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STATE-OF-THE-ART REVIEW

Roles of neutrophil granule proteins in orchestrating inflammation and immunity

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Neutrophil granulocytes form the first line of host defense against invading pathogens and tissue injury. They are rapidly recruited from the blood to the affected sites, where they deploy an impressive arsenal of effectors to eliminate invading microbes and damaged cells. This capacity is endowed in part by readily mobilizable proteins acquired during granulopoiesis and stored in multiple types of cytosolic granules with each granule type containing a unique cargo. Once released, granule proteins contribute to killing bacteria within the phagosome or the extracellular milieu, but are also capable of inflicting collateral tissue damage. Neutrophil-driven inflammation underlies many common diseases. Research over the last decade has documented neutrophil heterogeneity and functional versatility far beyond their antimicrobial function. Emerging evidence indicates that neutrophils utilize granule proteins to interact with innate and adaptive immune cells and orchestrate the inflammatory response. Granule proteins have been identified as important modulators of neutrophil trafficking, reverse transendothelial migration, phagocytosis, neutrophil life span, neutrophil extracellular trap formation, efferocytosis, cytokine activity, and autoimmunity. Hence, defining their roles within the inflammatory locus is critical for minimizing damage to the neighboring tissue and return to homeostasis. Here, we provide an overview of recent advances in the regulation of degranulation, granule protein functions, and signaling in modulating neutrophil-mediated immunity. We also discuss how targeting granule proteins and/or signaling could be harnessed for therapeutic benefits.

Introduction

Neutrophil granulocytes are the most abundant white blood cells in human circulation and play a central role in host defense against invading pathogens and tissue injury. Typically, neutrophils are rapidly recruited to sites of infection or injury, where they deploy an impressive chemical and enzymatic array of

Abbreviations
ANCA, antineutrophil cytoplasmic antibody; CCL5, C-C motif ligand 5 (aka RANTES); CR1, complement receptor 1; CR3, complement receptor 3 (CD11b/CD18); DAG, diacylglycerol; GPCR, G protein-coupled receptor; IP_3, inositol-(1, 4, 5)-trisphosphate; JAM-C, junctional adhesion molecule-C; LTB_4, leukotriene B_4; MMP-9, matrix metalloproteinase 9; MPO, myeloperoxidase; NE, neutrophil elastase; NET, neutrophil extracellular trap; PAD-4, peptidyl arginine deiminase 4; PAR-2, protease-activated receptor 2; PIP_3, phosphatidylinositol-(3, 4, 5)-trisphosphate; PKC, protein kinase C; PLC, phospholipase C; SLPI, secretory leukocyte peptidase inhibitor; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; SOC, store-operated channel; TEM, transendothelial migration; TLR9, Toll-like receptor 9; TNF, tumor necrosis factor; VAMP, vesicle-associated membrane protein.

mechanisms, resulting in the elimination of the offending agents and necrotic tissues [1,2]. A key component of this weaponry is the mobilization and release of preformed cytosolic granules with each granule type containing multiple microbicide proteins and proteases at unique signaling thresholds [3,4]. Granules can fuse with pathogen-containing phagosomes to destroy ingested microorganisms. Neutrophil granules can also fuse with the plasma membrane in response to pathogens that cannot be phagocytosed, leading to extracellular release of their content [1,5]. Release of granule proteins to the extracellular milieu can inflict damage to the surrounding tissues and prolong the inflammatory reaction [6–8]. Over the past two decades, neutrophil-driven inflammation has been recognized as a common mechanism underlying many pathological conditions, including atherosclerosis, cardiovascular, respiratory, autoimmune and neurodegenerative diseases, sepsis, and cancer [8,9]. Neutrophils are also involved in the resolution of inflammation [10]; hence, the balance between their deleterious and beneficial effects will likely be one of the key determinants of the outcome of the inflammatory response.

Research over the last decade has documented the heterogeneity of neutrophils and their functional versatility, extending far beyond their antimicrobial function [11,12]. Neutrophils interact with innate and adaptive immune cells and orchestrate immune responses [9]. Emerging evidence indicates the involvement of granule proteins, in addition to *de novo* synthesis of cytokines in this crosstalk [4]. In this paper, we aim to summarize recent advances in our understanding of degranulation, granule protein function, and signaling in neutrophil-mediated immunity. We also discuss how targeting granule proteins and/or signaling could be harnessed for therapeutic benefits.

Granule formation and composition

Neutrophils contain different granule subsets that are mobilized by stimulation. Based on physical properties and expression markers, at least four different types of secretory compartments have been identified: azurophilic or primary granules [specific markers: myeloperoxidase (MPO) and CD63], specific or secondary granules (markers: lipocalin 2 and CD66b), gelatinase (GG) or tertiary granules (markers: gelatinase B and CD11b), and secretory vesicles (SV) (Fig. 1) [13]. Neutrophils acquire granules and SV sequentially during their maturation in the bone marrow. The 'targeting by timing' hypothesis postulates that the synthesis of granule proteins at the time of formation of granule subset would determine granule content [14]. Appropriate timing of mRNA expression during granulopoiesis coincides with granule protein distribution in most proteins used to identify granule subsets [3]. However, the discrepancy in the timing of mRNA expression for a minority of proteins and overlaps in granule content among different subsets (e.g., lysozyme in all three granule types) suggest the involvement of mechanisms in addition to the timing of protein synthesis in the regulation of granule content. Primary granules are the earliest to be formed in promyelocytes (hence their name), stain by the dye azure A (i.e., azurophilic). They contain a large number of antimicrobial proteins, including MPO, serine proteases (elastase, proteinase 3, cathepsin G, and azurocidin), α-defensins, lysozyme, and Cap57 (bactericidal/permeability-increasing protein, BPI) [3,15]. Azurophilic granule proteases are activated by proteolytic processing prior to incorporation into granules, resulting in a highly toxic readily available cargo upon release [16]. Proteomic analysis has identified at least 850 proteins associated with azurophilic granules, of which 135 show the highest relative amounts among granules or are exclusively expressed in this granule subset [3]. Azurophilic granules are heterogeneous in their protein content, which may determine their trafficking to the cell surface or fusion with phagosomes. Plasma membrane-targeted azurophilic granules express Rab27 and Slp1/JFC1, whereas primary granules targeted to the phagosome lack these proteins [17]. Intriguingly, a subset of primary granules does not contain α-defensin, while expressing all other granule proteins [18]. The origin and function of this subset remain to be investigated.

