Proteases and antiproteases in chronic neutrophilic lung disease - relevance to drug discovery.

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Summary. Chronic inflammatory lung diseases such as cystic fibrosis and emphysema are characterised by higher than normal levels of pulmonary proteases. Whilst these enzymes play important roles such as bacterial killing, their dysregulated expression or activity can adversely impact on the inflammatory process. The existence of efficient endogenous control mechanisms that can dampen or halt this overexuberant protease activity *in vivo* is essential for the effective resolution of inflammatory lung disease. The function of pulmonary antiproteases is to fulfil this role. Interestingly, in addition to their antiprotease activity, protease inhibitors in the lung also often possess other intrinsic properties that contribute to microbial killing or termination of the inflammatory process. This review will outline important features of chronic inflammation that are regulated by pulmonary proteases and describe the various mechanisms by which antiproteases attempt to counterbalance exaggerated protease-mediated inflammatory events. These proteases, antiproteases and their modifiers represent interesting targets for therapeutic intervention.

Keywords: protease, antiprotease, lung, cystic fibrosis, chronic obstructive pulmonary disease (COPD), neutrophil elastase, alpha-1 antitrypsin, secretory leucoprotease inhibitor, elafin

Abbreviations: A disintegrin a metalloprotease, ADAM; alpha-1 antitrypsin, A1AT; arginine-threonine-arginine, RTR; cystic fibrosis, CF; chronic obstructive pulmonary disease, COPD; epidermal growth factor, EGF; EGF receptor, EGFR; epithelial lining fluid, ELF; human cathelicidin precursor-18, hCAP18; human beta defensin, HBD; interleukin-1 type 1 receptor, IL-1RI; IL-1R-associated kinase, IRAK; lipoteichoic acid, LTA; lipopolysaccharide, LPS; MyD88 adaptor like protein, Mal; matrix metalloprotease, MMP; monocyte-neutrophil elastase inhibitor, MNEI; myeloid differentiation factor 88, MyD88; neutrophil elastase NE; NET, neutrophil extracellular trap; proline-gylcine-proline, PGP; secretory leucoprotease inhibitor, SLPI; tissue growth factor-α, TGFα; tissue inhibitor of MMP, TIMP; toll-like receptor, TLR; TNFα converting enzyme, TACE/ADAM17; TNF receptor-associated factor, TRAF; whey acidic protein, WAP.

Proteinases (herein referred to colloquially as proteases) have a key role in the lung in health and disease. In the healthy lung proteases fulfil basic homeostatic roles and regulate processes such as regeneration and repair. Chronic inflammatory lung diseases are associated with higher than normal levels of proteases. Functionally this can positively impact on both infection and inflammation. However unless a perfect balance can be struck between the protective and harmful effects of pulmonary proteases by an appropriate antiprotease protective screen, damage can occur. Thus effective resolution of inflammation in the lung is associated not only with protease activity but also with appropriate antiproteolytic control mechanisms. Here we will focus on the mechanisms by which pulmonary proteases regulate innate immunity and the role of specific antiproteases in fine tuning these responses.

The principal classes of protease present in the human lung are the serine, cysteinyl, aspartyl and metalloproteases. These can function either intracellularly or extracellularly to regulate processes as diverse as tissue remodelling, mucin expression, neutrophil chemotaxis and bacterial killing. Members of these protease classes orchestrate a diverse range of changes with respect to infection and inflammation in the lung, with the serine protease neutrophil elastase (NE) occupying an important position at the apex of a specific protease hierarchy. NE has a number of important intrinsic proteolytic properties. However it can also directly control the inducible expression and biological properties of other pulmonary proteases. For example NE regulates expression of cathepsin B and MMP-2 in alveolar macrophages (Geraghty *et al.*, 2007b) and also activates proMMP-2, MMP7 and MMP-9 (Ferry *et al.*, 1997; Imai *et al.*, 1995; Shamamian *et al.*, 2001). Thus in addition to being a protease NE also behaves as a proinflammatory mediator. In certain circumstances NE also controls elegant signalling mechanisms regulating innate immunity (Bergin *et*

al., 2008; Devaney *et al.*, 2003; Kohri *et al.*, 2002; Nakamura *et al.*, 1992; Shao *et al.*, 2005a; Shao *et al.*, 2005b; Walsh *et al.*, 2001); its pluripotency distinguishes it as a unique factor controlling many aspects of infection and inflammation in the lung.

Neutrophil elastase

NE, as its name suggests, is a neutrophil-derived elastolytic protease. It is expressed as a 267 amino acid pre-proenzyme that is packaged in a processed and activated form in neutrophil primary (azurophilic) granules. Substrates of NE fall into many categories and include elastin and other extracellular matrix proteins, plasma proteins, cell surface receptors, cytokines protease inhibitors and proteases (Table 1). Other serine protease stored in the primary granules of neutrophils are proteinase 3 and cathepsin G. Similar to NE, these enzymes are released by activated and disintegrating neutrophils and are detectable at higher than normal levels in the airways during chronic inflammation (Witko-Sarsat *et al.*, 1999) (Goldstein *et al.*, 1986).

