Neutrophils: critical components in experimental animal models of cancer

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Abstract

Neutrophils have a crucial role in tumor development and metastatic progression. The contribution of neutrophils in tumor development is multifaceted and contradictory. On the one hand, neutrophils prompt tumor inception, promote tumor development by mediating the initial angiogenic switch and facilitate colonization of circulating tumor cells, and on the other hand, have cytotoxic and anti-metastatic capabilities.

Our understanding of the role of neutrophils in tumor development has greatly depended on different experimental animal models of cancer. In this review we cover important findings that have been made about neutrophils in experimental animal models of cancer, point to their advantages and limitations, and discuss novel techniques that can be used to expand our knowledge of how neutrophils influence tumor progression.

Keywords

neutrophil; tumor development; animal models

1. Introduction

There is growing evidence indicating that neutrophils have an important role in tumor development – from the inception of tumor formation and throughout the malignant progression [1-3]. Neutrophils both promote and prevent tumor progression (reviewed in [1]). The contradictory role of neutrophils in tumor development might be explained be the fact that neutrophils can have a pro- or anti-inflammatory phenotype [4-6]. Depending on their phenotype neutrophils appear to be either antitumoral (N1) or protumoral (N2) [4]. The occurrence of N1/N2 neutrophils has only been shown in murine tumor models and will have to be confirmed in other experimental animal models and more importantly in humans.

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However, neutrophils with an activated phenotype, producing pro-inflammatory factors and stimulating T cell proliferation has been described in early human lung cancer, while immunosuppressive neutrophils has been reported in human colorectal cancer [5, 6].

Tumor-associated neutrophils (TANs) with a N1 phenotype have a pro-inflammatory cytokine profile, producing nitrate oxide and hydrogen peroxide (H_2O_2), and are cytotoxic towards tumor cells [4]. N2 TANs, on the other hand, are characterized by high levels of arginase 1 (ARG1) and by their ability to inhibit effector T-cell functions [4, 6].

The tumor microenvironment, evolving with tumor progression, can influence the polarization state of TANs [7]. Transforming growth factor-β (TGFβ) can differentiate neutrophils toward a protumoral phenotype [4]. Intriguingly, neutrophils initially recruited during the early stages of tumor development have an antitumoral N1 phenotype. However, with tumor progression the tumor-infiltrating neutrophils become more protumoral and acquire a N2 phenotype. In line with the polarization paradigm only depletion of neutrophils at a later stage of tumor development reduces tumor growth, further pointing to the fact that neutrophils can have an anti- or protumoral phenotype [7]. Transcriptional profiling of TANs from different mouse tumor models furthermore has revealed that TANs can have strikingly different protumorigenic transcriptional profiles [8]. Thus, even though neutrophils can be polarized into either N1 or N2, they presumably make up a continuum of different phenotypes.

Our understanding of the polarization of neutrophils and how neutrophils contribute to tumor development has largely been elucidated from experimental mouse models. However, alternative models, for example, the zebrafish model, have provided key insights into how neutrophils influence tumor development. In this review, we cover important findings that have been made about neutrophils in experimental animal models of cancer and point to the advantages and limitations of different models. We point to how different experimental animal models and novel techniques can be used to expand our knowledge of how neutrophils influence tumor progression.

1.1 From men to mice

Experimental mouse tumor models have contributed to our understanding of tumor biology and the intricate role of neutrophils in tumor development. There are many advantages with mice; they are small in size, breed in captivity and have biological similarities with humans. However there are important differences between humans and mice [9, 10]. While neutrophils in humans make up the majority (50-70%) of leukocytes in the peripheral blood, they only account for 15-20% of the leukocytes in the peripheral blood of mice [11]. Furthermore, humans live longer, have a lower metabolic rate and undergo more cell divisions. One might therefore anticipate that cancer would be more common among humans, but, interestingly, the lifetime risk of developing cancer is comparable between humans and mice. Mice, however, more often get tumors of mesenchymal origin such as sarcomas and lymphomas, while tumors in humans most often originate from epithelial cells giving rise to carcinomas in, for example, colon, breast and lungs [12].

Different mouse tumor models provide powerful experimental tools to study various cancers that normally develop in humans [13-18]. There are three major experimental mouse tumor models: 1) genetically engineered, 2) transplantable, and 3) humanized tumor models. Each model has different advantages and limitations for elucidating the role of neutrophils in tumor development.

1.1.1 Genetically engineered tumor models

Genetic engineering has allowed for tissue-specific and time-specific induction of various oncogenes or dominant-negative tumor-suppressor genes triggering spontaneous tumor development in mice (Table 1) [13-15]. Spontaneous tumor growth in genetically engineered mouse models faithfully depicts the multistage tumor development seen in humans and, more importantly, the evolving interplay between the tumor microenvironment with neutrophils and initially premalignant tumor cells that with time will become malignant and evolve to full-blown cancer.

