Invited Review Article

Mechanisms of toxicity mediated by neutrophil and eosinophil granule proteins

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Abbreviations:
a1-PI, a1-proteinase inhibitor; a1-ACT, a1-antichymotrypsin; ALP, anti-leukoprotease; AMP, antimicrobial protein; BPI, bactericidal/permeability-increasing protein; CLC, Charcot-Leyden crystal protein; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EET, eosinophil extracellular trap; EPO, eosinophil peroxidase; ET, extracellular trap; fMLP, N-formylmethionyl-leucyl-phenylalanine; GPI, glycosylphosphatidylinositol; hCAP, human cationic antimicrobial protein; HNP, human neutrophil peptide; LBP, LPS-binding protein; MBP, major basic protein; MMP, metalloproteinase; MPO, myeloperoxidase; mtDNA, mitochondrial DNA; NET, neutrophil extracellular trap; NGAL, neutrophil gelatinase-associated lipocalin; PAF, platelet-activating factor; PMN, polymorphonuclear leukocytes; OS, reactive oxygen species; SLPI, secretory leukoprotease inhibitor; TIMP-1, tissue inhibitor of metalloproteinase-1; TULIP, tubular-lipid binding protein

A B S T R A C T

Neutrophils and eosinophils are granulocytes which are characterized by the presence of granules in the cytoplasm. Granules provide a safe storage site for granule proteins that play important roles in the immune function of granulocytes. Upon granulocytes activation, diverse proteins are released from the granules into the extracellular space and contribute to the fight against infections. In this article, we describe granule proteins of both neutrophils and eosinophils able to kill pathogens and review their anticipated mechanism of antimicrobial toxicity. It should be noted that an excess of granules protein release can lead to tissue damage of the host resulting in chronic inflammation and organ dysfunction.

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Introduction

Granulocytes are polymorphonuclear (PMN) leukocytes that comprise neutrophils, eosinophils, and basophils, of which neutrophils are the most frequent type found in blood. These white blood cells are an essential part of the innate immune system and
play important roles in fighting against infections, but, under pathological conditions, might also cause tissue injury.1–3 Granules are formed during the differentiation and described as the hallmark of granulocytes that store “pre-packed” proteins which can be released into the extracellular space upon stimulation. These diverse mediators, once released to the extracellular space, are able to encounter and destroy all types of microorganism, and, in the case of eosinophils and basophils, also known to orchestrate hypersensitivity reactions.7 Granule proteins form together with a fibrous scaffold build of mitochondrial DNA (mtDNA) extracellular structures called extracellular traps (ETs) that can be formed by all three granulocyte types. ETs comprise an efficient mechanism of host defense through facilitating the binding and killing of invading bacteria and fungi in the extracellular space. Granule proteins with a high affinity for DNA are delivered into ETs and cause a high local concentration of antimicrobial proteins and simultaneously reduce the spread of harmful proteins such as proteinases to adjacent tissues.4–10

The formation of granules (granulopoiesis) in neutrophils occurs sequentially during myeloid cell differentiation and is regulated by several myeloid transcription factors that are active at specific stages of neutrophil development. Four morphologically distinct granule populations can be distinguished: azurophilic (primary), specific (secondary), gelatase (tertiary) and secretory granules.11,12 Azurophilic granules are formed during the promyeloctytic stage and represent the largest granules in neutrophils. They contain the most toxic mediators including elastase, myeloperoxidase (MPO), cathepsins as well as defensins, and contribute mainly to the antimicrobial effects of neutrophils.13–16 In the metamyelocyte stage, specific granules emerge that are characterized by a high content of the glycoprotein lactoferin. In addition, they also contain antimicrobial compounds, including neutrophil gelatinase-associated lipocalin (NGAL), human cathelicidin (hCAP-18) and lysozyme.16 The smallest granules, the gelatinase granules, are the last population formed during neutrophil maturation. They contain metalloproteinases, including gelatinase and leukolysin, as well as of few antimicrobials.16 These three classical granules are formed by budding from the Golgi.16 In contrast, the secretory vesicles are formed through endocytosis at the end of neutrophil maturation, accordingly, they incorporate mainly plasma-derived proteins such as albumin.16

Eosinophil granules are subdivided in primary and secondary granules. Primary granules contain Charcot-Leyden crystal protein and eosinophil peroxidase (EPO). Secondary granules are composed of matrix with a crystalline core. Eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and EPO are found in the matrix, meanwhile major basic protein (MBP) is restrained in the crystalline core.17