Whilst neutrophils play an important role in many inflammatory lung diseases, cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) are considered to be the classical chronic neutrophil-dominated diseases of the airways. The abundance of neutrophils in the CF and COPD lung generates a milieu rich in NE (Doring, 1994; Griese *et al.*, 2008). Neutrophil accumulation is believed to be due in part to their inability to effectively clear pathogens and thus accumulate and undergo secondary necrosis. This leads to the liberation of NE and other intracellular components (Griese *et al.*, 2008). Foreign organic molecules that have been phagocytosed by neutrophils are degraded by NE intracellularly. NE also contributes not only to the intracellular killing of Gram-negative bacteria by neutrophils but also,

once released extracellularly, can play a role in bacterial killing by comprising a key component of neutrophil extracellular traps (NETs). NETs are involved in host defense (Brinkmann *et al.*, 2004). They bind Gram-positive and Gram-negative bacteria and allow neutrophils to deliver high concentrations of serine proteases that degrade virulence factors and kill bacteria. Recently, bacterial virulence factors that counteract NETs have been identified. The mechanisms identified include the expression of DNAses that degrade the NET-backbone, expression of capsule which can reduce bacterial trapping and modulation of cell-surface charge (Buchanan *et al.*, 2006; Wartha *et al.*, 2007).

In addition to direct killing of microbes, NE has important roles in innate immunity and inflammation in the lung particularly in the processes of neutrophil recruitment and mucin gene expression (Bergin *et al.*, 2008; Shao *et al.*, 2005b). Interestingly both of these processes are controlled via similar mechanisms. NE also regulates the expression of other classes of proteases.

Hierarchy of protease expression

Bronchoalveolar lavage fluid sampled from individuals with chronic inflammatory lung disease almost invariably contains significant quantities of proteases. The primary families to be released into the extracellular space following cell activation are the serine, MMP and cysteinyl cathepsin groups. There is evidence demonstrating that NE, and possibly other serine proteases, can transcriptionally regulate expression of other classes of proteases. In human macrophages, for example, transcription of both MMP-2 and cathepsin B has been shown to be increased in response to NE in an NFκB-dependent manner (Geraghty *et al.*, 2007b). This is one mechanism contributing to the positioning of serine proteases at the apex of one

hierarchy of protease regulation. Toll-like receptor 4 (TLR4) has also been implicated in NE-induced expression of MMP-2 and cathepsin B however its precise role in NE-induced changes in gene expression is less clear in macrophages than in airway epithelial cells (see below). In addition to its ability to induce the transcription of specific proteases, NE and other serine proteases can also activate MMPs. For example NE, proteinase 3 and cathepsin G can activate the latent 72 kDa MMP-2 zymogen via membrane type I MMP (Ferry *et al.*, 1997; Imai *et al.*, 1995; Shamamian *et al.*, 2001). NE can also activate proMMP-7, MMP-9 and procathepsin B and members of the ADAM (a disintegrin a metalloprotease) and meprin families (Bergin *et al.*, 2008; Dalet-Fumeron *et al.*, 1993; Ferry *et al.*, 1997; Imai *et al.*, 1995; Kohri *et al.*, 2002).

Regulation of mucin production and neutrophil recruitment.

The epidermal growth factor receptor (EGFR, alternatively known as Erb1 or HER1) is a receptor tyrosine kinase that can regulate expression of mucin and IL-8 gene expression. In human airway epithelium EGFR forms homodimers or heterodimers with Erb2 or Erb3 in response to activation by a range of diverse stimuli, including NE (Bergin *et al.*, 2008; Holbro *et al.*, 2004; Kohri *et al.*, 2002). EGFR is directly activated by binding of the EGFR ligands epidermal growth factor (EGF), transforming growth factor-α (TGF-α), heparin binding (HB)-EGF, amphiregulin, betacellulin or epiregulin. These ligands are expressed as membrane-tethered proligands on airway epithelial cells, eosinophils, neutrophils, mast cells and macrophages and are released as bioactive molecules in a metalloprotease-dependent manner. Whilst TNF-α converting enzyme (TACE/ADAM17) was originally thought to be uniquely responsible for EGFR ligand generation it is now clear that there is

redundancy between TACE, other MMPs, ADAMs and the metzincin meprin-α in this regard (Choudry *et al.*, 1991; Merlos-Suarez *et al.*, 2001; Schlondorff *et al.*, 2001). To date several *cis*-acting enzymes including ADAM10, ADAM12, ADAM15, ADAM17, MMP2, MMP9 and meprin-α, amongst others, have been implicated in EGFR ligand generation (Ohtsu *et al.*, 2006).