Genetically engineered mice can have an intact immune system, hence making the models suitable for studying the interaction, not only between tumor cells and immune cells, but also the intricate interplay between innate and adaptive immune cells during tumor development [19].

Genetic engineering can target different genes expressed by neutrophils and thereby depict how genes of interest contribute to the function of neutrophils in tumor development. By taking advantage of the Tie2:Cre deleter, Finisguerra *et al.* recently revealed that deletion of *Met* (coding for MET; the receptor for hepatocyte growth factor (HGF)) in neutrophils attenuated the recruitment of antitumoral, but not protumoral, neutrophils [20, 21]. MET is a prerequisite for the cytotoxic ability of antitumoral neutrophils and reduces tumor growth and metastasis [21].

1.1.2 Transplantable tumor models

While tumor growth in genetically engineered mice can take from a few months up to over a year, transplantable tumor models have the advantage that they can develop full-blown tumors after only a few weeks. A wide range of different cancer cell lines has been extensively used in transplantable tumor models (Table 1). These cancer cell lines have either originated spontaneously or from carcinogen-, transgene- or gene knockout-induced tumors [18].

Transplantation with cancer cell lines into syngeneic mice has the advantage that the mice have an intact immune system. However, the fact that the mice are of the same inbred mouse strain means that the model will not reflect the genetic heterogeneity found among human patients. Also, since the injected cells already are malignant and are put into a naïve microenvironment, the growth of the tumor will not reveal multistage tumor development and the evolving interaction between tumor cells and the tumor microenvironment.

Different strains of immunodeficient mice have made transplantation of human tumor cells, i.e., xenograft transplantation, feasible (Table 1) [22]. A major drawback of the xenograft transplantation model is that, to enable growth of human tissue and prevent rejection, the mice have to be devoid of functional immune system. Hence, even though the xenograft

models have greatly contributed to our knowledge of how human cancer cells behave during tumor progression, they are not able to fully depict the functional contribution of tumor cells, innate- and adaptive immune cells and their bidirectional communicate.

1.1.3 Humanized mice model—'Humanized mice' are immunodeficient mice that have gained a functional human immune system through the engraftment of human primary hematopoietic cells or tissues. Humanized mice are derived from various mouse strains of immunodeficient mice, which have in common that they have mutations in the interleukin-2 (IL-2) receptor common γ-chain locus *(Il2rg)*. Targeted mutation of *Il2rg* gives major impairments in the development and function of T, B and natural killer (NK) cells [23]. Currently, immunodeficient mice strains can be humanized with human adaptive immune cells and various innate immune cells including NK cells, monocytes and macrophages, however successful establishment of human neutrophils is a challenge yet to be conquered [24-27].

1.2 Neutrophils and Gr-1$^+$ immune cells

Neutrophils in mice are recognized as CD11b$^+$Ly6G$^+$ cells [28, 29]. However, until recently Gr-1 was broadly used as a marker for bone marrow-derived cells including immune cells of the neutrophil lineage. Gr-1 recognizes the two antigens, Ly6C on monocytes and Ly6G on neutrophils. Even though neutrophils are Gr-1^{high} (while monocytes are Gr-1^{int}), Gr-1 is not able to differentiate between monocytes and neutrophils efficiently [2, 28]. Moreover, Gr-1 has been used to classify immature myeloid derived suppressor cells (MDSCs) as immune cells that primarily exert their protumoral effect through inhibition of antitumoral T-cells [30, 31]. Two subpopulations of MDSCs have been characterized; monocytic-MDSCs (Mo-MDSCs), which are CD11b$^+$Ly6C$^+$, and granulocytic-MDSCs (G-MDSCs), which are CD11b$^+$Ly6G$^+$ [32, 33]. In addition to being classified by the same surface markers, G-MDSCs are morphologically similar to immature neutrophils and have functional overlap with N2 neutrophils (both having immunosuppressive capacity) [32, 34]. The immunosuppressive function of G-MDSCs is however contradictory and accumulating evidence indicate that only Mo-MDSCs and not G-MDSCs mediate T-cell suppression [35-37]. Hence an important question to be unraveled is the exact role of G-MDSCs in tumor development and whether or not neutrophils and GMDSCs are two distinct immune cell populations or if they in fact are the same immune population but with different phenotypes. Due to the lack of definite markers for each population the relationship between neutrophils and G-MDSCs is still largely undefined. However, work by Youn *et al.* showed that tumor-derived neutrophils (CD11b$^+$Ly6C^{low}Ly6G$^+$) have more functional characteristics in common with splenic G-MDSCs (CD11b$^+$Ly6C^{low}Ly6G$^+$) from tumor-bearing mice than with neutrophils from non-tumor bearing mice, which could imply that G-MDSCs migrate to the tumor site and that TANs acquire a N2 phenotype prior to arriving at the tumor site [38]. On the contrary, transcriptomic analysis, comparing TANs (CD11b$^+$Ly6G$^+$), splenic GMDSCs (CD11b$^+$Ly6G$^+$) from tumor-bearing mice and naïve bone marrow neutrophils, revealed that TANs make up a unique neutrophil population quite distinct from splenic G-MDSCs, which were more closely related to naïve bone marrow-derived neutrophils [39]. The lack of apparent transcriptomic similarity between TANs and splenic G-MDSCs could imply that factors, e.g., TGFβ, in the local tumor

