Tumor-associated neutrophils: Critical regulators in cancer progression and therapeutic resistance (Review)

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Abstract. Cancer is the second leading cause of death among humans worldwide. Despite remarkable improvements in cancer therapies, drug resistance remains a significant challenge. The tumor microenvironment (TME) is intimately associated with therapeutic resistance. Tumor-associated neutrophils (TANs) are a crucial component of the TME, which, along with other immune cells, play a role in tumorigenesis, development and metastasis. In the current review, the roles of TANs in the TME, as well as the mechanisms of neutrophil-mediated resistance to cancer therapy, including immunotherapy, chemotherapy, radiotherapy and targeted therapy, were summarized. Furthermore, strategies for neutrophil therapy were discussed and TANs were explored as potential targets for cancer treatment. In conclusion, the need to explore the precise roles, recruitment pathways and mechanisms of action of TANs was highlighted for the purpose of developing therapies that precisely target TANs and reverse drug resistance.

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Abbreviations: TME, tumor microenvironment; TANs, tumor-associated neutrophils; TGF-β, transforming growth factor β; scRNA-seq, single-cell RNA sequencing; NE, neutrophil elastase; IL, interleukin; EMT, epithelial-mesenchymal transition; JAK2/STAT3, janus kinase 2/signal transducer and activator of transcription 3; GM-CSF, granulocyte-macrophage colony-stimulating factor; PD-L1, programmed death-ligand 1; MMP-9, matrix metalloproteinase 9; VEGF, vascular endothelial growth factor; NETs, neutrophil extracellular traps; HCC, hepatocellular carcinoma; ROS, reactive oxygen species; TNF, tumor necrosis factor; ELANE, neutrophils released catalytically active neutrophil elastase; TAMs, tumor-associated macrophages; ICC, intrahepatic cholangiocarcinoma; PDAC, pancreatic ductal adenocarcinoma; PD-1, programmed cell death protein-1; CAFs, cancer-associated fibroblasts; SRGN, serglycin; CRKL, CT10 regulator of kinase-like; Ly6G, lymphocyte antigen-6 complex, locus G; CRC, colorectal cancer; MDSCs, myeloid-derived suppressor cells; Gas6, expressed growth arrest specific 6; IFP, interstitial fluid pressure; NLRP3, pyrin domain-containing protein 3; EGFR, epidermal growth factor receptor; HER-2, human EGFR 2; TGF-α, transforming growth factor-α; KRAS, Kirsten rat sarcoma viral oncogene; GLUT1, glucose transporter 1; HMGB1, high-mobility group box 1; ADCC, antibody-dependent cell-mediated cytotoxicity; NLR, neutrophil-to-lymphocyte ratio

Key words: tumor-associated neutrophils, tumor microenvironment, drug resistance, tumor progression

Contents

1. Introduction

Cancer is the second leading cause of human mortality worldwide, with >190,000 new cases diagnosed and 100,000 associated deaths occurring each year, accounting for one in six global deaths and representing a significant public health concern (1,2). The current standard of care for cancer involves a multimodal approach that includes surgical resection, radiotherapy, chemotherapy, targeted therapy and immunotherapy (3-5). Advances in surgical techniques and the development of targeted therapeutic agents have led to significant progress in cancer treatment (6). However, emerging drug resistance and metastasis of malignant tumors remain the primary causes of mortality in patients with cancer, representing a significant challenge in the field of oncology (7). >90% of cancer-related deaths are attributed to drug resistance (8,9). Therefore, exploring drug resistance during cancer treatment and identifying effective low-toxicity therapeutic targets that can reverse drug resistance are crucial for reducing adverse effects and improving overall prognosis.

Although the exact mechanisms underlying drug resistance in cancer cells remain elusive, current findings indicate the involvement of numerous genes associated with drug efflux, DNA repair, apoptosis and diverse cellular signaling pathways (10,11). In addition to research asserting that mutations are responsible for tumor development and drug resistance, recent studies have revealed the tumor microenvironment (TME) as an integral part of tumorigenesis. The TME, which refers to the microenvironment surrounding tumor cells, including blood vessels, immune cells, fibroblasts and extracellular matrix (12), is strongly associated with the development of therapeutic resistance in tumor cells through complex signaling pathways (7,13).

Neutrophils were originally considered as first responders of the innate immune system against extracellular pathogens (14). Increasing evidence suggests that neutrophils also play an important role in the TME (15-17). Tumor-associated neutrophils (TANs) are immune cells that infiltrate the TME (18) and act directly or indirectly on tumor cells by releasing a variety of pro-inflammatory factors, immunomodulatory factors and angiogenic factors, which either promote or inhibit tumor occurrence, progression and metastasis (19). In the current article, the role of TANs in tumorigenesis and cancer progression in the TME was summarized, their contribution to therapeutic resistance was explored and existing TAN-targeted therapeutic strategies were reviewed.

2. TANs and cancer progression

Formation and plasticity of TANs. The migration of neutrophils from bone marrow to the tumor site involves three stages (Fig. 1): Maturation of neutrophils in the bone marrow, circulation in the blood and chemotaxis to the tumor site (20). Increasing research on the body's immune functions have shown that TAN regulation is highly reprogrammable. Neutrophils can acquire different phenotypes based on environmental signals (21); these phenotypic differences determine functional differences among neutrophils and govern whether the cells are involved in pro- and/or anti-tumor responses.

Fridlender *et al* (22) described a dichotomy in TANs, whereby neutrophils reaching the vicinity of tumors in mice can develop into either anti-tumor N1 cells or pro-tumor N2 cells (23). This choice seems to depend on the production of transforming growth factor β (TGF-β) in the TME, which promotes the generation of pro-tumorigenic N2 cells (22). This differentiation may reflect plasticity rather than the true subtype of neutrophils.

Single-cell RNA sequencing (scRNA-seq) provides an unprecedented view of cellular heterogeneity in the TME. However, owing to the relatively low RNA levels and high RNase levels of neutrophils, neutrophil research is challenging. With the development of scRNA-seq, multiple phenotypes of TANs have been identified in a variety of cancers (19,24,25). Furthermore, pseudotime analysis has revealed differentiation trajectories along neutrophil states (26). In non-small cell lung cancer, Salcher *et al* (24) identified four TAN subsets (TAN-1 to TAN-4) and showed that the overall TAN phenotype was characterized by high expression of oxidized low-density lipoprotein receptor 1, vascular endothelial growth factor A, CD83, intercellular adhesion molecule 1 and C-X-C motif ligand receptor (CXCR)4 but low expression of CXCR1, CXCR2, prostaglandin-endoperoxide synthase 2, selectin L (CD62L), colony-stimulating factor (CSF)3R and Fc gamma receptor IIIb (CD16B). In pancreatic cancer (27), TANs were divided into five heterogeneous subgroups: A terminally differentiated pro-tumor subpopulation (TAN-1) associated with poor prognosis, an inflammatory subpopulation (TAN-2), a transitional population recently migrated to the TME (TAN-3) and a subpopulation preferentially expressing interferon-stimulated genes (TAN-4). Xue *et al* (19) stratified patients with liver cancer into five tumor immune microenvironment subtypes, including immune activation, immune suppression mediated by myeloid or stromal cells, immune exclusion and immune residence phenotypes, where differences in the tumor immune microenvironment steer the development of at least six different types of TAN in liver cancer. Furthermore, Wu *et al* (26) generated and integrated single-cell neutrophil transcriptomes from 17 cancer types and identified 10 distinct states, including inflammation, angiogenesis and antigen presentation. Notably, non-TANs could differentiate into TANs (24,25), indicating that the TME can induce plasticity.