In the airways NE has been shown to activate EGFR via generation of TGF-α. This event involves activation of TACE or meprin-α by NE and leads to intracellular signalling cascades that culminate in the enhanced expression of the mucin genes, MUC2 and MUC5AC, or the neutrophil chemokine IL-8. Hypersecretion of mucus is a common pathophysiological feature of CF and other inflammatory lung diseases. In CF, asthma and chronic bronchitis mucus obstruction of the airways contributes significantly to mortality and morbidity in these conditions. Consisting mostly of water and ions, mucus also comprises approximately 5% protein. It plays an important role in host defence by binding bacteria and ensuring their removal via the mucociliary escalator to the upper airways and oesophagus for expectoration or ingestion. To date nine MUC genes have been described in the lung – MUC1, 2, 4, 5AC, 5B, 7, 8, 13 and 19 (Chen *et al.*, 2004; Rose *et al.*, 2001). MUC2, 5AC and 5B are secreted, gel-forming mucins. MUC5AC represents the most prominent mucin in normal airway secretions and its expression is increased in nasal epithelium of individuals with CF and allergic rhinitis (Voynow *et al.*, 1998). In airway inflammation mucin gene expression can be activated by IL-9 via the human calcium-activated chloride channel, hCLCA1 (Hauber *et al.*, 2004) but also by NE via TACE and EGFR (Fischer *et al.*, 2002; Kohri *et al.*, 2002). Other stimuli that regulate MUC5AC or MUC2 expression via the TACE-EGFR pathway include cigarette

smoke, lipopolysaccharide (LPS) (Dohrman *et al.*, 1998; Shao *et al.*, 2004) and Gram-positive lipoteichoic acid (LTA) (Lemjabbar *et al.*, 2002).

Mechanisms regulating NE-induced IL-8 production.

IL-8 is a neutrophil chemokine and consequently represents a key factor present in the lungs during neutrophil-dominated inflammatory disease. Elevated levels of IL-8 are typically present in the bronchoalveolar lavage fluid of patients with COPD, emphysema and CF. Early studies identified a link between high concentrations of IL-8 and NE in the airways in CF (McElvaney *et al.*, 1992; Nakamura *et al.*, 1992). The mechanism by which this correlation exists has been studied in detail leading to the elucidation of the molecular mechanism by which NE can regulate the transcriptional induction of the IL-8 gene in airway epithelial cells. Preliminary investigations to determine how this may be occurring identified three important features of the regulatory cascade (Walsh *et al.*, 2001). Firstly in order for NE to induce IL-8 expression from airway epithelial cells it must retain its biological activity; inactivation of its protease activity with specific serine protease inhibitors abrogated the effect. Secondly, as the effect could be blocked using actinomycin D, NE was affecting the transcription of IL-8. Thirdly, and most surprisingly, NE was shown to induce IL-8 via the transcription factor NFκB in a manner that was dependent on MyD88, IRAK and TRAF6 – known transducers involved in TLR and interleukin-1 type 1 receptor (IL-1R) signalling. Subsequent studies revealed that a dominant-negative version of MyD88 (ΔMyD88) could inhibit the expression of multiple NFκB-dependent cytokines in response to NE. Furthermore the adaptor protein Mal, known to be involved in TLR2/TLR4 signalling, had similar inhibitory capacity as ΔMyD88 when expressed as an inactive transgene (Carroll *et al.*, 2005;

Greene *et al.*, 2005). Taken together these data implicated TLR2 and/or TLR4 in NE-induced IL-8 expression. Geraghty and colleagues (Geraghty *et al.*, 2007b) observed a similar phenomenon in macrophages in their studies on NE induction of MMP2 and cathepsin B. Others (Koff *et al.*, 2008) also recently provided evidence for a role for TLRs in EGFR-mediated signalling whilst Bergin et al demonstrated a direct association between EGFR and TLR4 in response to stimulation with NE (Bergin *et al.*, 2008). The mechanism by which NE regulates IL-8 expression in human bronchial epithelial cells is depicted in Figure 1.