microenvironment, indeed, dictate the fate of neutrophils and that TANs are not derived from G-MDSCs but from a peripheral neutrophil population. Indeed, different subpopulations of neutrophils exist in the circulation. Circulating high-density neutrophils are mature and predominant during early tumor development, while low-density neutrophils are immunosuppressive and accumulate with tumor progression [40]. Taken together the exact relationship between neutrophils and G-MDSCs is still largely unclear and more work is needed to determine how they relate. Similarities and differences between neutrophils and G-MDSCs have been reviewed in detail by Pillay *et al* [41]. Since there currently are no definite markers to distinguish neutrophils from G-MDSCs we cover both in this review and refer to them as neutrophils.

2. Inflammation and tumor inception

Chronic inflammation, i.e., unresolved inflammation with infiltration of immune cells, tissue remodeling and angiogenesis, can prompt the genetic mutation and proliferation rate in mutated cells or directly initiate genomic instability through e.g. DNA damage or intruding on DNA repair pathways. Neutrophils, accompanying chronic inflammation, are involved in creating a mutagenic environment capable of initiating and promoting tumor development [42-45].

The Mutatect tumor model, which allows for the detection of mutagenic incidents in murine fibrosarcoma cells, has been used to study how neutrophils contribute to genetic instability [46]. The genetic damage is detected in a particular target gene, namely *Hprt* (hypoxanthine phosphoribosyltransferase). Mutatect cells that become mutated, i.e., gain inactivation of *Hprt*, have acquired a resistance to the cytotoxic drug 6-thioguanine (6-TG) and can be identified with a clonogenic assay. Initially it was revealed that the mutation frequency in this model was elevated *in vivo* compared to *in vitro*, implying that factors in the microenvironment contributed to the genetic instability [47]. Indeed, when various Mutatect cell lines are subcutaneously injected into immunocompetent syngeneic C57BL/6 mice, neutrophils are the predominate leukocyte population infiltrating the tumors and the mutation frequency strongly correlates with the number of neutrophils [46].

The significance of neutrophils in tumor inception related to chronic inflammation has further been shown in a model of ulcerative colitis (UC). UC is a form of inflammatory bowel disease that gives an elevated risk of colon cancer [48]. The administrating of azoxymethane (AOM) and dextran sulfate sodium (DSS) to mice recapitulates the chronic inflammation seen in the colon of UC patients [49, 50]. One of the main inflammatory mediators to recruit neutrophils in this model is CXCL2 [51, 52]. CXCL2 attracts CXCR2+ neutrophils, and the infiltration of neutrophils precedes the development of multiple tumors in the colon [51]. Moreover deletion of *Cxcr2* in genetically engineered *Cxcr2*$^{-/-}$ mice impairs AOM/DSS induced colonic chronic inflammation, reduces tumor burden and inhibits malignant progression [52, 53].

Yet another model that has linked neutrophils to tumor inception in chronic inflammation is a model of skin inflammation where mice are topically treated with 7,12-dimethylbenz [a] anthracene (DMBA), followed by repeated application of 12-*O*-tetradecanoylphorbol-13-

acetate (TPA) [53]. The combined treatment induces skin inflammation with persistent recruitment of neutrophil and papillomas eventually develop in the site of tissue perturbation [54]. Importantly, inhibition of CXCR2^{+} neutrophils prevents the inflammation driven tumor development in this model [53]. Common for both the UC and skin model is that CXCR2^{+} neutrophils promote early tumor development through the inhibition of antitumoral T-cell immunity.

2.1 Neutrophils and adaptive immune cells

Adaptive immune cells are part of cancer immunosurveillance and can, once they are activated, efficiently eradicate premalignant tumor cells [55-58]. Hence, for a tumor to thrive it must gain capabilities that enable it to evade antitumoral adaptive immune control. Tumors can, for example, recruit protumoral neutrophils that will inhibit the generation and function of cytotoxic T-cells [52, 53]. Since neutrophils are key producers of ARG1, an enzyme that is involved in the impairment of antitumoral T-cells, they can modulate T-cell immunity and thereby control tumor progression. However, adaptive immune cells can exert an effect on neutrophils, emphasizing the two-directional communication between innate and adaptive immune system [2, 59, 60].

