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## Neutrophil serine proteases fine-tune the inflammatory response

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### Abstract

Neutrophil serine proteases are granule-associated enzymes known mainly for their function in the intracellular killing of pathogens. Their extracellular release upon neutrophil activation is traditionally regarded as the primary reason for tissue damage at the sites of inflammation. However, studies over the past several years indicate that neutrophil serine proteases may also be key regulators of the inflammatory response. Neutrophil serine proteases specifically process and release chemokines, cytokines, and growth factors, thus modulating their biological activity. In addition, neutrophil serine proteases activate and shed specific cell surface receptors, which can ultimately prolong or terminate cytokine-induced responses. Moreover, it has been proposed that these proteases can impact cell viability through their caspase-like activity and initiate the adaptive immune response by directly activating lymphocytes. In summary, these studies point to neutrophil serine proteases as versatile mediators that fine-tune the local immune response and identify them as potential targets for therapeutic interventions.

### Keywords

neutrophil; cathepsin G; neutrophil elastase; proteinase 3; inflammation

### Introduction

Neutrophils are the first line of defense against infection and their ability to phagocytose and kill invading microorganism is critical for the host defense. In response to infection, neutrophils engulf pathogens into an intracellular membrane-bound organelle called a phagosome where these microorganisms are destroyed by a combination of reactive oxygen species (ROS), proteases and antimicrobial peptides, aided by the acidic and nutrient deprived environment of the phagosome (Reeves et al., 2002; Stuart and Ezekowitz, 2005). Disorders of neutrophil number and function lead to increased risks of infection, as seen in patients with severe chronic neutropenia, and confirm that neutrophils are vital components of the immune defense (Newburger, 2006). However, once recruited to the site of inflammation, neutrophils must undergo apoptosis and their clearance is essential for the resolution of the infection (Eyles et al., 2006). Therefore, prolonged neutrophil accumulation can damage the host tissue and has been linked to chronic inflammation, which in turn is thought to contribute to the development of autoimmunity (Nathan, 2006). Indeed, neutrophils are a common feature of several inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, and chronic lung diseases, to name a few (Weiss, 1989). The importance of neutrophils in initiating

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and maintaining inflammatory response is demonstrated by several experimental animal models. Depletion of neutrophils abrogates the inflammation in experimental arthritis (Tanaka et al., 2006; Wipke and Allen, 2001), experimental allergic airway disease (Park et al., 2006), experimental colitis (Qualls et al., 2006), and several ischemia/reperfusion injury models (Crinnion et al., 1994; Kyriakides et al., 2000; Sisley et al., 1994). However, neutrophils are more than destructive cells whose main function is to kill and digest pathogens while damaging the host's tissues. Recent literature indicates that neutrophils have the unique capacity to directly and specifically shape the immune response (Nathan, 2006).

Neutrophils express a family of neutral serine proteases including cathepsin G (CG), neutrophil elastase (NE), and proteinase 3 (PR3) in their azurophil granules (Faurischou and Borregaard, 2003). In addition, human neutrophils also express azurocidin (AZU), an inactive serine protease homologue with chemotactic and antimicrobial activities (Almeida et al., 1996; Chertov et al., 1997). The bactericidal activity of neutrophils depends partly on the presence of these granule-associated proteases and the generation of neutrophil serine protease-deficient mice confirmed the relative importance of CG and NE in the killing of certain Gram-negative and Gram-positive bacteria (Belaouaj et al., 2000; Belaouaj et al., 1998; Reeves et al., 2002), as well as the clearance of certain fungi (Reeves et al., 2002; Tkalcevic et al., 2000). In addition, neutrophil serine proteases are implicated in several non-infectious inflammatory diseases, as indicated by animal models of inflammatory arthritis (Adkison et al., 2002), bullous pemphigoid (Liu, 2004; Liu et al., 2000), chronic inflammatory lung diseases (Chua et al., 2007; Shapiro et al., 2003), and tissue damage following ischemia/reperfusion injury (Shimoda et al., 2007). In some instances, the activity of these proteases goes beyond simple degradation of extracellular matrix. It is recently recognized that, although azurophil granules only undergo limited exocytosis, the extracellularly released serine proteases play an important role in the modulation of the inflammatory response by proteolytically modifying chemokines and cytokines, activating latent forms of cytokines and growth factors, and cleaving specific cell surface receptors. In addition, neutrophil serine proteases can induce caspase-independent cell apoptosis and initiate the adaptive immune response by activating lymphocytes. The aim of this review is to present the current knowledge regarding neutrophil serine proteases and their role as regulators of the inflammatory process and immune response.

## Neutrophil serine protease biosynthesis and secretion

Neutrophil serine proteases CG, NE, and PR3 are encoded by highly homologous genes in humans and mice. NE and PR3 are tightly linked in a cluster found on chromosome 19 (Zimmer et al., 1992) and chromosome 10 (Belaouaj et al., 1997) in humans and mice, respectively. In humans the gene for AZU is also encoded on chromosome 19. CG on the other hand is found in a separate cluster on syntenic regions of human and mouse chromosome 14 that include genes encoding for granzymes and mast cell chymases (Caughey et al., 1993; Heusel et al., 1993). The neutrophil granule-associated serine protease genes are tightly regulated and expressed only during the promyelocytic stage of myeloid development (Garwicz et al., 2005). Despite their distinct chromosomal localization, the expression of these proteases appear to be controlled at the transcriptional level by similar factors that bind to similar motifs in their proximal promoter regions (Faurischou and Borregaard, 2003; Lennartsson et al., 2005). The mRNAs of these serine proteases are expressed at the highest levels during the promyelocytic stage and then subsequently downregulated as the neutrophils mature.