Generation of bioactive molecules by pulmonary proteases

In addition to their roles in EGFR trans-activation MMPs have also recently been implicated as important factors regulating the expression of the novel proinflammatory chemotactic peptide proline-glycine-proline (PGP) in both CF and COPD. PGP is a breakdown product of the extracellular matrix protein collagen and shares sequence and structural homology with alpha chemokines (Weathington *et al.*, 2006). Indeed there is mounting evidence that fragments of a number of extracellular matrix proteins, including those derived from collagen and elastin, are important in regulating the recruitment of inflammatory cells to the lung. PGP (and N-acetylated PGP) has been shown to act as a neutrophil chemoattractant via CXC receptors 1 and 2 on neutrophils. PGP is generated from collagen via the combined activities of MMP-8, MMP-9 and the serine protease prolyl endopeptidase (Gaggar *et al.*, 2008; Lin *et al.*, 2008). In addition to higher than normal levels of NE in the CF and COPD lung, there is evidence that MMP-8, MMP-9 and prolyl endopeptidase are also elevated and that together these enzymes can degrade collagen *in vivo* to generate the PGP tripeptide. CF sputum has been shown to contain detectable levels of both PGP

and N-Ac-PGP. In addition to being chemotactic for human neutrophils, PGP has been linked to neutrophil superoxide production, alveolar enlargement and right ventricular hypertrophy which contribute to the pulmonary inflammatory manifestations of CF. Recent elegant studies have demonstrated that the corresponding tripeptide Arginine-Threonine-Arginine (RTR) can bind to PGP sequences, neutralize their effect and inhibit neutrophil infiltration in a model of COPD (van Houwelingen *et al.*, 2008). Furthermore, RTR completely inhibits PGP-induced lung emphysema assessed by changes in alveolar enlargement and right ventricular hypertrophy. Thus PGP antagonism via RTR is also likely to have therapeutic potential for CF. This represents another example of how protease interactions with structural proteins have an important effect on regulating innate immunity.

Pulmonary proteases and antimicrobial peptides

Important antimicrobial polypeptides of innate pulmonary host defense include lactoferrin, secretory leucoprotease inhibitor (SLPI), lysozyme, the defensins and the cathelicidin family (Zanetti, 2005). The human cathelicidin precursor protein, designated 18-kDa cationic antimicrobial protein (hCAP18) is expressed as a pro-protein composed of a conserved N-terminal pro-domain and a C-terminal antimicrobial peptide domain. In human neutrophils hCAP18 is processed to an active form by proteinase 3 to generate the cationic α-helical peptide LL-37 (Sørensen *et al.*, 2001; Zanetti *et al.*, 1995). Further proteolytic degradation of LL-37 can lead to the generation of peptide fragments with altered antimicrobial activity and reduced immunomodulatory potential. In addition to degradation of LL-37 a selection of pulmonary proteases have also been shown to be responsible for the degradation and

inactivation of other antimicrobial effector molecules, including human β-defensins 2 and 3, SLPI and lactoferrin (Rogan *et al.*, 2004; Taggart *et al.*, 2003; Taggart *et al.*, 2001). In particular the cysteinyl cathepsins B, L and S are implicated in cleavage and inactivation of innate immunity proteins *in vivo* during inflammatory lung disease.

Human β-defensins 1, 2, and 3 (HBD-1, -2, and -3) are antimicrobial peptides produced by epithelial cells lining the respiratory tract. They are active against Gram-positive and Gram-negative bacteria. In CF the antimicrobial activity of defensins is compromised therefore predisposing to bacterial colonization of the lung by *Pseudomonas aeruginosa* and other species. Whilst inactivation of HBDs by the high salt levels present in the CF lung represents one potential mechanism for the decreased antimicrobial protection (Goldman *et al.*, 1997), there also exists a protease-mediated mechanism which contributes significantly to this phenomenon. HBD-2 and HBD-3 have been shown to be susceptible to degradation and inactivation by the cysteine proteases cathepsins B, L, and S, with all three cathepsins present and active in CF bronchoalveolar lavage fluid (Taggart *et al.*, 2003). In addition to degrading HBDs these enzymes also cleave and inactivate human lactoferrin in CF, which plays an important role in inhibition of biofilm formation (Rogan *et al.*, 2004). Furthermore all three cathepsins have also been shown to cleave and inactivate SLPI in the context of pulmonary emphysema (Taggart *et al.*, 2001). The cleavage of SLPI by cathepsins B, L or S occurs between residues Thr(67) and Tyr(68). This cleavage results in loss of the active site of SLPI and the inactivation of its anti-neutrophil elastase capacity. Cathepsin L has also been shown to cleave and inactivate the serine antiprotease alpha-1 antitrypsin (A1AT) (Johnson *et al.*, 1986). MMP-7 fulfils a similar role in the CF lung where its expression is markedly upregulated (Sires *et al.*, 1994). Together these findings provide ample evidence for the involvement of

cathepsins in damaging the antimicrobial and antiprotease protective screens in the lung.

