



Neutrophil-derived granule cargoes: paving the way for tumor growth and progression

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Abstract

Neutrophils are the key cells of our innate immune system mediating host defense *via* a range of effector functions including phagocytosis, degranulation, and NETosis. For this, they employ an arsenal of anti-microbial cargoes packed in their readily mobilizable granule subsets. Notably, the release of granule content is tightly regulated; however, under certain circumstances, their unregulated release can aggravate tissue damage and could be detrimental to the host. Several constituents of neutrophil granules have also been associated with various inflammatory diseases including cancer. In cancer setting, their excessive release may modulate tissue microenvironment which ultimately leads the way for tumor initiation, growth and metastasis. Neutrophils actively infiltrate within tumor tissues, wherein they show diverse phenotypic and functional heterogeneity. While most studies are focused at understanding the phenotypic heterogeneity of neutrophils, their functional heterogeneity, much of which is likely orchestrated by their granule cargoes, is beginning to emerge. Therefore, a better understanding of neutrophil granules and their cargoes will not only shed light on their diverse role in cancer but will also reveal them as novel therapeutic targets. This review provides an overview on existing knowledge of neutrophil granules and detailed insight into the pathological relevance of their cargoes in cancer. In addition, we also discuss the therapeutic approach for targeting neutrophils or their microenvironment in disease setting that will pave the way forward for future research.

Keywords Neutrophil-derived granule cargoes · Effector functions · Tumor-associated neutrophils · Cancer therapeutics

1 Introduction

Neutrophils are the most abundant circulating immune cell types, constituting around 50–70% of all leukocytes in peripheral blood. They are constantly produced in the bone marrow through a process called granulopoiesis [1]. Interestingly, more than 50% of bone marrow is committed to the production neutrophils [2]. Being the first one to migrate at the site of infection, they are regarded as the first line of defense against invading pathogens. Neutrophils actively migrate from hematopoietic tissue and pass through the vasculature to reach the target site [3]. They exert their anti-pathogenic or pro-inflammatory roles *via* a wide range of effector functions, including phagocytosis, degranulation, and neutrophil extracellular trap (NET) formation [4]. To execute these effector

functions, neutrophils are heavily equipped with a stock of toxic, anti-microbial weapons (anti-microbial peptides and lytic enzymes) stored in their distinct granule subsets. Granules are the major attribute of neutrophil effector functions and are divided into several subsets depending upon their protein content and synthesis during granulopoiesis [5]. The controlled mobilization and release of granule content allow the transformation of neutrophils from inactive circulating cells to active effector cells of the innate immune system. Following an encounter with pathogenic targets, neutrophils are primed and activated for a swift release of their granule weaponry [6]. Neutrophils can destroy pathogens intracellularly by releasing granule content into phagosomes or extracellularly into the extracellular milieu in response to stimuli which is tightly regulated [7]. On the other hand, excessive release of granule content has been implicated in collateral tissue damage [8]. Traditionally, it was considered that neutrophils are present only during the initial phase of inflammation and their sole function is to eliminate a broad spectrum of invading pathogens. However, several reports have now demonstrated their functions beyond roles in eliminating infection

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[1, 9]. Emerging studies also suggest their persistent influx, hyper-activation, and excessive degranulation in the pathogenesis of several maladies, including chronic respiratory diseases, rheumatoid arthritis (RA), autoimmunity, and cancer [10]. Prolonged recruitment and activation of neutrophils can reflect a state of chronic inflammation, which is now a well-recognized hallmark of cancer. Importantly, cancer patients show remarkable increase in peripheral blood neutrophil count and their infiltration in tumors. Also, substantial reports suggest diverse phenotypic and functional heterogeneity of neutrophils in cancer. Neutrophil granules are the decisive mediators of neutrophil functionality and are regarded as double-edge swords [11]. Therefore, an understanding of granule cargoes, the mode of release, and their role is critical in understanding neutrophil function in the context of cancer. Here, we comprehensively revisit the expanding roles of neutrophil-derived granule cargoes in cancer. Further, we also highlight the potential of neutrophils as substantial therapeutic target in cancer.

2 Neutrophil dynamics: production, egression, and migration to the target site

2.1 Granulopoiesis

Neutrophils, eosinophils, and basophils collectively constitute a group of white blood cells defined as granulocytes. The process of production and development of granulocytes in the bone marrow is called granulopoiesis [12]. Neutrophils are the most plentiful cell population in the peripheral blood, with approximately 10^{11} cells produced per day [13]. They develop in the bone marrow from hematopoietic stem cells through a process involving multiple successive stages of neutrophil precursors [14] which includes myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell, and segmented granulocyte [15]. The mature segmented neutrophils are then finally mobilized from bone marrow to the circulation (Fig. 1). These circulating neutrophils are non-dividing cells and have a half-life of a few hours in peripheral blood [14]. In steady-state or normal conditions, only a small percentage, out of total neutrophils residing in the bone marrow, is released into the circulation. However, during inflammation or infection, their production and release increase rapidly in a process called emergency granulopoiesis. Various transcription factors play crucial roles during neutrophil maturation, such as E26 transformation-specific family transcription factor PU.1, C/EBP α (CCAAT enhancer binding protein α), C/EBP β , and C/EBP ϵ . PU.1 plays a decisive role in monocyte differentiation, while C/EBP protein promotes granulocyte differentiation. C/EBP α is an integral factor in the earliest stages of granulocyte differentiation [16]. It is also considered as a master regulator of steady-state granulopoiesis because it limits

the proliferation *via* inhibiting expression of cyclin-dependent kinases (cdk2 and cdk4) and *c-Myc*, whereas C/EBP β is known to promote emergency granulopoiesis, as it does not inhibit the expression of cdk2, cdk4, or *c-Myc*, thus allowing proliferation of granulocytic progenitors and increasing neutrophil count in peripheral blood. Several cytokines can also modulate neutrophil production, such as G-CSF (granulocyte colony-stimulating factor), GM-CSF (granulocyte-macrophage colony-stimulating factor), IL-6 (interleukin-6), and IL-3. Increased levels of these cytokines can switch steady-state granulopoiesis to emergency granulopoiesis [17]. Among them, G-CSF is known to be the primary regulator of steady-state as well as emergency granulopoiesis. It also influences the survival, maturation, and proliferation of the cells from the granulocyte lineage. Under normal conditions, the circulating level of G-CSF is very low, but this may increase during infection, inflammation or stress. This increased G-CSF activates JAK-STAT signaling pathway and further stimulates granulocyte differentiation in concert with C/EBP β [18, 19].

2.2 Neutrophil egress from primary niche

Neutrophils are matured and stored in the bone marrow until they are released into the circulation [13]. Their release is tightly controlled as only 1–2% of mature neutrophils are found in the circulation while their major population remains stored in the bone marrow [20]. Various factors can trigger their release from the bone marrow, such as cytokines, chemokines, and inflammatory stimuli. Further, their mobilization depends on various receptors expressed on the surface of neutrophils such as CXCR4, CXCR2, G-CSF-R, and their ligands on stromal cells. Among all, CXCR4 plays a crucial role in neutrophil retention in the bone marrow [21]. Deletion in CXCR4 shows increased release of neutrophils into the circulation, whereas high levels of CXCR4 and its ligand hold the neutrophil population in the bone marrow [22]. The major ligand for CXCR4 is stromal-derived factor 1 (SDF-1)/CXCL12, a CXC chemokine that is produced constitutively by stromal and endothelial cells of bone marrow [23]. The interaction between SDF-1 and CXCR4 maintains the neutrophil homeostasis in the bone marrow and circulation [24]. G-CSF is a principal cytokine and considered to be a potent stimulus which acts in several ways to induce neutrophil production and their release. It is known to promote neutrophil mobilization *via* downregulating SDF-1, thereby disrupting SDF-1 and CXCR4 interaction. In a study, the treatment of mice with G-CSF showed decreased SDF-1 production with high neutrophil mobilization from bone marrow. G-CSF treatment also reduced the surface expression of CXCR4 on myeloid cells. [25]. IL-23 produced by macrophages also increases neutrophil release through G-CSF, and therefore, the regulation of IL-23 production inhibits neutrophil release and thus maintains homeostasis of neutrophil