Richards *et al.* showed, in a model of subcutaneous injection of the mouse melanoma cell line B16FasL, that skin-resident regulatory T-cells (Treg) are rapidly mobilized to the site of injection where they limit early recruitment and survival of neutrophils [61]. Interestingly, neutrophils significantly contribute to early tumor rejection in this model, in line with previous data showing that neutrophils arriving during early tumor development are antitumoral [7, 61-63].

2.2 Acute inflammation – what can be learned from zebrafish

It is now generally appreciated that chronic inflammation can accelerate and induce development of cancer [64]. However, recent evidence from a zebrafish model indicates that even acute inflammation can be detrimental for tumor development (Figure 1) [65]. The zebrafish has emerged as an attractive animal model to study tumor biology and the role of neutrophils in tumor development [66]. An important advantage with the zebrafish model, besides being suitable for genetic manipulation, is the fact that their embryos and larvae are transparent. The transparency of the model allow for efficient live imaging revealing how tumor cells and infiltrating immune cells interact [67-69]. Compared to the mouse model, where the animal number is a limitation both physically and financially, large numbers of zebrafish can by included in each experiment, making the data from each experiment more powerful. Contrary to what one first might think, zebrafish tumor biology has important similarities with human cancers at both histological and genetic levels. Zebrafish neutrophils are an abundant leukocyte population in the circulation similar to humans [70, 71]. Moreover, zebrafish have both an innate and an adaptive arm of the immune system. During the embryonic and larvae stage they will, however, only have innate immune cells, making it an attractive model to study the specific role of neutrophils in tumor inception and early tumor development [72].

Using a zebrafish model where the melanocyte-specific promoter *mitfa* (microphtalmia-associated transcription factor a) drives oncogenic human HRas^{G12V} expression in melanocytes, Antonio *et al.* revealed that neutrophils are attracted from the site of acute injury toward premalignant cells where the neutrophils promote tumor progression through PGE$_2$ production (Figure 1) [65, 73, 74]. H$_2$O$_2$, derived from transformed cells but also from non-tumoral neighbors, is the key neutrophil-attractant and inhibiting the generation of H$_2$O$_2$ attenuates infiltration of neutrophils and the proliferation of transformed cells [75]. The finding that neutrophils, not only in chronic, but also in acute inflammation promote tumor development will have to be confirmed in other experimental animal models of cancer and could, if holding true, have important clinical implications.

3. Tumor progression

Tumor development starts in a single or a few cells that have acquired genetic and epigenetic alterations. The abnormal activation of oncogenes or the loss of tumor suppressor genes initiate stable blockade of cell proliferation, i.e., senescence. For a pre-malignant tumor to become malignant it will have to evade senescence [76]. In a model of prostatic tumor development inactivation of the tumor suppressor gene *Pten* in mouse prostate epithelium induces the formation of benign tumors. Benign *Pten*$^{-/-}$ tumors are characterized by strong senescence, but over time the tumors grow and become invasive [77, 78]. Intriguingly CD11b$^+$Gr-1$^+$ cells counteract the tumor senescence and promote the development of cancer. CD11b$^+$Gr-1$^+$ cells infiltrate the PTEN null tumors at the onset of senescence and secrete IL-1RA, which antagonizes senescence. Moreover, inhibition of CXCR2 and adoptive transfer of *Il1ra* knockout myeloid cells to *Pten*$^{-/-}$ mice enhances senescence of PTEN null tumors [79].

3.1 Neutrophil recruitment in spontaneous tumor models

Human cancer development is a multistage malady that can be depicted in different genetically engineered spontaneous tumor models (Table 1). An important advantage with spontaneous tumor models is that one can elucidate at what stage of tumor development neutrophils are being recruited.

The Kras^{G12D} pancreatic model faithfully recapitulates the development of human pancreatic ductal adenocarcinoma (PDA), which starts with noninvasive pancreatic intraepithelial neoplasia (PanIN) that progresses to invasive cancer [80]. Conditional expression of the mutated allele of *Kras* in pancreatic islets, specifically drives the progression from early pre-malignancy toward malignancy [81]. The mutational activation of Kras in pancreatic islets induces production of granulocyte-macrophage colony-stimulating factor (GM-CSF) recruiting protumoral CD11b$^+$Gr-1$^+$ cells that inhibit antitumoral T-cell immunity and thereby promote tumor progression. Inhibition of tumor-derived GM-CSF abrogates the recruitment of CD11b$^+$Gr-1$^+$ cells, which inhibits tumor development in an antitumoral T-cell dependent manner, once again emphasizing the important interplay between innate and adaptive immune cells [82, 83].