Antimicrobial proteins (AMP) are ancient molecules also present in granules of both neutrophils and eosinophils evolved as part of host defense to eliminate pathogenic bacteria, fungi, parasites and viruses.18–20 Their main modes of action consist of disruptions of bacterial membrane. Furthermore AMPs are able to vanish the electrochemical gradient of the membrane by pore formation or to interfere with DNA, protein or cell wall synthesis and inhibit protein folding after entering into the cytoplasm.10 AMPs share some structural features: 1) smaller length than 60 amino acids, 2) often a net positive charge, 3) broad-spectrum antimicrobial activity at physiological conditions and 4) hydrophobic and hydrophilic amino acids clusters adopted in an amphipathic shape.20

Granules of both neutrophils and eosinophils contain proteases which are important for physiological processes. However, their excessive, prolonged or inappropriate proteolytic activity is involved in the offset of diseases.21 Proteases are subdivided in four classes based on their biochemistry of the active site: 1) serine proteases, 2) metalloproteases, 3) thiol-proteases and 4) aspartate proteases. Serine proteases and metalloproteases are most active at neutral pH thus are involved in extracellular protein digestion.21 On the other hand, thiol-proteases and aspartate proteases are most active at acidic pH and participate in the degradation of intracellular proteins.21 Peroxidases in granulocytes are members of the heme peroxidase superfamily.22 Oxidants and reactive oxygen species (ROS) are required for many physiological processes, nonetheless their excessive production is involved in pathophysiological processes. Peroxidases are able to interact with H$_2$O$_2$ and are required for its cytotoxicity.23

In this article, we summarize our current understanding on the mechanisms of toxicity of neutrophil and eosinophil granule proteins (Fig. 1, Table 1) as well as their regulation to inhibit their cytotoxic potential.

**Eosinophil granule proteins**

**Primary granule proteins**

Charcot-Leyden crystal (CLC) protein, also known as galectin 10, belongs to the galectin superfamily24 and is only found in humans,
### Table 1
Antimicrobial mechanisms mediated by granule proteins of eosinophils and neutrophils.

| Granule Protein | Distribution | Mechanism of toxicity |
|-----------------|--------------|-----------------------|
| Eosinophil      |              |                       |
| CLC             | Primary granules | vesicular transport of cationic RNases; requirement for eosinophil granulogenesis; involvement in inflammation |
| EPO             | Primary & secondary granules | oxidative inactivation of pathogens; oxidative damage towards host endothelial cells; inhibition of LPS and lipid A in gram-negative bacteria membranes |
| EDN             | Secondary granules | ribonuclease activity against viruses; inactivation of extracellular viruses; ROS production & induction of apoptosis in keratinocytes |
| ECP             | Secondary granules | antibacterial & antiparasitic properties; involvement in EETs & bacterial cell membrane depolarization; neutralization of LPS; formation of amyloid-like fibrils; ROS production & induction of apoptosis in keratinocytes; upregulation of MMP9 expression |
| MBP             | Secondary granules | antibacterial & antiparasitic properties; cytotoxicity against host tissue; causes degranulation of human eosinophils; enhancement of production of proinflammatory IL-8; permabilization of cell membranes; formation of amyloid-like fibrils; involvement in EETs & non-toxic extracellular deposits |

| Granule Protein | Distribution | Mechanism of toxicity |
|-----------------|--------------|-----------------------|
| Neutrophil      |              |                       |
| MPO             | Azurophilic granules | oxidative inactivation of pathogens; chronic inflammation; involvement in NETs |
| Serine proteases (Elastase, Cathepsin G, Proteasease 3) | Azurophilic granules | antimicrobial activity against bacteria, yeast and fungi; non-infectious inflammatory diseases; degradations of bacterial virulence factors; involvement in NETs; proteolytic processing of antimicrobial proteins; induction of proapoptotic death pathway in neutrophils; proteolytic modification of chemokines; activation of NADPH oxidase |
| Defensins (HNPI–4) | Azurophilic granules | antimicrobial activity against bacteria, viruses and fungi; permeabilization of bacterial membranes; pore formation in lipid bilayers; inhibition of virus fusion to host plasma membrane; inhibition of serpins |
| BPI             | Azurophilic granules | neutralization of LPS; bacterial cell membrane damage; involvement in LPS uptake and antigen presentation by DCs |
| SLPI            | Azurophilic granules | inhibitor of serine proteases; protection of local tissues against inflammation; inhibition of bacterial translation; fungicidal activity |

