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Targeting proteases in cystic fibrosis lung disease: paradigms, progress and potential

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Abstract

Cystic fibrosis (CF) is the most common life-limiting hereditary condition of Caucasian populations and is characterised by chronic airways inflammation driving progressive structural lung damage. Despite tremendous advances in the treatment of CF and concomitant increased life expectancy for patients, chronic lung disease remains the main cause of morbidity and mortality among CF patients. While universal restoration of cystic fibrosis transmembrane conductance regulator activity remains a future hope, novel therapies aimed at reducing or preventing chronic airways inflammation and progressive structural lung damage are required.

It is well-established that proteolytic enzymes are important in the CF lung beyond the basic turnover of proteins and intracellular degradation of pathogens. When secreted, these enzymes play key roles in extracellular substrate modification implicated in important biological processes such as matrix and airway remodelling, goblet cell metaplasia and mucus hypersecretion, immune cell recruitment and dysregulation of epithelial ion channels. Importantly, the burden of proteases in the CF lung is significantly elevated, overwhelming the endogenous antiprotease shield. Indeed, free protease activity has emerged as a major risk factor of the onset and progression of bronchiectasis and lung function decline in patients with CF. Recent research has highlighted the importance of new players such as cathepsin S and matrix metalloprotease-12, as well as the membrane-associated activity of key proteases such as neutrophil elastase on the surface of neutrophils.

Here, we review the current knowledge and emerging concepts of the role of host proteases in the pathogenesis of CF lung disease and their potential as therapeutic targets.

Abstract word count: 250

Introduction

Cystic fibrosis (CF) is an autosomal recessive genetic condition, predominantly of Caucasian populations, which impacts multiple organ systems. However, it is the chronic progressive lung disease of CF that causes the greatest morbidity and mortality. The disease is caused by mutations of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. Consequently, the function of epithelial CFTR anion (chloride and bicarbonate) channels is compromised, leading to impaired anion and fluid secretion and airway surface dehydration, which in turn results in highly viscous airway mucus and impaired mucociliary clearance, setting the stage for mucus plugging, chronic inflammation and polymicrobial infection (1). Such a state causes progressive and irreversible damage of the airways and lung parenchyma, as recruited immune cells (predominantly neutrophils) release proteases, DNA and reactive oxygen species, and promote further immune cell recruitment by cytokine signalling.

The introduction of CFTR modulators (potentiators, correctors and amplifiers) in recent years has transformed the treatment of CF. Phase two trials of triple combination therapy suggest that a CFTR modulator therapy approach may be effective in up to 90% of CF patients (2, 3). While the emerging therapies show immense promise, there are still CF patients whose specific genotypes may not be amenable to these therapies. Furthermore, CFTR modulation alone may be insufficient to allow complete and lasting clearance of chronic airways infection and resolution of pulmonary inflammation, especially in the context of chronic CF lung disease with established structural lung damage (4). Importantly, it is unknown whether, or to what extent, these CFTR-directed therapies decrease protease activity. Until such a decrease has been demonstrated, novel antiprotease strategies are still highly relevant to limit tissue damage in CF lung disease.

The protease-antiprotease hypothesis is a simple paradigm that attempts to explain certain disease states as a product of an imbalance of proteases and cognate antiproteases, resulting in

elevated protease activity and damaging consequences for lung homeostasis (5). It is now well established that proteases play a significant role in the pathobiology of the CF lung (6), whether they are derived from immune cells or indeed the cells of the lung itself. The perception of these enzymes' roles has moved far beyond the terminal degradation of proteins; it is now recognised that proteases are key signalling molecules and that specific substrate cleavage can have myriad effects (7), beneficial or detrimental in the CF lung *(Figure 1)*. The use of protease inhibitor therapy may offer an alternative option in mitigating the disease state to regain homeostasis, which may go hand-in-hand with pharmacological rescue of mutant CFTR by emerging modulator therapies.