Neutrophils contain several subsets of granules that are formed sequentially during neutrophil differentiation in the bone marrow. The granules found in neutrophils are azurophil granules (also known as primary granules), specific granules (also known as secondary granules), gelatinase granules (also known as tertiary granules), and secretory granules (Faurischou and Borregaard, 2003). The segregation of proteins into different subsets of granules is determined

solely by the time of their biosynthesis and does not depend on individual sorting information present in the proteins (Le Cabec et al., 1996). The maturation-specific expression of serine proteases expression therefore explains why these enzymes are only present in azurophil granules, which are formed during the promyelocytic stage, and absent in granules formed at later stages of neutrophil differentiation. However, the mechanism by which these proteases are targeted to the granules instead of being constitutively secreted is still unknown. No sorting motifs have been found for these proteases. And although the major mechanism for sorting proteins to granules involve mannose-6-phosphate receptors, this pathway is not implicated in the sorting and targeting of proteins in neutrophils. Instead, adaptor protein 3 (AP3) seems to play an important role in targeting serine proteases to azurophil granules. AP3 is a cargo protein responsible for shuttling proteins from the *trans*-Golgi network to the granule compartment. The role of AP3 in granule sorting is seen in the Hermansky-Pudlak syndrome and in canine cyclic neutropenia where mutations in AP3 disrupt the normal trafficking of NE (Benson et al., 2003). Recent evidence also indicates that serglycin, the major intracellular proteoglycan, is required for NE localization to granules (Lemansky et al., 2007; Niemann et al., 2007). Serglycin has previously been shown to complex with the highly related cytotoxic T lymphocyte granule-associated serine proteases granzymes and may also act as a chaperone for these secreted proteases (Raja et al., 2002). The targeting of CG and PR3 to azurophil granules however is mediated by an unknown mechanism.

Serine proteases are synthesized as inactive zymogens that require two N-terminal proteolytic modifications to become active. After the signal peptide removal, the proenzyme is further processed by the lysosomal cysteine protease dipeptidyl peptidase I (DPPI, also known as cathepsin C) en route to the granules where they are stored as active enzymes (Adkison et al., 2002). Processing by DPPI is required for targeting and stable storage in granules, as most of the proform is either constitutively secreted (Garwicz et al., 2005) or degraded, as has been shown for the proform of granzyme A (Pham and Ley, 1999). In addition, neutrophil serine proteases also undergo C-terminal processing. Although this proteolytic modification is not required for enzymatic activity, the removal of this C-terminal sequence reveals a docking site that allows NE to interact with AP3 (Benson et al., 2003). The retention of this C-terminus apparently results in mistrafficking of NE and its relocation to the surface membrane (Benson et al., 2003). Because of this tightly regulated expression and storage, perturbation of the intracellular trafficking of NE to granules results characteristic disorders that will be discussed below. Whether C-terminal truncation is also required for the proper trafficking of CG and PR3 to granules is still unknown.

Although neutrophil serine proteases function mainly as intracellular antimicrobial agents, they are also released following limited granule exocytosis. It is well established that a hierarchy of mobilization exists for the exocytosis of different granule subsets, specifically regarding the extent to which the granule content is released in response to stimuli. Specific and tertiary granules are readily exocytosed upon cell activation with a variety of stimuli while azurophil granules mainly fuse with the phagosome and only release a limited amount of their content extracellularly (Borreagaard and Cowland, 1997; Mollinedo et al., 1999). The molecular basis for this hierarchy in exocytosis is still poorly understood but recent data suggest that different combinations of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), vesicle-associated membrane proteins (VAMPs), 23-kDa synaptosome-associated protein (SNAP-23), and syntaxin 4 control the differential mobilization of neutrophil granule subsets (Mollinedo et al., 2006). Thus, a higher ability to form diverse SNARE complexes renders the granules more prone to exocytosis. In addition, the actin cytoskeleton also regulates exocytosis of neutrophil granules. The actin cytoskeleton acts to limit the rate and extent of granule exocytosis for secretory, gelatinase, and specific granules and blocks fusion of azurophil granules to the plasma membrane under basal and certain stimulated conditions (Jog

et al., 2007). Thus, these different mechanisms act to prevent uncontrolled secretion of destructive proteases from intracellular granules unless neutrophils are appropriately activated.

Once released from the neutrophils these proteases remain active although the extracellular environment is replete with highly specific endogenous inhibitors. Under normal circumstances,  $\alpha$ 1-proteinase inhibitor and elafin (also known as skin-derived antileukoprotease) antagonize the excessive extracellular activity of NE and PR3 whereas secretory leukocyte protease inhibitor (SLPI) is a potent inhibitor of CG and NE. In addition, CG can also be inhibited by  $\alpha$ 1-antichymotrypsin (Fitch et al., 2006; Reid and Sallenave, 2001; Rest, 1988). However, there are several mechanisms by which neutrophil serine proteases evade endogenous inhibitors. Some studies suggest that the high local concentration of proteases at the sites of inflammation can overwhelm the action of protease inhibitors (Owen and Campbell, 1995). Others suggest that extracellular proteases remain bound to neutrophil cell surface through their interactions with chondroitin sulfate and heparan sulfate proteoglycans (Campbell and Owen, 2007) and this interaction may render the proteases inaccessible to circulating, high-molecular weight inhibitors (Campbell et al., 2000; Owen et al., 1995). More recent studies however indicate that cell membrane-bound NE can be fully inhibited by  $\alpha$ 1-proteinase inhibitor when the cells are in suspension (Korkmaz et al., 2005) and perhaps it is the tight adhesion between neutrophils and their substrates that leads to the compartmentalization of the membrane-bound proteases, thus excluding natural circulating inhibitors.