Specific and GG granules are peroxidase-negative and are formed throughout myelocyte, metamyelocyte, and band stages. These granules share similar contents and functions, though heterogeneity within these subsets is well recognized. Specific (secondary) granules contain lactoferrin and lipocalin (NGAL), but no GG [matrix metalloproteinase 9 (MMP-9)], whereas GG (tertiary) granules contain MMP-9, but lack lactoferrin and lipocalin [18]. Of 1024 proteins associated with specific granules (SG), 111 are maximally expressed in this subset [3]. GG granules express only 30 of 1123 proteins maximally. A hybrid type granule, constituting about two-thirds of the total peroxidase-negative granules, contains lactoferrin, lipocalin, and MMP-9 [18]. GG granules containing the microbe-binding lectin ficolin 1 have been proposed as a separate granule subset, termed ficolin 1-rich granules [19]. Besides the rapid exocytosis of these granules in response to the bacterial chemoattractant formyl-Met-Leu-Phe [19,20], little is known about the function of this granule type. SV are formed by endocytosis during the band and

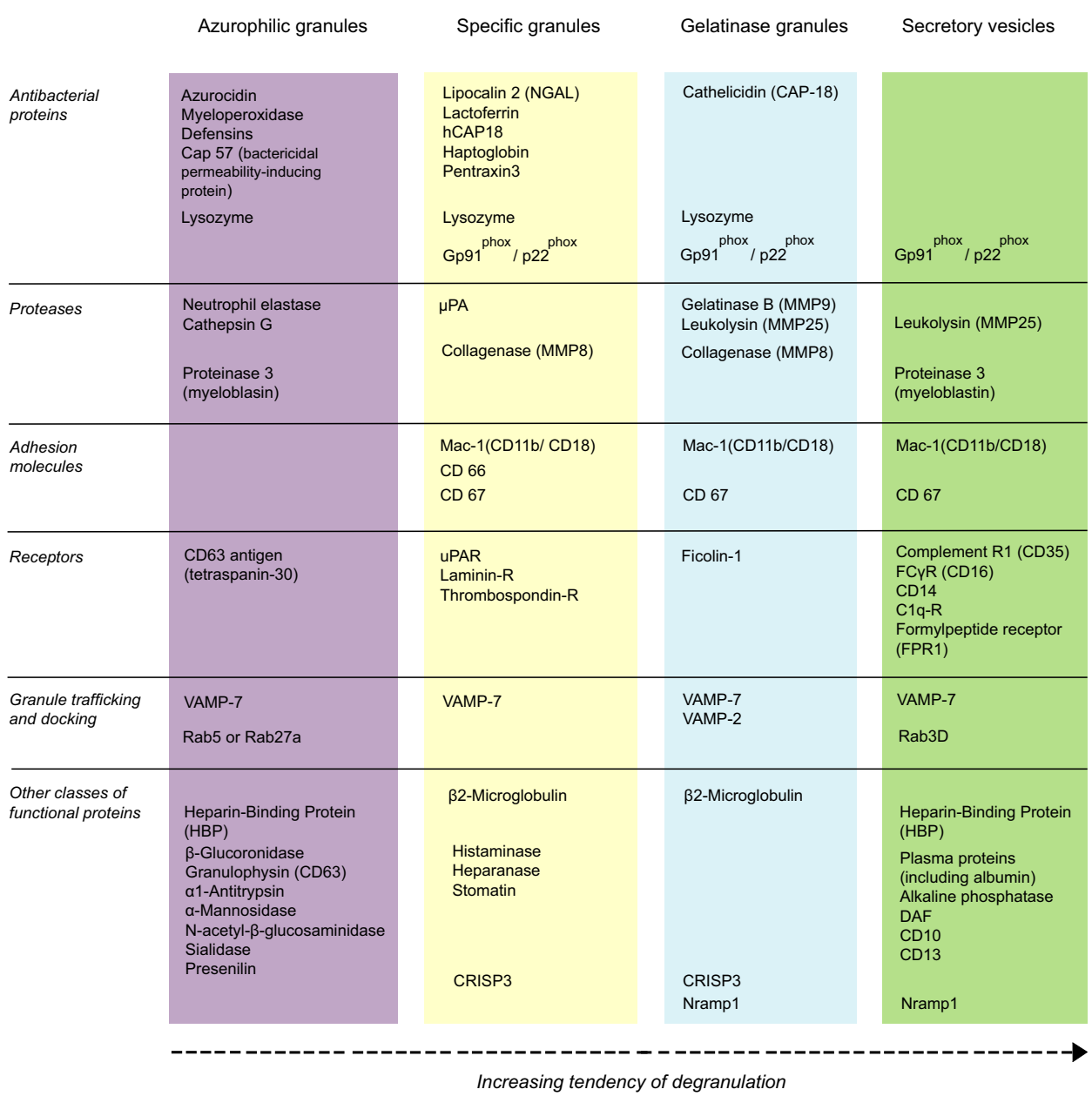

Fig. 1. Main constituents of neutrophil granules and secretory vesicles. The list depicts the most abundant proteins in human resting neutrophils. There are some species differences. For example, while mouse neutrophils contain most of these proteins, azurocidin, α-defensin 3, and Cap57 are specific for human neutrophils. In contrast, mouse neutrophils harbor abundant expression of neutrophil granular protein, high-mobility group 1, heat-shock protein A8, and chitinase-like protein 3 [4]. In human neutrophils, specific and gelatinase granules exist as a continuum bearing markers of SG (lactoferrin$^+$, gelatinase$^-$), gelatinase granules (lactoferrin$^-$, gelatinase$^+$), and hybrid granules (lactoferrin$^+$, gelatinase$^+$) [3]. A fourth granule type, ficolin 1-enriched granules have also been described [19].

segmented stages [21]. These vesicles (markers: albumin, CD45, Mac-1/CD11b, and CD13) are enriched in numerous proteins also present in the cell membrane, including phagocytic, chemoattractant and cytokine receptors, adhesion molecules, membrane components of NADPH oxidase, and plasma proteins [3,22]. Like specific and GG granules, the release of the content of SV is restricted to the plasma membrane [21] and is thought to facilitate neutrophil adherence to the activated vascular endothelial cells (EC), the initial step in

neutrophil trafficking into inflamed or injured tissues. Following firm adhesion of neutrophils to the endothelium, chemokine signaling and outside-in signaling through β_2 integrins induce exocytosis of GG and SG [18].

A recent case report shows apical polarization of neutrophil granules in both mature and band neutrophils from patients with severe sepsis [23], which may coincide with their antimicrobial activity. Several granule proteins, such as MPO, lactoferrin, lysozyme, cathepsin G, and α-defensin, were detected in thin cellular protrusions (termed cytonemes) that are likely specialized for delivering signaling proteins to neighboring cells [24,25]. Another communication structure, extracellular vesicles also contain granule proteins, including MPO, lactoferrin, cathepsin G, and α-defensin in a context-dependent fashion [26]. Little is known of the molecular links between the formation of these structures and neutrophil granules.

Regulation of degranulation

Neutrophil granules can fuse with the nascent phagosome, releasing their cargo into the vacuole to destroy ingested pathogens or with the plasma membrane, resulting in secretion of granule proteins extracellularly (degranulation). The release of granule contents is a tightly regulated receptor-coupled process that is mediated by distinct signaling events for each granule type, allowing the selective release of granule subsets (Fig. 2) [27]. Ligation of G protein-coupled receptors (GPCRs), Fcγ receptors, or the β_2 integrin Mac-1 (CD11b or CR3) by a wide range of stimuli, such as bacterial formyl-peptides, chemokines, cytokines, complement fragments, or EC adhesion molecules, triggers a degranulation response [28], which is markedly enhanced by prior exposure of neutrophils to a priming stimulus, such as granulocyte–macrophage colony-stimulating factor, tumor necrosis factor (TNF), or platelet-activating factor [29]. Although these receptors activate different sets of downstream signaling molecules, they converge on two signaling pathways, the activation of Rac2 and the induction of a calcium flux [30]. Importantly, the different granule subsets can be selectively released for each granule type requires a different intracellular calcium concentration to trigger its exocytosis [18,30]. SV have the highest sensitivity to calcium followed in order of decreasing calcium sensitivity by GG, SG, and azurophilic granules.