Overall, existing research demonstrates the remarkable plasticity and re-editable nature of TANs, which is an important factor to consider when designing anti-tumor therapies. To date, scRNA-seq has provided reliable support for improved disease outcome prediction and targeted therapy for specific differentiation pathways. However, as different clusters of neutrophils cannot currently be sorted, their functions cannot be verified. Therefore, isolating these cells will be an important step forward in neutrophil research.

Role of TANs in the TME. TANs play a dual role in the TME that depends on the neutrophil phenotype, the timing and the tumor type. TANs participate in pro-tumor inflammation by promoting tumor growth, metastasis and angiogenesis, as well as in remodeling of the extracellular matrix (15). Conversely, TANs can also mediate anti-tumor responses by directly killing tumor cells and participating in cellular networks that mediate anti-tumor resistance (28,29).

Pro-tumor effects

i) TANs regulate tumor growth and progression. TANs secrete a variety of molecules that can stimulate tumor growth, such as TGF-β, neutrophil elastase (NE), interleukin (IL)-17a, C-C motif chemokine ligand (CCL)2 and IL-8 (20,30-32). Blocking TGF-β shifts TAN polarization from the N2 to N1 phenotype in the TME, increasing tumor cell apoptosis and suppressing tumor cell migration (33,34). In pancreatic cancer, a pro-inflammatory microenvironment can be generated by recruiting TANs and activating NE release, which contributes to the progression of pancreatic neoplasms (35). In gastric cancer, TANs induce the epithelial-to-mesenchymal transition (EMT) of gastric cancer cells by secreting IL-17a and activating Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling in gastric cancer cells (36). Furthermore, N2-polarized TANs reportedly promote gastric cancer metastasis through the exosomal miR-4745-5p/3911-mediated inhibition of slit guidance ligand 2 expression (37). In a mouse model of lung adenocarcinoma, TANs exhibit widespread survival and increased expression

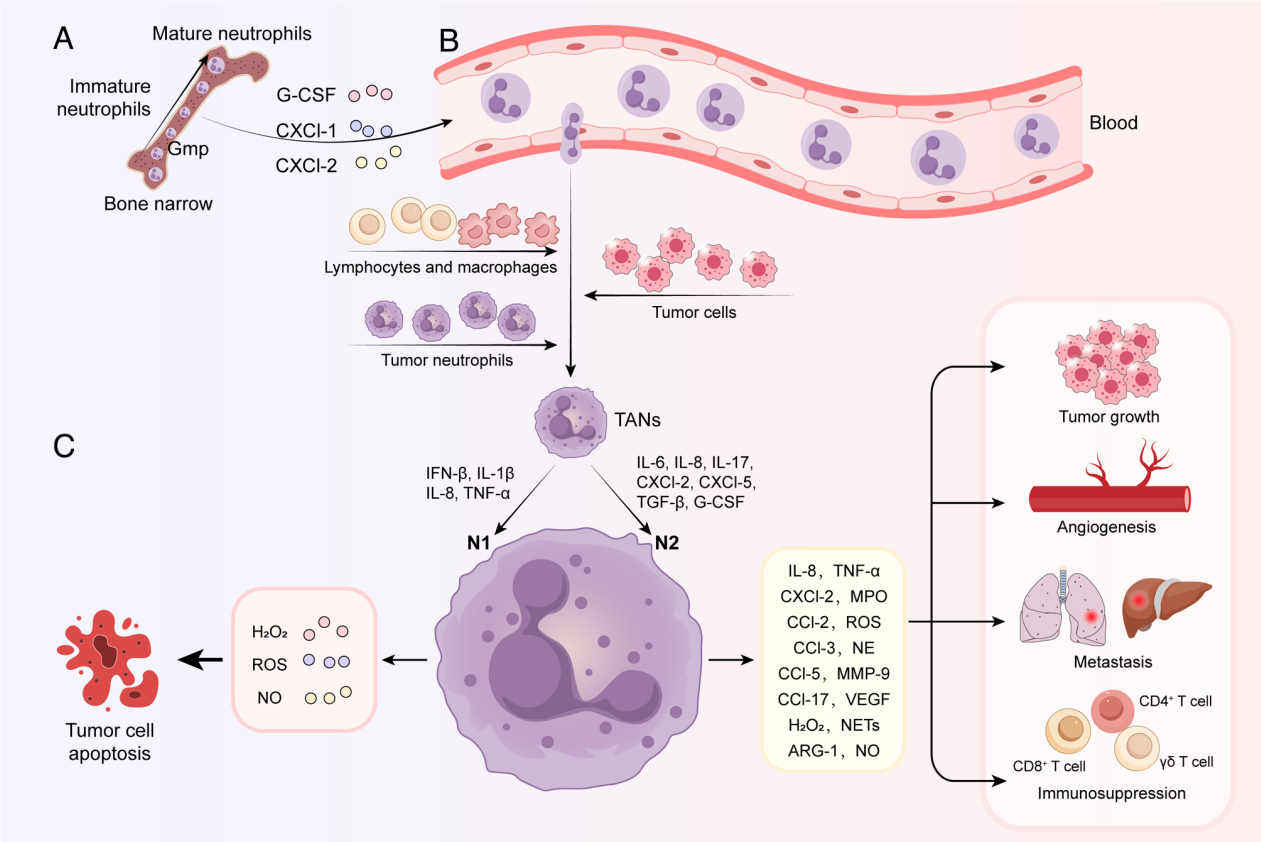

Figure 1. TANs formation and the role of the classical polarization phenotype in the TME. (A) Neutrophils undergo maturation within the bone marrow. (B) Neutrophils migrate into the bloodstream. (C) Neutrophils in the blood enter the TME after being affected by other cells and polarize into either N1 or N2 phenotypes under the action of various factors, consequently exerting anti-tumor or pro-tumor effects. TANs, tumor-associated neutrophils; TME, tumor microenvironment; ROS, reactive oxygen species; NO, nitric oxide; IFN-β, interferon-β; TNF, tumor necrosis factor; TGF-β, transforming growth factor β; G-CSF, granulocyte colony-stimulating factor; MPO, myeloperoxidase; NE, neutrophil elastase; MMP-9, matrix metalloproteinase 9; VEGF, vascular endothelial growth factor; NETs, neutrophil extracellular traps; ARG-1, arginase-1.

of the anti-apoptotic protein Bcl-xL to promote tumor growth via granulocyte-macrophage (GM)-CSF-induced JAK/STAT signaling (38). Another study found that TANs govern tumor progression in lung cancer through an IL-10/STAT3/programmed death-ligand 1 (PD-L1) feedback signaling loop (39).

ii) Neutrophils are responsible for producing pro-angiogenic factors, including Bv8 (also known as prokineticin 2), matrix metalloproteinase 9 (MMP-9) and VEGF, which play important roles in promoting tumor angiogenesis (40-42). Bv8 is a mediator of myeloid cell-dependent tumor angiogenesis and plays a role in the angiogenic switch by affecting the neoplastic vasculature and infiltration of $Gr1^+$ cells (43). MMP-9 is one of the most important mediators of tumor angiogenesis, with TANs thought to be a major source of MMP-9 (44,45). VEGF produced during the release of MMP-9 specifically stimulates the proliferation of vascular endothelial cells, inhibits the apoptosis of endothelial cells, promotes angiogenesis and increases vascular permeability (46).

iii) Neutrophils can also induce neutrophil extracellular traps (NETs), which are highly expressed in a variety of cancers and promote tumor proliferation, invasion and metastasis (47). Unlike the cell death programs of necrosis and apoptosis, NETs include MMP-9, NE, myeloperoxidase and cytoskeletal proteins (48). NETs contribute to the disruption of normal connections between endothelial cells, enabling tumor cell extravasation and metastasis (49-51). Furthermore, NETs encapsulate tumor cells, protecting them from the cytotoxic effects of surrounding immune cells (52). Although NETs promote tumor recurrence and metastasis in a number of ways, tumor cells, in turn, promote NETs via chemokines (53). In hepatocellular carcinoma (HCC), acetyl-CoA accumulation induces the H3 acetylation-dependent upregulation of CXCL1 gene expression, which can lead to TAN recruitment, NET formation and the promotion of HCC metastasis (54).