Bacterial proteases and inflammatory lung disease

Elastolytic activity within the lungs in CF is largely accounted for by the significantly elevated levels of NE, with one report suggesting that up to 90% of the activity in CF sputum is attributable to NE. According to Rees et al. proteinase 3 accounts for a further 7% of this activity whilst the remaining 3% derives from macrophage-derived metalloelastases but also elastolytic proteases expressed by *Ps. aeruginosa* (Rees et al., 1997). Although *Ps. aeruginosa* represents the classical pathogen associated with colonisation of CF airways, other opportunistic Gram-negative and Gram-positive bacteria such as *Haemophilus influenzae* and *Staphylococcus aureus*, respectively, are also important (Ramsey, 1996). However more is known regarding the function and activity of the *Pseudomonas*-derived metalloproteases in CF. Both *Pseudomonas* elastase and alkaline protease are present in CF airway surface liquid (Suter, 1994). *Pseudomonas* elastase has a number of important biological properties. It promotes secretion of mucus (Adler *et al.*, 1983), degrades surfactant proteins A and D (Mariencheck *et al.*, 2003), cleaves and inactivates A1AT (Morihara *et al.*, 1984), SLPI (Johnson *et al.*, 1982), elafin (Guyot N, 2008), lysozyme (Jacquot *et al.*, 1985) and LL-37 (Schmidtchen *et al.*, 2002) and it also impairs the function of cilia (Amitani *et al.*, 1991). Both P*seudomonas* elastase and alkaline protease can inactivate lactoferrin (Britigan *et al.*, 1993). These properties represent an ever-growing list and indicate that proteases expressed by bacterial pathogens colonising the airways should not be overlooked as important factors regulating the inflammatory process.

Pulmonary antiproteases

In order to counterbalance overexuberant and often harmful effects of pulmonary proteases a battery of antiproteases, exists in the lungs. Alpha-1 antitrypsin (A1AT), secretory leucoprotease inhibitor (SLPI) and elafin are three serine antiproteases present, in descending abundance, in the lungs. Monocyte/neutrophil elastase inhibitor (MNEI) is another pulmonary serine protease inhibitor with activity against NE, cathepsin G and proteinase 3 (Cooley *et al.*, 2001). The cysteinyl cathepsins are inhibited by the cystatins whilst the tissue inhibitors of metalloproteases (TIMPS) regulate the activities of MMPs and ADAMs.

Alpha-1 antitrypsin (A1AT)

A1AT is an acute phase 52kDa 418 amino acid glycoprotein that is primarily synthesised and secreted by hepatocytes (Rogers *et al.*, 1983) although it is also actively transcribed and secreted in smaller amounts by cells including neutrophils, mononuclear phagocytes, and enterocytes (Molmenti *et al.*, 1993). A1AT is also produced locally in the lung by bronchial epithelial cells (Cichy *et al.*, 1997; Hu *et al.*, 2002; Mason *et al.*, 1991; Mulgrew *et al.*, 2004; Venembre *et al.*, 1994). It is present in all tissues of the body and its primary role is to inhibit NE (Travis *et al.*, 1985). A1AT can also inhibit a range of other proteases including trypsin, chymotrypsin, cathepsin G, plasmin, thrombin, tissue kallikrein, factor Xa, plasminogen and proteinase 3.

Although A1AT is principally a serine protease inhibitor other of its properties include the ability to inhibit TNFα and MMP in alveolar macrophages in response to thrombin and cigarette smoke extract (Churg *et al.*, 2003), to impair LPS-induced

monocyte activation and to block apoptosis (Daemen *et al.*, 2000; Ikari *et al.*, 2001; Ikebe *et al.*, 2000). A1AT has also been reported to play an immunoregulatory role. It can inhibit neutrophil superoxide production, induce the release of macrophage-derived IL-1 receptor agonist and increase hepatocyte growth factor production in human lung fibroblasts. A1AT can bind to the secreted enteropathogenic *Escherichia coli* proteins EspB and EspD thereby reducing their haemolysis of red blood cells (Knappstein *et al.*, 2004). Thus A1AT may not only afford protection against proteolytic injury, but may also have the potential to neutralise microbial activities and to exert effects on the regulation of innate immunity. There is growing evidence that A1AT may also possess the ability to impair LPS-induced inflammatory responses both *in vitro* and *in vivo* (Nita *et al.*, 2005).

With respect to apoptosis, A1AT has been shown to have a direct pro-survival effect in a model of apoptosis-dependent emphysema (Petrache *et al.*, 2006b). The same group (Petrache *et al.*, 2006b) demonstrated that A1AT can inhibit apoptosis in alveolar epithelial cells following transduction of an A1AT-expressing adeno-associated virus in a mouse model of apoptosis-dependent emphysema. The mechanism by which A1AT mediates this effect is via direct inhibition of caspase-3 binding to its substrate (Petrache *et al.*, 2006a). Others have reported similar anti-apoptotic effects of A1AT in porcine pulmonary endothelial cells (Aldonyte *et al.*, 2008).