Table 1 (continued)

| Granule Protein | Distribution | Mechanism of toxicity |
|-----------------|--------------|-----------------------|
| Neutrophil      |              |                       |
| hCAP18/LL-37    | Specific granules | modification of bacterial surface; involvement in NETs; antimicrobial activity against intracellular pathogens |
| Lactoferrin     | Specific granules | involvement in NETs; bacteriostatic & bactericidal effect; inhibition of proliferation of fungi and viruses; modification of bacterial membrane; interference with bacterial cellular mechanism; increase of inflammatory cytokines & chemokines |
| NGAL            | Specific granules | decrease of MMP-9 activity; sequestration of neutrophil chemotacticants; effector of microbial killing; bacteriostatic effect; involvement in NETs |
| Lysozyme        | Specific granules | bactericidal effect; hydrolysis of bacterial cell wall; involvement in NETs |
| MMP-8           | Specific granules | degradation of collagen; modification of chemokines |
| MMP-9           | Gelatinase granules | degradation of collagen, elastin & gelatin; inactivation of SLPI; proteolytic processing of IL-1β & TNF precursor |
| Leukolysin      | Gelatinase granules | inactivation of IL-1, IL-18 |

but not in mouse eosinophils. Unlike other galectin members, CLC does not have the ability to bind β-galactosidase or any known mammalian glycan structures. CLC is found to interact with glycosylated EDN and ECP, two human eosinophil granule cationic RNases, but induces no inhibitory function upon binding. Upon IFN-γ activation of eosinophils, CLC associates rapidly with CD63 positive secondary granules and EDN, suggesting a role of CLC in vesicular transport of cationic RNases. The presence of CLC is required for eosinophil granulogenesis during differentiation. Furthermore, CLC has been shown to be involved in inflammation in allergic, parasitic and other eosinophil-associated diseases and inflammatory reactions. Eosinophil peroxidase (EPO, also known as EPX) catalyzes the two-electron redox reaction H₂O₂ + X− + H₂O → HO₂ + H₂O similar to other peroxidases. Unlike for MPO, the physiologic substrate for EPO is still mainly uncertain. Three rather unusual substrates including Br−, NO2 and the pseudohalide thiocyanate (SCN−) are involved in the oxidation by EPO and H₂O₂. During degranulation, EPO can be released into large cytoplasmic vacuoles (phagosome) or directly onto the surface of a target. Peroxidase-H₂O₂—iodide reaction leads to the iodination of protein and killing of bacteria. EPO interacts with the superoxide generated by the NADPH oxidase to provide the bactericidal activity of eosinophils. EPO exhibits cytotoxicity through the release of peroxidase-derived oxidants towards mammalian tumor cells, HIV-1 and schistosomula of Schistosoma mansoni when the peroxidase is combined with H₂O₂ and a halide. Eosinophils adhere to schistosomula coated with antibody and complement. Upon release EPO binds to the surface of schistosomula. The binding itself is non-toxic but the presence of surface-bound EPO can enhance the eosinophil-mediated toxicity against the parasite. Similar effect mediated by EPO–H₂O₂—halide system occurs towards host endothelial cells and is observed in eosinophil endocarditis. Mycobacterium tuberculosis treated...
with EPO exhibit alterations of the cell wall followed by cell wall fragmentation and cell lysis within 120 min, particularly in the presence of H$_2$O$_2$. Binding of EPO to gram-negative bacteria membranes and subsequent inhibition of LPS and lipid A occurs in a haloperoxidase-independent manner."