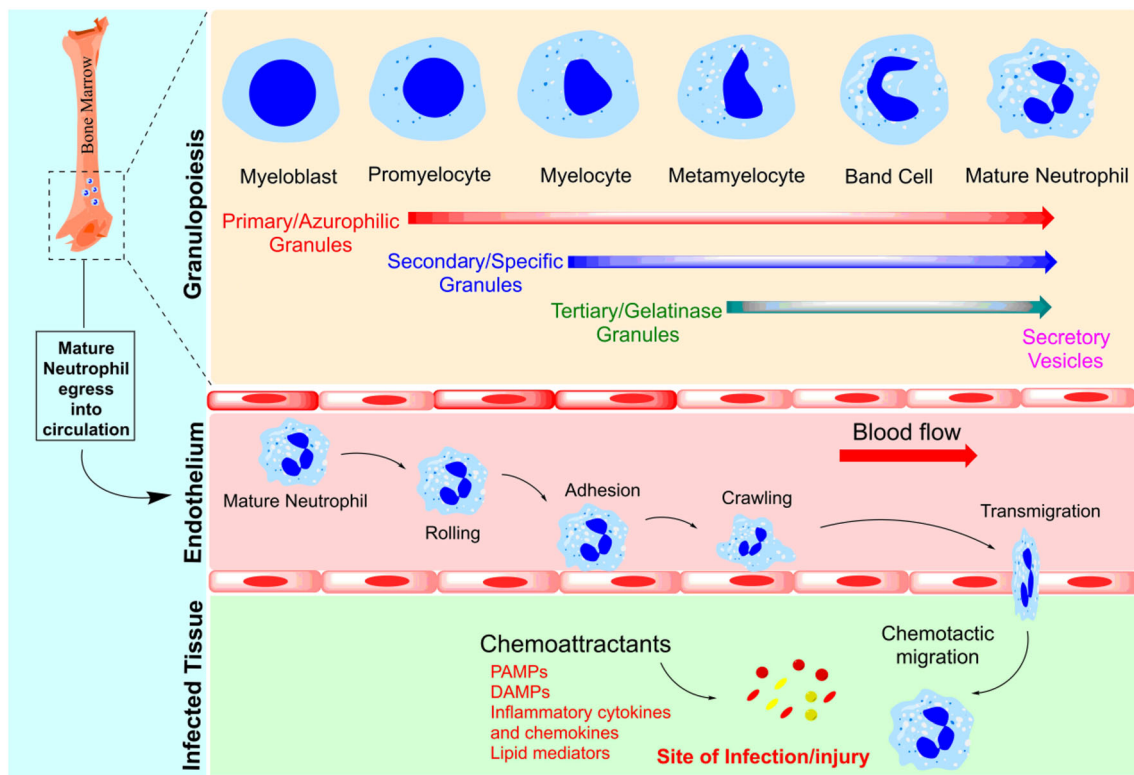


Fig. 1 Neutrophil granulopoiesis and recruitment to the target site. Granulopoiesis is characterized by the sequential formation of neutrophil granules. Myeloblast is the first cell of committed granulopoiesis that further differentiates into promyelocyte, myelocyte, metamyelocyte, band cell, and finally into mature neutrophil. Azurophilic or primary granules are synthesized during the promyelocytic stage and then gelatinase or tertiary granules are formed during the

metamyelocyte stage. Finally, secretory vesicles (SVs), which are exocytoseable membrane-bound organelles, are formed at the late stage of neutrophil maturation. Mature neutrophils now egress from the bone marrow into circulation. Upon sensing any chemoattractant, mature neutrophils actively migrate from circulation to the site of infection or injury in a process called extravasation that is a multi-step process including rolling, adhesion, crawling, and transmigration

number [26]. CXCR2 signaling on the other hand acts as CXCR4 antagonist. Upregulation of CXCR2 expression promotes neutrophil mobilization from bone marrow to the circulation [27]. Moreover, inflamed peripheral tissues produce several mediators that can influence mobilization of neutrophils from the bone marrow. For instance, in chronic peritonitis, LIX (CCL5) and MIP-2 act as ligand for CXCR2 and have shown to promote neutrophil release [28]. Similarly, in immune complex-induced arthritis, neutrophils present in the joint release LTB₄, MIP-1a (CCL3), MIP-2, and IL-1b which act as potent chemoattractants [29]. Moreover, MMPs (metalloproteinases) such as MMP8 and MMP9 can cleave collagen to a peptide proline-glycine-proline (PGP), which can activate CXCR2 on neutrophils [30]. Besides this, many tumor cells can produce CXCR2, which has been associated with tumor migration and invasion [31].

2.3 Migration to the battle fronts

Neutrophils are the first cells to arrive at the site of infection or inflammation to eliminate invading pathogens. The inflamed

site or damaged tissue generates a range of stimuli, such as PAMPs (pathogen-associated molecular patterns), DAMPs (damage-associated molecular patterns), lipid mediators, inflammatory cytokines, and chemokines, to initiate neutrophil migration [32, 33]. To reach at the site of inflammation, neutrophils must sense the inflammatory cues and extravasate from blood vessel into the tissues [34]. The extravasation is a multi-step process that involves rolling, adhesion, crawling, and trans-endothelial migration [35] (Fig. 1). In response to inflammatory signal, endothelial cells express P-selectin and E-selectin which are cell surface adhesion molecules. These molecules then bind to the glycosylated ligands expressed on the surface of neutrophils [36]. Neutrophils express multiple receptors such as cytokine receptors, pattern recognition receptors (PRRs), G protein-coupled receptors (GPCRs), adhesion receptors, and Fc receptors that can sense the pro-inflammatory mediators [37]. The interaction of selectins with their ligands allows the rapidly moving neutrophils to be captured from the circulation and stick to the endothelium, which is known to be a temporary interaction or fast rolling. Once the cells sense the inflammatory signal within the tissue and

microvasculature, there is slowing down of the rapidly moving cells, also called slow rolling. The activated neutrophils now tightly stick on to the endothelium *via* spreading their pseudopods [33, 38, 39]. Neutrophils constitutively express integrins such as LFA-1 (lymphocyte function-associated antigen 1) and MAC-1 (macrophage receptor 1), which bind to ICAM-1 (intercellular adhesion molecule)-1 and ICAM-2. The interaction between ICAM-1 and MAC-1 mediates the crawling of neutrophils within blood vessels. Crawling helps neutrophils to seek the most appropriate site for transmigration [40]. Trans-endothelial migration or diapedesis is the final step wherein cells breach the endothelial layer. Neutrophils can either migrate between endothelial cells (paracellular route) or through the endothelial cells (transcellular route); however, paracellular route is considered to be the efficient one [41, 42]. Neutrophil integrins such as LFA-1 and MAC-1 interact with endothelial adhesion molecules ICAM-1 and VCAM-1 (vascular cell adhesion molecule)-1 to facilitate transmigration. In addition, endothelial junctional adhesion molecules such as JAM-1, JAM-2, JAM-3, CD31, and CD99 also play crucial roles in trans-endothelial migration [43]. After passing through the endothelial cell barrier, neutrophils breach the pericyte layer and basement membrane with the help of proteases such as elastase, MMP8, and MMP9 stored in the granule subsets of neutrophils [44, 45].

Interestingly, the cell adhesion molecules also play an important role in many inflammatory processes including cancer and therefore represent key therapeutic targets [46]. Tumor cells can induce expression of E-selectin on vascular walls through the release of cytokines that stimulate E-selectin gene transcription. In addition, E-selectin also promotes tissue-specific metastasis of carcinomas. High levels of soluble form of E-selectins have been detected in serum of patients suffering from bronchial asthma, eczema, psoriasis, and allergic dermatitis, all examples of chronic inflammation [47]. During inflammation, the cytokines such as $\text{IFN}\gamma$, $\text{IL-1}\beta$, and $\text{TNF}\alpha$ can increase the expression of ICAM-1 and VCAM-1 [48]. Similarly, overexpression of junction adhesion molecules is also reported in many cancer types [49].

3 Neutrophil granules: major tools of neutrophil effector functions

3.1 Granule subsets: the vast armory

The versatile functions of neutrophils are dedicated to the different cytoplasmic granules of a mature neutrophil. They are equipped with three unique types of granule subsets namely primary (azurophilic) granules, secondary (specific) granules, and tertiary (gelatinase) granules [11]. These granules are classified as primary, secondary, and tertiary due to the fact that they are formed at different stages of neutrophils

maturation (Fig. 1) [50]. The primary or azurophilic granules are the first one to be synthesized at the promyelocytic stage. They are also called as peroxidase-positive granules due to the abundance of oxidant-producing enzymes, myeloperoxidase (MPO). In addition, they also store the most toxic and proteolytic mediators, including elastase, cathepsin G, proteinase 3, azurocidin, and defensins. These proteases are capable of degrading a vast range of ECM components such as elastin, fibronectin, and type IV collagen. Next in the line are secondary or specific granules synthesized during the myelocyte stage. They include collagenase, gelatinase, lactoferrin, lysozyme, lipocalin/NGAL, and membrane receptors. Tertiary or gelatinase granules are formed during the metamyelocyte stage and include gelatinase, cathepsin, acyl transferase, and collagenase. Notably, secondary and tertiary granules share some common granule contents that are discriminated based on their densities. They are also called as peroxidase-negative granules as they lack MPO [51]. In addition, there is another granule subset called as secretory vesicles (SVs) that are actually exocytoseable membrane-bound organelles. SVs are formed at late stage of neutrophil maturation and play an important role in delivering membrane-associated receptors to cell surface [15]. Unlike the rest three granule subsets, SVs do not acquire its proteins from Golgi compartment and completely rely upon endocytosis for their formation [52]. SVs derived proteins are responsible for neutrophil extravasation from the vasculature and also for phagocytosis [5]. Interestingly, upon external stimulations, neutrophil granules are mobilized in the reverse order of their formation. SVs formed at last are the first one to be mobilized to the cell surface. Next, in the order of mobilization, are the tertiary or gelatinase granules followed by secondary or specific granules and, finally, the azurophilic granules. Primary granules majorly release their contents into phagolysosomes and undergo limited extracellular release of their toxic contents. This delay in exocytosis upon external stimulations could be due to the fact that they contain a huge cargo of toxic mediators that would have detrimental effects on surrounding tissues as well. On the contrary, secondary and tertiary granules are exocytosed most readily [53, 54].