### Neutrophil serine proteases in infections

Studies using loss-of-function mutations in neutrophil serine proteases indicate that mice deficient in CG are more susceptible to infections with Gram-positive *Staphylococcus aureus* (Reeves et al., 2002). NE-deficient mice on the other hand have impaired host defense against infections with *Escherichia coli* and *Klebsiella pneumoniae* (Belaouaj et al., 1998). NE kills *E. coli* by degrading their outer membrane protein A (OmpA) (Belaouaj et al., 2000). In addition, NE also cleaves the virulence factors of enterobacteria *Salmonella enterica*, *Shigella flexneri*, and *Yersinia enterocolitica* (Weinrauch et al., 2002). Moreover NE and CG are important for the antifungal activity against *Candida albicans* and *Aspergillus fumigatus* (Reeves et al., 2002; Tkalcevic et al., 2000). Recently, a novel mechanism has been described where stimulated neutrophils release serine proteases and chromatin to form structures called neutrophil extracellular traps (NETs) that capture and kill pathogens (Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2007). In contrast, several studies suggest that neutrophil serine proteases may also interfere with the host normal defense and promote viral infection. Indeed CG has been shown to inhibit the host's ability to clear *Pseudomonas aeruginosa* from the lung, as indicated by a reduction in bacterial load recovered from infected CG-deficient mice (Sedor et al., 2007). NE on the other hand facilitates reovirus infection in U937 cells (Golden and Schiff, 2005) and CG increases the susceptibility of macrophages to acute human immunodeficiency virus type 1 (Moriuchi et al., 2000), thus raising the possibility that neutrophil serine protease release during inflammation may actually exacerbate certain infections by promoting viral spread. In summary, loss-of-function mutations in the mouse system reveal a prominent role for neutrophil serine proteases in the clearance of microorganisms. However, direct comparisons between mice and men are difficult, as murine neutrophils do not contain the broad-spectrum antimicrobial peptides defensins that are abundant in the human system (Eisenhauer and Lehrer, 1992).

### Neutrophil serine proteases in human diseases

Although deficiencies in specific neutrophil serine proteases have not been described in humans, relative deficiencies of these enzymes are associated with distinct disorders. Chediak-

Higashi syndrome is a disease characterized by recurrent bacterial infections, bleeding defects, and partial albinism (Holt et al., 2006). Mutations in these patients have been mapped to the CHS or “Lyst” protein that is believed to function in membrane interactions and vesicle transport (Ward et al., 2000). Neutrophils from patients and the beige mice with the same mutation form giant granules with a severe defect in exocytosis and significantly reduced serine protease activity. It is believed that the defect in granule secretion and the reduced protease activity may in part contribute to the increased susceptibility to infections. Loss-of-function mutations in DPPI leading to defects in neutrophil serine proteases have also been identified in patients with Papillon-Lefevre syndrome (PLS) (Hart et al., 2000; Toomes et al., 1999). Neutrophils from these patients have minimal residual activities in all three serine proteases (de Haar et al., 2004; Pham et al., 2004). Loss of neutrophil serine protease activity may also affect the processing of human 18-kDa cationic antimicrobial protein (hCAP18) to the antimicrobial peptide LL-37 (Sorensen et al., 2001), which may also potentially explain the susceptibility of PLS patients to periodontal diseases (de Haar et al., 2006). However, not all patients with PLS have defective antimicrobial activity against common microorganisms (Pham et al., 2004), indicating that human neutrophils do not rely solely on serine proteases to kill invading pathogens.

Antineutrophil cytoplasmic antibodies (ANCA) are diagnostic markers found in several forms of small vessel vasculitis, including Wegener’s granulomatosis, microscopic polyangiitis, and Churg-Strauss syndrome. ANCA are directed primarily against myeloperoxidase (MPO, another major protein found in azurophil granules) and PR3 (Bosch et al., 2006; Kallenberg et al., 2006). ANCA have been shown to activate cytokine-primed neutrophils to release ROS and pro-inflammatory cytokines *in vitro* and anti-MPO ANCA directly induce pauci-immune glomerulonephritis and systemic vasculitis *in vivo* (Xiao et al., 2002). Although anti-PR3 ANCA are found in 90% of the cases of Wegener’s granulomatosis and they activate neutrophils *in vitro*, it is unclear whether they are directly pathogenic *in vivo* (Pfister et al., 2004).

Other disorders mediated by neutrophil serine proteases include the hereditary neutropenia. Cyclic neutropenia (CN) and severe congenital neutropenia (SCN) are diseases characterized by maturation arrest of myelopoiesis leading to neutropenia. The molecular basis of CN and SCN has been identified as heterozygous mutations in the gene *ELA2* encoding NE (Horwitz et al., 2007). Recent observations indicate that mutations in *ELA2* disrupt normal intracellular trafficking of NE, leading to the accumulation of mutant NE protein intracellularly and resulting in apoptosis through the induction of the unfolded protein response (Kollner et al., 2006).

## Neutrophil serine proteases in the modulation of chemokines and cytokines

Growing evidence suggests that neutrophil serine proteases are closely involved in the regulation of chemokine and cytokine bioavailability. Indeed several studies have shown that neutrophil serine proteases proteolytically modify the activity of several chemokines and provide an alternative mechanism to convert cytokine precursors into their active forms. Chemokines are a family of small chemoattractant proteins that play an important role in recruiting leukocytes to the sites of inflammation. Studies have shown that limited truncation of chemokines by enzymatic proteolysis can enhance their activity and stability. In fact, N-terminal proteolytic modification of CXC-chemokine ligand 8 (CXCL8, also known as IL-8) by PR3 (Padrines et al., 1994) and CXCL5 (also known as ENA-78) by CG (Nufer et al., 1999) regulate their biological activity as the truncated variants have higher potency and efficiency for neutrophil chemotaxis than the full-length forms. Likewise, the N-terminal processing of CCL15 (also known as MIP-1 $\delta$ ) by CG leads to significant increased potency in its ability to induce calcium fluxes and chemotactic activity on monocytes (Richter et al.,



2005). CG also converts connective tissue-activating peptide III (CTAP-III) into CXCL7 (also known as neutrophil-activating peptide 2 or NAP-2) by limited proteolysis (Schiemann et al., 2006). In addition, CG and NE convert prochemerin to active chemerin, a novel chemoattractant factor that specifically attracts antigen-presenting cells, through the removal of a C-terminal peptide (Wittamer et al., 2005). Human NE and CG cleave the alternatively spliced form of CCL23 by both N-terminal and C-terminal processing, producing a CC chemokine “body” that can engage two distinct chemokine receptors with high affinity (Miao et al., 2007). In contrast, several studies have also shown that proteolytic processing of chemokines by serine proteases can result in decreased activity. CG digests CCL5 (also known as RANTES) into a variant lacking three N-terminal residues and exhibiting lower chemotactic activity (Lim et al., 2006). Proteolysis of CCL3 (also known as MIP-1 $\alpha$ ) by all three neutrophil serine proteases (Ryu et al., 2005) and proteolysis of CXCL12 (also known as SDF-1 $\alpha$ ) and its cognate receptor CXCR4 by NE and CG (Delgado et al., 2001; Rao et al., 2004; Valenzuela-Fernandez et al., 2002) abrogate their chemotactic activity. In addition to proteolytic modifications, studies also indicate that neutrophil serine proteases control the release of chemokines from neutrophils. Neutrophils themselves are a source of chemokines (Cassatella, 1995; Scapini et al., 2000) and the release of CXCL2 (also known as MIP-2) from neutrophils depends on the activity of CG (Raptis et al., 2005). The ability of neutrophil serine proteases to control chemokine release and bioactivity therefore suggests that they may play a crucial role in orchestrating the subsequent recruitment of different leukocyte subsets to the inflamed site.