**Secondary granule proteins**

Eosinophil granule proteins EDN/RNase 2 and ECP/RNase 3 are reported to be closely related proteins and part of RNase A superfamily. RNase A superfamily itself is an important part of the human AMP family. Members of the RNase A superfamily share a similar primary sequence and structural similarities such as 6–8 conserved cysteine residues forming characteristics distinct disulfide bonds and conserved histidines, as well as a lysine in the active center to catalyze the ribonuclease activity. The ribonucleases are reported in mucosal secretions, in several types of immune cells as well as in major organs.EDN exhibits ribonuclease activity against single stranded RNA viruses including respiratory syncytial virus, hepatitis B virus and HIV. On the other hand, ECP has antibacterial and antiparasitic properties. EDN has been shown to be less cationic and less cytotoxic than ECP and was originally described as a neurotoxin. In contrast, ECP is uniquely expressed in eosinophils, more cationic and exhibits significant less ribonuclease activity compared to EDN or RNase A. In fact, the cytotoxic activity of ECP is independent of its ribonuclease activity. EDN displays the ability to penetrate the viral capsid thus gaining access to the viral genome and inactivating extracellular virions by its ribonuclease activity. Both EDN and ECP mediate cytotoxic effects on keratinocytes through the production of ROS and induction of apoptosis. Additionally, ECP has been shown to promote keratinocyte cell–matrix detachment and to upregulate MMP9 expression. ECP is released in association with mitochondrial DNA in the context of forming eosinophil extracellular traps (EETs) which are able to bind to and kill bacteria in the extracellular space. High-affinity binding of ECP to lipopolysaccharide (LPS) and peptidoglycan causes destabilizing of the bacterial cell wall and subsequently cell membrane depolarization. Moreover, ECP-LPS interactions trigger LPS aggregation and neutralization of LPS-stimulated TNF-$\alpha$ production. ECP binds to the membrane phospholipid polar heads by electrostatic interactions. Following clustering of the liposome–protein complexes, liposome aggregation and destabilization of the lipid bilayer occurs that lead ultimately to membrane disruption and release of the liposome content. ECP interacts preferably with anionic and zwitterionic phospholipids of cell membranes. The anionic lipids are exposed on the external side of microbial membranes but sequestered on cytoplasmic side of eukaryote host cell membrane explaining a potential mechanism of antibacterial properties. Under in vitro condition, ECP was shown to form amyloid-like fibrils. The amyloid-type aggregates of ECP led to cell agglutination and bacterial clearance preceding the cell death.

**Neutrophil granule proteins**

**Azurophilic granules**

MPO is a heme-containing peroxidase of the peroxidase superfamily. In combination with H$_2$O$_2$ and a halide, it catalyzes the formation of reactive oxygen intermediates. MPO is released from azurophilic granules of activated neutrophils either into the phagolysosome or directly into the extracellular space. Hypohalous acids formed by MPO exhibit antibacterial, antiviral as well as antifungal properties and are involved in chronic inflammation when produced in excess. MPO is found in colocalization with mDNA within NETs.

Serine proteases contain a catalytically essential serine residue at their active side. They comprise the largest class of mammalian proteases and are mainly synthesized as inactive precursors. The exception are the three human leukocyte serine proteases elastase, cathepsin G and proteinase 3 that are stored in an active form within the azurophilic granules. Neutrophil serine proteases exhibit antimicrobial activity against bacteria, yeast and fungi. Furthermore, they are involved in several non-infections inflammatory diseases, including arthritis, bullous pemphigoid, chronic inflammatory lung diseases and tissue damage following ischemia/reperfusion injury. Cathepsin G-knockout mice are more susceptible to Gram-positive bacteria whereas mice lacking neutrophil elastase are more susceptible to Gram-negative bacteria and enterobacteria species. Neutrophil elastase binds the bacterial membrane and directly degrades the outer membrane protein A of Escherichia coli or virulence factors of enterobacteria, resulting in the loss of bacterial integrity and cell lysis. The human neutrophil serine proteases are upregulated on the cell surface upon activation. They mostly remain bound to the plasma membrane during exocytosis of the granules allowing the neutrophils to modulate their inflammatory response through the preservation of their catalytic activity. Additionally, neutrophil serine proteases display extracellular activities. Serine proteinases are released in NET-forming neutrophils. After exocytosis, proteinase 3 is required for proteolytic processing of the human cathelicidin to its active form LL-37. Similarly, mature active IL-18 is released of epithelial cells after stimulation with IFN-$\gamma$ in combination of proteinase 3 and LPS without the activity of caspase-1. In contrast, neutrophil elastase downregulates the biological activity of IL-18 through proteolysis. Neutrophil elastase and cathepsin G abolish the pro-inflammatory effect of bacterial
flagellin on epithelial cells through cleavage.83 Interestingly, by cleaving caspase-8, cathepsin D is able to induce a proapoptotic death pathway in neutrophils themselves.4,85 Moreover, neutrophil serine proteases are able to regulate chemokine activities by proteolytic modifications. For example, the activity and stability of CXCL8 and CXCL5 are enhanced following their N-terminal proteolytic modification by protease 3 and cathepsin G.6,87 On the other hand, cleavage of CCL3, CXCL12 and CXCR4 results in lower chemotactic activity.6,88,89 Recently, it has been shown that fibroblast activation protein – α alpha, a serine protease, is expressed in neutrophils and involved in the activation of NAPDH oxidase.90