3.2 Effector functions of neutrophils: mechanisms of host protection

Once reached to the site of infection, activated neutrophils adopt diverse mechanisms to eliminate the invading pathogens. These mechanisms include phagocytosis, degranulation, and NETs (Fig. 2). Phagocytosis is an endocytosis process, wherein phagocytic cells such as macrophages and neutrophils engulf the microbes [55]. Phagocytic cells recognize the invading microbes *via* the interaction of neutrophil surface receptors with the opsonic receptors present on the surface of the microbes [56]. Neutrophils employ two different receptor

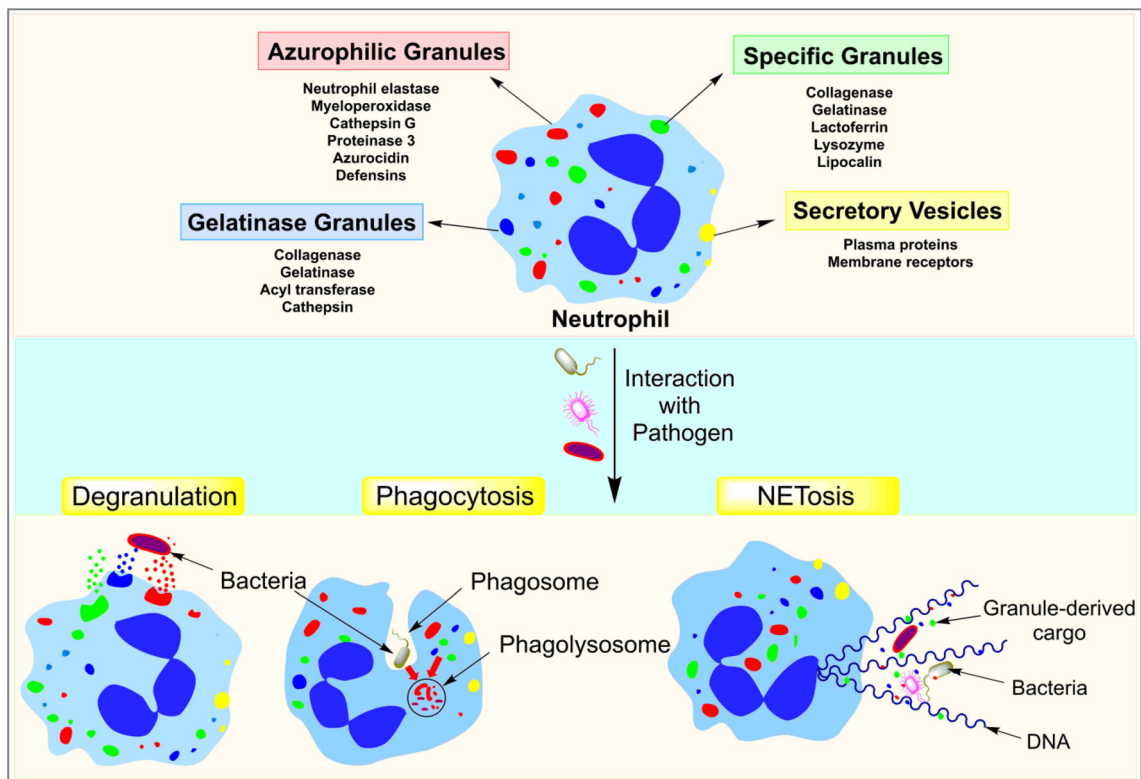


Fig. 2 Effector functions of neutrophils. Once reaching the battle front, neutrophils adopt diverse mechanisms to destroy pathogens, such as phagocytosis, degranulation, and NETosis. In phagocytosis, the pathogen is ingested into phagocytic vacuoles called phagosomes which become phagolysosome upon maturation. Further in the

phagolysosome, the pathogen is destroyed by the action of degrading enzymes. In degranulation, neutrophils release their toxic cargo, stored in the granule subsets. During NETosis, DNA fibers equipped with granule cargo are released in the form of neutrophil extracellular traps (NETs) to entrap and kill the large microbes that cannot be ingested

classes to perform phagocytosis. The first one is Fcγ receptors that include FcγRIIA (CD32) and FcγRIIIB (CD16). The second one is the complement receptors, CR1 (CD35) and CR3 (or CD11b/CD18 integrin) [57]. Microbes are internalized and enclosed within a part of the cell membrane in the form of a vacuole called a phagosome. To initiate the killing process, there is fusion of these phagosomes with the granules within the cells which is termed as phagosome maturation [58]. Phagocytosis triggers the process of oxidative burst in neutrophils which is a process of generation of reactive oxygen species (ROS) in order to kill internalized microbes. The activated neutrophils are highly efficient in generating ROS by utilizing the NADPH oxidase complex [59]. In resting neutrophils, the NADPH complex is disassembled and remains dormant. Phagocytic receptors that mediated downstream signaling trigger the assembly and activation of the NADPH complex on phagosomes [60]. Chronic granulomatous disease (CGD) is a rare genetic disorder that reveals the importance of NADPH oxidase. The disease involves a defect in the gene encoding one of the NADPH oxidase subunits resulting in the inactive form of NADPH oxidase. Therefore, CGD patients suffer from severe fungal and bacterial infections [61]. Activated NADPH complex catalyzes the formation of superoxides *via* transferring electrons from NADPH to

O₂ [62]. Superoxides can undergo further reactions to produce vast range of ROS, including H₂O₂ and HOCl. MPO, a heme protein localized in the azurophilic granules of neutrophils, catalyzes the reaction to generate HOCl from H₂O₂. HOCl is a highly toxic oxidant used by neutrophils to kill a wide range of pathogens [63].

During superoxide production by activating NADPH oxidase complex, the granules stored in the neutrophils fuse with the membrane and release their enzymatic contents in a process called degranulation. It is a receptor-mediated process adopted by neutrophils to kill the invading microbes *via* the release of toxic mediators (proteases, anti-microbial peptides and inflammatory substances [64]. Among the vast library of primary granule contents, neutrophil elastase (NE), cathepsin G, and proteinase 3, collectively called as neutrophil serine proteases, are critical for host defense. They effectively act against bacterial infections *via* direct killing of bacterial cells, cleaving host proteins or attenuating the bacterial virulence factors. Lactoferrin present in the secondary granules also possesses a broad range of anti-bacterial activities such as blocking the entry and adhesion of bacterial pathogens on host cells [56]. Similarly, defensins can also exert anti-microbial activities *via* disrupting the target cell membranes and neutralizing a range of enzymes secreted by bacteria. It can also

inhibit viral transcription by blocking the intracellular signaling cascade [65]. Moreover, MMPs such as collagenases and gelatinases stored in secondary and tertiary granules are potent ECM degrading agents which facilitate neutrophils migration through basement membranes [66]. These granule contents are rich in tissue destructive proteases, and their excessive release can also cause severe tissue damage. Degranulation, therefore, is a tightly regulate process which is initiated once the receptor in the phagosomal membrane get activated and signal the granules for their mobilization and release of cargoes [67]. The molecular basis for granule exocytosis remains to be fully understood, but SNARE proteins including SNAP-23, VAMP 2, and syntaxins 4 seem to play an important role [66]. Earlier, neutrophils were known to destroy pathogens by phagocytosis, which is the engulfment of microbes or by the release of anti-microbial contents. But it was in 2004 that NETosis was recognized as another excellent method of pathogen killing adopted by neutrophils. NETs are extracellular fibers or meshes equipped with decondensed DNA, histones, and granular proteins of neutrophils such as MPO, elastase, and lactoferrin [68, 69]. Some reports propose that NETosis is adopted by neutrophils to encounter large pathogens that cannot be phagocytosed [70]. NETs entrap and neutralize microbes to promote their extracellular killing. NETosis has been categorized as suicidal NETosis and vital NETosis. Neutrophils undergo several morphological changes during suicidal NETosis which include alterations in the nucleus structure, increased permeability of nuclear and granular membranes, and inactivation of histones. These alterations then leads to chromatin expansion, mixing of chromatin and granule content, and release of NETs into the extracellular spaces through permeable plasma membrane [71, 72]. In vital NETosis, neutrophils remain functionally active after NET release [73]. NETosis can be triggered by PAMPs from microbes, auto antibodies, inflammatory mediators [64], and mitogenic stimuli like PMA (phorbol 12-myristate 13-acetate) and concanavalin A [74]. PAMPs triggered NETosis require active NADPH oxidase complex [75]; otherwise, ionomycin- and nicotine-triggered NADPH-independent NETosis is also reported which rely on mitochondrial ROS [76, 77].

4 Neutrophils in pathogenesis: actions against the host

4.1 Neutrophils in prevalent chronic diseases

Neutrophil number, infiltration, and activation have to be tightly controlled as dysregulated release of toxic mediators can lead to severe tissue damage and inflammation. Altered neutrophil functioning has been the cause of various diseases such as infection, cardiovascular diseases, respiratory diseases, and neuroinflammation [26] (Fig. 3). Sepsis is a life-

threatening condition resulting from heightened immune response and severe tissue damage. Neutrophil reprogramming during sepsis leads to diminished recruitment of activated neutrophils at the infection site and concomitant increase in their number in circulation with enhanced release of effector molecules, thus causing tissue damage and several organ failure [78]. Neutrophils are also involved in various respiratory disorders such as chronic obstructive lung disease (COPD), adult respiratory distress syndrome (ARDS), and cystic fibrosis. Patients suffer from bronchial inflammation due to elevated recruitment of neutrophils and release of neutrophil-derived proteolytic mediators [79–82]. Atherosclerosis, a condition of narrowing of arteries due to accumulation of plaque, is now considered as an inflammatory disease. Recent studies suggest an important contribution of neutrophils in triggering inflammatory response in atherosclerosis [83]. Ionita and colleagues reported a high number of neutrophils in human atherosclerotic plaques [84], whereas some studies have also reported the involvement of NETs in atherosclerosis [85]. Neutrophils migrate early to the ischemic site and are known to promote inflammation and release of proteolytic granule contents [86–88]. NETs are also thought to be involved in the thrombus formation in venous and arterial systems in conditions such as sepsis and cancer [89, 90]. In recent years, neutrophils have emerged as important participants in various systemic autoimmune diseases also. Rheumatoid arthritis (RA) is a chronic inflammatory disorder that leads to bone erosion, chronic synovitis, and joint deformity. Neutrophils are found in flocks in the synovial fluid of the affected joints of RA patients [91]. Similarly, circulating anti-citrullinated peptide autoantibodies (ACPAs) are also known to contribute to RA pathogenesis. Neutrophil-derived NETs contain citrullinated histones and serve as potential sources of autoantigens that can trigger the production of autoantibodies in RA [92]. Psoriasis is another chronic autoimmune disease characterized by skin lesions or patches. Overstimulation of neutrophils along with other immune cells such as dendritic cells, T cells, fibroblasts, melanocytes, and mast cells has been reported in psoriasis pathogenesis [93–96]. Neutrophils mobilize to the psoriatic site and trigger oxidative burst, degranulation, and NETosis, thus contributing to disease pathogenesis. Neutrophils isolated from psoriasis patients showed high ROS release compared with healthy individuals, whereas depletion of neutrophils and suppression of oxidative burst significantly relieved psoriasis patients [97]. Involvement of NETs has been reported in systemic lupus erythematosus, an autoimmune disorder wherein the body's immune system attacks its own tissues and organs system [98, 99]. Anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis is inflammation of blood vessels in which both arteries and veins are affected. ANCA is directed against MPO and proteinase 3, which are present in the primary granules of neutrophils [100]. ANCA-stimulated neutrophils also release NETs,