Rac2 is a member of the Rho GTPase subfamily, which plays multiple roles in degranulation. It mediates the remodeling of the actin cytoskeleton required for mobilization of granules from the cytosol to the target membrane and clearing of the cortical actin cytoskeleton that would otherwise block granule docking to the plasma membrane [31–34]. Genetic deletion of Rac2 in mice leads to a loss of primary granule release without affecting degranulation of specific and GG granules, suggesting a role restricted to primary granules [31,32,35]. However, actin reorganization is required for the degranulation of all granule types [36], but it likely depends on granule-specific signaling mechanisms, such as the Src family of nonreceptor tyrosine kinases. Neutrophils express 3 Src-family members, Hck, Fgr, and Lyn. Ligation of GPCRs or the phagocytic receptor FcγRIIA (CD64) induces Hck translocation to azurophilic granules and Fgr to SG, albeit some degree of functional overlap between these kinases has also been reported [37–39]. Hck activates the ELMO-Dock2 pathway leading to Rac2 activation and degranulation of azurophilic granules [40]. Src-family kinases also activate p38 MAPK, which through a yet unidentified pathway mediates degranulation of azurophilic and specific/ GG granules [41]. Of note, p38 MAPK can induce actin rearrangements via Hsp27 [42]; hence, it may function in a manner similar to Rac2. Recent results indicate that Hck activation following ligation of Toll-like receptor 9 (TLR9) with bacterial DNA, a pathogen-associated molecular pattern or the damage-associated molecular pattern mitochondrial DNA, leads to the release of primary granule contents, MPO, neutrophil elastase (NE), and proteinase 3 [43]. TLR9 activation induces NF-κB-mediated transcription of inducible nitric oxide synthase (iNOS) in human neutrophils [44], which, in turn, could activate Src kinases as observed in macrophages [45]. TLR9 signaling does not activate Lyn or Fgr and does not require neutrophil priming [43].

An increase in intracellular calcium is indispensable for degranulation of all granule subtypes and is required to form the fusion pore between granules and cell or phagosome membranes. Calcium signaling can be induced at multiple points during neutrophil trafficking to inflamed loci and phagocytosis. The most studied receptors driving calcium release are GPCRs. Ligation of GPCRs induces the dissociation of the β/γ heterodimer from the Gα subunit [46]. The ß/γ subunit transduces the majority of the neutrophil signaling through activation of phospholipase C (PLC), which generates inositol-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases calcium stored in the endoplasmic reticulum (ER), leading to modest increases in cytosolic calcium [47]. DAG induces protein kinase C (PKC)-mediated activation of store-operated channels (SOCs), producing a more robust and prolonged increase in intracellular calcium. Activation of GPCRs

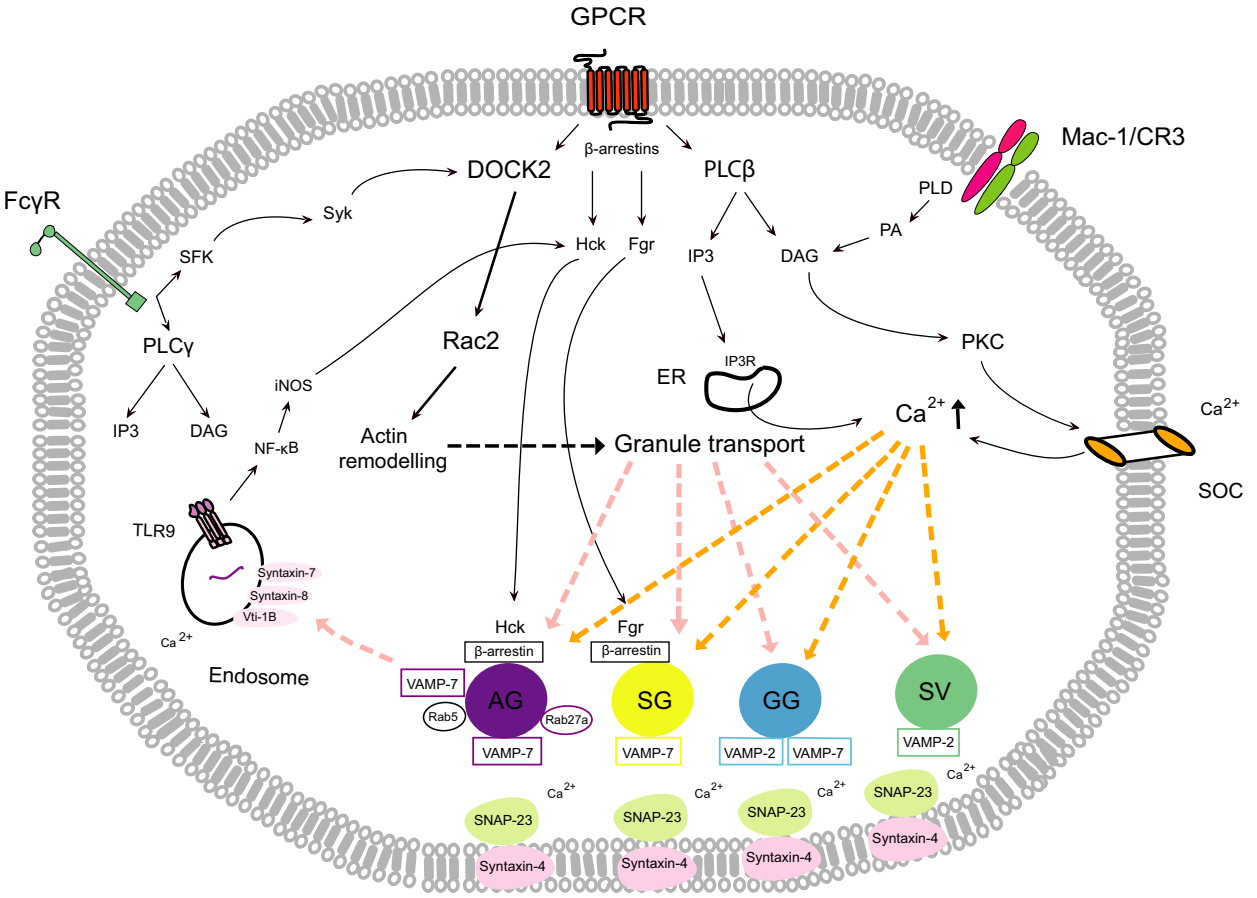

Fig. 2. Major signaling pathways regulating neutrophil degranulation. Ligation of GPCRs, Fc receptors, or the phagocytic β_2-integrin Mac-1/CR3 triggers degranulation through multiple, partially overlapping downstream signaling pathways, converging on activation of Rac2, and increasing intracellular calcium. Rac2 is activated through GPCR-activated guanine nucleotide exchange factors or the SFK (Src family of tyrosine kinases)-Syk-DOCK2 pathway following ligation of Fcγ receptors. Calcium signaling is driven by influx of extracellular calcium via DAG/PKC-regulated SOCs or calcium release from intracellular stores via the IP3 receptor. Chemokine binding to GPCRs leads to direct binding of β-arrestins to the receptor, which along with the Src-family kinases Hck or Fgr translocate to the azurophilic and SG, respectively. Activation of TLR9 in the endosome also triggers degranulation through NF-κB/iNOS-mediated activation of Hck. DOCK-2-Rac2 signaling induces actin remodeling and granule transport from the cytosol to the plasma membrane. Fusion of granules with the plasma membrane is governed by calcium-dependent formation of SNARE complexes, consisting of granule VAMPs and cognate SNAPs/syntaxins on the plasma membrane. The Rab GTPase, Rab5, expressed on a subset of azurophilic granules, mediates their fusion with endosomes. AG, azurophilic (primary) granules; IP_3R, IP_3 receptor; SG, secretory (secondary) granules.