In neutrophils, four small cationic peptides of α-defensins called human neutrophil peptides 1, 2, 3 and 4 (HNP1-4) are secreted upon activation and exhibit antibacterial, antiviral as well as anti-fungal activity.91,92 Defensins are characterized by a β-sheet-rich fold, a high cathodal electrophoretic mobility and a framework of six disulphide-linked cysteines. Three subfamilies are identified: α- and β-defensins that are formed by a triple-stranded β-sheet with a “defensin” fold and θ-defensins.93 HNP1 and HNP3 differ only at the first residue, while HNP2 is proteolytically processed of one of these two defensins.94 HNP1 and HNP3 have been characterized as NET-associated proteins.95 Defensins are produced as propeptides. First steps of maturation include removing of the signal peptide and packaging of the inactive propeptides in azurophil granules. The full process occurs within the granules mediated by neutrophil elastase and protease 3.95 HNPs are acting against Gram-positive and Gram-negative bacteria through membrane permeabilization.21 Defensins decrease the membrane potential of target cells within minutes through the creation of small membrane channels followed by the permeabilization of the cells after a short lag period. Interestingly, cells can be rescued by removing the HNP-containing media after 30 min, hence a second phase of injury and continuing presence of HNP is necessary for cell lysis.96 HNP2 has been shown to form multimeric pores in lipid bilayers.96 Defensin-mediated bactericidal activity occurs by outer membrane permeabilization following inner membrane permeabilization.24 Synchronous inhibition of RNA, DNA and protein production, as well as loss of cellular metabolites and altered ionic cellular environment lead to loss of the bacterial colony-forming potential.97 Defensins interfere and also disrupt the virus fusion to the host plasma membrane through inhibition of the interactions between viral glycoproteins and cellular receptors.98 In contrast to antibacterial and antifungal mechanism, direct disruption of the virus lipid bilayer does not occur. Viral envelopes are mainly derived from host cell membranes helping the enveloped virus to escape the defensins.99 Moreover, at sites of inflammations where defensins are present at high concentrations, they can bind serine proteinase inhibitors (serpins), particularly z1-proteinase inhibitor (z1-PI) and z1-antichymotrypsin (z1-ACT), to prevent their activity against serine proteases.100 Within the phagolysosome of neutrophils, increased proteolytic activity of serine proteases through defensin-serpin binding may lead to enhanced phagocytic digestion of microbes.101

Bactericidal/Permeability-Increasing Protein (BPI) belongs to the lipid transfer/LPS binding protein family that is characterized by the binding of lipid substrates102 as well as to the tubular-lipid binding protein (TULIP) family.103 BPI exerts bactericidal activity against Gram-negative bacteria through the neutralization of LPS by binding to the lipid A moiety.104 Penetration of the inner bacterial cell membrane induces membrane damage leading eventually to cell death.105 BPI-mediated toxicity occurs in two stages. In an early, reversible sublethal phase, BPI acts on the outer bacterial membrane and induces growth arrest. Penetration of the cell membrane leads to a time- and pH-dependent lethal stage with the involvement of cytoplasmic membrane damage.106 BPI is able to reduce LPS-mediated neutrophil stimulation, pyrogenicity and LPS-induced TNF-α production.107,108 BPI enhances the interaction of bacterial outer membrane vesicles with dendritic cells (DCs) and is involved in the LPS uptake and antigen presentation by DCs.109 In Gram-positive infections, binding of BPI to bacterial lipopeptides and lipoproteins, as well as lipoteichoic acid enhances their inflammatory effects.110 The LPS-binding protein (LBP) moiety of the TULIP family binds to the same component of LPS on gram-negative bacterial membrane as BPI. However, LBP helps to initiate the host immune response by bringing small amounts of LPS to effector cells.111,112