Likewise, most of the important cytokines produced at the sites inflammation also require proteolytic processing to be released from an inactive precursor form. Interleukin-18 (IL-18), originally named interferon-gamma (IFN- $\gamma$ )-inducing factor, is constitutively secreted by a variety of cells as a pro-IL-18 precursor that is activated by caspase 1 (Ghayur et al., 1997; Gu et al., 1997). However, stimulation of epithelial cells with IFN- $\gamma$  in the presence of PR3 and lipopolysaccharide (LPS) leads to the release of biologically active mature IL-18, independent of caspase 1 (Sugawara et al., 2001). In contrast, it has been shown that mature IL-18 released by neutrophils can be proteolysed by NE leading to diminished biological activity (Robertson et al., 2006).

Tumor necrosis factor alpha (TNF- $\alpha$ ) exists as a proform bound to the surface membrane and is proteolytically activated by the membrane-bound metalloproteinase TNF- $\alpha$  converting enzyme (TACE) (Black et al., 1997). Similarly, pro-IL-1 $\beta$  is converted to its active, soluble form by caspase 1 (also known as interleukin-1 converting enzyme or ICE) (Thornberry et al., 1992). However, studies indicate that serine proteases may alternatively process TNF- $\alpha$  and IL-1 $\beta$ . It has been shown that serine protease inhibitors suppress the release of TNF- $\alpha$  from the surface of activated macrophages (Armstrong et al., 2006; Scuderi, 1989). Moreover, pre-treatment with a serine protease inhibitor leads to suppression of TNF- $\alpha$  release *in vivo* and protects mice against the effects of LPS (Wendel, 1990). *In vitro* studies confirm that PR3 processes the cytokine precursor to generate a biologically active form of TNF- $\alpha$  (Coeshott et al., 1999; Robache-Gallea et al., 1995). In contrast, the effect of CG and NE on TNF- $\alpha$  processing is controversial. Some studies indicate that CG and NE degrade TNF- $\alpha$ , resulting in a loss of activity (Scuderi et al., 1991). Others suggest that at the appropriate concentrations CG and NE can process the membrane-bound TNF- $\alpha$  into a soluble and biologically active form (Mezyk-Kopec et al., 2005). Although the enzymatic activity of PR3 closely resembles that of NE, only PR3 has been shown to convert the precursor of IL-1 $\beta$  into its biologically active form (Coeshott et al., 1999). The fact that there are converting-enzyme-independent pathways for TNF- $\alpha$  and IL-1 $\beta$  release and that IL-1 $\beta$  production is not entirely abolished in the absence caspase 1 (Fantuzzi et al., 1997) suggests that alternative processing of these cytokines by neutrophil serine proteases may be operative at sites of inflammation.

IL-32 is a pro-inflammatory cytokine originally found as a transcript termed NK4 in activated natural killer cells and T lymphocytes (Dahl et al., 1992). IL-32 expression is increased in a variety of inflammatory diseases, including rheumatoid arthritis, crohn's disease, psoriasis, and chronic obstructive pulmonary disease (Dinareello and Kim, 2006). It has been shown that PR3 is a binding protein for IL-32 and limited proteolysis of IL-32 by PR3 enhances the activity of the cytokine (Novick et al., 2006). In contrast CG, NE, and PR3 have all been shown to degrade and inactivate IL-6 at sites of inflammation (Bank et al., 2000). Since IL-6 has both pro- and anti-inflammatory effects (Tilg et al., 1997), it is unclear whether IL-6 inactivation has limiting or pro-inflammatory consequences. Thus, neutrophil serine proteases can fine-tune the local inflammatory response by modulating the biological activity of chemokines and cytokines through limited proteolysis. In some cases proteolysis enhances biological activity. In others, proteolysis leads to inactivation and may provide a negative feedback mechanism to terminate immunostimulating signals.

### Neutrophil serine proteases in growth factor regulation

Mucus hypersecretion is a prominent feature of chronic inflammatory lung diseases and stimulation of the epidermal growth factor receptor (EGFR) has been shown to be a major cause of mucus secretion (Nadel, 2007). NE promotes mucous cell hyperplasia and MUC5AC mRNA expression by modulating the cleavage of the membrane-bound transforming growth factor alpha (TGF- $\alpha$ ), releasing a soluble form that binds to and activates EGFR (Kohri et al., 2002; Voynow et al., 2004). The TGF- $\alpha$  soluble form can also be released from the surface membrane by TACE, which is activated by ROS (Nadel, 2007). NE is also thought to activate EGFR through an indirect pathway involving protein kinase C (PKC) activation and ROS production that in turn activate TACE, leading to the cleavage of pro-TGF- $\alpha$  into its active soluble form (Shao and Nadel, 2005). In addition, TGF- $\alpha$  liberated by NE also induces keratinocyte proliferation via EGFR activation, suggesting that NE may play a role in epidermal proliferation (Meyer-Hoffert et al., 2004). The fact that NE activity is elevated in psoriatic lesions suggests that it may be a relevant stimulus for epidermal proliferation in this inflammatory disease (Wiedow et al., 1995). Transforming growth factors, such as TGF- $\beta$ , exist in latent form complexed with different binding proteins. The latent form of TGF- $\beta$  consists of the mature TGF- $\beta$  homodimer, the latency-associated protein (LAP), and a latent TGF-beta-binding protein (LTBP), which mediates the association of TGF- $\beta$  to the extracellular matrix. Through the proteolysis of LTBP, NE releases the active growth factor and therefore contributes to the tissue remodeling that accompanies inflammation in the lung (Taipale et al., 1995). NE has also been shown to stimulate the release of TGF- $\beta$  through a myeloid differentiation primary-response gene 88 (MyD88)/IL-1 receptor-associated kinase (IRAK)/nuclear factor  $\kappa$ B (NF- $\kappa$ B) pathway from airway smooth muscle cells, suggesting another mechanism by which NE can cause airway mucus hypersecretion (Lee et al., 2006). The role of NE in growth factor release was also confirmed *in vivo* where intra-tracheal instillation of elastase into mice leads to a time-dependent increase in the active form of TGF- $\beta$ 1 in the bronchoalveolar lavage (Buczek-Thomas et al., 2004).