A1AT is susceptible to both cleavage and oxidative inactivation *in vivo*. Cathepsin L and *Pseudomonas* elastase are known to cleave A1AT (Johnson *et al.*, 1986; Morihara *et al.*, 1984). A1AT contains nine methionines, two of which are readily oxidizable, Met(351) and Met (358). Met(358) is a key residue located in the active site of A1AT (Johnson *et al.*, 1978). When oxidation occurs A1AT's anti-NE

capacity is abolished and its association rate constant for NE is reduced 2000-fold. Cigarette smoke and inflammatory cells in the lower respiratory tract can oxidize Met(358). Studies by Taggart et al have demonstrated that Met(351) is also susceptible to oxidation, and site-directed mutants of A1AT with alanines substituted for these key methionines are resistant to oxidative inactivation (Taggart *et al.*, 2000).

Augmentation therapy with A1AT is the current treatment for the pulmonary manifestations of A1AT deficiency, a genetic form of emphysema. This approach has the potential not only to redress the protease/antiprotease imbalance and dampen the inflammatory response on the airway surface but also could potentially inhibit apoptosis associated with the development of emphysema by inactivating caspase-3.

Hartl et al. recently described how CXCR1 fragments released from the surface of neutrophils *in vivo* in individuals with CF or COPD can act as bioactive molecules signalling via TLR2 in airway epithelial cells (Hartl *et al.*, 2007). *In vivo* inhibition of proteases by inhalation of A1AT restored CXCR1 expression and improved bacterial killing in individuals with cystic fibrosis. These findings support a novel role for A1AT as a therapeutic for CF and possibly COPD.

Secretory Leukoprotease Inhibitor (SLPI)

SLPI is a 11.7 kDa cationic, non-glycosylated serine proteinase inhibitor that is present in fluids lining mucosal surfaces (McElvaney, 1997). It inhibits a variety of proteinases, including NE, cathepsin G, trypsin, chymotrypsin, chymase and tryptase (Doumas *et al.*, 2005). The molecule is composed of two highly homologous cysteine-rich domains, and it is the C-terminal domain that contains the elastase-inhibitory activity. SLPI is constitutively expressed at many mucosal surfaces and is produced by a number of cell types, including neutrophils, macrophages, and

epithelial cells lining the respiratory and alimentary tracts. The physiological concentration of SLPI in lung epithelial lining fluid can be as high as 670 nM/ ELF (McNeely *et al.*, 1995; Taggart *et al.*, 2001). In the lung SLPI is expressed by clara cells and goblet cells of the surface epithelium and the serous cells of the submucosal glands (Hiemstra, 2002).

In addition to its antiprotease activity SLPI is well recognised as an antimicrobial factor. Its antimicrobial activity is encoded by the N-terminal domain of the protein (Hiemstra *et al.*, 1996). It has been postulated that due to its high cationicity, SLPI can disrupt microbial cell membranes and that this is the mechanism by which it can inhibit such pathogens as *Staphylococcus aureus*, *Staphylococcus epidermidis, Pseudomonas aeruginosa* and *Candida albicans* (Wiedow *et al.*, 1998; Williams *et al.*, 2006). SLPI also displays anti-viral activity and can inhibit human immunodeficiency virus (HIV) replication in monocytes and interfere with HIV infection of macrophages via binding to annexin II (Ma *et al.*, 2004; McNeely *et al.*, 1997).

An important property of SLPI is its immunomodulatory activity. SLPI can regulate a variety of important inflammatory processes including decreasing the production of prostaglandin H synthase-2, prostaglandin E2, and MMP-1 and -9 by monocytes (Zhang *et al.*, 1997), inhibiting interferon-γ-induced cathepsin S expression (Geraghty *et al.*, 2007a) and antagonizing the pro-inflammatory activity of bacterial LPS (Ding *et al.*, 1999; Jin *et al.*, 1997). Whilst it has been reported that SLPI can interfere with the interaction between CD14 and LPS (Ding *et al.*, 1999) other reports provide evidence for an intracellular role for SLPI (McNeely *et al.*, 1997; Taggart *et al.*, 2005). It has been shown that SLPI can be internalised by monocytic cells and be distributed throughout the cytoplasm and nucleus. In addition

to its ability to impair the LPS response, SLPI can also inhibit lipoteichoic acid (LTA)-induced NFκB activation in monocytic cells (Greene *et al.*, 2004; Taggart *et al.*, 2002) . Overall the inhibition has been shown to occur via two mechanisms; firstly by preventing the proteolytic degradation of IRAK-1, IκBβ and IκBα and secondly as a direct result of binding of SLPI to NFκB consensus sequences and competing with p65 for occupancy of the promoters of NFκB-regulated genes (Taggart *et al.*, 2005; Taggart *et al.*, 2002).