also generates phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3) via protein kinase AKT. Hypoxia through activation of PI3Kγ also evokes PIP_3 and AKT signaling and enhances degranulation [48]. A hierarchy of granule exocytosis exists in response to elevated intracellular calcium concentrations with an order of release of SV > GG granules > SG > azurophilic granules [49,50]. The exocytosis of primary granules depends on ATP and high cytosolic calcium concentration [50]. Activation of neutrophil receptors other than GPCRs also induces calcium fluxes. For instance, FcγRIIA-mediated phagocytosis of antibody-opsonized pathogens induces a potent calcium flux through the recruitment of Src-family kinases, which activates the Syk-PLC pathway, leading to IP_3 and DAG synthesis [51]. Engagement of complement receptor 1 (CR1) and CR3 with C3b-opsonized pathogens induces phospholipase D (PLD)-mediated release of phosphatidic acid (PA), which is readily converted into DAG [18].

Rapid mobilization of granules is controlled by effector molecules that include small GTPases and their interacting proteins [52]. The final step of degranulation, docking, and fusion of granule membrane to the target membrane is mediated by the binding of vesicle soluble N-ethylmaleimide-sensitive factor

attachment protein receptors (SNAREs, also referred to as vesicle-associated membrane protein or VAMP) to a cognate SNARE (t-SNARE) expressed at the plasma membrane or phagosome at a ratio of 1 : 3 [53]. Human neutrophils express a range of vSNARES, including VAMP-2 and VAMP-7, and t-SNARES, such as syntaxin A1, 3, 4, 5, 6, 7, 9, 11, and SNAP-23 [54], though degranulation largely depends on SNAP-23 and syntaxin 6. VAMPs may impart granule-specific degranulation. Thus, VAMP-2 mediates exocytosis of GG granules [55], whereas VAMP-1 and VAMP-7 direct release of azurophilic granules [56,57]. The selective recruitment of VAMP-7 to azurophilic granules may account for their capability to fuse with both the plasma membrane and phagosomes [56]. VAMP-7 is thought to interact with phagosome-resident syntaxin 7, syntaxin 8, and/or Vti-1B to mediate azurophilic granule–phagosome fusion [18].

The Rab GTPases Rab5 and Rab27a have also been implicated in controlling the degranulation of human neutrophils. Rab5 appears to be selectively associated with azurophilic granules and mediates their fusion with phagosomes, although the underlying mechanism has not been explored in detail [38]. In contrast, Rab27a and its two effectors Munc-13-4 and JFC1 are required for extracellular release of azurophilic, SG, and GG granules [18]. JFC1 transports granules to the plasma membrane, where it docks granules to the plasma membrane through bridging Rab27a. Munc13-4 immobilizes Rab271-positive granules at the plasma membrane and controls the secretion of azurophilic and tertiary granules, for example MPO release in response to bacterial lipopolysaccharide. Deficiency in Rab27a and Munc13-4 has been linked to impaired neutrophil functions in some hereditary immune deficiency diseases such as the Griscelli syndrome type 2 and familial hemophagocytic lymphohistiocytosis type 3 [58]. Munc13-4 was also reported to regulate TLR9 activation directly, upregulate surface expression of Mac-1 (CD11b), and bind to Rab11, enabling Rab11a-positive vesicles to dock and presumably fuse with the plasma membrane [59,60]. At least four additional Rab GTPases have been identified in neutrophils, but their function remains largely unexplored [18,52].

Biological roles of granule proteins

Much has been written on the mechanisms of neutrophil trafficking into inflamed tissues and the roles of emigrated neutrophils in host defense and inflicting tissue injury [1,2,6,28,61–64]. While degranulation at specific times during neutrophil transendothelial migration (TEM) and engagement with invading pathogens equips neutrophils with efficient effectors, the roles of granule proteins are not restricted to antimicrobial and tissue-damaging actions. In the following sections, we aim to provide a focus on how neutrophils utilize granule proteins to interact with innate and adaptive immune cells and shape innate and adaptive immunity (Fig. 3).

Neutrophil trafficking into tissues and reverse transendothelial migration

Rapid neutrophil infiltration into microbe-infected and damaged tissues is a critical component of the innate immune response and a hallmark of acute inflammatory reactions. Neutrophils egress from the blood to tissues is a multistep process involving a sequence of events orchestrated by well-characterized adhesive and stimulatory pathways, followed by migration through the EC barrier via both paracellular and transcellular paths and chemotactic migration toward the inflammatory locus [61–64].

Neutrophil activation results in secretion of NE, proteinase 3, and cathepsin G from the azurophilic granules, leading to degradation of extracellular matrix and neutrophil transmigration [1,63]. *In vivo*, the activity of these proteases is inhibited by protease inhibitors, such as leukocyte protease inhibitor (SLPI), antichymotrypsin, and α1-antitrypsin [4], thereby restricting protease activity to the infected or injured locus. Proteinase 3 binds to the neutrophil surface through an association with NB1 (CD177), a heterophilic binding partner for platelet–EC adhesion molecule 1 [65,66]. This interaction protects proteinase 3 from proteolytic inactivation. Thus, NB1 may direct proteinase 3 to EC junctions where transmigration occurs [67] and facilitates neutrophil diapedesis through degradation of junctional proteins or the extracellular matrix [66] or by proteolysis of endothelial cadherin [68]. Acting through the endothelial protease-activated receptor 2 (PAR-2), proteinase 3 counters the actions of PAR-1 agonists on the endothelium and contributes to restoring junctional integrity and barrier function following neutrophil TEM [69]. Proteinase 3 was also reported to maintain calcium transients in EC independent of its proteolytic activity [69]. This action may contribute to protecting ECs from permeability changes, though the underlying molecular mechanism requires future investigations.

Studies in zebrafish embryos [70] and mouse cremaster circulation [71] reported the ability of neutrophils to exhibit motility away from inflamed tissues back to the vessel lumen, known as reverse TEM [71,72]. Neutrophil reverse TEM is most prevalent in tissues

