of papers demonstrating the opposite effect of neutrophils on tumor growth [55-58]. Moreover, there are conflicting data regarding neutrophil function even within the same type of tumor. For instance, some studies reported a pro-tumorigenic role of neutrophils in lung cancer development in mice [59,60], whereas other demonstrated the opposite result [55,56].

Other potentially confounding factors that could dramatically alter tumor development and neutrophil behavior in mice versus humans are the substantial differences in the organization of the immune system that have been alarmingly emphasized in many recent reviews [48,61,62]. Importantly, these differences are even more dramatic between humans and the inbred Specific Pathogen Free laboratory mice

used for tumor models [63]. Mouse and man use two different immunological defense strategies; in humans, immune resistance mechanisms dominate, while in mice tolerance mechanisms are highly favored [64]. Importantly, the use of inbred strains creates a wealth of homozygous recessive defects (many of which are still to be discovered) that could skew the regulation of the immune response.

In addition to mouse-to-human differences in tumor evolution and immunity, there are significant intrinsic differences in the biology of neutrophils that might affect their function during tumor development in mice versus humans. Below, we highlight several of the known differences between human and mouse neutrophils.

1. Neutrophils represent the majority of white blood cells in humans (50-70%), but are less common in mice (10-30%) [61].
2. In mice, Gr-1 and Ly-6G are well-established markers to identify granulocytes. In humans, granulocytes do not express the Gr-1 and Ly-6G antigens, making the direct comparison of murine and human granulocytes impossible.
3. The structure and physicochemical properties of key molecules such as selectins, PI3 kinases, and serine proteases are different, which might affect the migration of neutrophils into tumor sites, intracellular cell signaling, and neutrophil effector mechanisms [65-67].
4. Mice do not express Fc α RI – one of the most powerful Fc receptors that triggers effector functions such as oxidative burst, cytokine release, NETosis and phagocytosis in neutrophils as well as sufficient ADCC [68,69].
5. The granule content in human neutrophils is very different than in mice, which might dramatically alter neutrophil effector functions in mice versus human. For

instance, mouse neutrophils do not produce defensins, while human neutrophils do [70]. Moreover, the expression of bactericidal/permeability-increasing protein (BPI), myeloperoxidase (MPO), β -Glucuronidase, Lysozyme, Alkaline phosphatase, and Arginase-1 is much higher in humans than in mice [71].

6. The production of immunoregulatory cytokines also differs. For example, secretion of IL-10 is well established in murine neutrophils, while human neutrophils appear not to secrete detectable amounts of IL-10 [72]. Additionally, whether human neutrophils can express IL-6, IL-17A, IL-17F and IFN- γ like their mouse counterparts is the subject of conflicting reports [73].

7. The activation pathways of reactive oxygen species (ROS) production differ between human and mouse neutrophils [74].

8. Many chemokines and their receptors, including those that affect neutrophil trafficking, are absent in mice. The chemokines IL-8 (CXCL8) and its receptor CXCR1, neutrophil-activating peptide-2 (CXCL7), IFN-inducible T cell chemoattractant (CXCL11), monocyte chemoattractant protein (MCP)-4 (CCL13), HCC-1 (CCL14), hemofiltrate CC chemokines-2 (CCL15), pulmonary and activation-regulated chemokine (CCL18), myeloid progenitor inhibitory factor-1 (CCL23), and eotaxin-2/3 (CCL24/CCL26) have all been identified in humans but not in mice [75]. Thus, targeting CXCR2 to modulate the recruitment neutrophils in murine tumors might not represent a reliable system to study neutrophils in humans.

9. The delayed type hypersensitivity (DTH) reaction in mice tends to be more neutrophil rich after the antigen challenge, despite the fact that murine peripheral blood has a relative paucity of neutrophils compared with humans. In humans, DTH

is characterized by a rapid neutrophil response, followed by a dramatic influx of mononuclear cells, T cells, and macrophages [61,76].