Anti-tumor effects. In addition to pro-tumorigenic properties, neutrophils also exhibit anti-tumor responses. However, few studies have reported the anti-tumor effects of neutrophils.

i) Neutrophils can exert anti-proliferative effects. Direct cell contacts between Fas ligand on human neutrophils and Fas on tumor cells suppresses tumor cells by causing cell cycle arrest *in vitro* (55). In a mouse model of phosphatase and tensin homolog-deficient uterine cancer, neutrophils impeded early-stage tumor growth and retarded malignant progression by inducing tumor cell detachment from the basement membrane (56).

ii) Neutrophils can kill tumor cells by releasing a series of effector molecules. For instance, reactive oxygen species (ROS) are secreted through neutrophil degranulation, mediating cell killing by opening the transient receptor potential

ion channel M2, which leads to an influx of calcium ions into the target cells (57,58). In addition to ROS, TANs also release nitric oxide and tumor necrosis factor (TNF), which are further involved in tumor suppression (59). Neutrophil-derived TNF-related apoptosis-inducing ligand reportedly induces apoptosis of leukemic T cells (60). Furthermore, Cui *et al* (61) reported that neutrophils release catalytically active neutrophil elastase (ELANE) to kill numerous types of cancer cell both *in vitro* and *in vivo*. ELANE proteolytically liberates the CD95 death domain, which interacts with histone H1 isoforms to selectively eradicate cancer cells. ELANE can also attenuate primary tumor growth and produce a $CD8^+$T cell-mediated abscopal effect to attack distant metastases. In melanomas, specific neutrophil subpopulations play an important role in preventing the immune escape of tumor cells, either through the release of inducible NO synthase or the direct phagocytosis of antigen-deficient melanoma cells (62).

Interaction of TANs with other immune cells in the TME. TANs engage in complex bidirectional interactions with macrophages and lymphocytes in the TME through the expression of multiple cytokines, as well as immunosuppressive and stimulatory molecules (63,64).

Macrophages. TANs and tumor-associated macrophages (TAMs) are functional partners in the inflammatory process, hypothesized to synergize and interact in the TME to promote tumor progression through similar molecular forms (65). When macrophages take up TAN-derived factors, they stimulate TAMs toward M1 polarization (66). NETs induce mononuclear macrophages to secrete interleukins, such as IL-1β and IL-6, and recruit progenitor cells of TANs, with activated neutrophils further releasing IL-8, among other cytokines, to recruit macrophages (67). In addition, neutrophils secrete myeloperoxidase, which binds to the mannose receptor and dominates the secretion of GM-CSF in chronic inflammatory environments (68). GM-CSF is an important factor mediating the recruitment and development of TAMs and critical for TME macrophage recruitment and polarization (69,70). In early luminal breast cancers, TAN density correlates with $CD163^+$ M2-like TAM density. Furthermore, TANs are a negative prognostic factor in tumors with an elevated M1/M2 TAM ratio, whereas this impact on patient outcome is lost in tumors with a low M1/M2 ratio (71). Thus, the recruitment and function of TANs and TAMs are inextricably linked. In intrahepatic cholangiocarcinoma (ICC), the interaction between TANs and TAMs produces higher levels of oncostatin M and IL-11, respectively, which then activate STAT3 signaling in ICC cells. STAT3 knockdown attenuates the pro-tumorigenic effects of TANs and TAMs in ICC (63).

Lymphocytes. TANs can lead to the suppression and depletion of T-cell function in several ways; this phenomenon will be elaborated on further below. In addition, activated neutrophils can recruit type 1 T-helper (Th1) and Th17 cells by releasing multiple chemokines (72-74). CCL17 released by TANs may also support tumor growth by promoting the recruitment of regulatory T (Treg) cells to tumors and inhibiting anti-tumor immune activity (75). In pancreatic ductal adenocarcinoma (PDAC), polarized TANs upregulate CCL5 secretion, which promotes cancer cell migration and invasion and enhances Treg cell infiltration in the tumor (76). Furthermore, TANs reduce the cytotoxic and infiltration capacity of natural killer (NK) cells and regulate the expression of programmed cell death protein-1 (PD-1) and PD-L1 through the G-CSF/STAT3 and IL-18 signaling pathways, thereby inhibiting the anti-tumor immune activity of NK cells (77). G-CSF-mobilized neutrophils inhibit NK-cell activation (78-80). By contrast, NK cells control the tumor-promoting and angiogenic functions of neutrophils in an interferon γ-dependent manner by inhibiting VEGF expression (81).

Others. Other components of the TME, such as cancer-associated fibroblasts (CAFs), reportedly secrete IL-8, which further recruit neutrophils into the TME. The infiltrated neutrophils upregulate Serglycin (SRGN) expression in gastric cancer cells via the regenerating family member 4. SRGN secreted by tumor cells then activates the CD44/c-Myc pathway to upregulate Lysine[K]-specific demethylase 5B expression, thereby promoting IL-8 production in CAFs. Thus, the SRGN-IL-8-TANs-SRGN loop facilitates gastric cancer progression (82). In the TME of HCC, CAF-derived cardiotrophin-like cytokine factor 1 increases CXCL6 and TGF-β secretion in tumor cells, which subsequently promotes tumor cell stemness in an autocrine manner, as well as TAN infiltration and polarization in a paracrine manner (83). Furthermore, fibroblast growth factor 19 secreted by tumor cells induces the formation of inflammatory CAFs via the fibroblast growth factor receptor-JAK2-STAT3 pathway, and the release of C5α and IL-1β from inflammatory CAFs promotes the formation of NETs, leading to liver metastases in colorectal cancer (CRC) (84). Other studies have shown that mast cell-derived TNF promotes the extravasation of neutrophils (85).

In summary, TANs exhibit dual pro-tumor and anti-tumor effects. Next, the molecular mechanisms underlying TAN-mediated resistance to cancer therapy will be explored, including resistance to immunotherapy, chemotherapy, targeted therapy and radiotherapy.

3. TANs and therapeutic resistance

Anti-tumor drugs mainly include chemotherapeutic drugs, molecularly targeted drugs and immune checkpoint inhibitors, with clinical treatment plans based on a combination of drug regimens (86). As TANs can induce resistance to specific drugs through various different mechanisms, the specific mechanisms of TAN-mediated drug resistance have been studied in the context of anti-tumor therapy.