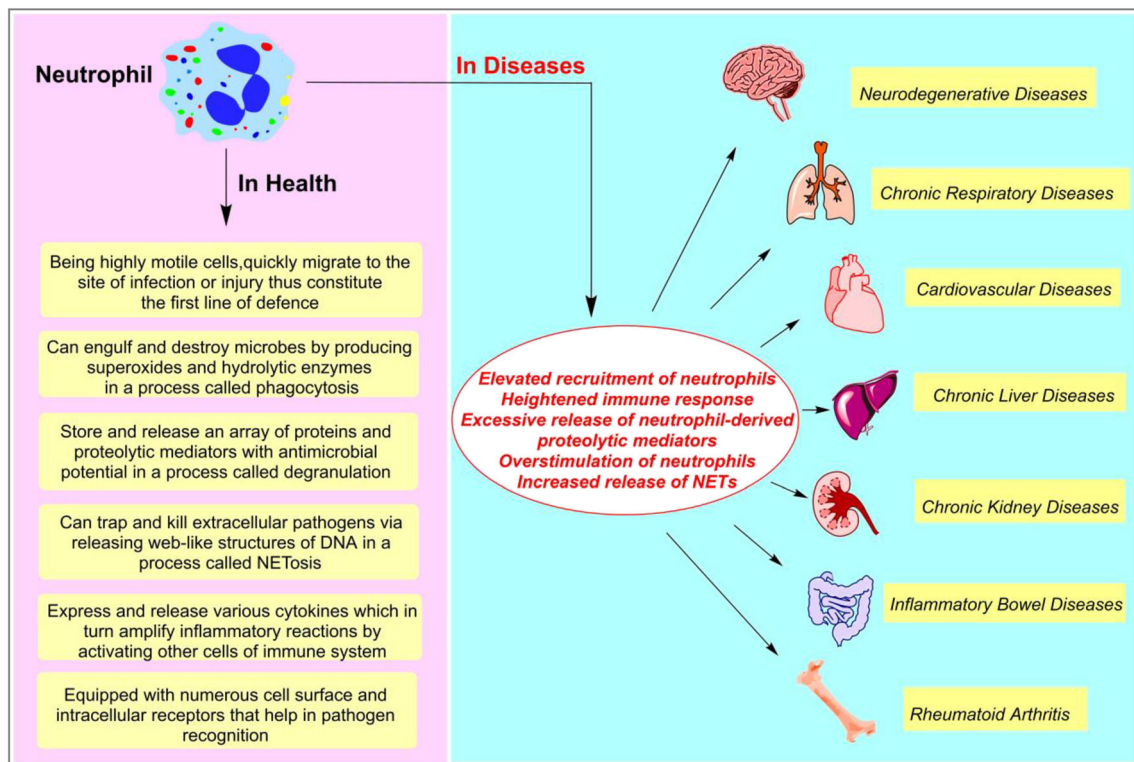


Fig. 3 Neutrophils in health and diseases. Neutrophils act as first line of defense and are equipped with diverse mechanisms such as phagocytosis, degranulation, and NETosis to eliminate pathogens. However, abnormal

neutrophil count and function are associated with multiple diseases affecting vital organs ranging the brain, lungs, heart, liver, kidney, intestine, and bones

which are known to promote vasculitis pathogenesis [101]. Similarly, neutrophil-mediated pathogenesis has been reported in mice models of neuroinflammatory and neurodegenerative diseases such as multiple sclerosis and Alzheimer disease. Increased release of NETs is observed in the circulation of multiple sclerosis patients which results in disruption of the blood-brain barrier [102]. Moreover, high accumulation of neutrophils and NETs release has been reported around amyloid- β plaques found in brains of Alzheimer patients [103, 104].

4.2 Neutrophils in cancer: not-so-neutral

Chronic inflammation is now well recognized as a major hallmark of cancer, and neutrophils are believed to be a central component of this process. At the site of infection, once neutrophil function is over, their clearance is essential for resolution of inflammation to maintain tissue homeostasis. But, failure in the resolution machinery and prolonged neutrophil accumulation can damage the host tissue and reflect a state of chronic inflammation [105]. According to traditional immunology, the sole function of neutrophils was in host defense, in immune modulation, and in tissue injury [12]. However, emerging research negates this traditional idea and proved that these cells function in a more complex way and display clear phenotypic and functional heterogeneity [106, 107].

Emerging studies have well documented the role of neutrophils in various chronic inflammatory disorders including cancer [107]. Though, their role in cancer was earlier ignored due to their short life span but several recent reports validate their dominant pro-tumoral role [108]. Intriguingly, an increasing number of clinical as well as preclinical observations have reported frequent accumulation of neutrophils in tumors. In clinical studies, a bulk of correlation reports have suggested poor prognosis of patients with high peripheral neutrophil count and high neutrophil infiltration at tumor sites leading to high neutrophil-to-lymphocyte (NLR) ratio. This state of neutrophilia has been principally observed in patients with advanced cancer [109]. For instance, in human HNSCC, enhanced neutrophil infiltration was observed in more aggressive tumors than in less aggressive tumors [110]. Also, high NLR ratios were correlated with low survival probability in patients suffering from liver, lung, colon, and pancreatic cancers [111–114]. Importantly, NLR has been introduced as a simple and inexpensive biomarker for many tumor types, including colorectal cancer [115], breast cancer [116], non-small cell lung cancer [117], and hepatocellular carcinoma [118]. Neutrophils present in the tumor microenvironment are referred to as tumor-associated neutrophils (TANs), which are further categorized as anti-tumoral (N1 type) and pro-tumoral (N2 type) and thus regarded as double-edged sword in cancer. The anti-tumoral role of neutrophils is governed by

either direct tumor cell killing or indirect killing by activation of other immune cells. In direct killing, neutrophils interact with tumor cells in ADCC-dependent manner. After the physical contact, neutrophils secrete cytotoxic mediators, such as H_2O_2 to induce apoptosis within tumor cell [119]. Also, neutrophils can directly inhibit tumor cell proliferation and survival through production of TRAIL, a TNF superfamily member that binds to its receptor in tumor cells and induces apoptosis [120]. In indirect killing, neutrophils release various pro-inflammatory or immunostimulatory cytokines, such as $TNF-\alpha$, IL-12, and chemokines such as CCL3, CXCL9, and CXCL10. These factors further facilitate recruitment and activation of other immune cells including $CD8^+$ T cells, B cells, NK cells, and dendritic cells [121]. On the contrary, the pro-tumoral neutrophils promote tumor invasion, metastasis, and angiogenesis *via* releasing various factors such as oncostatin M (OSM) [122], hepatocyte growth factor (HGF) [123], neutrophil elastase (NE), and matrix metalloproteinases (MMPs) [124]. They also show strong immunosuppressive activity by releasing high levels of arginase which in turn suppress the activity of $CD8^+$ T cells [125]. Studies conducted in mouse lung carcinoma and mesothelioma models suggest that TANs show N1 phenotype at the initial stage of tumor growth, whereas they convert into N2 phenotype with tumor progression [126]. Fridlender et al. suggested an important role of factors produced by cancer cells or other immune cells in mediating phenotypic transformation of TANs within tumor microenvironment. They were first to show the role of immunosuppressive cytokine, $TGF-\beta$ in neutrophil polarization. In the presence of $TGF-\beta$, neutrophils are skewed towards an N2 phenotype. It also blocks the production of H_2O_2 and restricted the migration of neutrophils towards tumor cells, thus inhibiting the anti-tumoral functions of neutrophils. In contrast, the blockade of $TGF-\beta$ enhances the development of neutrophils into an anti-tumoral N1 phenotype [125]. Besides $TGF-\beta$, various other cytokines have also been implicated in regulation of TANs plasticity. For instance, type I interferon can also regulate neutrophil polarization but its effect opposes to that of $TGF-\beta$. The presence of type I interferon polarizes neutrophils into an N1 phenotype, whereas impaired endogenous type I interferon signaling polarizes neutrophils into an N2 phenotype [127]. In a study with murine tumor model, $IFN-\beta$ -deficient mice showed faster tumor growth, enhanced vascularization, and higher infiltration of neutrophils as compared with the wild-type mice [128]. Similarly, IL-12 is known to activate anti-tumoral N1 phenotype [129] while GCSF and IL-6 activate pro-tumoral N2 phenotype [130]. During physiological condition, neutrophils are in inactive state in circulation and get activated during any condition of infection or inflammation. Once they reach to the site of infection, they can polarize depending upon the factors present in their microenvironment. Whether these

TANs can be irreversibly polarized or not is still unclear and require further studies.