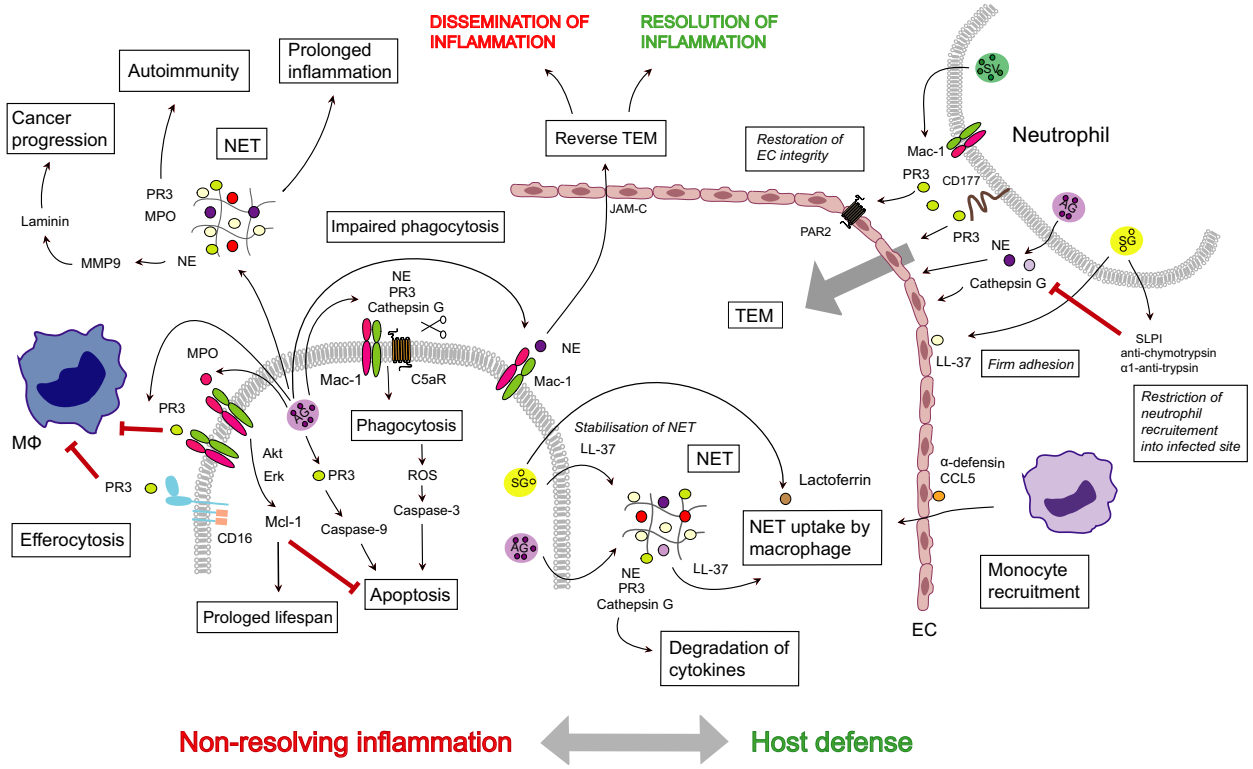

Fig. 3. Roles of neutrophil granule proteins in host defense, nonresolving inflammation, autoimmunity, and cancer progression. Release of content of SV and LL-37 (cathelicidin) bound to ECs allows firm adherence of neutrophils to ECs. PR3, NE, and cathepsin G are required for TEM and extravasation into the inflamed tissue. Neutrophils also secrete protease inhibitors that by cleaving NE and cathepsin G restricts neutrophil recruitment into the infected locus. The α-defensin-CCL5 complex immobilized on the EC surface contributes to monocyte recruitment. PR3 through PAR-2 contributes to restoration of EC integrity following TEM. NE binds to Mac-1 and may direct neutrophil reverse TEM through interacting with JAM-C. NE, PR3, and cathepsin G are components of NET. LL-37 stabilizes NET and together with lactoferrin may promote NET uptake by macrophages. NE, PR3, and cathepsin G can cleave C5aR and by altering the Mac-1/C5aR ratio impairs phagocytosis, bacterial clearance, and phagocytosis-induced cell death. MPO binds Mac-1 and generates survival signals for neutrophils, consistent with prolongation of inflammation. PR3 bound to Mac-1 or CD16 functions as a 'don't eat me signal' for macrophages and attenuates efferocytosis. Extensive NET formation is associated with many pathological conditions. NET-associated PR3 and MPO are potent autoantigens to trigger autoimmunity. NE can activate MMP-9, which cleaves laminin, thereby may awaken dormant cancer cells. AG, azurophilic granules; MΦ, macrophage; PR3, proteinase 3.

subjected to ischemia–reperfusion injury and is driven by a leukotriene B_4 (LTB_4)- NE axis [72] in a paracellular manner [73]. Under ischemia-reperfusion, locally generated LTB_4, through the LTB_4 receptor BLT1, induces elastase release from neutrophils [72]. NE binds to Mac-1 [74], which is also a ligand for junctional adhesion molecule-C (JAM-C), and then cleaves JAM-C [72]. Reversely transmigrated neutrophils display a phenotype (ICAM-1^{high}, $CXCR1^{low}$) distinct from tissue-resident (ICAM-1^{low}, $CXCR1^{low}$) or circulating neutrophils (ICAM-1^{low}, $CXCR1^{high}$) and have increased capacity to produce superoxide [71,75]. The pathophysiological relevance of neutrophils undergoing reverse TEM remains unclear. Reverse TEM might be a protective response as it may dampen the inflammatory response through facilitating the removal of neutrophils from the inflamed site [70,76]. Alternatively, retrograde neutrophil migration might disseminate local inflammation and lead to distant organ injury [71,72].

Regulation of neutrophil life span, apoptosis, and efferocytosis

Neutrophils are typically short-lived with a half-life in the range of 1–8 h in the circulation [77,78]; however, some reports estimated that their life span can extend to 5.4 days [79], especially once activated. Blood neutrophils die via apoptosis [80]. At sites of inflammation, neutrophil life span is extended through delaying intrinsic apoptosis by prosurvival cues from neutrophil adhesion to ECs, inflammatory mediators, and

microbial constituents [4,12]. Upon their release, granule proteins act in an autocrine/paracrine fashion to modulate neutrophil function, life span, and mode of cell death. Neutrophil subsets may undergo apoptosis, NETosis [81], or necroptosis [82]. Cell death is an important mechanism to remove neutrophils from inflamed tissues for assuring the timely resolution of inflammation. Pathways that lead to apoptosis have been extensively studied, and the execution of this intrinsic death program depends on activation of the caspase cascade [83,84]. However, in mouse neutrophils, activation of caspase 3 may occur independently of the canonical caspase 8 or caspase 9-triggered pathway. Instead, proteinase 3 was detected in the cytosol of aging neutrophils, where it cleaves procaspase 3 at a site upstream of the canonical caspase 9 cleavage site [85]. Consistently, pharmacological inhibition or knockdown of proteinase 3 delays neutrophil death *in vitro* and in a murine model of peritonitis [85].

MPO can regulate neutrophil function through its nonenzymatic actions. MPO binds to Mac-1 on human neutrophils [86]. Ligation of Mac-1 evokes release of additional MPO and leads to upregulation of Mac-1 expression [86,87]. MPO also activates the MAPK/ERK and PI3K/Akt pathways, resulting in the preservation of the expression of the anti-apoptotic protein Mcl-1, a key regulator of neutrophil life span [88], and delay of intrinsic apoptosis [87]. Thus, activation of a MPO-centered feed-forward loop would amplify neutrophil responses and perpetuate the inflammatory response [87]. Consistently, genetic deletion of MPO in mice attenuates local neutrophil accumulation and distant organ damage after renal ischemia–reperfusion [89] and reduces *Escherichia coli* septicemia-induced lung injury and mortality [90]. Administration of exogenous MPO prolongs neutrophil-mediated acute lung injury in mice [87]. Decomposition of MPO, which consists of two identical protomers, into the monomers results in a partial loss in prolonging neutrophil life span and inducing degranulation [91], suggesting a mechanism that may control MPO-mediated neutrophil activation. Such mechanism may be operational *in vivo* as suggested by the detection of monomeric MPO in the serum of patients with acute inflammation [91]. However, the mechanism of MPO decomposition remains to be investigated.

Proteinase 3 is constitutively expressed on the surface of a subset of human neutrophils [92,93] and is externalized through association with phospholipid scramblase 1 in neutrophils undergoing apoptosis [94]. Membrane-bound proteinase 3 decreases macrophage phagocytosis, indicating that proteinase 3 may function as a 'don't eat me' signal, thereby delaying neutrophil clearance [94].