10. The mechanisms and relative importance of various modes of immunosuppression (e.g., NO, ROS, Arginase-1) are different between humans and mice. In contrast to murine neutrophils, Arginase-I is constitutively expressed in human granulocytes and is not modulated by a variety of pro-inflammatory and anti-inflammatory stimuli in vitro [77]. Human Arginase-I is localized and well-preserved in azurophil granules and it appears that human neutrophils do not significantly metabolize extracellular arginine in the milieu [77]. Thus, the arginine-dependent suppression of T cell responses observed in mouse tumor models might not take place in humans.

Therefore, for the multiple reasons outlined above it is important to consider the possibility that the neutrophil response in mouse tumor models may not occur in precisely the same way as it does in humans.

Challenges in the Study of Human Tumor–Associated Neutrophils

Murine experimental studies have highlighted opposing roles of neutrophils in cancer, however there is no direct evidence yet that neutrophils tend to be pro-tumoral in human tumor tissue and it is still unproven whether N1/N2 polarization of tumor-infiltrating neutrophils occurs in humans. The majority of experimental approaches to study neutrophils in cancer patients have relied on circulating blood neutrophils and make the assumption that these cells have a similar function in the tumor. Results of these studies have been recently summarized and reviewed [8,78]. Unfortunately, there is virtually no functional data relating to TANs in humans. The primary challenges that

have limited progress in this field in humans include technical difficulties in obtaining fresh tumors, inefficient digestion of human tumor tissue, failure to isolate TANs while preserving cell surface markers and functionality, and the lack of cognate mouse myeloid-cell markers in humans. The logistical, ethical, and regulatory difficulties in obtaining human tumor tissue for research also act to discourage such studies.

Given all these obstacles, the characterization of neutrophils within human tumor tissue has been mostly limited to detection of only single or double granulocytic markers by immunohistochemistry and correlations of neutrophil counts to clinical prognosis. Intratumoral accumulation of neutrophils is associated with poor prognosis for patients with head and neck cancer [79], RCC [3], hepatocellular cancer [80], and some other types of cancers [81]. In contrast, a high neutrophil count has been associated with a favorable prognosis in patients with gastric cancer [82]. The results in lung cancer have been mixed. In one recent study, the presence of TANs in NSCLC did not correlate with overall survival [1]. In another study, an increased intratumoral neutrophil-to-CD8 T cell ratio was a poor prognostic factor [2]. Similarly contradicting results were reported for colorectal cancer [83,84]. Importantly, there have been no reports directly demonstrating a pro-tumoral role of TANs in any type of human tumors.

There are several explanations as to why the density of TANs is an inconsistent prognostic factor for the clinical outcome of cancer patients. First, all clinical studies have used immunohistochemical analyses of tumor tissues to correlate the presence of granulocytes with prognosis. The limitations of this type of analysis prevent comprehensive descriptions of the neutrophil phenotype, as well as evaluation of the functional state of these cells in the tumor. The heterogeneous expression of many

surface receptors on TANs suggests that different subpopulations of TANs exist in the tumor and might perform different functions [27,85,86]. Thus, the variation in the accumulation of different subsets of neutrophils in tumor tissue might explain why the percent of total neutrophils in the tumor is an inconsistent predictor for survival of cancer patients. Second, an immunogenic “switch” from anti-tumor to pro-tumor phenotype might take place [87]. Specifically, TANs might lose or change their functions as the tumors progress [85,86]. Accordingly, a simple neutrophil count in tumor tissue at any one time point (where the pro- versus anti-tumor status of the neutrophils is not known) may not be an accurate parameter for clinical prognosis.

To study human TAN function, the generation of high quality single-cell suspensions from human tumor tissues is required. However, to date no standardized techniques exist for the disaggregation of human tumor tissue. Currently available methods may cause alterations in the true immune cell profile and thus can provide misleading results. We have conducted a study in which we critically evaluated current techniques used to prepare human tumors for immunologic studies [88] and found that many of these approaches use an unbalanced composition of enzymes that artificially cleave multiple cell-surface markers. This digestion-induced effect might lead to false conclusions about the presence of specific cellular populations and their biologic characteristics. To overcome this technical difficulty, we optimized a disaggregation method designed for human lung tumors that preserves the phenotype and function of the immune cells, including neutrophils [85,88]. Our data suggest that a similar approach should be applied to any type of human tumors to be analyzed, as conditions vary depending on tumor type.