Signals from tumor cells or tumor microenvironment are known to influence infiltrating neutrophils to release their effector molecules like ROS, cytokines, chemokines, NETs, and granule contents which then impact tumorigenesis [22] (Fig. 4). Neutrophil-derived ROS can induce DNA damage and thereby enhance mutation rates contributing to increased tumor cell proliferation by deregulating tumor suppressor genes and oncogenes. Studies also correlated an increase in the number of TANs with high ROS activity and mutation rates [131, 132]. Additionally, neutrophil-derived ROS can produce a range of highly reactive mediators such as lipid hydroperoxides and epoxides which can also cause DNA damage [133]. Besides this, ROS, particularly H_2O_2 , can act as a messenger in cell signaling which can regulate the PI3K/Akt, IKK/NF- κ B, and MAPK/Erk1/2 signaling pathways in cancer [134]. In addition to ROS, neutrophils are capable of *de novo* synthesis and secretion of a range of cytokines and chemokines at tumor sites which not only enhances their own recruitment but also promotes the infiltration of other tumor-supportive immune cells [135]. Although neutrophils typically produce lower amounts of cytokines per cell compared with other immune cells such as macrophages, they are so abundant at inflammatory sites that their contribution to total cytokine level is quite significant [136]. IL-8, the most abundantly produced cytokine, can enhance neutrophil influx and support tumor cell proliferation by autocrine and paracrine mechanisms. Also, pro-inflammatory cytokines such as IL-1 β and $TNF-\alpha$ can induce other cells to produce neutrophil chemoattractants and promote their extravasation. IL-6, another pro-inflammatory cytokine, released by neutrophils was reported to promote VEGF expression, thereby impacting angiogenesis. Tumor-infiltrating neutrophils can also produce cytokines such as IL-17, APRIL, BAFF, OSM, and HGF all of which have been implicated in tumor progression [137]. Furthermore, in a study, TANs showed active secretion of CCL17 which promoted recruitment of immunosuppressive regulatory T cells (Tregs) cells in the tumor microenvironment, whereas neutrophil depletion significantly reduced their recruitment to tumors [138].

Furthermore, NETs have been recognized as a new add-on to the anti-microbial action of neutrophils [139]. Released by the activated neutrophils, they are pathogen-trapping fibers equipped with chromatin and neutrophil proteolytic enzymes. Their role in cancer has recently been demonstrated [140]. They can trap the circulating tumor cells and aid their transfer to distant sites, hence acting as possible mediators of metastasis [141]. They can also impact tumorigenesis by enhancing tumor cell proliferation either by releasing granule proteases such as NE, cathepsin G, and MMP9 on the NETs, or by activating signaling machinery such as NF- κ B pathway. Higher levels of NETs were found in the plasma of lung,

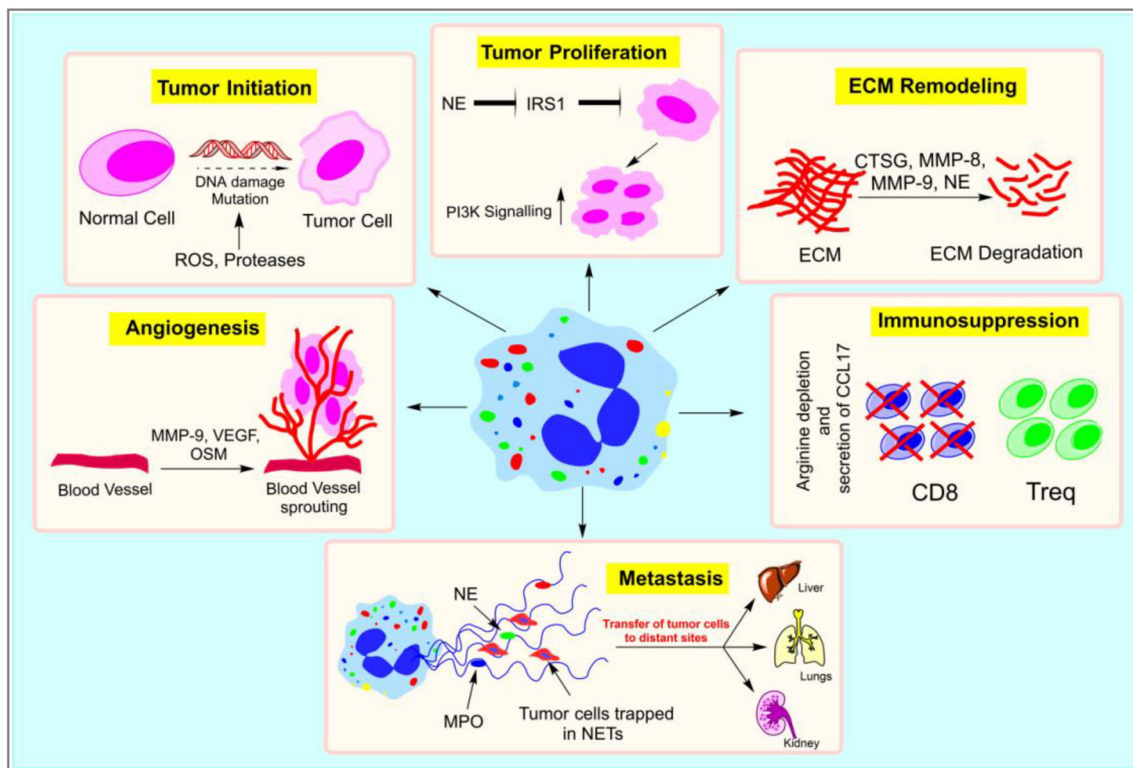


Fig. 4 Neutrophils in cancer. Neutrophils can promote tumorigenesis in several ways. NE (neutrophil elastase) can degrade insulin receptor substrate (IRS-1) and upregulate PI3K (phosphatidylinositol 3-kinase) signaling, thus inducing tumor cell proliferation. Similarly, neutrophil-derived ROS and proteases can induce DNA damage and enhance mutation rates in normal cells that can instigate initiation of tumors. Neutrophils can support tumors by stimulating tumor angiogenesis by releasing proangiogenic factors such as MMP-9, VEGF (vascular

endothelial growth factor), and OSM (oncostatin M). They can also promote recruitment of immunosuppressive regulatory T cells (Tregs) cells in the tumor microenvironment. Neutrophil-derived proteolytic enzymes like MMP-8, MMP-9, NE, and CTSG (cathepsin G) can degrade a range of ECM (extracellular matrix) components, thus facilitating tumor cell migration. Further, NETs (neutrophil extracellular traps) can entrap tumor cells and aid their transfer to distant sites

pancreatic adenocarcinoma, and bladder cancer patients as compared with healthy controls [142]. Moreover, adverse patient outcomes are also reported to be associated with increased NETs production [143]. Besides this, granule cargoes also play a pivotal role in deciphering tumor aggressiveness which we will discuss in detail in the upcoming section.

5 Granule cargoes in cancer: paving the way forward

As in case of other chronic inflammatory diseases, the role of neutrophil granules seems to be important in cancer as well. Most of the granule components of neutrophils act as a salient protagonist in tumor progression (Table 1). By far, the most well-studied ones in cancer include NE, MPO, cathepsin G, MMP8, and MMP9 which play diverse pro-tumoral roles. Similarly, neutrophil- α defensins and oncostatin M can also pave the way for tumor growth and progression. In the subsequent section, we discuss the role of these granule cargoes in the context of cancer.

5.1 Neutrophil elastase

NE is a 29-kDa serine protease of the chymotrypsin family and a key effector molecule encapsulated in the primary (azurophil) granule of neutrophils [144]. It is synthesized as a precursor in promyelocytes in bone marrow and becomes active in mature neutrophils. It is the most abundant enzyme present in neutrophils, wherein a single neutrophil contains 1 pg of NE [145]. Activated neutrophils release NE into the extracellular spaces *via* degranulation or NETs formation [146]. It plays an important role in mounting an inflammatory response for host defense and pathogen clearance during infection. It is also considered as an important regulator of leukocyte transmigration and emergency myelopoiesis [147]. NE hydrolyzes variety of substrates, including elastin and other ECM components such as cadherins, fibronectin, collagen, proteoglycan, lung surfactant, and growth factors [148]. In addition to its role in host defense, evidences suggest an important contribution of NE in various chronic inflammatory diseases, including cancer [149].

Table 1 Neutrophil-derived granule cargoes and their role in different types of cancer

Neutrophil-derived granule cargoes	Types of cancer	Role	References
Neutrophil elastase (NE)	Lung cancer	High elastase levels in serum and bronchoalveolar lavage fluid, correlated with disease progression	[153]
Myeloperoxidase (MPO)	Pancreatic adenocarcinoma, acute promyelocytic leukemia	MPO polymorphism leading to abnormal MPO expression	[183] [184]
	Breast cancer	High MPO levels correlated with high cancer risk	[188]
Cathepsin G (CTSG)	Breast cancer	Mediate tumor cell adhesion by stimulating E-cadherin/catenin complex formation	[209]
Neutrophil collagenase (MMP8)	Pancreatic adenocarcinoma, uterine cancer, head and neck squamous carcinoma cells, ovarian cancer and colorectal cancer	High expression of MMP8 correlated with tumor progression	[215–219]
	Ovarian cancer, melanoma	MMP8 gene polymorphism associated with high cancer risks	[221, 222]
Gelatinase B (MMP9)	Myxofibrosarcoma, epithelial carcinogenesis	Tumor invasion, angiogenesis, hyper-proliferation	[234, 236]
Neutrophil α -defensins	Renal, bladder, oral squamous cell carcinoma, and breast cancer	Over expression of α -defensins leading to tumor cell proliferation, migration and invasion	[253–256]
Oncostatin M (OSM)	Breast cancer	High OSM expression correlated with tumor angiogenesis and neovascularization	[262]