So far, the serine protease class has drawn the most attention in CF, in particular neutrophil elastase (NE), with its free extracellular form previously thought to be the major player in CF lung disease pathogenesis. Indeed, free NE in sputum has long been known to correlate with FEV_1 in children with CF (8) and elevated NE activity in bronchoalveolar lavage fluid at 3 months of age was found to be associated with persistent bronchiectasis by the Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF) (9). However, several novel concepts are unfolding in pulmonary protease biology, which have led investigators to broaden their view beyond NE. These concepts include the redundancy of function between proteases *(Table 1)*, the *trans*-class activation of proteases, the discovery of highly active membrane-bound proteases and the emergence of new key players from the cysteine and matrix metalloprotease (MMP) classes. This review will summarise the current knowledge of host protease function in CF lung disease and how this may inform therapeutic intervention. The role of bacterial proteases in CF lies beyond the scope of this review but we direct readers to the following recent references (10, 11).

The actions of serine proteases in CF

The neutrophilic nature of CF airways inflammation gives intuitive significance to the group of proteases known collectively as the neutrophil serine proteases (NSPs). Its members are NE, proteinase-3 (PR-3), cathepsin G (CTSG) and the more recently discovered, lesser studied, NSP-4. The NSPs may be activated by the cysteine protease cathepsin C (CTSC) (12) and are involved in the intracellular degradation of neutrophil-phagocytosed microbes, a particularly important process in the chronically infected CF lung. The NSPs are harboured in primary neutrophil granules and their exocytosis is increased in CF, even during early CF disease prior to the onset of bronchiectasis (13). Extracellular NSPs have been shown to actively mediate the recruitment of immune cells to the site of inflammation by processing an array of cytokines including members of the IL-1 family (14), upregulating neutrophil chemoattractants such as IL-8 (15) and triggering the release of damage associated molecular patterns (DAMPs) such as High Mobility Group Box 1 (HMGB1), which can act as a biomarker for CF lung disease severity (16, 17). This modulation of neutrophil chemotaxis leads to enhanced inflammatory cell infiltration, continuing the vicious cycle of CF inflammation. Furthermore, highly chemotactic, truncated forms of various chemokines including IL-8 can be produced by NSP-mediated cleavage (18).

Neutrophil extracellular traps (NETs), the complex matrix of secreted DNA, proteases and other cellular contents released by neutrophils in CF airways (19) are important reservoirs of NSPs in CF. It has been demonstrated that this DNA-based matrix effectively maintains protease activity by preventing interactions with cognate endogenous or administered antiproteases (20). While intended as a protective mechanism, NSP activity can adversely affect the body's innate response mechanisms to infection including antimicrobial peptides (AMPs) and surfactant proteins. A number of proteases including NE can cleave AMPs such as lactoferrin and midkine (21, 22) and degrade surfactant proteins (23) thereby compromising

the host response and/or susceptibility to infection. NE also cleaves extracellular haem-containing proteins such as ferritin, liberating sequestered iron into the airway. Not only does this increase oxidative stress in the airway epithelium but it also promotes bacterial proliferation and biofilm formation as iron is made accessible for microbial nutrition (24, 25). NSPs may also play an important role as regulators of other proteases, particularly the MMPs. This role is especially relevant when considering the tissue-destructive nature of the proteases; NSP-activated MMP-9 and MMP-12, as well as the NSPs themselves, contribute directly to extracellular matrix (ECM) remodelling and bronchiectasis that is characteristic in CF (9, 26, 27). The protease-mediated loss of elastin limits elastic recoil, while the loss of collagen creates a structural deficit, leading to the emphysematous phenotype that can occur in adolescent and adult CF patients (28). The body's endogenous protection against aberrant NSP activity is also compromised, as NE inactivates tissue-protective antiproteases (some of which also possess antimicrobial properties) such as secretory leukocyte protease inhibitor (SLPI) (29).