Secretory Leukoprotease Inhibitor (SLPI), also known as anti-leukoprotease (ALP), is an endogenous serine protease inhibitor (serpin) produced by epithelial cells and some myeloid cells including neutrophils.113 SLPI is mainly involved in the protection of local tissues against inflammatory consequences that are caused by proteases including cathepsin G, elastase and trypsin from neutrophils.114 Furthermore, some studies propose antibacterial and antifungal capacity for SLPI115–117. While the protease inhibitory activities are shown to be located at the COOH-terminal domain, the antibacterial capacity against Gram-negative and Gram-positive bacteria depend on the NH2-terminal domain.111 SLPI induces toxicity against E. coli by binding to DNA and mRNA leading to inhibition of translation.110 Fungicidal activity of SLPI emerge against metabolically active Aspergillus fumigatus and Candida albicans. On the other hand, metabolically quiescent A. fumigatus has been reported to be resistant against SLPI.112

Specific granule proteins

LL-37 is expressed by immune cells including neutrophils, monocytes, mast cells, NK cells, B and T cells, as well as stem cells.113 LL-37 is the mature peptide of 37 amino acids released from the C-terminus of the propeptide hCAP18.114 hCAP18 is processed by extracellular proteolysis of the C-terminal end of the human cationic antimicrobial protein (hCAP).115 hCAP is the only cathelicidin family member, one of the major AMP families, known in humans. They are comprised of a highly conserved N-terminal signal peptide — “cathelin domain” — and a structurally variable cationic AMP at the C-terminus.116 LL-37 is active against the anionic membrane of Gram-positive and Gram-negative bacteria, similar to other cationic AMPs. The positive charge of the peptide enhances the binding to negatively charged bacterial membranes rather than mammalian cell membranes.117 The importance of the electrostatic attraction for the antibacterial capacity is underlined by the fact that modification of the bacterial surface influences the susceptibility of the cells to LL-37.118–120 LL-37 and its propeptide hCAP18 are released within NETs. LL-37 binds to the DNA scaffold of the extracellular traps,121 reportedly leading to the loss of its antibacterial capacity.122,123 Stephen et al. reported the internalization of LL-37:DNA complexes derived from NETs by macrophages enabling antimicrobial activity of LL-37 against intracellular pathogens.124

Lactoferrin, also known as lactotransferrin, is a basic iron-binding glycoprotein of the secondary granules in neutrophils.125,126 Leukocytes, macrophages, platelets and bacteria, as well as the gastrointestinal tract exhibit lactoferrin receptors.127 Lactoferrin is found to be associated to NETs. Lactoferrin exerts a bacteriocidal effect through iron sequestering inhibition of the bacterial proliferation.127 Additionally, lactoferrin is reported to hold a bactericidal effect and inhibits the proliferation of fungi and viruses.125 The protein modifies outer membrane of Gram-negative bacteria by binding and releasing LPS. This way, lactoferrin also enhances the entry of lysozyme into Gram-negative bacteria.128 The modulation of bacterial cell membrane can be blocked by the
addition of Ca\(^{2+}\) and Mg\(^{2+}\).[129] Through its iron-binding ability, lactoferrin may interfere with cellular mechanisms including DNA and RNA synthesis, protein synthesis, expression of lymphocyte surface markers, immunoglobulin secretion and interleukin-2 expression.[125] Lactoferrin serves also as ligand for TLR4 of rheumatoid arthritis synovial fibroblasts stimulating increased expressions of inflammatory cytokines and chemokines.[130] Furthermore, lactoferrin has been reported to inhibit spontaneous apoptosis in human neutrophils if the iron saturation status is low.[137]

Neutrophil gelatinase-associated lipocalin (NGAL, also known as lipocalin–2) is a member of the lipocalin family that is characterized by the ability of transporting small lipophilic substances, binding to specific cell-surface receptors and forming complexes with soluble macromolecules.[131] NGAL was first identified to be covalently associated with MMP-9.[135] Furthermore, NGAL-MMP-9 complex can be observed in a ternary complex with tissue inhibitor of metalloproteinase-1 (TIMP-1) that results in a 10-fold lower activity of MMP-9.[133] NGAL is able to bind to MBL,[134] PAF,[135] and leukotriene B\(_4\).[135] NGAL may also serve as a cofactor of microbial killing by other proteins through the binding to microbial, lipophilic ligands or modulating inflammatory responses induced by neutrophils.[136] Furthermore, NGAL has been found to bind to bacterial catecholate-type ferric siderophores preventing growth and spread of *Myobacterium tuberculosis*.[137] and *E. coli*. NGAL has also been identified in NETs and to be involved in their bactericidal effect in vitro.[138]