SLPI has been administered by aerosolisation to CF patients to suppress respiratory epithelial NE levels and reduce bronchoalveolar lavage fluid IL-8 levels (McElvaney *et al.*, 1992). A major drawback to its therapeutic potential however is its susceptibility to degradation by pulmonary proteases (Taggart *et al.*, 2001).

Elafin

The peptide elafin, also known as SKALP (skin-derived antileukoprotease) or ESI (elastase-specific inhibitor), is a cationic 6-kDa non-glycosylated serine antiproteinase. Elafin belongs to the chelonianin family, a distinct group of canonical inhibitors also including SLPI (Zani *et al.*, 2004). Its compact structure is characteristic of whey acidic proteins (WAPs) and is maintained by four conserved disulphide bonds. Elafin shares 40% sequence identity with SLPI. Tryptase releases elafin from a larger pre-protein molecule called trappin-2 or pre-elafin (Guyot *et al.*, 2005). Trappin-2 possesses an N-terminal WAP domain and a cementoin domain containing repeating GQDPVK motifs that act as a transglutaminase substrate, facilitating the cross-linking of trappin-2 to extracellular matrix proteins.

Elafin is a secreted protein principally expressed by epithelial surfaces such as skin (Alkemade *et al.*, 1994; Nonomura *et al.*, 1994; Pfundt *et al.*, 1996) or lung

epithelium (Sallenave *et al.*, 1994; van Wetering *et al.*, 2000) but also by inflammatory cells including alveolar macrophages (Mihaila *et al.*, 2001) and neutrophils (Sallenave *et al.*, 1997). It is found in plasma (Alkemade *et al.*, 1995), urine (Streit *et al.*, 1995) and bronchial secretions (Nara *et al.*, 1994; Sallenave *et al.*, 1992) and constitutes up to 20% of the total antielastase activity retrieved from bronchoalveolar lavage fluid in healthy individuals.

First identified by Hochstrasser, elafin was described as an acid-stable inhibitor present in human bronchial mucus that differed from SLPI in that it exerted inhibitory activity towards porcine pancreatic and human granulocytic elastase, but not against trypsin, chymotrypsin, or granulocytic cathepsin G (Hochstrasser *et al.*, 1981). Later the anti-protease spectrum of elafin was found to include activity against proteinase 3 (Wiedow *et al.*, 1991). Based on these properties elafin was thought to protect tissue from degradation by these enzymes.

Several studies have demonstrated that expression of elafin is inducible and its expression is significantly upregulated by TNF-α or IL-1β in the airway epithelial cell lines NCI-H322 and A549 (Sallenave *et al.*, 1994). Its expression is also induced in response to other proinflammatory stimuli such as LPS and NE (Reid *et al.*, 1999; Simpson *et al.*, 2001). In addition to its antiprotease properties, elafin also possesses both anti-inflammatory and anti-bacterial activities. Elafin/Trappin-2 can inhibit growth of both *Pseudomonas aeruginosa* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) (Simpson *et al.*, 1999) with reported significant killing of both organisms by doses of 2.5-25μM elafin, concentrations which are potentially achievable in the airway epithelial lining fluid. With respect to its anti-inflammatory activity, the elafin precursor trappin-2 has been shown to dose dependently reduce LPS-induced neutrophil influx into alveoli, to inhibit LPS-induced MMP-9

production and to prevent the generation of CXCL1 and CXCL2 (chemokine ligands 1 and 2) (Simpson *et al.*, 1999). Trappin-2 can also attenuate IL-8 secretion by endothelial cells and/or macrophages in response to TNF, LPS or oxidized low density lipoprotein via inhibition of NFκB (Henriksen *et al.*, 2004). Recently, Butler et al reported that elafin also inhibits LPS-induced MCP- 1 production in monocytes by inhibiting both AP-1 and NFκB activation (Butler *et al.*, 2006).

Notwithstanding elafin's favourable qualities as an antiprotease, anti-bacterial and anti-inflammatory molecule, in a milieu containing high levels of NE, elafin is known to undergo cleavage at Val(5)-Lys(6) and Val(9)-Ser(10). Although this does not impair elafin's anti-NE capacity it does diminish its ability to be immobilised by transglutamination and also to bind LPS (Guyot *et al.*, 2008). This has important implications for the immunmodulatory properties of elafin *in vivo* at sites characterised by a high-NE burden such as the CF lung.

Cystatins and TIMPs

The activity of cysteinyl cathepsins is regulated by endogenous protein inhibitors called cystatins. Three subfamilies exist based on sequence homology and structure; type 1, 2 and 3 (Rawlings *et al.*, 2004). These are located predominantly intracellularly, extracellularly and intravascularly, respectively. The naturally-occurring inhibitors of MMPs are the TIMPs. These are small proteins ranging from 21 to 28 kDa in size which inhibit MMPs in a 1:1 stochiometry. TIMPs are also able to inhibit the metalloproteinase activity of several members of the ADAM family (Huovila *et al.*, 2005). Readers are directed elsewhere for comprehensive reviews of the cysatins and TIMPs (Nagase *et al.*, 2006; Turk *et al.*, 2008).