Prompt removal of apoptotic cells, including neutrophils by tissue-resident phagocytes (such as macrophages and dendritic cells), is central for returning to homeostasis. The molecular mechanisms that underpin the recognition and clearance of apoptotic cells are increasingly better defined [95]. Recent data imply the involvement of granule proteins in this process. During the resolution of experimental peritonitis, macrophages process and cleave lactoferrin initially released by neutrophils [96]. A 17 kDa lactoferrin fragment was found to enhance efferocytosis of apoptotic neutrophils and to stimulate secretion of the proresolution cytokine IL-10 from macrophages, thereby facilitating the resolution of peritonitis in mice [96]. Apoptotic human cells of diverse lineage can also secrete lactoferrin, which selectively inhibits migration and activation of neutrophils but not mononuclear phagocytes [97], suggesting a regulatory role for lactoferrin in dampening the inflammatory response.

Neutrophil apoptosis and subsequent efferocytosis are important mechanisms of removing neutrophils from inflamed sites. Clinical studies documented extended neutrophil life span through delayed apoptosis in a wide range of pathologies, including acute coronary artery disease [98], severe asthma [99], acute respiratory distress syndrome [100], and sepsis [101] and its association with disease severity and poor prognosis. Consistent with the clinical observations, in experimental models, delaying neutrophil apoptosis, for example with administration of exogenous MPO, can adversely affect the outcome of the inflammatory response [87,102].

Phagocytosis and phagocytosis-induced cell death

Phagocytosis of opsonized bacteria or necrotic cells is initiated by lateral clustering of Mac-1 (complement receptor 3 or CR3) [51] and governed by a delicate balance between Mac-1 and the complement C5a receptor (C5aR or CD88) [103,104]. C5aR is highly expressed in cells of myeloid origin [105]. Diminished expression of Mac-1 [106] or pharmacological blockade or genetic deletion of C5aR leads to defective phagocytosis and intracellular killing of bacteria by both human and mouse neutrophils [104]. Neutrophil serine proteases have been shown to proteolytically reduce the expression of several molecules involved in immunity, including CXCR1 (IL-8 receptor), CD16, CR1, and C5aR. NE, proteinase 3, and cathepsin G can also cleave C5aR [43,107], though their

involvement appears to be context-dependent. For example, C5a reduces C5aR expression through inducing NE release [107], whereas TLR9 activation-induced reduction of C5aR and defective phagocytosis are mediated by the release of NE and proteinase 3 [43]. Bronchoalveolar lavage fluid from patients with cystic fibrosis was found to contain NE and cathepsin G at concentrations sufficient to cleave C5aR [107]. Typically, phagocytosis of opsonized bacteria accelerates neutrophil apoptosis through ROS-dependent activation of caspase 8, which counters the survival signals generated by ligation of Mac-1 [12,108,109]. Consistently, activation of TLR9 with either bacterial or mitochondrial DNA impaired bacterial phagocytosis and delayed apoptosis in both human and mouse neutrophils, and prolonged *E. coli*-evoked lung injury in mice [43].

Reduced C5aR expression is a common finding *ex vivo* in neutrophils from patients with sepsis [110,111] and in experimental models of sepsis [112,113]. C5a-induced neutrophil serine protease-mediated cleavage of C5aR may explain why high levels of activated C5a in the serum are associated with neutrophil unresponsiveness to C5a in sepsis [103]. TLR9-evoked cleavage of C5aR may explain defects in neutrophil function to clear bacteria under pathological conditions that are associated with release of bacterial or mitochondrial DNA, as observed in sepsis [114], acute respiratory distress syndrome [115], inflammation, and tissue injury [116,117].

Neutrophil extracellular trap formation

Neutrophils target invading pathogens through several processes, including phagocytosis, degranulation, and formation of neutrophil extracellular traps (NETs) [1,118]. NETs capture and kill an extensive range of microbes [119], degrade cytokines and chemokines [120], and immobilize activated platelets [121], microparticles [122], and coagulation factors [123]. NETs are a meshwork of chromatin decorated with granule proteins, including MPO, NE, azurocidin, proteinase 3, and LL-37 (cathelicidin), and absorb penetrating 3 [124] and complement [125]. Recent reviews detail NADPH oxidase 2-dependent and NADPH oxidase 2-independent pathways leading to NET formation as well as differences in its composition [126–128]. Quantitative proteomics of NET proteins produced by neutrophils from healthy subjects and patients with rheumatoid arthritis or systemic lupus erythematosus indicates that the nature of the stimulant rather than neutrophil physiology may determine NET protein profiles irrespective of the disease background [129].

Neutrophil extracellular traps may be formed by the release of a DNA scaffold consisting of mitochondrial DNA that binds granule proteins without affecting cell viability [130]. This process depends on glycolytic ATP production for cytoskeletal rearrangements that are essential for releasing both mitochondrial DNA and granule proteins. By contrast, the process commonly designated as NETosis is a form of programmed necrotic cell death, involving the release of nuclear DNA [131]. This process involves ROS generation through PKC-dependent activation of NADPH oxidase, followed by MPO-dependent release and migration of elastase from azurophilic granules to the nucleus [131], where it cleaves histones to elicit chromatin decondensation [132]. While the molecular mechanisms eliciting the release of nuclear or mitochondrial DNA appear to differ [130], the pathological relevance of these processes to innate immunity remains to be investigated. Of note, phagocytosis of bulky phosphatidylserine-exposing particles, such as apoptotic bodies or platelets, renders neutrophils unable to release NET [121,133]. Hence, impaired phagocytosis by MPO, proteinase 3, or NE may represent another mechanism to govern NET formation.

Complexes of extracellular DNA and LL-37 can form stable structures that protect NET against degradation by bacterial nucleases and activate TLR9 in monocytes and dendritic cells [134,135]. NETs can trigger thrombus formation, which prevents the dissemination of microorganisms [123,136]. Neutrophil serine proteases may function as a reciprocal link between coagulation and innate immunity [123]. However, the role of serine proteases may be context-dependent. For instance, NE is not required to form NETs in a mouse model of deep vein thrombosis [137]. NETs are eventually degraded by DNase 1 released predominantly from dendritic cells [138,139] or following engulfment by macrophages through the cytosolic exonuclease TREX1 (DNase III) [139,140]. LL-37 is required for the uptake of NET by macrophages [139].

Neutrophil extracellular traps formation plays an important role in host defense and limits the extent of infected areas [136,141]. However, aberrant NET formation is increasingly being recognized to participate in a range of human pathologies, including atherosclerosis, thrombosis [142], and sepsis-associated or COVID-19-associated acute respiratory distress syndrome [143–145]. Excessive NET formation and/or impaired NET degradation have also been implicated as a trigger of autoimmune diseases [119].