### Neutrophil serine proteases in the activation of cell surface receptors

In addition to the proteolytic modulation and release of cytokines and growth factors, neutrophil serine proteases also contribute to the inflammatory response through the specific activation of cell surface receptors. Integrins are a large family of cell surface receptors that mediate the interaction between cells and the environment. NE has previously been shown to proteolytically activate the platelet integrin  $\alpha$ IIb $\beta$ 3 through limited cleavage of the carboxyl terminus of the  $\alpha$ IIb subunit heavy chain, a process that enhances platelet aggregation (Si-Tahar et al., 1997). Recently the presence of neutrophil serine protease CG on the surface of cells has been shown to modulate neutrophil spreading, clustering of integrins,

which lead to optimal neutrophil effector functions (Raptis et al., 2005). The action of CG requires its proteolytic activity but does not involve direct cleavage of integrins. The mechanism by which CG modulates neutrophil spreading is unclear; however, it has been shown that shedding of CD43 (also known as leukosialin) is required for neutrophils to fully spread (Nathan et al., 1993). Furthermore, it has been shown that NE cleaves the ectodomain of CD43 (Remold-O'Donnell and Parent, 1995). Whether CG also cleaves CD43 remains to be studied.

On the other hand, studies indicate that integrins may provide binding sites for neutrophil serine proteases on the cell surface. Cai and colleagues have shown that NE binds directly to integrin CD11b/CD18 (CR3, Mac-1) and regulates integrin-mediated cellular attachment and detachment (Cai and Wright, 1996). Others also report that PR3 can co-immunoprecipitate with CD11b and Fc gamma receptor IIIb (FcγRIIIb) (David et al., 2005; David et al., 2003). However, recent evidence indicates that neutrophil serine proteases bind to proteoglycans on the surface of cells in a saturable and reversible manner (Campbell and Owen, 2007). Rather than being contradictory, these studies suggest that serine proteases likely bind to proteoglycans on the cell surface and can be found in the same complex that includes Fc gamma receptor and CD11b/CD18 (Fridlich et al., 2006).

Protease-activated receptors (PARs) belong to a family of G-protein-coupled receptors that undergo N-terminal cleavage to reveal a tethered ligand. The role of PARs in inflammation is well documented (Coughlin and Camerer, 2003). It has been shown that CG directly activates PAR4 on platelets and this activation may mediate neutrophil-platelet interactions at the sites of inflammation (Sambrano et al., 2000). In contrast, the role of neutrophil serine proteases in the activation of other PARs is more controversial. Activation of PAR2 has been shown to be both pro- and anti-inflammatory (Chignard and Pidard, 2006) and neutrophil serine proteases can simultaneously activate and disarm this receptor. Uehara *et al.* have shown that *in vitro*, all three neutrophil serine proteases activate PAR2, which then induces the release of the chemokines CXCL8 and CCL2 (Uehara et al., 2002; Uehara et al., 2003; Uehara et al., 2004). On the other hand, these proteases have also been shown to disarm PAR2 through different cleavage sites (Dulon et al., 2003). Likewise, NE, CG, and PR3 cleave PAR1 at distinct sites from thrombin and inhibit subsequent thrombin-induced activation of the receptor (Renesto et al., 1997). Thus it is clear that *in vitro* neutrophil serine proteases can display opposite effects on PARs but the precise role of these enzymes in the activation of these receptors *in vivo* requires further studies.

Previous studies have shown that subcutaneous injection of CG into mice induces the recruitment of monocytes and neutrophils, suggesting that CG is a potent chemoattractant *in vivo* (Chertov et al., 1997). Neutrophil serine protease inhibitors abolish the chemotactic activity of CG, indicating a requirement for its enzymatic activity. In addition, alpha-1-antichymotrypsin and antibodies to CG inhibit the bacterial chemotactic peptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) and C5a-induced chemotaxis, indicating that CG also modulates leukocyte chemotaxis *in vitro* (Lomas et al., 1995). The fact that CG inhibits the action of fMLP suggests that the two molecules may share a common receptor. Indeed, Sun *et al.* recently show that the chemotactic activity of CG is mediated through the G-protein-coupled formyl peptide receptor (FPR). CG binds to FPR, inducing calcium fluxes and translocation of protein kinase C- $\zeta$  (PKC $\zeta$ ), a step that is essential for CG-mediated chemotactic activity (Sun et al., 2004).

NE has been shown to induce the release of IL-8 (Devaney et al., 2003), cathepsin B, and matrix metalloproteinase 2 (MMP2) (Geraghty et al., 2007) through a MyD88/IRAK/TNF-receptor-associated factor 6 (TRAF-6)-dependent pathway that also implicates toll-like receptor 4 (TLR4) (Devaney et al., 2003; Walsh et al., 2001). Neutralizing antibodies to TLR4 and a



dominant negative IRAK-4 abrogate the NE-induced upregulation of cathepsin B and MMP2 (Geraghty et al., 2007), further confirming the involvement of this pathway; but how NE activates TLR4 is unknown.