Resistance to immunotherapy. Immunotherapy, which predominantly includes immune checkpoint inhibitors and chimeric antigen receptor (CAR)-T cell therapy, utilizes the immune system of the patient to attack the tumor (87). Neutrophils play an important role in immunotherapy and have a significant impact on the outcome of tumor treatment.

Resistance to anti-PD-1/PD-L1 therapy. Anti-PD-1/PD-L1 therapies have become an important part of numerous cancer treatments and showed remarkable success. These therapies effectively inhibit tumor growth and metastasis by inhibiting the immune checkpoint molecules PD-1/PD-L1 and restoring the T cell-mediated immune response to the

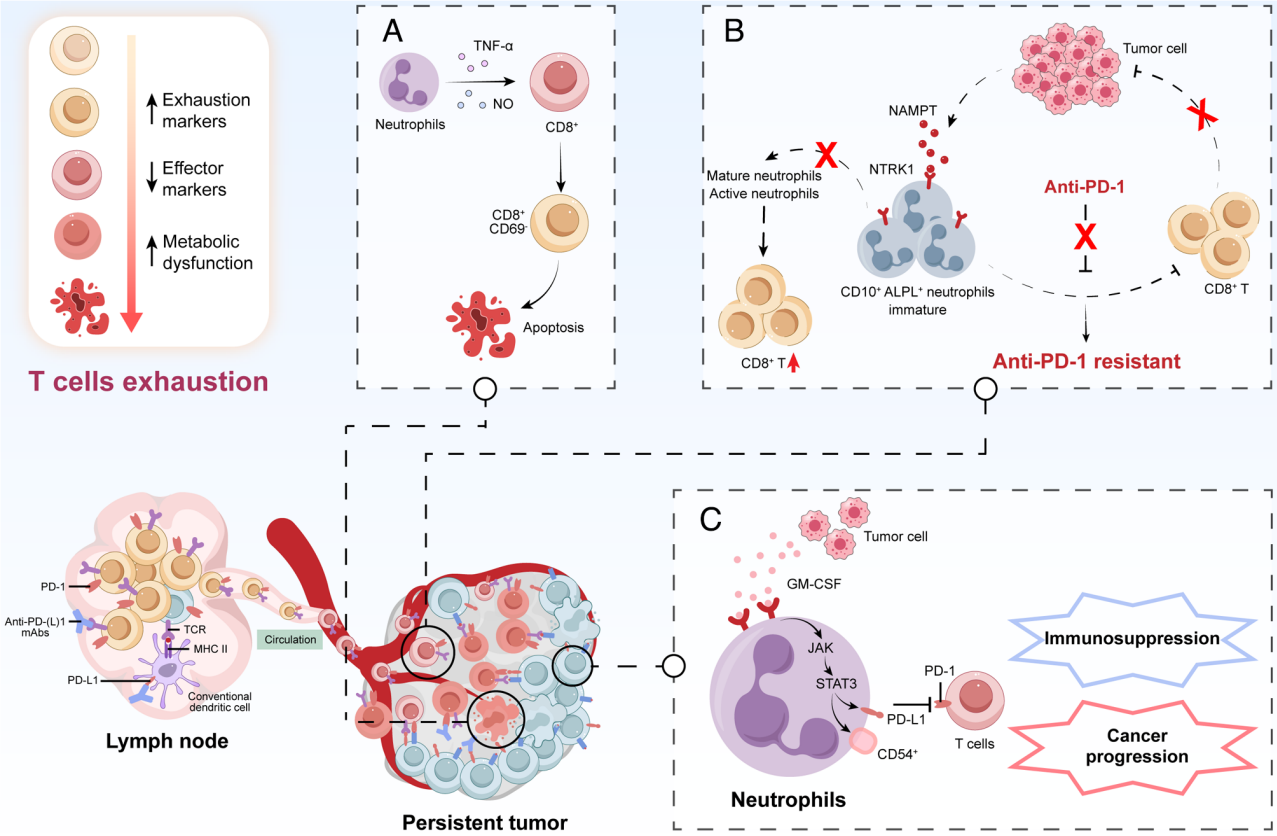

Figure 2. Multiple mechanisms of T-cell exhaustion induced by neutrophils in the TME. (A) Isolated TANs induce apoptosis of CD8+ T cells in the TME via TNF-α and NO contact-dependent mechanisms. (B) Tumor-secreted nicotinamide phosphoribosyl transferase reprograms CD10+ALPL+ neutrophils via neurotrophic tropomyosin receptor kinase 1, maintaining their immaturity and inhibiting their maturation and activation. These neutrophils induce T-cell exhaustion and resistance to anti-PD-1 treatment. (C) Release of granulocyte-macrophage colony-stimulating factor induces TAN activation, which is accompanied by the induction of PD-L1 expression on these cells via JAK-STAT3 signaling pathway activation. These activated neutrophils contribute to immunosuppression and cancer progression by inhibiting T-cell immunity in a PD-L1/PD-1-dependent manner. TANs, tumor-associated neutrophils; NO, nitric oxide; TME, tumor microenvironment; PD-1, programmed cell death 1; PD-L1, programmed cell death ligand 1; TNF, tumor necrosis factor; NAMPT, nicotinamide phosphoribosyl transferase; NTRK1, neurotrophic tropomyosin receptor kinase 1; TCR, t cell receptor; MHC, major histocompatibility complex; GM-CSF, granulocyte-macrophage colony-stimulating factor; JAK-STAT3, janus kinase-signal transducer and activator of transcription 3.

tumor (88-90). However, T-cell depletion, with which TANs are closely associated, is a major impediment to immunotherapy (91-93). Numerous studies have revealed that TANs influence anti-PD-1/PD-L1 therapy primarily by promoting an immunosuppressive TME (94,95). Tumor-secreted nicotinamide phosphoribosyl transferase reprograms CD10+ alkaline phosphatase, biomineralization associated+ neutrophils via neurotrophic tropomyosin receptor kinase 1, maintaining their immaturity and inhibiting their maturation and activation, which induces apparent 'irreversible' exhaustion of T cells in terms of their cell number, frequency and gene profile (96). In HCC, overexpression of CT10 regulator of kinase-like (CRKL) shapes the immunosuppressive TME by recruiting TANs through the upregulation of CRKL/β-catenin/VEGFα and CXCL1 axes. A decrease in the proportion of activated CD8+ T cells was accompanied by an increase of depleted CD8+ T cells in the CRKL overexpression group. PD-L1+ TANs, a potential subset of TANs regulated by CRKL, are significantly upregulated in CRKL-overexpressing tumor tissues, exerting an immune-suppressive effect and resulting in poor patient prognosis. Studies in mice have verified that lymphocyte antigen-6 complex, locus G (Ly6G) restores the efficacy of anti-PD-1 treatment following CRKL overexpression-induced anti-PD-1 resistance, revealing that CRKL regulates PD-1 resistance by mediating the infiltration of TANs in HCC (97). Michaeli *et al* (98) isolated TANs from different mouse tumor models, which induced apoptosis of CD8+ T cells in the TME via TNF-α and NO contact-dependent mechanisms. Wang *et al* (99) found that tumors isolated from patients with gastric cancer were infiltrated with CD54+ TANs, expressing high levels of PD-L1. Furthermore, GM-CSF secreted by tumor cells activates TANs and induces PD-L1 expression on TANs through the JAK-STAT3 signaling pathways (99). These activated neutrophils contribute to immunosuppression and cancer progression by inhibiting T-cell immunity in a PD-L1-PD-1-dependent manner. The multiple mechanisms of T-cell exhaustion induced by neutrophils in the TME are shown in Fig. 2.