The inability of such endogenous antiproteases, even when intact, to perform their inhibitory function has also been highlighted in recent years. This may be due, in part, to membrane-association of NSPs such as NE and CTSG (30). More recently, novel Förster resonance energy transfer (FRET)-based probes were used to analyse membrane-bound activity of proteases such as NE on the surface of neutrophils (31). In this surface-bound form, proteases are less accessible for their prospective inhibitors, which are unable to access the enzyme's active site. Indeed, surface-bound NE has been found to correlate with severity of lung disease and various inflammatory markers in CF (32).

As well as influencing both inflammatory cell recruitment and tissue destruction, NE contributes to increased mucus production in the CF lung by upregulating mucin expression and inducing goblet cell metaplasia, a process thought to be mediated through tumour necrosis factor-α converting enzyme (33, 34). In addition, NE induces secretion of mucins from airway

epithelial cells, augmenting mucus plugging in the CF lung (35). NE has also been shown to decrease the frequency of ciliary beat and damage the airway epithelium (36) which may contribute to impaired mucociliary clearance and hence mucus plugging. Further, NE may directly impact on airway ion transport by degrading CFTR (37) and activating epithelial sodium channel (ENaC) (38), thereby aggravating the basic ion transport defect and airway surface dehydration in CF airways. While CF is caused by mutations in *CFTR*, CF airways are characterized by increased ENaC-mediated sodium absorption in addition to deficient CFTR-mediated chloride secretion. Mimicking the hyperactivity of ENaC by airway-specific overexpression in mice can produce a phenotype that is strikingly similar to that found in CF patients, demonstrating that airway surface dehydration is a key disease mechanism in CF lung disease (1, 39) and that increased ENaC activity contributes critically to this abnormality. In this context, proteolytic activation of ENaC by NE and other proteases may be a key mechanism leading to increased ENaC activity that aggravates airway surface dehydration in CF airways.

Collectively, these studies show that NE is a key mediator in each of the major pathologies contributing to CF lung disease. However, the roles of the other NSPs have been less well studied and more research into these and their relative importance in CF is warranted.

The actions of cysteine proteases in CF

The predominant group of cysteine proteases in CF is the cysteine cathepsins. These papain-like proteases are lysosomally derived and hence display optimal activity in a reducing and acidic environment; only cathepsin S (CTSS) is thought to maintain its activity in the neutral-alkaline pH range (40). In the intracellular context, cathepsins are involved in the degradation of host and pathogen proteins as well as the processing and presentation of antigens. These

functions are crucial in homeostatic protein turnover, fighting infection and in the development of adaptive immune responses to infections. However, like the NSPs, certain members can be found in the extracellular milieu of the CF lung. Cathepsins are secreted by macrophages but may also be sourced from neutrophils, other antigen-presenting cells, lung epithelial and endothelial cells; this secretion may be associated with acidification of the pericellular space (41).

While only more recently recognised as major players in CF, the cysteine proteases mirror many the actions of the NSPs. Like all classes of proteases, the cysteine cathepsins are capable of degrading various ECM components, contributing to the tissue-destructive web of proteases involved in CF. A series of studies demonstrated the potential of cathepsins B (CTSB), L and S to compromise mucosal immunity in the CF lung via mechanisms similar to those mentioned already for the NSPs. They were shown to cleave AMPs including lactoferrin, LL-37, surfactant protein A and the human β-defensins (42–45). Thus, by the loss of active airway AMPs, the ability to maintain a pathogen-free airway may be undermined in CF. Several cathepsins have also demonstrated the ability to process CXCL chemokines *in vitro*, though it has yet to be determined whether these modifications occur or are highly relevant *in vivo* (46). The role of CTSC in the activation of NSPs makes it an interesting candidate for therapeutic intervention in the context of neutrophilic CF lung disease and although inhibitors are in early phase clinical trials, their efficacy and potential for CF remains to be determined (12).