## Neutrophil serine proteases in the shedding of surface receptors

Proteolytic shedding of cell surface receptors provides a mechanism for controlling cytokine bioactivity at the sites of inflammation. Shedding of receptors renders the cells less responsive to the cytokine effects and provides a source of soluble proteins in the body fluids. Porteu *et al.* show that *in vitro* stimulation of neutrophils with chemotactic factors, such as fMLP and C5a, and pharmacological agonists, such as calcium ionophores, leads to the loss of TNF receptors from the cell surface (Porteu and Nathan, 1990). This TNF receptor sheddase was identified as NE (Porteu et al., 1991). NE acts on the p75 TNF receptor, releasing a 32-kDa soluble fragment, while the 55-kDa TNF receptor is completely resistant to the activity of NE. The shedding of TNF receptor by NE may provide an additional mechanism for the control of cellular response to TNF at sites of inflammation.

Urokinase-type plasminogen activator receptor (CD87) is a glycosylphosphatidylinositol (GPI)-anchored protein that participates in cell migration and tissue remodeling. Cleavage of CD87 by CG and NE reduces the ability of the cell to bind urokinase, leading to reduced pericellular proteolysis and thus affecting cellular migration in the context of tissue remodeling. Moreover, proteolysis of CD87 generates soluble chemotactic fragments that can influence the inflammatory response (Beaufort et al., 2004).

CD14 is the main bacterial LPS cell surface receptor. Recognition of LPS occurs mainly through the TLR4-MD2-CD14 complex (Miller et al., 2005). CG and NE released by neutrophils cleave CD14 from the cell surface in a time- and concentration-dependent manner (Le-Barillec et al., 1999; Le-Barillec et al., 2000; Nemoto et al., 2000). Proteolysis of CD14 suppresses LPS-induced cell activation and CXCL8 production, suggesting that these neutrophil serine proteases might provide a negative feedback mechanism to downmodulate the inflammatory response. On the other hand, cleavage of CD14 by NE has been shown to reduce the ability of macrophages to recognize apoptotic cells (Henriksen et al., 2004). Moreover, NE can also disrupt apoptotic cell phagocytosis by cleaving the phosphatidylserine receptor (Vandivier et al., 2002). Since the resolution of inflammation requires the recognition and phagocytic removal of apoptotic cells by macrophages, impaired apoptotic cell recognition may contribute to prolonged inflammation.

Neutrophils and complement are crucial components of the innate immunity and the interaction of complement and neutrophils modulates the inflammatory response. Complement receptor 1 (CR1, also known as CD35 or C3b/C4b receptor) is a transmembrane protein of many hematopoietic cells. CR1 is cleaved from the surface of neutrophils by NE and NE-like activity, releasing the functional soluble fragment sCR1 that inhibits complement activation and prevents further complement-mediated injury (Hamacher et al., 1998; Sadallah et al., 1999). In addition, previous studies also show that membrane bound CG can generate active fragments of C3 (Maison et al., 1991) and NE cleaves C5 in the presence of C6, leading to the formation of an active C5b6-like complex that lyse non-sensitized cells upon the addition of the terminal components C7, C8, and C9 (Vogt, 2000). In addition, the cleavage of C5 by NE modulates the relative yield of C5a-like fragment, a chemotactic peptide. In contrast, NE and CG have been shown to inhibit the responsiveness of neutrophils to C5a, thus potentially down-regulating the acute inflammatory response (Tralau et al., 2004).

Neutrophil serine proteases have also been implicated in the release of the cell surface-bound ligand-binding chains of IL-6 receptor (Bank et al., 2000). The soluble IL-6 receptor subunits retain ligand-binding capacity and therefore may influence cellular responsiveness to IL-6. In

addition, CG and NE can release soluble CD23 fragments that stimulate monocytes to produce oxidative burst and pro-inflammatory cytokines without any co-stimulatory signals (Brignone et al., 2001). In summary, these studies suggest that neutrophil serine proteases have the potential to activate and shed many receptors. However, the consequences of these activities *in vivo* remain a matter of further investigation.

## Neutrophil serine proteases in cell adhesion and transmigration

Recruitment of neutrophils to sites of inflammation requires firm adhesion and transmigration of the cells across the endothelium. Studies have previously shown that inhibition of NE alone was sufficient to reduce neutrophil infiltration (without affecting neutrophil adherence) after ischemia/reperfusion in skeletal muscle (Carden and Korthuis, 1996). Also, incubation of neutrophils with a combined inhibitor of CG and NE markedly reduces the adhesion of neutrophils to various surfaces (Delyani et al., 1996; Murohara et al., 1995). Due to their proteolytic properties, it was originally thought that neutrophil serine proteases degrade components of the extracellular matrix, thus allowing cells to crawl through the gaps. Studies using serine protease inhibitors subsequently show that neutrophil transendothelial migration *in vitro* does not require serine proteases (Mackarel et al., 1999). The generation of serine protease-deficient mice further confirms the fact that neutrophil serine proteases are not required for *in vitro* chemotaxis and certain *in vivo* inflammatory models. (Hirche et al., 2004; MacIvor et al., 1999; Raptis et al., 2005). However, more recently Young *et al.*, using NE-deficient mice, demonstrate that NE in fact plays a non-redundant role in zymosan-induced leukocyte firm adhesion and transmigration (Young et al., 2004). The defect in transmigration in NE-deficient mice is accompanied by reduction in the levels of pro-inflammatory chemokines and cytokines. Furthermore the authors show that NE cooperates with platelet/endothelial–cell adhesion molecule 1 (PECAM-1) and alpha6 integrins in mediating neutrophil migration through the perivascular environment (Wang et al., 2005). CG and NE have also been shown to cleave vascular endothelial cadherins (E-cadherins) (Hermant et al., 2003; Mayerle et al., 2005), intercellular adhesion molecule 1 (ICAM-1) (Champagne et al., 1998; Robledo et al., 2003), and vascular cell adhesion molecule 1 (VCAM-1) (Levesque et al., 2001; Xu et al., 2005). Since these adhesion molecules play important role in cell-cell contact formation, their extracellular cleavage may induce formation of gaps through which neutrophils transmigrate. Thus how neutrophil serine proteases actually participate in leukocyte adhesion and transmigration remains controversial.