Lysozyme is a conserved hydrolytic antimicrobial protein that is well known for its importance to host defense. Lysozyme is expressed in phagocytes, hepatocytes, and epithelial cells of mucosal surfaces as well as in body fluids such as blood, tears, urine, saliva and milk.[140] Almost 100 years ago, Fleming et al. observed the bactericidal effect mediated by lysozyme.[141] Lysozyme provokes bacterial cell lysis through targeted hydrolysis of the bacterial cell wall.[141] Lysozyme is identified as a NET-associated protein using a proteomic approach.[79] Lysozyme is supposed to contribute to the antimicrobial activity of NETs due to its action on the bacterial wall.[142]

Matrix metalloproteinases (MMPs) are characterized by their dependence on intrinsic Zn\(^{2+}\) ions and extrinsic Ca\(^{2+}\). Their many function consist of degradation of all constitutes of the extracellular matrix.[68] Several MMPs are involved in the cleavage and inactivation of z1-antitrypsin, an inhibitor of neutrophil elastase, cathepsin G and proteinase 3.142 Likewise, serine proteases are able to inactivate protease inhibitor like TIMP-1,144 and cystatin C[145] that serve as inhibitors of MMPs. Metalloproteinase-8 (MMP-8; also known as neutrophil collagenase or collagenase 2) is a potent collagenase highly expressed by neutrophils[146] as well as rheumatoid synovial fibroblasts,147 endothelial cells,147 activated macrophages,148 smooth muscle cells,149 bronchial epithelial cells,150 mast cells150 and chondrocytes.151 MMP-8 are secreted in tuberculosis-associated inflammation driving the tissue destruction through degradation of the collagen.152 MMP-8 mediates responsiveness of neutrophils to LPS through chemokine modification.[153] Cleavage of collagen by MMP-8 facilitates neutrophil migration to sites of inflammation and injury behind collagen-rich barriers.[146]

**Gelatinase granule proteins**

Metalloproteinase-9 (MMP-9; also known as gelatinase B) is a member of the MMP family and is secreted by endothelial cells,[154] monocytes/macrophages[155] mast cells,[156] eosinophils,[157] and neutrophils.[158] MMP-9 degrades collagen, elastin and gelatin of the extracellular matrix upon exocytosis.[159] In allergic asthmatic patients MMP-9 is released by neutrophils after exposure to allergens.[160] MMP-9 cleaves and inactivate SLPI at sites of inflammation preventing its binding to LPS and inhibition of neutrophil elastase activity.[161] Several MMPs including MMP-9 process human IL-1β and TNF precursor into its bio logically active form regulating their appearance at inflammatory sites.[162,163]

Leukolysin, also known as membrane-type 6 metalloproteinase (MT6-MMP, MMP25), is a GPI-anchored proteinase of the MMP family.[164] Leukolysin is expressed in leukocytes,[165] in SW480 colon carcinoma cells and several brain tumors including anaplastic astrocytomas and glioblastomas.[166] Leukolysin is the most efficient proteinase in the inactivation of z1-PI. z1-PI is found in high concentrations in interstitial spaces and circulating plasma to protect tissue from neutrophil serine proteases. Action of leukolysin against z1-PI releases neutrophil elastase, proteinase 3 and cathepsin G from its inhibitor leading ultimately to tissue damage.[167]

**Conclusions**

Granule proteins are secreted from eosinophils and neutrophils into the extracellular space upon activation and consist of a variety of important mediators that are crucial for the function of granulocytes within the innate immunity. The released granule proteins display a great variety of weapons and diverse antimicrobial mechanism that are directed against different kind of pathogens (Table 1). On the other hand, excessive production of granule proteins of eosinophils and neutrophils plays a significant role in the pathogenesis of diverse human diseases. These findings arise the possibility to use granule proteins as diagnostic marker and drug targets for the development of new therapeutics against infectious and chronic inflammatory diseases. The understanding of the functional consequences of the molecular interactions between DNA and granule proteins within NETs and EETs requires additional experimentation.

**Conflict of interest**

The authors have no conflict of interest to declare.

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