The immunosuppressive potential of NETs is highlighted by the recent discovery of PD-L1 in NETs. PD-L1 is a ligand that affects adaptive anti-cancer immune responses and metastasis combined with hepatic ischemia or reperfusion by inducing T-cell depletion and dysfunction within the TME in a murine liver model. In a mouse study, treatment with DNase I to digest NETs attenuated tumor growth and increased functional T-cell levels (100). Furthermore, treatment with an

adeno-associated virus gene therapy vector expressing DNase I in the liver inhibited liver metastases of CRC by inhibiting neutrophil infiltration and NET formation, as well as restoring local immune responses at the tumor site by increasing the percentage of $CD8^+$ T cells (101). NETs have also recently emerged as powerful modulators of immunotherapy outcomes. In a CRC xenograft model, although the digestion of NETs with DNase I and treatment with PD-1 reduced tumor growth, combination of the two strategies had a synergistic effect. Mechanistically, inhibition of NETs with DNase I reverses resistance to anti-PD-1 blockade by increasing $CD8^+$ T-cell infiltration and cytotoxicity (102).

Resistance to CAR-T therapy. CAR-T therapy has changed the therapeutic landscape for hematological malignancies. Current challenges of CAR-T cell therapy are mainly related to side effects, toxicity, T-cell depletion and a malignant TME (103-105). The most significant difference between hematological and solid tumors is the presence of the TME in solid tumors, with the immunosuppressive nature of the TME being the likely reason why CAR-T cellular immunotherapy has not been successful in solid tumors (106,107). The TME includes highly infiltrating mesenchymal stromal cells, and immunosuppressive cells such as TANs, myeloid-derived suppressor cells (MDSCs), TAMs, mast cells and regulatory T cells (108). All of these cellular components contribute to establishing an immune-suppressive TME capable of interfering with the efficacy of CAR-T cell therapy (109,110). However, the exact mechanism requires further investigation.

Resistance to chemotherapy. Chemotherapy is currently the primary clinical option for tumors, but its efficacy is often limited by drug resistance. This phenomenon results in the failure of chemotherapeutic drugs as well as the development of multidrug resistance, which is the main cause of tumor recurrence, metastasis and death in most patients (111,112). Specifically, TANs can interact with other immune cells or modulate the TME to alter the efficacy of chemotherapy.

NETs. During chemotherapy, neutrophils respond to chemotherapy by infiltrating the TME and releasing NETs, thereby promoting chemoresistance (113,114). Mousset *et al* (113) reported that upregulation of the CXCL1/5-CXCR2 axis following chemotherapy is involved in the recruitment of neutrophils to metastatic lungs. Eliminating NETs through peptidylarginine deiminase 4 inhibitors or DNase I significantly improved the response to chemotherapy, suggesting a direct causal relationship between NETs and chemoresistance. In terms of the mechanism, NETs promote EMT and chemotherapy resistance by binding and activating TGF-β (a classical inducer of EMT) (113). In addition, neutrophils can induce EMT by releasing NETs (115-118). Specifically, neutrophils induce epithelial stabilization and transcription of zinc finger e-box-binding homeobox 1 by releasing NETs, thus promoting EMT and chemotherapy resistance. Furthermore, lung cancer with breast metastases treated with chemotherapy released CXCL1/5 and IL-1β, which promoted neutrophil recruitment and NET formation, respectively, leading to chemoresistance (119). Another study highlighted the role of neutrophils and NETs in chemotherapy resistance in multiple myeloma, demonstrating for the first time that neutrophils promote cancer cell survival by secreting soluble factors in patients treated with doxorubicin and melphalan (120). Moreover, NETs effectively trap and inhibit the spread of adriamycin in a two-compartment system, which may attenuate its ability to induce apoptosis in ovarian cancer cells (121). The clinical relevance of NETs in chemotherapy resistance has also been demonstrated. Plasma NET levels are significantly higher in patients with metastatic breast cancer exhibiting progressive disease 15 days after chemotherapy (113). The mechanisms through which neutrophils induce chemotherapy resistance by infiltrating the TME and releasing NETs are shown in Fig. 3.

In conclusion, chemotherapy-induced activation of the NET pathway is a major mechanism of tumor chemoresistance.

Chemokines. Chemokines represent a subset of chemoattractant cytokines that control the directed migration of immune cells and play a multifaceted role in tumor cell proliferation, tumor heterogeneity, stemness, senescence, angiogenesis and tumor metastasis (122-124). Increasing evidence has revealed that neutrophil-related chemokines exert a crucial impact on tumor progression and chemotherapy resistance. In the TME, cancer cells regulate neutrophil recruitment to tumor sites through the expression of various chemokine ligands (CXCL1, CXCL2, CXCL5, CXCL6 and CXCL8) for neutrophil receptors CXCR1 and CXCR2 (125,126). Host CXCR2 inhibition by genetic ablation prevents neutrophil accumulation in pancreatic tumors and leads to T cell-dependent suppression of tumor growth (127,128). In a zebrafish model of glioblastoma, CXCR1 mediates the recruitment of neutrophils and supports the proliferation of tumor-initiating astrocytes (129,130); in melanoma-bearing mice, CXCL1 and CXCL2 chemokines enhance neutrophil recruitment and induce angiogenesis (131); in HCC, overexpression of CXCL5 mediates neutrophil infiltration and indicates poor prognosis (132-134), whereas CXCL6 secretion in tumor cells promotes TAN infiltration and polarization to accelerate HCC progression (83); and in chemotaxis assays and mouse models of thyroid cancer, elevated concentrations of CXCL8 promote TAN recruitment and cancer progression (135).

Chemokines play essential roles in TME changes induced by chemotherapy. Apoptotic CRC cells induced by chemotherapy release abundant neutrophil-attracting chemokines, notably CXCL8, thereby attracting neutrophils into the tumor, where their interaction with neighboring macrophages can promote an immunologically unfavorable TME (136). CXCL1 and CXCL2 secretion by metastatic tumor cells recruited neutrophils to the metastatic liver in mouse models of PDAC. These recruited neutrophils expressed growth arrest specific 6 (Gas6), which led to AXL receptor activation on tumor cells, enabling their regrowth. Furthermore, disruption of neutrophil infiltration or inhibition of the Gas6/AXL signaling axis in combination with chemotherapy inhibited metastatic growth (137). To date, growing evidence has revealed that targeting the chemokine/chemokine receptor axis is a promising approach to reverse chemoresistance and improve efficacy. In an orthotopic PDAC model, CXCR2 blockade prevented neutrophil mobilization from the circulation and augmented chemotherapeutic efficacy. Targeting both $CXCR2^+$ TANs and $CCR2^+$ macrophages disrupted myeloid recruitment and improved the response to FOLFIRINOX chemotherapy in PDAC (138). Overexpression of CXCL1/2 in breast cancer led to metastasis and resistance to chemotherapy in a paracrine