CTSS is emerging as an important player in early CF lung disease with extracellular CTSS levels correlating significantly with lung function decline and neutrophil recruitment into the airways (47). A recent study, using the βENaC-overexpressing mouse with CF-like lung disease, elucidated roles for CTSS in the *in vivo* pathogenesis of several key CF pathologies (48). In this study, genetic and pharmacological knockdown of CTSS was associated with a reduction in neutrophil recruitment and amelioration of airway mucus obstruction and lung

tissue destruction. It also highlighted that CTSS may mediate inflammatory cell recruitment and mucin expression via protease-activated receptor 2. In relation to airway ion transport, both CTSS and CTSB have been reported to activate ENaC (49, 50). As such, in concert with NE-mediated CFTR degradation, the cysteine cathepsins may accentuate the mucus dehydration intrinsic to CF airways pathology.

The actions of matrix metalloproteases in CF

The members of the MMP class are not abundant in the healthy lung; however, they are produced by lung and inflammatory cells in response to inflammatory chemokines, noxious stimuli and free oxygen radicals (51). These zinc and calcium-dependent endopeptidases are loosely numbered in order of discovery up to MMP-28 and, as their name suggests, are potent ECM-degrading enzymes (52). While some MMPs are mainly tissue-derived, MMP-8 and MMP-9 are predominantly derived from neutrophils, making them proteases of particular interest in CF (53). The degradation of interstitial collagen is key to the development of bronchiectasis and other aberrant structural formations of the CF lung. In addition, this cleavage process generates matrix fragments, which can produce secondary downstream effects. During airway inflammation the proline-glycine-proline (PGP, a potent neutrophil chemoattractant) fragments produced by collagenase activity are not matched by a concomitant rise in PGP-degradation by leukotriene A_4 hydrolase, causing PGP accumulation which contributes to CF neutrophilia (54, 55). In addition, MMP-9 is capable of truncating IL-8 into a highly chemoattractive form (56).

While it is clear that the dominant immune cell population in the CF lung is the neutrophil, macrophage-derived proteases are gaining reputation in CF, particularly as regards their membrane-associated activity. A noteworthy example of this is macrophage elastase (also

known as MMP-12). Recent studies in βENaC-overexpressing mice with CF-like lung disease and paediatric CF patients suggest that mucostatic conditions in the CF airways may trigger elevated membrane-associated MMP-12 activity via macrophage activation (57). Interestingly, a functional polymorphism in the *MMP12* promoter (rs2276109) that decreases *MMP12* expression was positively associated with FEV_1 % predicted in patients with CF (57). This work opens interesting lines of inquiry: what are the specific signals which precipitate protease release from specific cell types? Are they to be found in CF mucus? Can they be targeted therapeutically? Can protease gene expression be targeted?

It is worth noting that there is evidence of positive roles for macrophage MMPs in the inflamed lung. MMP-10, for example, is highly expressed in CF patient macrophages and appears to have a protective role in acute bacterial infection by moderating macrophage inflammatory responses (58). MMPs continue to draw most attention for their contribution to lung tissue damage, though there is an emerging sense that this may not be the limit of their influence in CF.

Therapeutic strategies targeting the protease-antiprotease imbalance

The combined contribution of proteases to the pathology of CF makes them promising targets for novel therapeutics. Endogenous protease inhibitors are overpowered as a consequence of quantitatively elevated levels of secreted 'free' protease and have limited efficacy in the inhibition of surface-bound activity in CF lung disease. Importantly, current CF therapy relies heavily on mucolytic agents like dornase alfa, which are known to markedly increase NE activity in CF sputum (59). Therefore, antiproteases may constitute an important adjunct therapy to help limit further lung injury. Indeed, it has been shown that certain antiproteases are most effective when used along with mucolytics (59). Increased protease secretion and

membrane-associated activity are likely already initiated during infancy and early childhood, even in the absence of detectable bacterial infection (5, 9), strengthening the case for early antiprotease treatment.