Upregulated activity and expression of NE have been observed in various cancer types, and its concentration is often correlated with cancer grade, stage, and survival of patients. Notably, α 1-anti-trypsin, a secretory glycoprotein produced in the liver, is a natural inhibitor of NE. An imbalance in the levels of NE and α 1-anti-trypsin is also associated with several diseases such as lung emphysema, RA, bronchiectasis, asthma, and chronic liver diseases. Similar imbalance has been reported in the development and progression of lung, liver, and colorectal cancers [150]. Yamashita and the group in their study showed that lung cancer patients with high elastase concentration had shorter survival and poor prognosis compared with those with low elastase levels [151]. Similar results were also observed in mice model of lung adenocarcinoma, wherein mice lacking NE showed longer survival [152]. Elevated elastase levels in serum and bronchoalveolar lavage fluid (BALF) are also correlated with disease progression in lung cancer patients [153]. Several studies have reported that NE can directly promote tumor cell proliferation by hyper activating phosphatidylinositol 3-kinase (PI3K). Clathrin-coated pit and neuropilin-1 mediate endosomal internalization of NE, which enzymatically degrades insulin receptor substrate-1 (IRS-1). It further increases the interactions between PI3K and PDGFR, a potent mitogen that triggers tumor cell proliferation [154, 155]. It is also involved in tumor cell invasion and migration by degrading ECM proteins and activating MMPs such as MMP2 and MMP3 [156, 157]. Other studies also suggest that NE promotes the release of tumor promoting factors such as VEGF, PDGF, and TGF- α into the ECM, thus directly or indirectly supporting tumor growth and progression [158].

5.2 Myeloperoxidase

MPO is found in the primary granules and makes up to 5% of neutrophil's dry weight [159]. It is also found in monocytes, but in a much lesser amount than neutrophils and eventually lost during monocyte-to-macrophage differentiation in tissues [160, 161]. This enzyme is one of the important players in neutrophil function which is released into the ECM *via* degranulation, apoptosis, necrosis, and NETosis [101, 161, 162]. It acts as a powerful pro-inflammatory agent which catalyzes the formation of ROS including hypochlorous acid (HOCl), hypobromous acid (HOBr), and hypothiocyanous acid (HOSCN) [163, 164]. These intermediates are toxic agents for pathogens and act as important players in the immune response. On the contrary, their unregulated production can also damage host tissues and causes several diseases [165]. Recent reports have very well documented the role of MPO in the initiation and progression of several diseases including cardiovascular diseases [166], neurodegenerative diseases [167], RA [168], asthma [169], and cancer [170].

Genomic instability is one of the hallmarks of cancer which leads to either epigenetic alterations or mutations collectively termed as the hypermutagen state. An inefficient DNA repair system and increased sensitivity to mutagens are the salient features of this state. MPO is known to play an important role in promoting hypermutagen environment through its enzymatic actions [171]. HOCl, particularly, is known to be a potent oxidizing agent that can oxidize proteins, lipids, and DNA [172]. It can further interfere with DNA repair and promote DNA cross-links and formation of pyrimidine oxidant products. MPO-derived oxidants can also activate inhaled carcinogens such as polycyclic aromatic hydrocarbons (PAHs), leading to mutagenesis [173]. Besides DNA modifications,

MPO-derived HOCL can also encourage tumor cell metastasis *via* activation of MMPs such as MMP2, 7, and 8 [174]. In addition, MPO-dependent activation of MMPs also contributes to vascular dysfunction leading to cardiovascular [175] and renal disorders [176]. Moreover, ROS generated by MPO is known to inhibit the activity of NK cells against tumor cells [177]. Furthermore, MPO gene polymorphism is also associated with altered MPO expression and susceptibility to cancer risks [178]. Single nucleotide polymorphism in the promoter region of MPO can affect transcription as well as translation [179]. MPO-463G/A promoter polymorphism are considered in this respect wherein G allele is associated with higher MPO gene transcription and A allele with lower gene transcription. Various reports have associated the 463A allele variant of MPO with lower risk of lung, breast, and bladder cancer [180–182], whereas 463G allele variant is known to promote pancreatic adenocarcinoma [183] and acute promyelocytic leukemia [184]. Moreover, in neurodegenerative diseases such as multiple sclerosis [185] and Alzheimer's disease [186], G/G allele expression is correlated with a high possibility of disease development and in cystic fibrosis patients [187]; it is associated with severe lung infections.

A high MPO level in biological fluids is also correlated with several malignancies. For instance, high MPO level has been directly linked to the risk of breast cancer development and is considered as an efficient marker in premenopausal women suffering from breast cancer [188]. Similarly, patients suffering from acute myeloid leukemia (AML) showed an increase in plasma MPO levels (1.0–9514 ng/mL) as compared with the controls (3.5–20.6 ng/mL) pointing towards its clinical relevance [189]. An increased level of MPO was also detected in serum and BALF of patients suffering from COPD and lung cancer [153]. Furthermore, in a mice model of lung cancer, MPO inhibition showed a 50% reduction in MCA-induced lung carcinogenesis [190]. Thus, MPO can efficiently contribute to cancer initiation and progression; notably, its high levels can enhance cancer susceptibility.

5.3 Cathepsin G

Cathepsins are an extensive family of proteases which are known to participate in many physiological and pathological processes [191]. They are transported either to the nucleus to regulate gene expression or to the cell surface to regulate cell signaling as well as secreted outside to degrade ECM [192]. These enzymes are actively involved in development, differentiation, angiogenesis, wound healing, bone remodeling, antigen processing, and reproduction. On the contrary, they are also involved in diseases such as bronchial asthma, atherosclerosis, RA, and cancer [193–197]. They are broadly categorized into serine, aspartic, and cysteine cathepsins on the basis of the amino acids present at their active sites [198]. Neutrophils are equipped with cathepsin G (CTSG), a serine

cathepsin which is stored in their azurophilic granules together with NE and proteinase 3 (PR3) [144]. Being a degradative enzyme, CTSG can kill the ingested pathogen and perform extracellular functions such as degradation of ECM, hydrolysis of host plasma proteins, and hormonal factors [199]. It can also perform immunomodulatory actions by cleaving certain chemokines and their receptors [200]. CTSG is also involved in transmigration of leukocytes at the infection site [201], platelet activation [202], conversion of angiotensin I to angiotensin II [203], and induction of airway sub mucosal gland secretion [204]. Besides its anti-microbial role, CTSG has been identified to play a very important role in inflammation, tumor growth, and progression. Pancreatic ductal adenocarcinoma (PDAC) patients showed a 2.4-fold increase in CTSG protein expression. Similarly, in patients suffering from chronic pancreatitis, a 1.9-fold upregulation of CTSG protein as compared with controls was reported [205]. CTSG is known to enhance the activity of IL-8 which is a pro-inflammatory cytokine and strong neutrophil chemoattractant. Furthermore, CTSG can also activate other pro-inflammatory cytokines such as TNF- α and IL-1 β , thus contributing to inflammatory disorders. It can promote tumor cell invasion *via* degradation of ECM and increasing the permeability of endothelial cells [206, 207]. In another report, upregulated expression of CTSG is known to activate pro-MMP-2 and MMP-9 at the tumor-bone interface and subsequently activates TGF- β which further promotes tumor growth and bone destruction through osteoclast activation [156, 208]. In addition, it can also mediate tumor cell adhesion by stimulating E-cadherin/catenin complex formation in breast cancer cells and thus promoting tumor growth [209]. The molecular mechanism involved in these processes remains poorly understood; however, a study showed the role of IGF-1R signaling. CTSG stimulated IGF-1 release from MCF-7 cells by activating IGF-1R signaling which further promoted cell aggregation [210]. Together, these reports suggest a crucial role of CTSG in tumorigenesis and can be a potential therapeutic target.

5.4 Neutrophil collagenase

Neutrophil collagenase is also known as MMP8 or collagenase-2. It is highly expressed in neutrophils and stored as proenzyme in specific granules. Beyond the capacity to degrade ECM, MMP8 has diverse biological roles such as in innate immunity, modulation of chemokines, production of chemotactic peptides, and regulation of repair response [211–213]. Activated neutrophils quickly release MMP8 to ensure its availability at the site of infection or inflammation. Neutrophils release only 20–30% of its cellular MMP8 content into extracellular spaces, while the rest are solely localized at the cell surface. MMP activity is regulated by its inhibitor TIMP (tissue inhibitor of metalloproteinases) but, being

resistant to TIMP-mediated inactivation, MMP8, majorly membrane bound, shows high efficiency in cleaving type I collagen [214]. Various studies have reported the upregulation of MMP8 in a wide range of inflammatory disorders including cancer. For instance, high expression of MMP8 was observed in tissue samples of pancreatic adenocarcinoma [215] and uterine cancer patients [216] as compared with normal tissues. Similarly, high expression of MMP8 was correlated with tumor progression as well as poor prognosis in patients with head and neck squamous carcinoma, ovarian cancer and colorectal cancer [217–219]. In hepatocellular carcinoma cells, MMP8 exhibits pro-tumorigenic effects by regulating its signaling cascade *via* activating the PI3K/Akt/Rac-1 pathway. It upregulates TGF- β expression, which stimulates epithelial-mesenchymal transition (EMT), thereby increasing invasion and migration of the cancer cells [220].