Given that human tumor development represents an ongoing process of evolution, it is critical to understand the complex interaction of TANs with the tumor microenvironment during all stages of tumor growth. One of the major challenges in TAN biology is deciphering the complex interaction of activated neutrophils with T cells in the tumor microenvironment. Understanding the role of TANs in the regulation of the T cell response is especially important because cytotoxic T lymphocytes are the major effector cells mediating antigen-driven anti-tumor immunity [89]. There is increasing evidence that activated neutrophils can interact with T cells in dichotomous ways. Several studies have shown that neutrophils can present antigens and provide accessory signals for T cell activation [90,91]. Other studies have demonstrated that peripheral blood neutrophils (PBN) can suppress antigen-nonspecific T cell proliferation through the depletion of arginine and the production of ROS [92,93]. To date, the suppressive function of granulocytic cells in cancer patients has generally been attributed to a circulating low-density PMN-MDSC population [94]. However, there has been some uncertainty whether PMN-MDSCs are present in human tumors and whether the majority of TANs are actually PMN-MDSCs.

We have begun initial studies with the goal of phenotypic and functional characterization of human TANs at early stages of tumor development. Although the presence of a minor suppressive subpopulation of TANs cannot be excluded, our data suggest that in patients with early stage lung cancer, TANs do not significantly contribute to the inhibition of T cell responses [85]. In fact, the TANs isolated from the vast majority of small size, early-stage tumors were actually able to stimulate T cell responses to varying degrees. We found that direct cell-cell contact was important for the

neutrophil-mediated stimulation of T cell proliferation. An important feature of this interaction was crosstalk between TAN and T cells resulting in mutual activation. During their interaction, T cells further up-regulated their activation markers and produced more IFN- γ , whereas TANs increased their survival time and expressed the co-stimulatory molecules CD86, CD40, CD54, OX40L, and 4-1BBL.

Our data are consistent with some literature showing the anti-tumor potential of neutrophils during tumor growth in mouse models [95,96]. For instance, Suttman and colleagues demonstrated that polymorphonuclear neutrophils were an indispensable subset of immunoregulatory cells and orchestrated T cell chemotaxis to the bladder during *Bacillus Calmette-Guérin* immunotherapy [19]. Augmentation of T cell proliferation and/or survival by tumor-infiltrating neutrophils was found to be critical in the establishment of antitumor immunity following photodynamic therapy [18]. Liu et al., have also demonstrated that neutrophils could control early tumorigenesis in lung by direct cytotoxic effects [55].

We also identified a specialized subpopulation of TANs with “hybrid” characteristics of neutrophils and antigen-presenting cells [86]. These APC-like hybrid TANs accumulated only in small sized, early stage tumors and dramatically contributed to the strong T cell stimulatory effect of all TANs observed in small tumors. Based on the observation that TANs lost their T cell stimulatory ability in large tumors, we postulate that this anti-tumor TAN response could represent the footprint of incomplete or ongoing elimination or equilibrium phases of immunoediting during the early stages of lung cancer. It appears that the anti-tumoral potential of neutrophils diminishes during tumor progression, supporting the concept of an immunogenic “switch” from anti-tumor to pro-

tumor phenotype during tumor progression (Fig.1) [87,97]. On the other hand, these T cell-stimulatory TANs secrete factors such as MIP-1 α , FGF, HGF, and EGF that could be pro-tumorigenic and act as growth and survival factors for tumor cells, as well as support angiogenesis [85,98]. Thus, it is difficult to accurately determine if the TANs in early stages of human tumors are actually N1 or N2 neutrophils.

We can also speculate that although small size early-stage tumors in humans have already reached a considerable size and represent an escape stage of immunoediting process, the process of tumor evolution is still ongoing, because the profound immunosuppressive environment is not fully developed yet and anti-tumoral neutrophils still prevail in small size tumors (early escape stage). The resistant tumor clones, which survived the immunoediting process, likely further develop immunosuppressive mechanisms in order to sustain tumor growth into more advanced stages. At these advanced tumor stages, the suppressive environment appears to disable anti-tumoral N1 neutrophils and perhaps even convert them into the pro-tumoral N2 type (late escape stage). Thus, it is very possible that N2 pro-tumoral TANs represent the consequences of tumor progression, but not a cause. This hypothesis has not been experimentally confirmed, but we are trying to test it with TANs from patients with advanced lung cancer (stages III and IV). This has turned out to be logistically challenging since these individuals do not routinely undergo tumor resection and are managed with chemotherapy and radiation therapy.