The MMTV-PyMT is another genetically engineered mouse model that depicts spontaneous tumor development in humans. The mouse mammary tumor virus long terminal repeat

(MMTV-LTR) drives mammary gland-specific expression of the oncogenic polyoma virus middle T antigen (PyMT) and, at the age of 6 weeks, MMTV-PyMT mice have developed hyperplasia in their mammary glands, that progresses to adenoma two weeks later, followed by early carcinoma (week 10); by week 11-15 the mice have late-stage carcinoma with metastatic burden, primarily in the lungs [84, 85]. Even though breast cancer is not preceded by chronic inflammation it recruits inflammatory cells. Recently, our group revealed that neutrophils start to expand systemically at the time of malignant transformation from adenoma to early carcinoma. The only hematopoietic growth factor elevated in MMTV-PyMT mice at this time point is granulocyte-colony stimulating factor (G-CSF). G-CSF expands multi-potent progenitors in the bone marrow that give rise to Rb^{low} T-cell immunosuppressive $CD11b^+Ly6G^+$ neutrophils [84].

The MMTV promoter has also been used to drive the transforming rat oncogene cerbB-2 (HER2/neu) [86]. The main attractant for neutrophils in this model is the major proangiogenic factor vascular endothelial growth factor (VEGF) [87].

3.2 Angiogenic switch

For a tumor to thrive and grow beyond 1-2 mm^2 in size it must acquire an angiogenic phenotype [88, 89]. Neutrophils have a prominent role in tumor angiogenesis both directly and indirectly (reviewed in [90]). Neutrophils have a large intracellular pool of VEGF that can become available upon degranulation. Interestingly, neutrophils in mice deficient in Hck and Fgr ($hck^{-/-}fgr^{-/-}$), two Src family protein tyrosine kinases expressed by granulocytes and monocytes, have impaired VEGF degranulation [91].

Using the transgenic multistage pancreatic tumor RIP-Tag2 model, where the rat insulin promoter (RIP) induces production of simian virus 40 (SV40) large T-antigen (Tag) oncoproteins in pancreatic islets, Nozawa *et al.* revealed that the initial angiogenic switch is mediated by MMP-9 derived from infiltrating neutrophils [92, 93]. Also in transplantable tumor models of murine melanoma (B16F10), fibrosarcoma (L929), Lewis lung carcinoma (LLC) and human prostate cancer cells (PC-3), infiltrating neutrophils are the major source of MMP-9 [94]. Importantly unlike other immune cells that also produce MMP-9, neutrophils supply the tumor with a TIMP-free form of MMP-9 that more efficiently induces angiogenesis [94-97]. Activate MMP9 remodels the extracellular matrix and thereby enhances the bioavailability of VEGF and fibroblast growth factor (FGF-2), another angiogenic factor, that otherwise are captured in the extracellular matrix [98, 99]. Intriguingly, neutrophil-derived MMP-9 is also a potent, direct and VEGF-independent angiogenic factor [100]. Transplantable tumor models and the RIP-Tag2 model have furthermore revealed that prokineticin-2 is expressed by $CD11b^+Gr-1^+$ cells and promotes not only $CD11b^+Gr-1^+$ cell mobilization but also angiogenesis [101, 102].

In contrast, interferon-β (IFNβ) negatively regulates neutrophil-induced angiogenesis [103]. In a tumor mouse model where melanoma B16F10 cells and MCA205 fibrosarcoma cells are transplanted in $Ifnb1^{-/-}$ or $Ifnar1^{-/-}$ syngeneic C57BL76 mice, the tumors grow faster compared to syngeneic control mice. The acceleration in tumor growth in IFNβ deficient mice is due to an increase of proangiogenic neutrophils recruited by CXCR2 ligands [103, 104]. The researchers concluded that adaptive immune cells are not involved in the IFNβ-

dependent inhibition of tumor angiogenesis by repeating the experiments in $Ifnb1^{-/-}Rag2^{-/-}$ and $Rag2^{-/-}$ mice that lack T and B-cells [103, 105]. Moreover, in the $Ifnb1^{-/-}$ mice model deficiency of IFNβ delays the apoptosis of pro-angiogenic neutrophils infiltrating the tumor [106].

4. Neutrophils in metastatic progression

Metastatic progression begins with tumor cells in the primary tumor gaining invasive and migratory capabilities that enable them to intravasate local blood and lymphatic vessels and thereby reach distant organs with the circulation. Neutrophils play a crucial role during metastatic progression – beginning in the primary tumor where they favor tumor invasion.