Autoimmunity

Many granule proteins (e.g., MPO, proteinase 3) are recognized autoantigens in autoimmunity [119,146]. Since these molecules are externalized through NETosis together with other well-known autoantigens, such as double-stranded DNA and histones, aberrant NET formation, or degradation has been implicated in the initiation of systemic autoimmune responses in susceptible individuals [119]. Some granule proteins are target antigens in antineutrophil cytoplasmic antibody (ANCA)-associated vasculitides. MPO is the main target antigen in microscopic polyangiitis and Churg–Strauss syndrome, whereas Wegener's granulomatosis is predominantly associated with ANCA directed against proteinase 3 [147]. MPO might trigger autoimmunity during uncontrolled inflammation [148], though it is unclear whether this response would also involve NET formation. The functions of proteinase 3 depend on its localization [92,93]. Membrane-bound proteinase 3 likely contributes to neutrophil activation in co-operation with its binding partners, CD16, Mac-1, and CD177 [93,149], and inhibits macrophage phagocytosis [94]. Proteinase 3 on apoptotic neutrophils instructs plasmocytoid dendritic cell-driven generation of Th9/Th2 cells, disrupting immune silencing [150]. Antiproteinase 3 ANCA can be triggered by cPR3(105-201), a complimentary protein translated from proteinase 3 antisense DNA [151]. Neutrophils from vasculitis patients exhibit an altered gene expression profile (e.g., re-expression of mRNA for several azurophilic granule proteins), and newly synthesized proteinase 3 partner proteins that are not expressed in neutrophils from healthy subjects might associate with proteinase 3 and function as autoantigens [93]. An alternative possibility is that NET-associated proteinase 3 or proteinase 3 released during cell death other than apoptosis [152] could act as sources of modified autoantigens.

The antimicrobial protein LL-37 (cathelicidin) was identified as an autoantigen in psoriasis [153]. Recent studies with the cathelicidin protein CRAMP (a truncated form of the mouse homolog of human CAP18) have linked this protein to atherosclerosis as a potential self-antigen in ApoE-deficient mice [154]. Immunization with CRAMP resulted in differential outcomes depending on the dose used; at a lower dose reducing atherosclerosis, whereas at a higher dose exacerbating the disease. While clinical studies indicate an association between psoriasis and cardiovascular disease, the pathological relevance of LL-37 to human atherosclerosis remains to be investigated.

Regulation of cytokine/chemokine activity

Human neutrophils produce cytokines belonging to different families, including pro- and anti-inflammatory cytokines, chemokines, colony-stimulating factors (e.g., G-CSF), angiogenic, and fibrinogenic factors [4,155]. A peculiar characteristic of neutrophils is their ability to regulate cytokine activity extracellularly through the release of serine proteases. Neutrophil serine proteases can either negatively or positively modulate the activity of cytokines/chemokines produced by neutrophils or other cells. For example, elastase, cathepsin G, and proteinase 3 bound to aggregated NETs degrade cytokines and chemokines and protect from antiproteases, thereby facilitating the resolution of inflammation [156]. These serine proteases, either individually or collectively, can inactivate mature IL-1β and IL-33, while they process immature IL-1α, IL-33, and IL-36 to yield mature active cytokines [157]. Other studies showed proteinase 3 to cleave IL-32 synthesized by natural killer cells, T lymphocytes, and epithelial cells, yielding a more potent form than the parent molecule to stimulate macrophage differentiation [158]. Upregulation of cathepsin G expression in neutrophils led to increased formation of active IL-1β, which, in turn, promoted tumor progression in a murine model of lung cancer [159]. NET released during inflammation can also awaken dormant cancer cells. Thus, NET-bound elastase and MMP-9 were shown to sequentially cleave laminin, uncovering an epitope that triggered the proliferation of dormant tumor cells in a mouse model of lung injury [160]. These findings suggest another intriguing link between chronic inflammation and cancer.

Neutrophil granule proteins can also synergize with chemokines. For example, MMP-9 (gelatinase B) cleaves the chemoattractant cytokine CXCL8 (interleukin-8) at the N terminus to yield a truncated form that displays increased binding affinity to its cognate receptor and more potent biological activities than the parent chemokine [161,162]. The truncated CXCL8 enhances MMP-9 release and promotes neutrophil chemotaxis, forming a feed-forward circuit to amplify the inflammatory response [163]. Cathepsin G or cathelicidin immobilized on the surface of ECs promotes firm adhesion of rolling monocytes in the vasculature [164,165] as reported in mouse models of atherosclerosis [166]. Another granule protein α-defensin (also known as HNP1) forms a heterodimer with platelet-derived C-C motif ligand 5 (aka RANTES) (CCL5), which enhances monocyte adherence in the mouse microvasculature [167].

Granule constituents and neutrophil phenotypic heterogeneity

Consideration of neutrophil functional versatility and novel paradigms in neutrophil biology (as discussed above and reviewed in [4,11,12]) provide clues to characterizing neutrophil phenotypic heterogeneity. Recent reviews detailed distinct neutrophils subsets, characterized by expression of various surface markers, such as NB1 (CD177), olfactomedin 4, IL-17$^+$, CD63$^+$, or variable T-cell receptor-like immune receptors [11,12]. Neutrophil populations with different density properties have also been identified (reviewed in Ref. [168,169]). The spectrum of neutrophil density may partially link to their stage of maturation with high-density and low-density populations representing mature and immature neutrophils, respectively [169]. Low-density neutrophils (also known as granulocytic myeloid-derived suppressor cells) consist of a heterogeneous population, which may either indicate *in vivo* activation/degranulation of mature neutrophils or represent a distinct lineage of cells (i.e., immature neutrophils released from the bone marrow) [169]. Expression of certain granule markers on the surface of neutrophils, such as CD63 (azurophilic granules), CD66b (SG), or CD11b (GG granules), has been proposed as indicators of cell activation [169]. Alternatively, the activated phenotype might be acquired within inflamed tissues, thus indicating neutrophils that underwent reverse TEM [71,75]. Low-density neutrophils have been detected in pregnancy [170] and in patients with sepsis [171], diabetes [172], cancer [173,174], or autoimmune diseases [168]. Intriguingly, some low-density neutrophil subsets share the ability to suppress immune response, whereas systemic lupus erythematosus-associated low-density neutrophils display pro-inflammatory properties and are highly susceptible to form NET [168]. The phenotypical and functional divergence of low-density neutrophil subsets requires further investigations.

Potential therapeutic modulation of granule proteins

The role of neutrophils in the initiation and progression of pathological processes underlying tissue damage makes these cells attractive therapeutic targets. However, global reduction of neutrophil numbers or functional responses limits the usefulness of these therapies because of compromised host defense to bacterial infections. Considering the importance of granule proteins in mediating neutrophil responses, an attractive alternative approach may be targeting the release of these proteins and/or their actions without impairing the ability of neutrophils to contain the microbial invasion. Accumulating data indicate the feasibility of this approach.

Inhibition of granule trafficking and docking

Azurophilic granules contain the most toxic protein cargo and their exocytosis is selectively regulated by Rab27a through interaction with the effector Slp1/JFC1 [18]. Structural modeling and high-throughput screening analysis led to identifying small molecule neutrophil-specific exocytosis inhibitors, termed Nexinhibs, which selectively interrupt the Rab27a-JFC1 interaction and release of the azurophilic granule cargo without interfering with phagocytosis [57,175]. Nexinhib 20-treated mice were reported to exhibit decreases in plasma levels of neutrophil granule proteins [176] and reduced neutrophil accumulation in the kidney and liver in a mouse model of LPS-induced systemic inflammation [175], similar to the phenotype observed in Rab27a-deficient mice [177]. Peptide aptamers derived from SNARE domains that compete for binding between intact SNARE proteins have also been developed [57]. SNARE mimicking peptides were fused with the cell-penetrating peptide HIV TAT to facilitate uptake by neutrophils [57]. Fusion proteins containing the N-terminal SNAP-23 SNARE domain (TAT-SNAP-23) efficiently inhibited formyl-Met-Leu-Phe-stimulated degranulation of SG, GG granules, and SV, but not azurophilic granule exocytosis, whereas fusion proteins containing syntaxin 4 SNARE domain (TAT-STX-4) inhibited degranulation of all four granule subsets *in vitro* [57]. In preclinical models, TAT-SNAP-23 reduced neutrophil-mediated acute lung injury induced by pulmonary immune complex deposition [178] or sepsis [179]. Since SNARES expression is not restricted to neutrophils, SNARE inhibitors lack selectivity for neutrophils [57]. Further studies are needed to define the cell types other than neutrophils affected by SNARE inhibitors *in vivo*.