Importantly, various reports suggest the association of MMP8 gene polymorphism with cancer risk. For example, single nucleotide polymorphism (SNP rs11225395) in the promoter region of MMP8 gene increases the transcription rate of MMP8 which is reported to be associated with high risk of developing ovarian cancer and melanoma [221, 222]. Also, MMP8 gene polymorphism (MMP8 78K/E) is associated with high risk of bladder cancer [223]. MMP8 is also considered as potential diagnostic biomarker and therapeutic targets for various diseases. Its level can be easily quantified in oral fluids, plasma and serum. In patients with colorectal cancer, high serum MMP8 levels were correlated with increased malignancy, systemic inflammation and reduced overall survival [224, 225]. Similarly high level of MMP8 in fluids of ovarian cysts was associated with tumor development [226]. These aforementioned reports suggest a pivotal role of MMP8 in cancer; however, the tumor-promoting mechanism of MMP8 requires more extensive studies to understand the overall role of MMP8 in different steps of tumorigenesis.

5.5 MMP9/gelatinase B

MMP9 is expressed by different cell types, including neutrophils, macrophages, and mast cells. In neutrophils, it is synthesized during their maturation in the bone marrow and stored as pro-MMP9 in the secondary and tertiary granules from which it is released upon activation or chemotactic stimulations [227, 228]. In cancer microenvironment, MMP9 is known to be a major contributor to ECM degradation but it is tightly regulated by the linked TIMP-1. Interestingly, neutrophils do not produce endogenous TIMP-1 and therefore neutrophil-derived MMP9 remains active. In cancer milieu, this TIMP-1 free MMP9 is majorly known to enhance angiogenesis and tumor invasion in multiple ways such as by activating angiogenic mitogen present in matrix stores, regulating recruitment and proliferation of pericytes and mobilizing bone marrow-derived angiogenic factors to tumor stroma

[229–232]. According to a study, 1.5 ng of MMP9 released by as few as 5×10^4 neutrophils can lead to a 5-fold raise in angiogenesis level suggesting its strong angiogenic potential [233].

In patients with myxofibrosarcoma, high number of neutrophils also correlated with increase in microvessel density [234]. Similarly, they were abundantly present in the angiogenic islets of tumors and dysplasia and the frequency of angiogenic response was significantly reduced upon transient depletion of the neutrophils [227]. Pahler and colleagues reported that in a transgenic mice model with impaired monocyte function, angiogenesis and tumor progression were enhanced by MMP9⁺ neutrophils [235]. In a mouse model for epithelial carcinogenesis (K14-HPV16), upregulation of neutrophil-derived MMP9 was associated with angiogenesis, hyperproliferation, and advancement towards more aggressive stages of cancer [236]. MMP9⁺ neutrophils also prevent tumor cell apoptosis and support the establishment of pulmonary tumors as shown in MMP9 knockdown mice [237].

Additionally, MMP9 shows broad catalytic activity against components of ECM. It can cleave various non-matrix proteins and activate tumor-supporting cytokines such as IL-8 and IL-1 β [238, 239]. IL-8 acts as a potent chemoattractant for neutrophils. In a study, anti-IL-8 treatment blocked the infiltration of MMP9⁺ neutrophils and significantly inhibited tumor angiogenesis and invasion [240]. Neutrophils can promote tumor angiogenesis either directly *via* release of vesicle-stored proteolytic enzymes, growth factors, pro-angiogenic factors (VEGF, FGF), and cytokines or indirectly *via* activating signaling cascade. Ardi and colleagues showed that neutrophil-derived pro-MMP9 induce angiogenesis *via* involvement of FGF-2/FGFR-2 signaling pathway. MMP-9 activity results in increased bioavailability of FGF-2, which then becomes the essential downstream angiogenic inducer acting through its specific receptor, FGFR-2 [241]. These findings suggest that infiltrating neutrophils have a significant contribution in MMP9 levels and are critical determinant of angiogenesis and tumor invasion.

5.6 Neutrophil α -defensins

Defensins are anti-microbial peptides that belong to a unique class of cysteine-rich cationic polypeptides and play a pivotal role in innate and adaptive immunity. Based on the structure and cysteine pairing, defensins are categorized into three sub-families: α defensins, β defensins, and θ defensins [242]. Six α -defensins are known in humans out of which four are produced by granulocytes and rest two are epithelial defensins [243]. Human neutrophil α -defensin are also defined as human neutrophil peptides (HNP). Out of the four HNPs, HNP1, HNP2, and HNP3 have potent bactericidal role and abundantly stored in the azurophil granules and make upto 5–7% of total cellular protein content in neutrophils. They lack

enzymatic activity and utilize the oxygen-independent mechanism to eliminate pathogens, which makes them unique to neutrophil serine proteases [244]. HNPs move to phagolysosome upon phagocytosis or released into extracellular spaces by the activated neutrophils, which can be detected in the biological fluids [245]. In addition to potent antimicrobial activity, defensins also display cytotoxic, chemotactic and stimulatory activities towards eukaryotic cells [243]. In a healthy individual, the concentration of HNP1–3 in human plasma is approximately 40 ng/mL, and the concentration can increase several folds in various inflammatory diseases. [246]. An elevated level of neutrophil defensin is reported in various inflammatory diseases. In BALF of cystic fibrosis patients, there is 500–10,000-fold increase in the HNP concentration suggesting their crucial role in pulmonary infections. Similarly HNP-1 levels were significantly higher in the saliva of patients suffering from oral inflammation [247]. Various studies have also reported increased levels of α -defensins in several neutrophil-dominated inflammatory disorders such as idiopathic pulmonary fibrosis and meningitis [248, 249]. Furthermore, defensin also stimulates the production of IL-8 and LTB-4 by alveolar macrophages, which are potent neutrophil chemoattractants [250]. Over the past few years, an altered expression as well as secretion of α -defensin has been reported in various tumor types. Elevated HNP1 level is reported in lung tumors, oral squamous cell carcinoma, and colorectal cancer [251]. Elevated accumulation of HNP-1 was seen in the malignant human pancreatic tissue, and similar expression was also observed *in vitro* in human pancreatic epithelial cells [252]. HNPs are also known to promote proliferation, migration, and invasion of malignant cells in renal, bladder, oral squamous cell carcinoma, and breast cancer [253–256]. Some studies also observed expression of HNP1–3 in the endothelial cells of tumor capillaries suggesting their role in angiogenesis [253]. In addition, HNP-1 over-expression is also correlated with reduced survival in intestinal-type gastric cancer [257]. On the contrary, some studies have reported the positive role of defensin, such as cytotoxic role towards cancer cells and induction of adaptive immune response [258, 259]. Still the crucial involvement of defensins in a variety of diseases including cancer makes them a fascinating target for diagnosis and therapy which warrants further studies.

5.7 Oncostatin M

OSM is a 28-kDa glycoprotein that belongs to the IL-6 family of cytokines [260]. Also secreted by monocytes and activated T cells, neutrophils represent the predominant cells in the circulation to express OSM. It can be readily mobilized from the granule stores of activated neutrophils or can be synthesized upon stimulation with inflammatory mediators [261]. The role of neutrophil-derived OSM in cancer is still limited to a study

by Queen et al. wherein they showed OSM release by neutrophils upon stimulation by breast cancer cells. This released OSM further increases VEGF secretion in breast cancer cells thus promoting tumor angiogenesis and neovascularization [262]. However, there are numerous reports of OSM in the pathogenesis of other inflammatory disorders like asthma [263], RA [264], and acute lung injury [265]. Hurst and group showed that infiltrating neutrophils were the major source of OSM in bacterial infection leading to acute inflammatory conditions [266]. OSM can impart pro-inflammatory effects by inducing adhesion and chemotaxis in neutrophils [267]. It can also induce chemokine and adhesion molecules synthesis by endothelial cells which further helps in the transmigration of neutrophils [268]. Neutrophil presence was also correlated with high levels of OSM in BALF of patients with severe pneumonia [269]. Similarly, in acute lung injury, OSM was majorly released by the infiltrating neutrophils. Grenier and colleagues showed that a combination of LPS and CSF2 treatment *in vitro* can induce production of OSM in neutrophils and suggested a crucial role of neutrophils in the modulation of acute inflammation [261]. Pothoven et al. showed that in patients with mucosal airway disease, neutrophils were the major source of OSM that resulted in epithelial barrier disruption. The group also reported that majority of OSM+ neutrophils expressed Arg1, which is a N2 marker, indicating that OSM+ neutrophils resemble more towards pro-tumoral phenotype of neutrophils [270]. OSM can utilize JAK/STAT, PI3K, and MAPK pathways to initiate signal transduction [266, 271, 272]. It is also known to be an effective activator of STAT1, STAT3, and STAT5 transcription factors [273]. Hence, considering the diverse role of OSM in other chronic inflammatory diseases, OSM is proposed as a putative protagonist in cancer. Further studies are warranted to understand its pathological relevance in cancer.

6 Targeting neutrophils: the therapeutic strategy underway

Neutrophils are not-so-neutral in cancer and their pro-tumoral side provides a rationale for development of neutrophil-targeted therapeutic interventions. Researchers across the globe are passionately exploring the strategies that could limit deleterious effects of neutrophils in cancer. One of the strategies deals with the inhibition of expansion and production of neutrophils in the bone marrow. In this context, substantial studies have targeted G-CSF, a key regulator of granulopoiesis and inducer of neutrophil production [274]. It binds to G-CSF receptors, which are highly expressed on mature neutrophils [275]. Multiple studies have reported a crucial role of G-CSF in various inflammatory ailments and high levels are correlated with disease severity [276, 277]. Thus G-CSF and its receptor could be a potential therapeutic target

which can regulate neutrophil production as well as its activation [278]. This strategy has shown promising results in some preclinical models [279]. Inhibition of upstream regulator of neutrophil production is another approach to limit neutrophil production. IL-17 and IL-23 stimulate neutrophil production by regulating G-CSF [280]. Biological therapeutics targeting IL-17 and IL-23 axis has been developed for multiple inflammatory disorders [281].