Murine breast cancer cells with $Tgfbr2$ deletion produce the CXCR2 ligand, CXCL5, and their tumors have enhanced infiltration of CD11b+Gr-1+ cells, which promote tumor invasion through MMP production [107]. Similar results have been obtained in the RETAAD model, a model of uveal melanoma where mice are transgenic for the activated oncogene RET [108]. In the RETAAD model CXCR2+ neutrophils infiltrate the primary tumors where they favor melanoma cell migration and induce an invasive phenotype of melanoma cells [109]. In yet another model of invasive and metastatic melanoma (due to transgenic overexpression of HGF and oncogenic mutation of CDK4(R24C) giving an impaired cell cycle control) ultraviolet radiation induces inflammation. The inflammation is mediated by neutrophils recruited by high mobility group box 1 (HGMB1) produced by UV-damaged epidermal keratinocytes. The infiltration of neutrophils promotes metastatic progression to the lungs through enhanced growth of melanoma cells along the abluminal side of endothelial cells with occasional perivascular invasion [110].

In addition to promoting metastatic progression in the primary tumor neutrophils expand systemically in tumor-bearing mice [33, 84, 111, 112]. Intriguingly, the recruitment of neutrophils begins before circulating tumor cells actually reach the metastatic sites. Hence, neutrophils are part of the pre-metastatic niche [113-115]. Tumor-derived G-CSF is an important attractant for neutrophils in many experimental models of cancer and recruits neutrophils to the lung pre-metastatic niche, while tumor-derived COX2 and PGE$_2$ accumulate CD11b+Gr-1+ cells in the pre-metastatic niche of the brain [116, 117]. Recent work by Wculek and Malachani has further revealed that neutrophils accumulate in the lungs prior to tumor cell arrival; at the metastatic site they produce leukotrienes, which facilitate the colonization of a particular population of tumor cells that have high tumorigenic capacity [113]. The metastatic colonization of circulating tumor cells can further be facilitated by β2-integrin and Mac-1 on neutrophils, which bind ICAM on circulating tumor cells and thereby anchor the circulating tumor cells at the metastatic site [118, 119]. Moreover, neutrophils promote metastatic colonization with neutrophil extracellular traps (NETs) [120]. NETs are extracellular neutrophil-derived DNA structures used by neutrophils to capture circulating tumor cells at the metastatic site. Intriguingly, neutrophils in tumor-bearing mice are more prone to form NETs [113-115].

Although mounting evidence points to the fact that neutrophils promote metastatic progression, there are contradicting data. In a model of renal cell carcinoma deletion of

important chemoattractants for neutrophils attenuates recruitment of neutrophils and increases metastatic tumor burden revealing an anti-metastatic role of neutrophils [121]. Furthermore, Granot *et al.* showed in a model of breast cancer that CCL2 derived from the primary tumor induces an anti-metastatic phenotype of neutrophils, which significantly inhibits the formation of lung metastases [122].

5. Future perspectives and conclusions

It is becoming evident that neutrophils have a prominent role, both preventing and promoting tumor development and metastatic progression. Their dual role indicates that neutrophils are not a homogenous population but make up a continuum of different phenotypes. Accordingly, there are different populations of circulating neutrophils and depending on the stage of tumor development, tumors appear to be infiltrated by either anti-(N1) or protumoral (N2) neutrophils [4, 7, 40]. Intriguingly, protumoral neutrophils can have strikingly different transcriptional profiles implying that there are far more distinct populations of neutrophils [8]. Single-cell analysis could provide a powerful tool to uncover the complex heterogeneity of neutrophils, not only in experimental animal models of cancer, but more importantly in human specimens, and give key insights into the plasticity of neutrophils [123, 124].

The differentiation of neutrophils is dictated by the tumor context. Tumor-derived TGFβ, for example, promotes the differentiation of N2 TANs and high-density neutrophils are able to differentiate into immunosuppressive low-density neutrophils in a TGFβ-dependent manner [4, 40]. Presumably, there is a plethora of other factors, acting autonomously or in combinations that can determine the fate of neutrophils. To develop therapeutics that can tune neutrophils into becoming antitumoral it will be vital to identify the factors that can induce a N1 phenotype. Importantly, N1 and N2 neutrophils have only been explored in murine tumor models; hence the classification of N1 and N2 neutrophils and the factors dictating the fate of neutrophils will have to be further confirmed in human cancers.

Tumor-derived factors will not only differentiate neutrophils but also cause their expansion and recruitment. It is however becoming evident that non-tumoral cells in the tumor microenvironment also attract neutrophils. UV-damaged keratinocytes recruit neutrophils that promote metastatic progression of melanoma cells; H_2O_2 derived from transformed melanocytes but also from their wounded non-tumoral neighbors attracts neutrophils in a melanoma zebrafish model and in a model of metastatic breast cancer, tumor cells produce IL-1β, activating γδ T-cells, which causes neutrophil expansion [74, 110, 125, 126]. Hence, not only tumor cells but also non-tumoral cells recruit neutrophils and have to be taken into account when elucidating how neutrophils are being recruited during tumor progression.