Modulation of NET formation or degradation

Neutrophil extracellular traps formation may contribute to the pathophysiology of many diseases; hence, modulating this process opens potential avenues for therapy. Preclinical studies showed that ROS scavengers, such as N-acetyl cysteine [180], and MPO inhibitors [180,181] could reduce NET release both *in vitro* and *in vivo*. Similar to MPO inhibitors, TLR4 [182] or

peptidyl arginine deiminase (PAD) inhibitors [183–185] reduced NET formation and tissue damage in murine models of arthritis, atherosclerosis, and lupus. Studies in PAD4-knockout mice [186,187], however, suggest that bacterial infections may shift the balance of the protective and deleterious effects of NETs in host defense. Another potential approach is to facilitate the degradation of already formed NETs with DNase 1 and enhance their clearance. Indeed, treatment with exogenous DNase 1 reduced tissue damage and mortality in tumor-bearing mice [188], lupus-prone mice [189], acid inspiration-induced lung injury [190], and transplantation-associated lung injury in mice [191]. Of note, the synthetic DNase 1 analog dornase-α is currently being tested in a phase III clinical trial in patients with severe trauma-associated respiratory failure [192].

Blocking the actions of granule proteins

Disrupting granule protein-mediated crosstalk and signaling circuits is another promising therapeutic avenue. Of particular interest is the members of the superfamily of specialized pro-resolving lipid mediators (SPMs), including lipoxins, resolvins, protectins, and maresins [193–195]. SPMs are formed within the inflammatory environment during the resolution phase of acute inflammation [193,195] and resolution of clots in venous thrombosis [196]. SPMs are typically generated from arachidonic acid and polyunsaturated fatty acids by transcellular biosynthesis in inflammatory exudates and by neutrophils and macrophages [195]. Aspirin and statins trigger the biosynthesis of R-epimeric forms of SPMs [193,197]. In addition to transcellular mechanisms, macrophages can also produce various SPMs when interacting with apoptotic neutrophils [198], pro-resolving microparticles [199], or high-density lipoprotein [200]. While each mediator carries defining biological functions with specific receptors and cell and organ-specific properties, their primary cellular targets are myeloid cells [201]. This intricate signaling network is mapped onto the searchable Atlas of Inflammation Resolution [202]. SPMs attenuate neutrophil accumulation in inflamed tissues partly by preventing upregulation of Mac-1 [193–195,202]. Resolvin D1 may limit neutrophil trafficking into tissues through countering the exocytosis of SV [203]. Aspirin triggered 15-epi-lipoxin A_4, acting through the lipoxin A_4/formyl peptide receptor 2, disrupts the MPO-based self-amplifying loop by attenuating MPO-induced Mac-1 upregulation and degranulation and redirects neutrophil to apoptosis [204]. 15-epi-lipoxin A_4 and 17-epi-resolvin D1 counter TLR9 activation-triggered release of NE and proteinase 3 and restore the balance between Mac-1 and C5aR expression to enhance phagocytosis of bacteria and phagocytosis-induced apoptosis in human neutrophils [43]. The therapeutic potential of these lipids is illustrated by the observations that treatment with these lipids limits neutrophil accumulation, accelerates bacterial clearance, and enhances neutrophil apoptosis and efferocytosis, resulting in accelerated resolution of inflammation in mouse models of MPO [204] or *E. coli*-induced acute lung injury [43]. Select SPMs, such as resolvin D4, limit NETosis in the formation of clots in murine models of deep vein thrombosis [196]. Other SPMs, such as resolvin E1, which signals through the LTB_4 receptor BLT1 [205], and resolvin D5, which acts through GPR32 [206], facilitate phagocytosis of bacteria by naïve neutrophils via distinct receptors and molecular mechanisms. Whether these SPMs could also modulate the actions of granule proteins remain to be investigated.

Due to the ability of NE to degrade extracellular matrix proteins, a critical event in the development of chronic inflammatory diseases, such as pulmonary emphysema, adult respiratory distress syndrome, cystic fibrosis, chronic obstructive pulmonary disease, and rheumatoid arthritis [57,207], extensive research efforts have been directed to develop inhibitors. Recombinant endogenous elastase inhibitors, including recombinant α1-proteinase inhibitor and secretory leukocyte inhibitor, became available [208], and some (e.g., sivelestat) were evaluated in clinical trials [209]. However, the toxicity and off-target effects of synthetic inhibitors considerably limit their clinical use. Several natural compounds (for example, flavonoids, tannins, and cinnamic acid derivatives) were found to exert direct inhibitory activity on NE *in vitro* [210], though little information is available on their *in vivo* actions. Of note, by degrading various pro-inflammatory cytokines, including IL-1, TNF, and IL-6 [211] and mediating neutrophil reverse TEM [72], NE may play a role in dampening the inflammatory reactions. In addition to NE, preclinical data imply a role for proteinase 3 in the development of chronic obstructive pulmonary disease [212] and possibly other chronic diseases characterized by tissue destruction. However, validating proteinase 3 as a relevant therapeutic target in patients requires additional investigation and development of selective inhibitors.

Concluding remarks

Proteins expressed and stored in neutrophils during granulopoiesis represent a readily mobilizable pool of

molecules that mediate neutrophil effector functions. In addition to microbicidal and tissue-damaging actions, neutrophil granule proteins are increasingly being recognized as mediators of neutrophil orchestration of innate and adaptive immunity.

Consideration of novel paradigms in functions of granule proteins provides a different vantage point of the neutrophils' roles in host defense, causing local tissue damage and systemic complications, in particular under chronic inflammatory conditions. However, whether neutrophil subsets (identified by phenotypic markers) exhibit differences in granule protein content and secretion remains to be defined. A better understanding of the roles of neutrophil granule proteins is essential to further the development of neutrophil granule-specific therapies. Results from preclinical models indicate that this can be accomplished by selective targeting of granule exocytosis or interference with signaling from granule constituents released to the extracellular environment with small molecule inhibitors, recombinant protein inhibitors, and SPMs, such as lipoxins and resolvins, which can dampen neutrophil-mediated inflammation without interfering with antimicrobial defense. Clinical trials with synthetic NE inhibitors yielded disappointing results due to toxicity. While large-scale clinical trials with selective inhibitors of other granule proteins seem distant, results from an ongoing trial targeting granule protein functions (i.e., promoting degradation of NETs) hold promise as a disease-modifying intervention. Further studies are needed to investigate whether therapeutic interventions aimed to counter the actions of individual granule proteins could limit the deleterious actions of neutrophils in inflamed tissues, perhaps in a partial tissue-specific manner, and whether this will facilitate the resolution of inflammation underlying many chronic diseases.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

AO, MS, and JGF wrote the review.

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