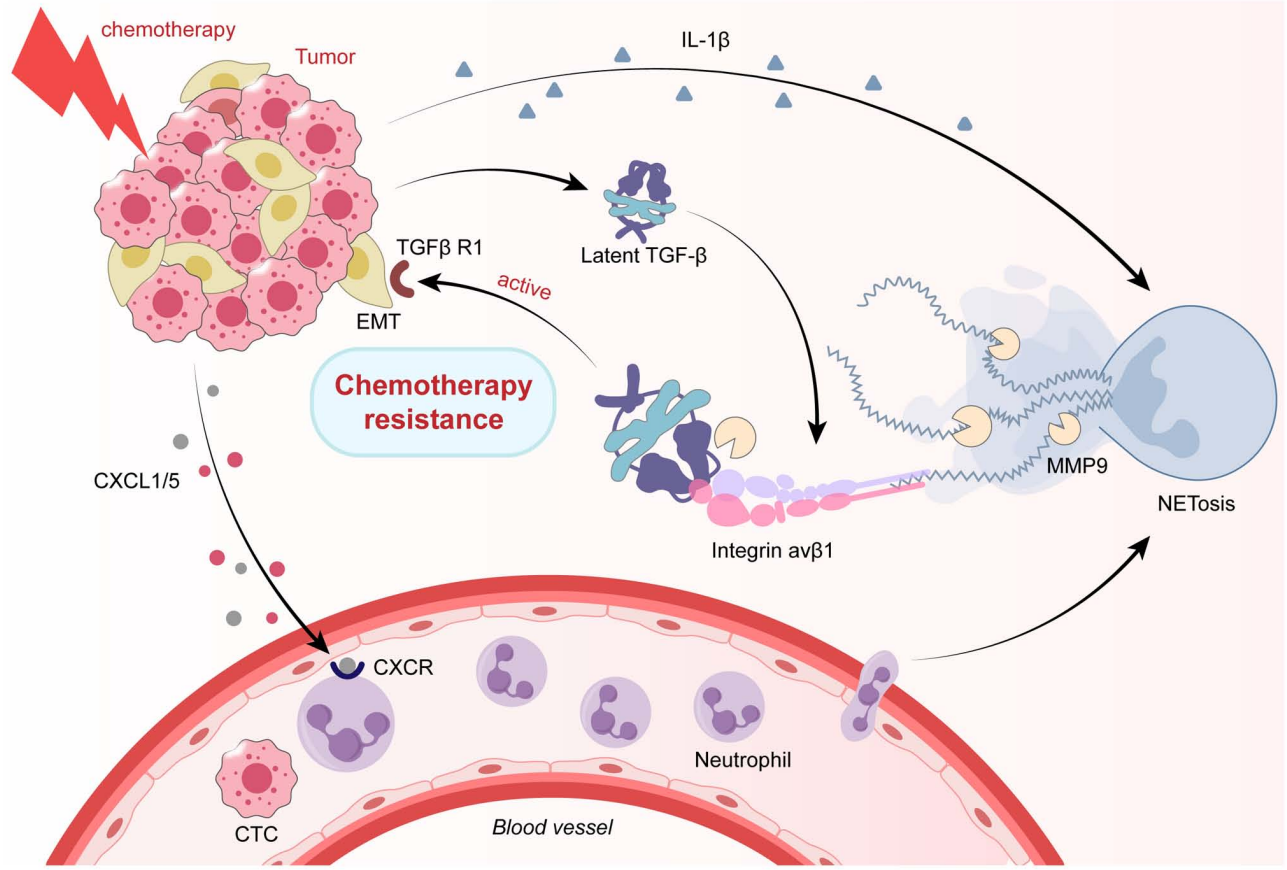

Figure 3. Neutrophils induce chemotherapy resistance by infiltrating the TME and releasing NETs. Chemotherapy-treated tumor cells secrete CXCL1/5 and IL-1β, which recruit neutrophils to infiltrate the TME, triggering NET formation. NET-associated protein integrin-avβ1 traps latent TGF-β, and MMP-9 cleaves and activates the trapped latent TGF-β. TGF-β activation causes tumor cells to undergo epithelial-to-mesenchymal transition, which is associated with chemoresistance. TME, tumor microenvironment; NETs, neutrophil extracellular traps; TGF-β, transforming growth factor-β; MMP-9, matrix metalloproteinase 9; CTC, circulating tumor cell; EMT, epithelial-mesenchymal transition.

manner involving the TME. However, CXCR2 blockade inhibited this vicious cycle, increasing the efficacy of chemotherapy against breast cancer (139). Reparixin, a small-molecule inhibitor of the CXCL8-CXCR1/2 axis, offers the possibility of chemotherapy-induced synergy in breast cancer. For example, a combination of reparixin with paclitaxel reduced brain metastasis as well as the population of cancer stem-like cells (140). Sequential treatment with first-line and second-line chemotherapy and reparixin inhibited tumor growth, reduced toxicity and prolonged survival in mouse models of gastric cancer (141). In addition, a CXCR2-specific small-molecule inhibitor, SB225002, decreased neutrophil infiltration and reduced tumor growth in lung cancer (142).

Resistance to targeted therapy. In recent years, advances in molecular biology and genetics research have shown that malignant tumors exhibit complex and specific biological defects, including oncogenes, oncogene mutations and chromatin modifications (143). Targeted therapy utilizes the specific structural molecules of tumor tissues or cells and drugs that specifically bind to them to precisely kill tumor cells (144). However, primary or acquired resistance limits their clinical use. In this chapter, an overview of the mechanisms of resistance associated with TANs in common molecular targeted therapies is presented.

VEGF. The occurrence and development of tumors depend on tumor angiogenesis, which provides oxygen and nutrients to tumor cells, removes metabolic waste and also enables tumor cells to metastasize (145). Angiogenic vessels contain irregular branching and form intermittent arteriovenous shunts, leading to discontinuous perfusion and disturbed blood flow patterns, resulting in an environment with abnormally high interstitial fluid pressure (IFP) (146).

VEGF has been identified as a key cytokine involved in tumor angiogenesis and metastasis (147). TANs release NE and MMP-9 to degrade the extracellular matrix and later activate VEGF, thus promoting angiogenesis in the TME (15,20,46). MMP-9 reportedly promotes the release of VEGF or inhibits the action of anti-angiogenic factors, exerting a key role in angiogenesis in breast cancer (23,148). In addition to MMP-9, G-CSF released by tumor and stromal cells upregulated the angiogenic peptide Bv8 on neutrophils, which promoted angiogenesis by mediating endothelial cell proliferation (149,150). TANs residing in hypoxic scars may contribute to distorted blood flow and a high IFP environment through MMP-9, VEGF and Bv8 (151). High IFP can be a barrier to both the effective delivery of anti-cancer drugs toward the TME or drug accumulation within the tumor area, thus promoting tumor resistance to therapy (152) (Fig. 4).