Therapeutics targeting pulmonary proteases

Considerable evidence for the importance of proteases in chronic inflammatory lung disease comes from knockout mouse studies. Both NE and MMP-12 (macrophage metalloelastase) knock-out mice are more resistant to cigarette-smoke induced emphysema (Hautamaki *et al.*, 1997; Shapiro *et al.*, 2003). Animal studies have also played a large part in developing our understanding of the therapeutic potential of antiprotease therapies. Cantin et al. performed a number of studies investigating the therapeutic potential of plasma-purified A1AT (Prolastin) and MNEI in rat agar bead models of chronic *Ps. aeruginosa* infection (Cantin *et al.*, 1999; Woods *et al.*, 2005). For example, significantly decreased elastase activity, lung neutrophil counts, bacterial colony counts and a marked decrease in lung inflammation were evident in the A1AT-treated animals compared to controls (Cantin *et al.*, 1999). Thiol-specific conjugation of A1AT with polyethylene glycol at Cys232 markedly improved its *in vivo* pharmacokinetic profile (Cantin *et al.*, 2002). Similar studies may prove useful in animal models of CF. Strangely CFTR knock-out mice show little signs of lung disease (reviewed by (Guilbault *et al.*, 2007), however mice with airway-specific overexpression of epithelial Na(+) channels (ENaC) show pulmonary characteristics very similar to CF, most likely due to their accelerated Na(+) transport, and represent a more appropriate model for testing antiprotease therapies for CF (Mall *et al.*, 2004).

The potential use of irreversible synthetic inhibitors of NE such as peptide chloromethyl ketones or reversible peptide aldehydes, tripeptide ketones, modified NE-specific β-lactams or peptide boronic acids has been largely superseded by the development of EPI-HNE-4, a rapid acting and potent NE inhibitor (Delacourt *et al.*,

2002) which can potentially be nebulised to CF patients (Grimbert *et al.*, 2003), however clear clinical efficacy remains to be demonstrated.

Most evidence to date exists for the use of A1AT as an antiprotease-targetted therapeutic for NE-dominated airways diseases. In addition to augmentation studies for A1AT deficiency (Hubbard *et al.*, 1989) a number of human studies have shown that A1AT aerosol therapy has many beneficial effects on airway inflammation in patients with CF (Cantin *et al.*, 2006; Griese *et al.*, 2007; McElvaney *et al.*, 1991). Delivery of SLPI to the lung has yielded less success (Vogelmeier *et al.*, 1990). Unlike A1AT, SLPI does not accumulate on the epithelial surface due to its degradation by cysteinyl cathepsins (Taggart *et al.*, 2001) and consequently relatively higher doses are required to inhibit NE. Elafin and its precursor trappin-2 have both antiproteolytic and anti-inflammatory potential however, like SLPI they too are susceptible to degradation by proteases in the CF lung (Guyot *et al.*, 2008).

Concluding remarks

There is a fine balance between the physiologic and deleterious effects of pulmonary proteases. When this balance is disturbed lung damage results as in the case of cystic fibrosis or COPD where there is dysregulated release of proteases or insufficient inhibition by antiproteases in A1AT deficiency. Therapeutics that target specific pulmonary proteases hold much promise for the treatment of chronic inflammatory lung disease not only with respect to protecting the lungs from protease-mediated tissue damage but also by controlling overexuberant inflammatory responses.

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Table 1. Classes and protein targets degraded by neutrophil elastse

Substrate	**Targets**
Immunoglobulin	IgA, IgG, IgM
Plasma protein	C3, C5, plasminogen, fibrinogen, factors V, VII, XII, and XIII, platelet IIb/IIIa receptor,
Matrix protein	Elasin, collagen I-IV, fibronectin, thrombomodulin, proteoglycan
Cytokine	IL-1, IL-2, IL-6, TNFα
Protease inhibitor	TIMP, elafin, SLPI
Protease	MMP-2, MMP-9, Cathepsin B, TACE, meprin α
Other	Cadherins, complement receptors, surfactant, ICAM1, gp120

Figure 1. Mechanism of NE induction of IL-8 in airway epithelial cells
Following its release from the azurophilic granules in response to a microbial insult, NE activates meprin α or TACE which in turn cleave proTGFα to generate soluble TGFα as a ligand for EGFR. EGFR co-localises with TLR4 and a signal transduction cascade is initiated via MyD88 or Mal, IRAKs, TRAF6, TAK1 and the IKKs leading to degradation of IκB proteins, activation of NFκB and increased IL-8 gene transcription.