To directly redress the protease-antiprotease imbalance, two principal strategies may be employed: antiprotease replacement/augmentation and pharmacological protease inhibition.

There is an attraction to using antiprotease-based therapies such as α_1-antitrypsin augmentation, especially considering the success of this strategy in α_1-antitrypsin deficiency. In 2015, a long-term, randomised control trial was reported with weekly α_1-antitrypsin administration for up to 48 months (60). RAPID (Randomized, Placebo-controlled Trial of Augmentation Therapy in Alpha-1 Proteinase Inhibitor Deficiency) demonstrated the slowing of lung parenchymal damage after redressing the protease-antiprotease imbalance and significantly, that this effect was most evident over the course of months and years, rather than short-term improvements over weeks (60). This was not a surprising finding, considering that antiprotease therapy is predicted to slow the progression of irreversible lung damage and bronchiectasis rather than producing short-term improvements in lung function; short trials are therefore unlikely to capture these therapeutic benefits. α1-antitrypsin augmentation has also been tested in CF, though this has been limited to short trials, predominantly powered to establish safety (61). Endogenous antiprotease augmentation is not without its pitfalls given their propensity to be degraded by proteases (host or pathogen). Recombinant variants of the endogenous antiproteases such as SLPI with reduced susceptibility to protease cleavage have shown efficacy in reducing inflammation (62). However, the size and complexity of these proteins, their generally broad antiprotease activity and the quantities required to address the substantial protease burden in CF are all factors to be overcome. These molecules have also yet to demonstrate efficacy against surface-bound proteases.

With these considerations in mind, perhaps it is the synthetic, low molecular weight, specific chemical inhibitors that hold the answer? As with all drugs, walking the tightrope between specificity and bio-reactivity has proven a challenge. For various reasons (including safety, a propensity for hapten formation and the struggle for target selectivity) it is no longer the consensus that rapid irreversible inhibition is necessarily the gold standard for these compounds (63). A new generation of highly specific, reversible inhibitors of NE or the emerging proteases (CTSC, CTSS and MMP-12) might help to shape the future of antiprotease therapy in CF. Many synthetic inhibitors have demonstrated potency *in vitro* and in the preclinical *in vivo* settings. However, NE inhibition, which has been the focus of clinical antiprotease work, has so far not proved overly effective in reducing key measures of disease in CF or other inflammatory lung diseases (64). Therefore, it may be that the inhibition of other proteases or a spectrum of proteases in combination with conventional therapies produces more promising results. Comprehensive studies will be required to ensure that patient susceptibility to infection is not increased by protease inhibition, though there is little preclinical evidence that this will be the case. Interestingly, the genetic ablation of NE in βENaC-overexpressing mice did not increase susceptibility to spontaneous airways infection in this model with CF-like lung disease (33). NSP-deficient mice have exhibited weakened host defence against certain respiratory pathogens (65, 66), though it should be noted that inhibitor-treated and full knockout mice are not direct corollaries and as such, further research is required to assess the effects of therapeutically relevant protease knockdown on host immunity.

An interesting alternative to the use of canonical antiproteases is inhaled heparin, which has been shown to improve pulmonary function in COPD (67). 2-O, 3-O-desulfated heparin, a modified polysulfated molecule, possesses both anti-NSP and anti-inflammatory properties with minimal anti-coagulant activity (16, 68). In some instances, repurposed drugs might offer a simpler and faster route to protease inhibition than the development of novel inhibitors,

especially regarding their safety profile. One drug which has emerged is the tetracycline antibiotic doxycycline. A 2017 study highlighted FEV_1 improvements following doxycycline treatment during acute pulmonary exacerbations in CF patients, seemingly independent of doxycycline's antibiotic properties, via MMP-9 neutralisation and TIMP-1 enhancement (69). Currently, there are no other licensed drugs that are known to fall into this category.