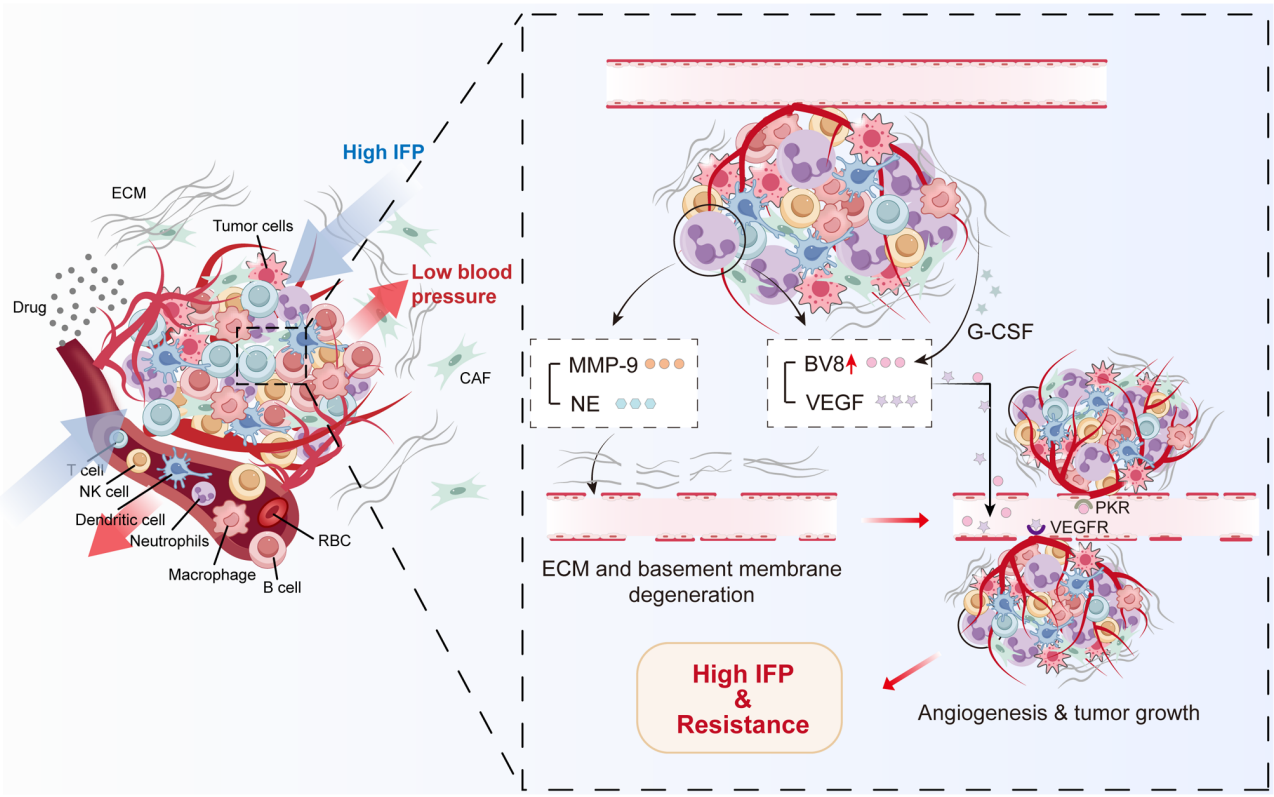

Figure 4. TANs aggravate angiogenesis to produce high interstitial fluid pressure, which induces targeted VEGF resistance. In the hypoxic TME, TANs release MMP-9, NE and VEGF. Additionally, granulocyte-macrophage colony-stimulating factor released by tumor and stromal cells promotes the expression of the angiogenesis peptide Bv8 on TANs. First, MMP-9 and NE released by TANs are involved in the degradation of extracellular matrix and vascular basement membrane. Next, Bv8 and VEGF bind to their respective receptors (PKR and VEGFR) to promote angiogenesis by mediating endothelial cell proliferation. These events contribute to distorted blood flow and high interstitial fluid pressure, thus impeding drug delivery. TANs, tumor-associated neutrophils; VEGFR, vascular endothelial growth factor receptor; NE, neutrophil elastase; ECM, extracellular matrix; RBC, red blood cell; CAF, cancer-associated fibroblast; MMP-9, matrix metalloproteinase 9; G-CSF, granulocyte colony-stimulating factor; PKR, protein kinase R; IFP, interstitial fluid pressure.

TANs can also promote angiogenesis through the direct secretion of IL-17 or indirect activation of the nucleotide-binding domain, leucine-rich repeat and pyrin domain-containing protein 3 (NLRP3), leading to increased IL-1β secretion (153). In mouse tumor models, treatment with gemcitabine plus 5-fluorouracil induced the release of proteinase B from TANs and MDSCs, which in turn led to an IL-1β-dependent increase in IL-17 production and angiogenic blood vessel formation through the action of NLRP3 (154). Furthermore, neutrophil-derived Bv8 is associated with resistance to anti-VEGF therapy, whereas inhibition of G-CSF may increase the efficacy of anti-VEGF therapy (149,153).

Overall, these studies support the role of neutrophils in the initial angiogenic switch during tumorigenesis and reveal the pathways through which TANs may reduce the efficacy of anti-angiogenic therapy.

Epidermal growth factor receptor (EGFR). EGFR-targeting drugs typically interfere with activation of the EGFR signaling pathway (155). When EGFR binds to its ligands, it activates numerous downstream signaling pathways, including Ras-Raf-MEK-Erk, PI3K-AKT-mTOR and STAT, thus promoting cell proliferation, growth, angiogenesis and metastasis (156-158). Experiments on A549 cells using an *in vitro* co-culture system have shown that elastase secreted by neutrophils stimulates proteinase-activated receptor 2 and induces EGFR trans-activation to promote drug resistance (159).

Human EGFR 2 (HER-2). HER-2 is associated with poor prognosis in numerous cancers but predominantly breast cancer. Drugs targeting HER-2 mainly fall into one of three categories: Monoclonal antibodies, including trastuzumab and pertuzumab; tyrosine kinase inhibitors, including lapatinib and eratinib; and antibody-drug conjugates (160) such as T-DM1. The results of an autocrine model showed that NE splits cell surface EGF or TGF-α from the cell membrane to activate signal transduction (161). TGF-α not only suppresses HER-2 downregulation by disrupting endocytosis and lysosome function, but also recruits HER-2 on the cell surface (162) and may affect the therapeutic effect of targeting HER-2. Furthermore, combining the CXCR1/2 inhibitor SCH563705 with lapatinib reduced cancer stem-like cell activity compared with either treatment alone in HER2-positive breast cancer via a novel SRC and EGFR/HER2-dependent mechanism (163).

Kirsten rat sarcoma viral oncogene (KRAS). KRAS mutations have been implicated in ~40% of CRC cases, as well as in numerous other types of human cancers, such as lung cancer, breast cancer and prostate cancer (164-167). Exosomal KRAS mutants exert stimulatory effects on IL-8 production and NET formation to promote the growth of CRC cells (168). Furthermore, when combined with anti-VEGF, neutralizing G-CSF activity and G-CSF-induced $CD11b^{+}Ly6G^{+}$ neutrophils is effective in reducing tumor

Table I. Clinical trials based on neutrophil-targeted tumor therapy.

Target/drug	Cancer applications	NCT no.
CXCR1/CXCR2 inhibitors		
Reparixin	Breast cancer	NCT02370238
	Breast cancer	NCT02001974
	Breast cancer	NCT01861054
AZD5069	Metastatic castration-resistant prostate cancer	NCT03177187
TGF-β pathway inhibitors		
Galunisertib	Breast cancer	NCT02672475
	Glioblastoma	NCT01582269
	Glioma	NCT01682187
CD47-SIRPα inhibitors		
TTI-621	Hematologic cancers	NCT02663518
IBI188	Advanced malignancies	NCT03717103
CC-90002	Solid and hematologic cancers	NCT02367196
Hu5F9-G4	Solid tumors	NCT02216409
TRAIL receptor 2 agonists		
Tigatuzumab	Breast cancer	NCT01307891
CS-1008	Metastatic colorectal cancer	NCT01220999

TGF-β, transforming growth factor-β; SIRPα, signal-regulatory protein α; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; NCT, national clinical trials registry.

growth and increasing survival in KRAS-driven PDAC, as indicated in a mouse model with knockout of pancreatic epithelial-specific TGFβ receptor type II and activated KRAS (169).