Another approach targets the migration of neutrophils to the tumor sites. Chemokines play a crucial role in orchestrating neutrophil migration. Chemokines such as CXCL1 and IL-8 (CXCL8) act as potent chemoattractants for neutrophils which further activate GPCRs, particularly, CXCR1 and CXCR2. Activation of CXCR2 by IL-8 triggers neutrophil migration to the site of infection [282], whereas receptor-ligand interaction of CXCR1 is responsible for neutrophil degranulation [283]. Currently, chemokine receptor antagonists are in different stages of clinical trials. For instance, SB-656933, a CXCR2 antagonist was found effective in cystic fibrosis by reducing the levels of inflammatory biomarkers in the sputum of patients [284]. Similarly, MK-7123 (navarixin), another CXCR2 antagonist, improved lung function in COPD patients [285]. In animal models, it inhibited recruitment of neutrophils, mucus production and goblet cell hyperplasia [286]. Furthermore, neutrophil-derived molecules, playing important role in neutrophil-mediated disease pathogenesis, are emerging as potential therapeutic targets. Granule contents are the major attributes of neutrophil effector functions and their functionality can be harmonized with specific drugs targeting their production, localization or the release of key granule cargoes. For example, NE has been proposed as a potential target in various pathologies because of its unique potential to hydrolyze variety of substrates, including elastin and other ECM components such as cadherins, fibronectin, collagen, proteoglycan, lung surfactant, and growth factors [148]. Moreover, it can cleave and activate several other cytokines, such as G-CSF [287], IL-1 [288], and

VEGF [289]. These attributes are known to contribute to the progression of chronic pulmonary disorders and different cancer types. Beside this, NE is known to be an independent prognostic factor in patients with lung, colon, prostate and breast cancer [290, 291]. Considering the role of NE in these pathologies, researchers have used various drugs to target NE which are currently in different phases of clinical trials. For instance, AZD9668 is an NE inhibitor which has been evaluated for its efficacy in clinical trials of various inflammatory diseases. In bronchiectasis, treatment improved the lung function with significant reduction in sputum inflammatory biomarkers [292]. Similarly, in cystic fibrosis, the patients showed reduced sputum inflammatory biomarkers including IL-6 and RANTES, together with urinary desmosine; however, the treatment had no effect on lung function [293]. Sivelestat, another NE inhibitor has been approved in Japan [294] which is known to inhibit neutrophil activation, reduce inflammation in the lungs, and induce competitive inhibition of neutrophils [295]. Sivelestat was also effective in reducing tumor growth in murine models of prostate and colorectal cancer [296, 297]. Similarly, granule contents like MPO and MMP9 are also gaining attention as potential therapeutic targets. However, the granule-mediated signaling pathways in various cancer types are poorly understood. Only few studies have been reported which shows the mechanistic pathway elucidated in cancer cell lines (Table 2) and require more extensive studies. If unraveled, these signaling mechanisms will uncover potential therapeutic targets. Additionally, NETs have also recently established their niche in sustaining tumorigenesis. Hence, targeting NETs can also be an easy and applicable strategy in cancer therapeutics. In some cases, NETs formation depends upon the active NADPH oxidase complex, thus targeting this enzyme could also inhibit NETosis. In experimental mice, DNA targeting enzyme, DNase I, was found effective against various inflammatory disease. Therefore, targeting NETs with DNase I can also be considered as a therapeutic option [140]. Another excellent

Table 2 Potential signaling pathways of neutrophil-derived granule cargoes in different cancer types

Neutrophil-derived granule cargoes	Cancer type	Signaling pathway involved	Pro-tumoral effects	References
Neutrophil elastase (NE)	Lung cancer Oral cancer	PDGF Signaling, Src/PI3K-Dependent Akt Signaling	Increased tumor cell proliferation and migration	[154] [300]
Myeloperoxidase (MPO)	T cell acute lymphoblastic leukemia	p38 MAPK and NF-kB signaling	Production of superoxides and increased degranulation of neutrophils	[301] [302]
Cathepsin G (CTSG)	Breast cancer	IGF-1R signaling	Promote tumor cell aggregation	[210]
Neutrophil collagenase (MMP8)	Hepatocellular carcinoma	PI3K/Akt/Rac-1 signaling	Stimulate epithelial-mesenchymal transition (EMT)	[220]
Oncostatin M (OSM)	Skin squamous cell carcinoma	JAK/STAT, PI3K, MAPK signaling	Promote tumor angiogenesis and neovascularization	[271, 272, 303]

therapeutic strategy could be the manipulation of neutrophil function in tumor microenvironment. TGF- β promotes N2 polarization of neutrophils, which initiates the pro-tumoral response [125]. Anti-TGF- β therapy could stimulate a robust anti-tumor response by eliciting activation of N1 neutrophils. Fresolimumab (an anti-TGF- β monoclonal antibody) and galunisertib (a TGF β R1 kinase inhibitor) are TGF- β pathway inhibitors which can promote the development of neutrophils with anti-tumor (N1 type) potential [298, 299]. Table 3 shows the list of antagonists affecting neutrophils or their microenvironment that are under different phases of clinical trials.

We are very well aware of the fact that neutrophils are crucial for host defense against microbial infections. Hence, their elimination or complete inhibition of their effector functions could not be the appropriate therapeutic strategy. This would cause neutropenia leading to a risky state of immunosuppression and render patients susceptible to secondary infections. Accordingly, better characterization of neutrophils,

targeting tumor-promoting subsets, and limiting the neutrophil effector functions is the need of the hour for developing precise therapeutic interventions without detrimental side effects.

7 Conclusion and future perspectives

Effector functions of neutrophils largely depend upon their granule cargoes and their regulated release. Together with a new realization of their role, which extends beyond just antimicrobial actions, neutrophil granules and their cargoes are emerging as key players in many chronic inflammatory disorders including cancer. While much is known about granule cargoes and their functions in host defense, the process governing granule formation, packaging of cargoes and the signaling mechanisms regulating their release is fragmentary and needs to be unraveled in a more systematic way. A better

Table 3 List of drugs targeting neutrophils or their microenvironment and the mode of action

Drug	Mode of action	Condition/disease	Clinical phase	Clinical Trials.gov identifier
AZD9668	Inhibitor of neutrophil elastase	Type 2 diabetes mellitus	Phase II	NCT02597101
Alvelestat (MPH966)		Cystic fibrosis	Phase II	NCT00757848
Sivelestat		Bronchiectasis	Phase II	NCT00769119
		Bronchiolitis obliterans syndrome	Phase II	NCT02669251
		Respiratory distress syndrome	Phase II	NCT00036062
		Adult acute lung injury		
GSK3196165 (otilimab)	Neutralizes the action of GM-CSF, thus incapable of binding to its targeted cells.	Rheumatoid arthritis	Phase III	NCT03980483
CAM-3001 (mavrilimumab)	Competitive antagonist of GM-CSF signaling	Rheumatoid arthritis	Phase II	NCT01050998
Pirfenidone	Reduced neutrophil infiltration <i>via</i> inhibiting TNF- α	Idiopathic pulmonary fibrosis	Phase III	NCT01366209
Navarixin (MK-7123)	Antagonist of CXCR2, reduces neutrophil chemotaxis	Solid tumors	Phase II	NCT03473925
SB-656933	Selective CXCR2 antagonist, inhibits CXCL1-induced CD11b on peripheral blood neutrophils	Colitis, ulcerative	Phase II	NCT00748410
SB-656933-AAA		Pulmonary disease, chronic obstructive	Phase I	NCT00551811
Navarixin (MK-7123, SCH 527123)	Antagonist of human CXCR1 and CXCR2, inhibits neutrophil recruitment, MPO release	Neutrophilic asthma	Phase II	NCT00632502
		COPD	Phase II	NCT01006616
Danirixin (GSK1325756)	CXCR2 antagonist, decreases neutrophil activation and transmigration to site of inflammation.	Virus diseases	Phase II	NCT02469298
QBM076		COPD	Phase II	NCT03170232
		COPD	Phase II	NCT01972776
SX-682	CXCR1 and CXCR2 antagonist	Metastatic melanoma	Phase I	NCT03161431
CCX168 (Avacopan)	Selective complement C5a receptor inhibitor	ANCA-associated vasculitis	Phase III	NCT02994927
RV1729	Phosphoinositide 3-kinase δ and γ isoform inhibitor	COPD	Phase I	NCT02140346
AZD1236	MMP-9 and MMP-12 inhibitor	COPD	Phase II	NCT00758706
AZD7986	Reversible inhibitor of DPP1, it inhibits the formation of active neutrophil serine proteases during neutrophil maturation	Healthy subjects	Phase I	NCT02653872
Nemiralisib (GSK2269557)	Phosphoinositide 3-kinase δ inhibitor	COPD	Phase II	NCT03345407
Ixekizumab	Selectively binds and neutralizes IL-17A thus inhibit IL-17A mediated neutrophil recruitment	Psoriasis, arthritic	Phase III	NCT01695239
Secukinumab	Target IL-17A	Chronic plaque psoriasis	Phase III	NCT01358578

understanding of these processes will aid in delineating the functional heterogeneity of neutrophils in tumor setting. Currently, drugs that can target neutrophils or their microenvironment in various inflammatory diseases are in the different stages of clinical trials. However, in cancer therapeutic, it is still limited by the emerging plasticity and heterogeneity of neutrophils in tumor conditions. How to specifically target the disease-promoting phenotype still remains a quest. Future studies are required to identify novel markers so that we can target specific neutrophil subsets in cancer and progress towards developing neutrophil subtype-specific therapeutics. This would aim to suppress the “against the host actions” of neutrophils while favoring their “against the pathogen actions.” Nonetheless, the growing interest in neutrophil biology will soon unravel this enigma and neutrophil-directed therapeutics without compromising the host immunity would become a better approach in cancer therapeutics.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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