The exact origin of recruited neutrophils is still unknown. One possibility could be that N1 TANs originate from high-density circulating neutrophils, which are more predominant during early tumor development, while N2 TANs emanate from circulating low-density neutrophils, which are immunosuppressive and accumulate with tumor progression[40]. On the contrary, TANs might be the origin of different circulating neutrophil populations (Figure 2). Indeed, retrograde migration of neutrophils, from the site of tissue perturbation,

back to the circulation has been reported in the context of inflammation but still has to be proven in experimental animal models of cancer [127-129]. Recently, a new genetically engineered mouse model exhibited successful intravital imaging of neutrophils giving it an important advantage when, e.g., attempting to visualize neutrophil-retrograde migration [130].

Taken together neutrophils have an important role in tumor progression, which cannot be overlooked, independent of whether it is anti- or protumoral. The contradictory role of neutrophils in tumor development and metastatic progression can be attributed to the heterogeneity within the neutrophil population. The identification and importance of different neutrophil populations will be uncovered with single-cell technology and functionally attributed with recent development of cluster regularly interspersed short palindromic repeats (CRISPR)-Cas9 [131, 132]. The use of experimental animal models has been and will hence continue to be important in our effort to expand our knowledge about how neutrophils influence tumor development and metastatic progression.

Acknowledgements

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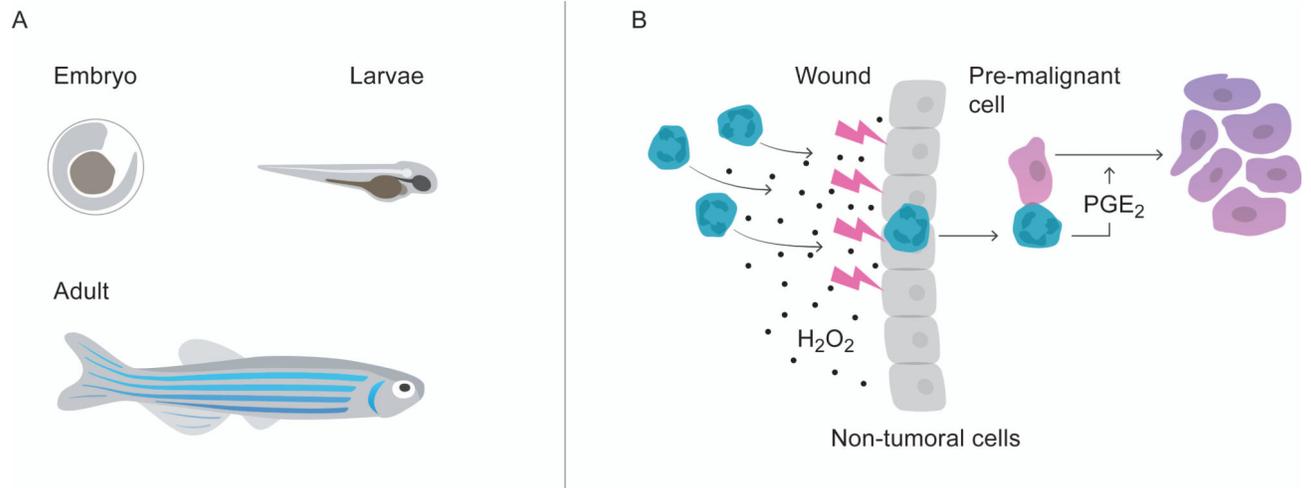
Highlights

- Neutrophils have a dual role in tumor development and metastatic progression

- Neutrophils are not a homogenous population but make up a continuum of phenotypes

- Mouse and zebrafish models provide key insights into how neutrophils influence tumor development