Summary and outlook

What is evident from the research to date is that proteases play a role in many of the most damaging facets of CF lung disease and as such could be targeted in combination with current antibiotic, mucolytic, bronchodilator and CFTR modulator therapies. A return to protease-antiprotease parity may indeed facilitate the breaking of the inflammatory cycle and slow the rate of structural and functional decline in CF. For proteases such as NE and CTSS, which are elevated from an early stage in the pathogenesis of CF lung disease, the age of CF patients at the start of protease inhibitor therapy and the frequency and duration of treatments may well be crucial factors to consider for the design of clinical trials. A significant challenge remains in developing protease inhibitors that retain specificity, stability and efficacy in the complex milieu of the CF lung and that are well tolerated over longer courses of treatment.

Our knowledge of the role and functions of proteases continues to evolve through the development and use of new experimental tools, reagents and pathobiological models. Because of their differential expression and activity profiles in CF lung disease, proteases (and their endogenous inhibitors) may serve as useful biomarkers for diagnostic and monitoring purposes to enable, for example, detection of lung disease severity and prediction of progression or response to treatment. Nonetheless, further work is needed to extensively characterise the lung

degradome, in addition to the status of endogenous antiproteases, activators, substrates and cleavage products in the CF lung.

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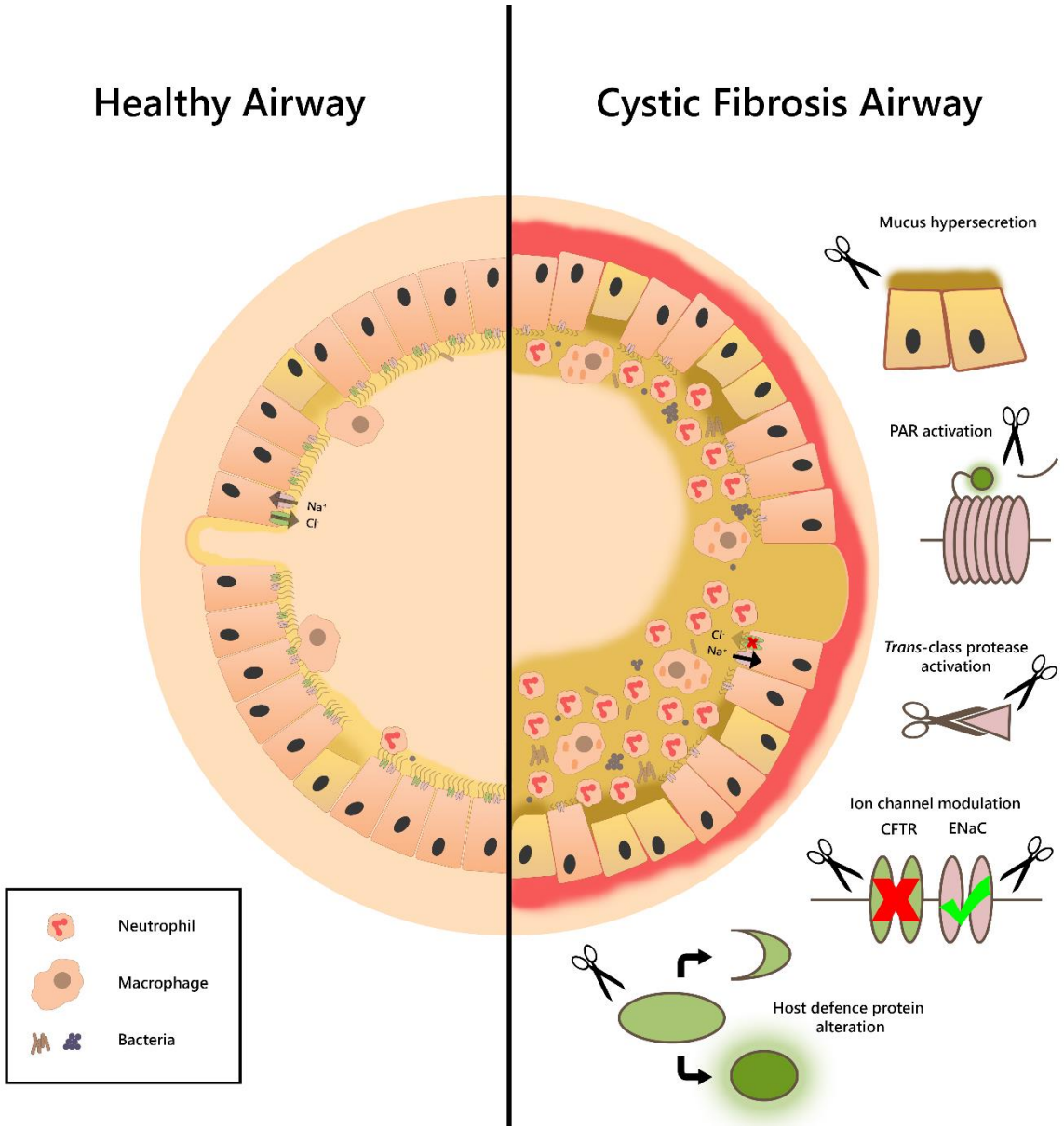
Figure legends