Concluding Remarks

The goal of this review is to raise awareness about some key issues relating to extrapolating data from mouse tumor models to human cancers. It is becoming increasingly obvious that critical species-specific differences in tumor evolution and immunity, as well as significant species differences in the biology of neutrophils create challenges and risks in translating data derived from mouse studies into humans. Moreover, there is a lack of data about the function of neutrophils in human tumors. Thus, to date, it is very difficult to reliably compare the phenotypes and functions of neutrophils during tumor progression in mice versus humans.

Since human tumor development represents a process of gradual evolution, it is critical to understand the complex interaction of TANs with the tumor microenvironment at all stages of tumor evolution, as their relationship appears to change over time. Although the first attempts have been made, there is a continuing need to develop new and innovative approaches to characterize granulocytes and their subpopulation in human cancers and to characterize the variety of their functions in the human tumor microenvironment (see outstanding questions). This knowledge will allow us to develop different therapeutic strategies to regulate the function of TANs depending on tumor stage. It is hoped that understanding how to direct and maintain the human TANs towards anti-tumor effector cells will open new therapeutic options in the future design of active immunotherapy to potentially boost natural or vaccine-induced anti-tumor immunity.

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Figure legend

Figure 1. Fundamental differences in genetic heterogeneity, tumor evolution and intrinsic biology of neutrophils might affect neutrophil phenotype and function in mouse versus human tumors.

In contrast to humans, laboratory mice used for tumor models are inbred and genetically homogeneous. Moreover, they live in abnormally hygienic specific pathogen free (SPF) barrier facilities. Undoubtedly, this does not reflect relevant aspects of the human immune and inflammatory responses occurred during tumor development. Human tumors evolve slowly and undergo all steps of cancer immunoediting (elimination, equilibrium

and escape). At the first steps of the tumor evolution, tumor clones initiation, proliferation and diversification occur along with selective pressure on these tumor clones by anti-tumor immune responses. Some clones may elicit immune responses and be killed, while other clones (brown and black color) may favor the immune tolerance and survive. At these stages anti-tumoral neutrophils (N1) would be expected to develop. Consequent immune selection pressure allows surviving tumor clones to acquire sufficient adaptations that permit immune evasion. Subclones resistant to an immune response will gradually become predominant. Surviving clones form the third phase of immune escape, where pro-tumor mechanisms predominate. At this stage, where anti-tumor response is weak against surviving clones, the accumulation of immunosuppressive N2 neutrophils and/or (Polymorphonuclear Myeloid-Derived Suppressor Cells) PMN-MDSC might be expected. In contrast to human, mouse tumor models use tumor cell lines that are originated from advanced tumors. These tumor cells have already undergone cancer immunoediting and thus are relatively resistant to an immune response. In addition, these tumor cell lines represent a homogenous tumor burden to the immune system that shortly enters the last phase of tumor evolution (escape) after transplantation in mice. This phase is mostly characterized by profound pro-tumoral response with accumulation of immunosuppressive N2 neutrophils and/or PMN-MDSC.

Outstanding Questions

1. Does the anti-tumoral N1/pro-tumoral N2 paradigm that appears to exist in mice occur in human cancer?
2. Are these N1/N2 neutrophils truly bona fide neutrophil subtypes or do they represent a continuum of activation and maturation states?
3. Is there the immunogenic "switch" from anti-tumor to pro-tumor neutrophil phenotype as tumors progress from early stages to advanced stages?
4. Can the neutrophil response observed in rapid growing murine tumors be extrapolated to human cancers that evolve very slowly?
5. Can anti-tumor functions of TANs be harnessed to treat cancer?

Human tumor

Mouse transplant tumor model