Pulmonary Perspective

Elastolytic Proteases

Inflammation Resolution and Dysregulation in Chronic Infective Lung Disease

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Proteases are enzymes that have the capacity to hydrolyze peptide bonds and degrade other proteins. They are classified according to the active groups of their catalytic center. In lung disease, the three major protease groups of interest are serine, cysteine, and the matrix metalloproteases (MMPs) (1). Another group is the ADAM ("a disintegrin and metalloprotease") family of proteases, which has an emerging role in mucin production and cytokine processing (2). We direct the reader to some of the many excellent reviews on the structural and functional aspects of the serine, cysteine, and MMP families and their cognate inhibitors (3–5).

The major sources of proteases within the lung are inflammatory cells, such as neutrophils, mast cells, macrophages, and lymphocytes (6–8). Other cells, including epithelial, endothelial, and fibroblasts, also synthesize proteases (9, 10). Serine proteases, including neutrophil elastase (NE), cathepsin G, and proteinase 3, are packaged in primary granules within neutrophils (11). Some of the metalloproteases, MMP-8 and MMP-9, are also packaged into specific and gelatinase granules, respectively, in the neutrophil (12). Neutrophil proteases are released either intracellularly into phagolysosomes or extracellularly after cellular activation (13). Differentiation of monocytes to macrophages results in the loss of the serine protease complement of these cells. However, this is replaced by an ability to synthesize other proteases, including a number of MMPs and elastolytic cathepsins (7).

In the lung, proteases function either intracellularly or after secretion into the extracellular environment. Arrayed against these proteases in the extracellular space are antiproteases, which inhibit proteases by interacting with their catalytic sites, thereby preventing them from damaging normal structures. The major antiproteases in the extracellular milieu of the lung are α_1-antitrypsin, secretory leukoprotease inhibitor, elafin, tissue inhibitor of metalloproteases (TIMP), and cystatins (14). In the normal lung, antiproteases prevent the deleterious effects of proteases, because they are present in higher concentrations than the proteases, providing an "antiprotease screen." However, when the protease concentration in the local milieu overwhelms the local antiprotease protective screen, such as occurs in chronic infective lung disease, this results in excessive extracellular activity—leading to degradation of lung tissue (15).

Although much research has centered on the destructive nature of proteases, recent evidence has emphasized the role of proteases in a number of key biological activities including the following: (*1*) inflammation, involving cell recruitment and transmigration; (*2*) innate immunity, incorporating cleavage of cell surface and soluble proteins involved in the innate immune response; and (*3*) infection, involving their role in bacterial killing, apoptosis, and phagocytosis and mucin production. The main emphasis of this perspective will be to address these nondestructive activities of the major lung proteases and describe how these processes become dysregulated during chronic lung infections.

INFLAMMATION

Cell Recruitment

Proteases have been implicated in the chemotaxis of all of the various inflammatory cell types to the lung. MMP-9 and serine proteases increase eosinophil chemotaxis, and MMP-12 is believed to play a role in eosinophil and macrophage accumulation (16, 17). However, the majority of work on proteases and cell migration in chronic infective lung disease has concentrated on neutrophil chemoattraction to the lung by interleukin 8 (IL-8) and leukotriene B4. Serine proteases such as NE can induce IL-8 expression by bronchial epithelial cells (Figure 1) and leukotriene B4 expression by macrophages (18, 19). NE appears to be the most important factor present in the cystic fibrosis (CF) lung responsible for IL-8 expression because inhibition of NE activity in CF bronchoalveolar lavage fluid almost completely prevents IL-8 message in bronchial epithelium (20). Interestingly, NE has been shown to act at least in part via an IL-1 receptor–associated kinase-1/myeloid differentiation factor-88/nuclear factor-κB–dependent pathway in bronchial epithelial cells; this can be inhibited by a dominant negative variant of myeloid differentiation factor-88 (18). This highlights the potential new therapeutic approaches aimed at inhibiting the NE-activated intracellular pathways rather than NE itself. Clearly, expression of IL-8 and leukotriene B4 has repercussions for neutrophil migration to the lung, and given the high neutrophil and NE burden present in the CF lung this has lead to the "vicious cycle" hypothesis whereby NE is the driving force behind IL-8 production and neutrophil influx into the CF lung. Further evidence that an initial inflammatory event can stimulate further inflammation are data showing that epithelial cell injury in mice leads to secretion of the murine homolog of IL-8, which in turn binds to an adhesive component of the extracellular matrix, syndecan-1 (21). Cleavage of this syndecan-1-murine IL-8 complex by MMP-7 is crucial in attracting neutrophils to the damaged epithelial surface (Figure 1).