Figure 1.
The zebrafish model. A) The transparency of the zebrafish embryo and larvae makes the model suitable for live imaging of neutrophil- and tumor interactions. B) Neutrophils are attracted from the site of acute injury toward pre-malignant cells where the neutrophils promote tumor proliferation through PGE_2 production.
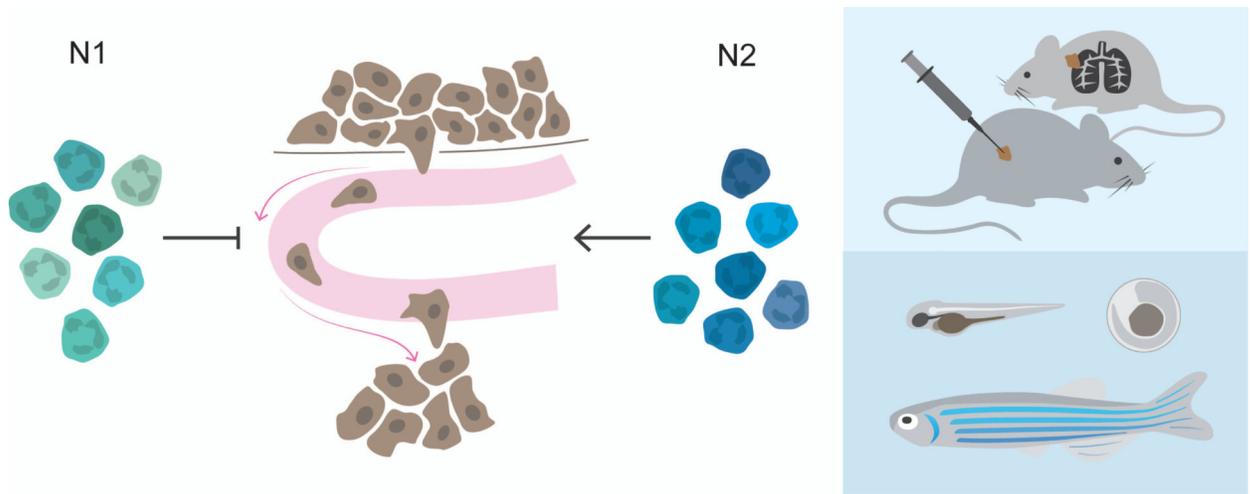
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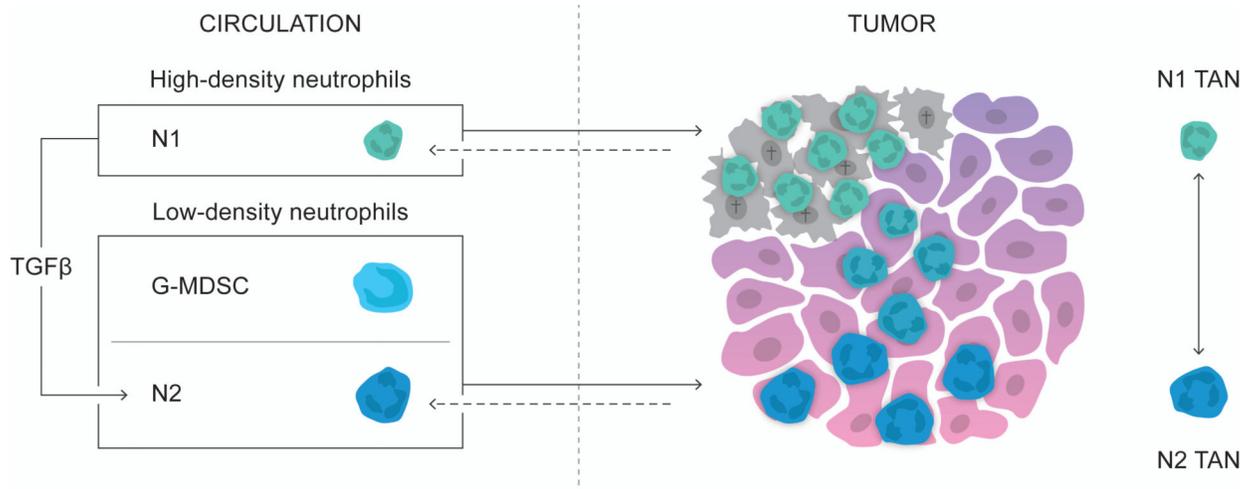
Figure 0003

Figure 2.

Neutrophils make up a continuum of phenotypes. The tumor microenvironment can influence the polarization state of TANs. TGFβ can differentiate neutrophils toward a protumoral N2 phenotype. The exact origin of recruited neutrophils is still unknown. N1 TANs might originate from high-density circulating neutrophils, while N2 TANs emanate from immunosuppressive low-density neutrophils. Another alternative could be that the local tumor microenvironment dictates the fate of recruited neutrophils. On the contrary, TANs might be the origin of different circulating neutrophil populations.

Table 1

Examples of experimental mouse models of cancer

Genetically engineered models	
Genetic model	*Cancer type*
RIP-Tag2	Pancreatic cancer
KrasGD12	Pancreatic cancer
K14-HPV16	Papilloma
MMTV-PyMT	Breast cancer
MMTV-Neu	Breast cancer
Transplantable models	
Transplantable mouse cancer cell lines	*Cancer type*
Lewis lung carcinoma	Lung cancer
B16	Melanoma
CT26	Colon cancer
4T1	Breast cancer
Transplantable human cancer cell lines	*Cancer type*
PC-3	Prostate cancer
MDA-MB-231	Breast cancer