## Neutrophil serine proteases and apoptosis

The role of granzymes in the induction of apoptosis is well established. However, emerging data suggest that neutrophil serine proteases also have caspase-like activity and can induce cellular apoptosis at the sites of inflammation. NE and PR3 entry into endothelial cells coincides with the sustained activation of proapoptotic-signaling events through extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) pathways (Preston et al., 2002). Inhibition of JNK blocks PR3-induced apoptosis; likewise, inhibition of p38 MAPK blocks PR3- and NE-induced apoptosis, indicating a direct role for serine proteases in controlling cell viability. NE and PR3 directly cleave NF- $\kappa$ B (Preston et al., 2002) and PR3 can cleave p21 (Waf1/Cip1/Sdi1) to induce endothelial cell apoptosis (Pendergraft et al., 2004) and modulate differentiation of monocytic cell line (Dublet et al., 2005). In addition, incubation of epithelial cells with NE leads to decreased mitochondrial membrane potential, release of cytochrome c to the cytosol, and cleavage of caspases 9 and 3, indicating that neutrophil serine proteases may induce apoptosis through the alterations of mitochondrial membrane permeability (Ginzberg et al., 2004). These studies suggest that neutrophil serine proteases might play specific functions in cell survival during the course of inflammation.

## Neutrophil serine proteases in lymphocyte activation

CG has been shown to bind to and activate lymphocytes and modulate antigen-specific humoral responses in mice (Hase-Yamazaki and Aoki, 1995; Tani et al., 2001; Yamazaki and Aoki, 1997). Co-injection of CG into mice immunized with an antigen results in significant increase in antibody production. *In vitro* restimulation of lymph node cells from immunized mice indicates that CG induces a marked increase in IFN- $\gamma$  production and antigen-specific production of IL-4. In addition, CG enhances the cytotoxicity of natural killer cells in a dose-dependent manner (Yamazaki and Aoki, 1998). Thus CG may act as an adjuvant to modulate the immune response.

## Concluding remarks

Neutrophil serine proteases have emerged as important regulators of the immune response and the generation of protease-deficient mice confirms their critical role in the perpetuation of inflammation. Studies over the last several years demonstrate that, rather than being simply degradative intracellular enzymes, neutrophil serine proteases maintain their enzymatic activity in the extracellular environment and may participate in the regulation of various biological pathways (Table 1 and Figure 1). However, most of the regulatory pathways summarized in this review come from *in vitro* studies. The true extent of neutrophil serine proteases involvement in these regulatory networks in specific diseases and under pathological conditions requires further studies. Nonetheless, these studies suggest that *in vivo* antagonism of serine protease activity may provide new therapeutic strategies in inflammatory diseases. Indeed, attempts have been made to suppress the activity of NE in hereditary emphysema (Dirksen et al., 1999; Doring, 1999), chronic obstructive pulmonary disease (COPD) (Luisetti et al., 1996; Ohbayashi, 2002), cystic fibrosis (Cantin et al., 2006; Doring, 1999; Martin et al., 2006), and the management of acute lung injury (Hoshi et al., 2005; Ono et al., 2007; Zeiher et al., 2004), all with varying results. To date, no inhibitors of serine proteases have been approved for use in patients as many practical problems associated with the synthesis and delivery of these agents still remain [Reviewed in (Chughtai and O'Riordan, 2004)].

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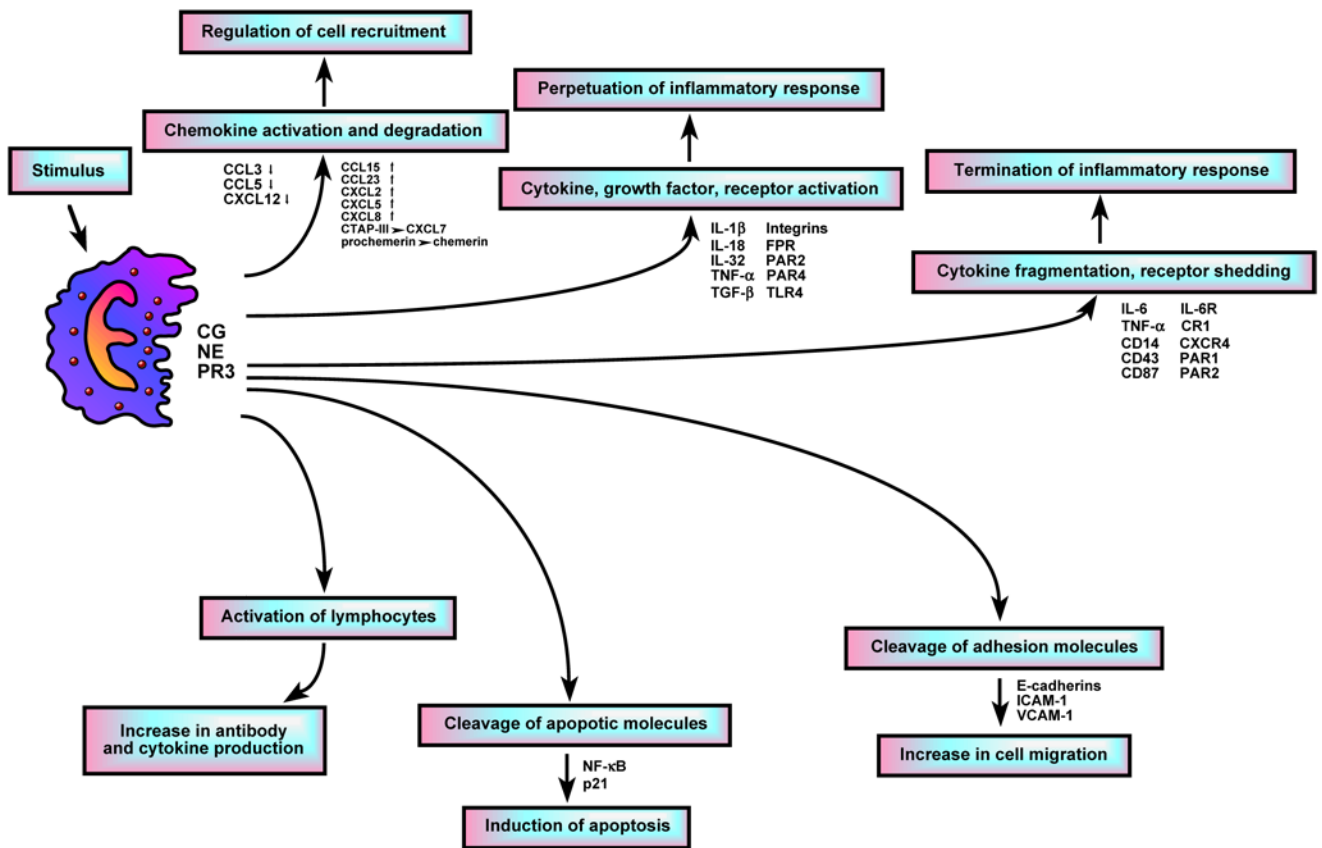
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## Abbreviations