Resistance to radiotherapy. Radiotherapy is one of the most effective approaches for achieving tumor control (170). Nearly two-thirds of patients with cancer are treated with radiotherapy, often with the intent to achieve complete and permanent tumor regression (local control) (171). However, innate or acquired radiotherapy resistance remains a significant challenge that markedly limits the therapeutic effects, leading to cancer relapse and poor prognosis (172,173). Several crucial aspects contribute to radiotherapy resistance, including radiation-induced DNA damage repair, apoptosis escape, cell-cycle arrest, abundance of cancer stem cells, modification of cancer cells and their microenvironment, metabolic reprogramming, presence of exosomal and non-coding RNA and ferroptosis (174-177).

Studies have shown that high neutrophil infiltration is associated with poor response to radiotherapy (20,171,178). Neutrophils promote radiotherapy resistance in various malignant tumors. For instance, in an irradiated glioblastoma model, $Ly6G^+$ inflammatory cells promoted the conversion of glioblastoma cells to glioblastoma stem cells through the NOS2-NO-ID4 regulatory axis. Treatment with Ly6G-neutralizing antibodies reduced the number of glioblastoma stem cells and prolonged survival in tumor-bearing mice after radiotherapy (179). In a model of local-regional failure for breast cancer after irradiation, high expression of ectonucleotide pyrophosphatase/phosphodiesterase 1 in circulating tumor cells enhanced the expression of haptoglobin, resulting in neutrophil infiltration, NET formation and tumor relapse (180). Ancey *et al* (181) showed that glucose transporter 1 (GLUT1) expression in TANs promotes lung cancer growth and resistance to radiotherapy in a mouse model of lung adenocarcinoma. Loss of GLUT1 accelerates neutrophil turnover in tumors and reduces a subset of TANs expressing sialic acid-binding immunoglobulin-like lectin F. In the absence of GLUT1 expression by TANs, tumor growth is diminished and the efficacy of radiotherapy is augmented (181). Nolan *et al* (170) found that off-target exposure to radiation promotes the formation of a premetastatic niche by neutrophils in a mouse model of breast cancer lung metastasis. By preventing neutrophil-dependent Notch activation by blocking degranulation, radiation-enhanced metastases are significantly offset (170). In bladder cancer, radiation induces cancer cells to release high mobility group box 1 (HMGB1), which promotes the formation of NETs through Toll-like receptor 4 signaling; subsequent inhibition of HMGB1 and NETs improved the overall radiotherapy response in mouse models (182). Furthermore, in an autochthonous mouse model of soft tissue sarcoma, neutrophil depletion prior to image-guided focal irradiation improved tumor response to radiotherapy. According to scRNA-seq, tumor radiosensitization by neutrophil depletion after radiotherapy is associated with the downregulation of oncogenic transcriptional programs (171).

Furthermore, Li *et al* (183) proposed an innovative approach that integrated peroxynitrite ($ONOO^-$)-mediated radiosensitization with TAN polarization (reprogramming of TANs from N2 to N1 phenotypes) to reverse an immunosuppressive TME, markedly amplifying the potency of radiotherapy in metastatic CRC. In conclusion, targeting

neutrophils represents a potential therapeutic strategy for modulating the efficacy of radiotherapy.

4. TAN-targeted therapeutic strategies

Given the impact of TANs on tumors, targeting and regulating TANs in the TME represents a promising new therapeutic approach.

Targeted neutrophil therapy. Targeted neutrophil therapy focuses on inhibiting tumor formation, metastasis and angiogenesis by inhibiting the polarization and recruitment of neutrophils in the TME, preventing the formation and aggregation of TANs (18,20,184). Several clinical trials of neutrophil-targeted tumor therapy are currently underway (Table I). In another study, antibody-dependent cell-mediated cytotoxicity (ADCC) antibody therapy with monoclonal antibodies (mAbs) was applied to enhance the ADCC potential of TANs. The results showed that, for two different tumor targets, EGFR and HER-2, a combination of IgG and IgA mAbs is more cytotoxic than the antibodies alone (185-187). In addition, as reported by Kumbhojkar *et al* (188), micropatch-loaded neutrophils provide a potent, scalable and drug-free approach to neutrophil-based cancer immunotherapy.

Biomarkers for potential tumor therapies. High infiltration of TANs is associated with poor prognosis in most human tumors. Neutrophils infiltrate to varying degrees, as assessed by routine immunohistochemical staining of neutrophil markers (CD66b in humans and Ly6G in mice) and neutrophil transcriptional profiles of solid tumors (20). Although TANs are a poor prognostic indicator of survival in a number of malignant tumors, such as HCC, cholangiocarcinoma, head and neck cancer and renal cancer (27,189,190), certain studies have found that TANs can improve the survival rate of patients with colon cancer (191,192).

The neutrophil-to-lymphocyte ratio (NLR) is valuable for determining the prognosis of patients with cancer, with a high NLR associated with poor prognosis in patients with colorectal, pancreatic, gastric and breast cancers (193-195). A large-scale meta-analysis of 8,500 patients with breast cancer found that a high NLR (1.9 to 5.0) was strongly associated with poor overall survival and reduced disease-free survival (196). In patients with CRC and liver metastases who underwent hepatic lobectomy, an elevated NLR was the only positive predictor of postoperative recurrence and was positively correlated with tumor recurrence but negatively correlated with postoperative survival in patients who underwent *in situ* liver transplantation for primary liver cancer (197,198). Furthermore, an elevated NLR during the postoperative follow-up period was an independent risk factor for shorter survival in a large number of patients with gastric cancer who underwent gastric resection (199,200).

Neutrophils have been reported to express ligand-activated immune checkpoints on T cells. For instance, PD-L1-expressing neutrophils have prognostic significance in both HCC and gastric cancer (99). Zhou *et al* (134) also demonstrated that intra-tumor neutrophils express high levels of CCL2 and CCL17, which correlate with disease progression and prognosis. The number of $CCL2^+$ and $CCL17^+$ TANs is also positively associated with tumor size, microvascular invasion, level of tumor differentiation and stage (201). In summary, TANs are potential biomarkers for tumor therapy.

5. Conclusions

TANs regulate tumorigenesis and progression by i) regulating the function of other immune cells, controlling NET formation and affecting the polarization state, and ii) mediating resistance to tumor therapy in various ways. However, the existence and interaction of different immune cell subpopulations in the TME, the different immune characteristics of the TME in different cancer types and individual patients, and the depletion of TANs, which can lead to a reduction of organism immunity, are urgent clinical problems that must be addressed. Combining existing effective tumor therapies with neutrophil-targeted therapies may represent a safer and more effective way to overcome tumor drug resistance. Future research should further investigate the exact roles, recruitment pathways and mechanisms of action of TANs to develop therapies that precisely target TANs and counter drug resistance.

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Availability of data and materials

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Authors' contributions

RH, HJ, HYW, JD and JX conceived the study. RH and HJ prepared the original draft of the manuscript and drew the figures. XW, CW, HF, YZ and HCW revised the manuscript. JX and HJ supervised and approved the final manuscript. Data authentication is not applicable. All the authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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