Figure 1. A model of the cystic fibrosis (CF) airway and associated protease-mediated pathologies. A healthy airway maintains a thin layer of well-hydrated mucus covering the airway surface. Invading pathogens and particulates are trapped and subsequently removed from the airway by mucociliary clearance. In CF airways, malfunction of cystic fibrosis transmembrane conductance regulator (CFTR) anion channels and increased activity of epithelial sodium channels (ENaC) results in airway surface dehydration, altered viscoelastic properties of airway mucus and impaired mucociliary clearance, which makes the airway susceptible to chronic infection and inflammation. Neutrophils recruited to the airway, along with macrophages and epithelial cells, secrete proteases which aggravate key aspects of the pathophysiology of CF. Active proteases compromise the structural integrity of the airway through the degradation of elastin and collagen, leading to bronchiectasis. In addition, other protease roles in CF include *(top to bottom)*: the enhancement of mucin/mucus production and secretion; the activation of protease-activated receptors (PARs) leading to pro-inflammatory signalling; the *trans*-activation of other proteases by cleaving pro-domains and degrading cognate antiproteases; the aggravation of basic CF ion transport defects by the proteolytic degradation of CFTR and activation of ENaC and; the cleavage of various host protein substrates precipitating either activation (in the case of some pro-inflammatory cytokines) or inactivation (in the case of some antimicrobial peptides and surfactant proteins).

602 **Figures**

603 Figure 1.



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Tables

Table 1. Protease functions and redundancy in CF lung disease

Function	Protease class involved	References
Matrix degradation	Serine, cysteine, MMP	(52, 54, 55, 70)
Cytokine processing	Serine, cysteine, MMP	(14, 18, 46, 56)
Cytokine upregulation	Serine, cysteine, MMP	(15, 48, 54, 55)
PAR activation	Serine, cysteine, MMP	(48, 71)
Trans-class protease activation	Serine, cysteine	(9, 12, 26, 27)
Host defence protein degradation (including antiproteases)	Serine, cysteine	(21–23, 29, 42–45)
ENaC activation	Serine, cysteine	(38, 49, 50)
CFTR degradation	Serine	(37)
Mucus modulation	Serine	(33–36, 48)
Iron liberation	Serine	(24, 25)

Definition of abbreviations: MMP = matrix metalloprotease; PAR = protease-activated receptor; ENaC = epithelial sodium channel; CFTR = cystic fibrosis transmembrance conductance regulator.