- CG, cathepsin G  
 NE, neutrophil elastase  
 PR3, proteinase 3  
 AZU, azurocidin  
 AP3, adaptor protein 3  
 DPPI, dipeptidyl peptidase I  
 SNAREs, N-ethylmaleimide-sensitive factor attachment protein receptors  
 VAMP, vesicle-associated membrane protein  
 SNAP, synaptosome-associated protein  
 SLPI, secretory leukocyte protease inhibitor  
 NET, neutrophil extracellular traps  
 CHS, Chediak-Higashi syndrome  
 PLS, Papillon-Lefevre syndrome  
 ANCA, antineutrophil cytoplasmic antibody  
 ROS, reactive oxygen species  
 CN, cyclic neutropenia  
 SCN, severe congenital neutropenia  
 MPO, myeloperoxidase  
 ENA78, epithelial neutrophil activating peptide 78  
 MIP, macrophage inflammatory protein  
 CTAP, connective tissue-activating peptide  
 NAP, neutrophil-activating peptide  
 RANTES, regulated upon activation, normal T cell expressed and secreted  
 SDF1, stromal cell derived factor 1  
 IFN- $\gamma$ , interferon gamma  
 TNF, tumor necrosis factor  
 TACE, TNF- $\alpha$  converting enzyme  
 ICE, IL-1 converting enzyme  
 LPS, lipopolysaccharide  
 IL, interleukin  
 EGFR, epidermal growth factor receptor  
 TGF, transforming growth factor  
 PKC, protein kinase C  
 LAP, latency-associated protein  
 LTBP, latent-TGF-beta-binding protein

IRAK, IL-1 receptor-associated kinase  
MyD88, myeloid differentiation primary-response gene 88  
NF- $\kappa$ B, nuclear factor  $\kappa$ B  
TRAF, TNF-receptor-associated factor  
PAR, protease-activated receptor  
FPR, formyl peptide receptor  
fMLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine  
TLR, toll-like receptor  
PECAM, platelet/endothelial cell adhesion molecule  
ICAM, intercellular adhesion molecule  
VCAM, vascular cell adhesion molecule  
ERK, extracellular signal-regulated kinase  
JNK, Jun N-terminal kinase  
MAPK, mitogenactivated protein kinase  
APC, antigen-presenting cell  
GPI, glycosphosphatidylinositol  
hCAP18, human 18-kDa cationic antimicrobial protein  
COPD, chronic obstructive pulmonary disease



**Figure 1.** Potential mechanisms by which extracellular neutrophil serine proteases may be involved in the regulation of the inflammatory process and the immune response.



**Table 1**

Effects of neutrophil serine proteases on selected biological targets

Targets	Proteases	Potential biological effects
<u>Chemokines</u>		
CCL3	CG, NE, PR3	Abrogation of chemotactic activity
CCL5	CG	N-terminal truncation and ↓ chemotactic activity
CCL15	CG	N-terminal truncation and ↑ chemotactic activity
CCL23	CG, NE	N- and C-terminal truncation and ↑ affinity to receptors
CXCL2	CG	↑ release from neutrophils
CXCL5	CG	N-terminal truncation and ↑ chemotactic activity
CXCL7	CG	Conversion of CTAP-III to CXCL7
CXCL8	PR3	N-terminal truncation and ↑ chemotactic activity
CXCL12	CG, NE	Abrogation of chemotactic activity
Chemerin	CG, NE	Conversion of prochemerin to chemerin
<u>Cytokines</u>		
IL-1β	PR3	Conversion of pro-IL-1β to soluble, active IL-1β
IL-6	CG, NE, PR3	Degradation and inactivation
IL-18	PR3, NE	Conversion of pro-IL-18 to mature IL-8 by PR3; proteolysis and abrogation of activity by NE
IL-32	PR3	Increase in activity
TNF-α	PR3, CG, NE	Activation of membrane-bound pro-TNF-α by CG, NE, and PR3; degradation by CG and NE
<u>Growth factors</u>		
TGF-β	NE	Release of membrane-bound TGF-β to activate EGFR; cleavage of LTBP to release latent TGF-β
<u>Antimicrobial peptides</u>		
LL-37	PR3	Conversion of hCAP18 to LL-37
<u>Receptors</u>		
αIIb3 integrin	NE	↑ platelet aggregation
CD14	CG, NE	↓ LPS-induced cell activation; ↓ ability of macrophages to recognize apoptotic cells
CD43	NE	↑ cell spreading
CD87	CG, NE	↓ urokinase binding; generation of chemotactic fragments
CR1	NE	Release of sCR1 and inhibition of complement activation
CXCR4	NE	N-terminal proteolysis, ↓ CXCL12 binding
FPR	CG	Induction of PKC $\zeta$ translocation, Ca <sup>+</sup> flux, and chemotaxis
IL-6R	CG	Inactivation
PAR1	CG, NE, PR3	Inactivation
PAR2	CG, NE, PR3	Activation and inactivation of the receptor through distinct cleavage sites
PAR4	CG	Activation
TLR4	NE	Activation through a MyD88/IRAK/TRAF-6-dependent pathway
TNFR	NE	Inactivation of the p75 receptor
<u>Adhesion molecules</u>		
E-cadherins	CG, NE	↑ neutrophil transmigration
ICAM-1	CG, NE	↑ neutrophil infiltration
VCAM-1	CG, NE	↑ mobilization of hematopoietic cells
<u>Apoptotic molecules</u>		
NF-κB	NE, PR3	Induction of apoptosis
p21	PR3	Induction of apoptosis