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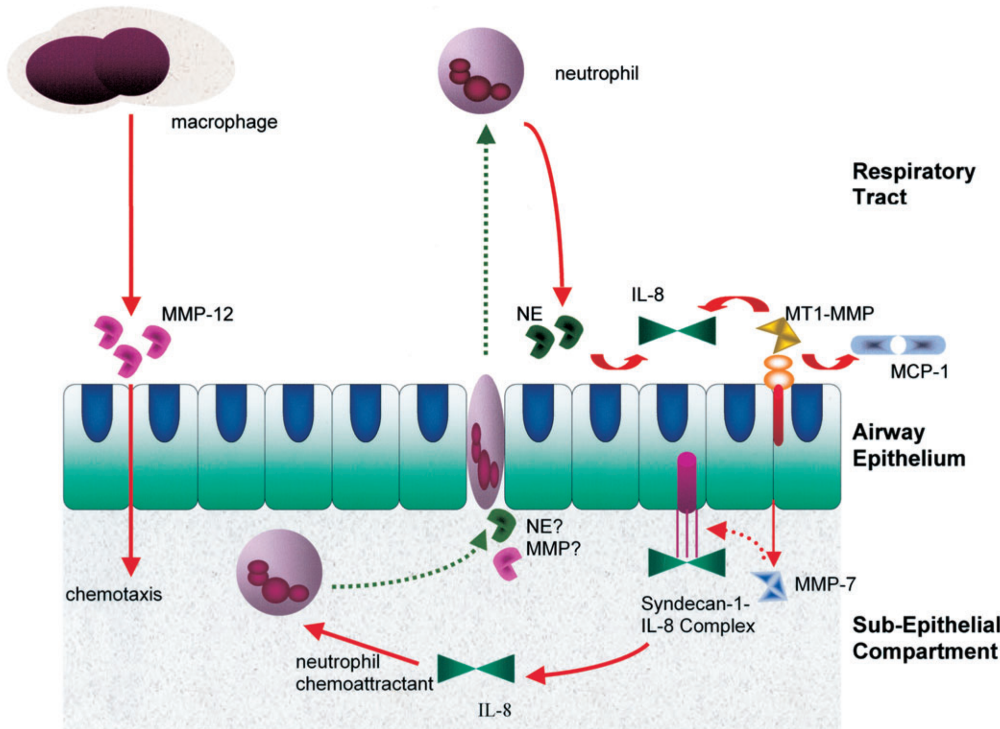

Figure 1. Protease-induced inflammatory responses in the respiratory tract. Neutrophil elastase (NE) can induce interleukin-8 (IL-8) synthesis, resulting in chemoattraction of neutrophils to the respiratory tract. MT1–matrix metalloprotease (MMP) 14 processes IL-8 and monocyte chemoattractant protein 1 (MCP-1), and MMP-7 cleaves the syndecan-1–IL-8 complex to generate active IL-8, which in turn acts as a neutrophil chemoattractant. MMP-12 released from macrophages can also act as an inflammatory cell chemoattractant.

Apart from inducing IL-8 expression, proteases also process this cytokine. MMP-14 (MT1-MMP) has been demonstrated to process IL-8 by removing a pentapeptide from the N-terminus of the protein, resulting in a more biologically active form (Figure 1). In addition, serine proteases such as proteinase 3 can process the mature form of IL-8 to a more active truncated form, as can MMP-9, which has been shown to process IL-8 to a form 20-fold more active as a chemoattractant (22, 23). Degranulation, intracellular calcium mobilization, and binding to the CXC chemokine receptor 1 are also more pronounced with the truncated form of IL-8 (23). In contrast, MMPs can also process monocyte chemoattractant proteins to produce antagonists of chemokine receptors, indicating a role for MMP-14 in dampening inflammation (Figure 1) (24). In the context of CF, it seems likely that because of the enormous neutrophil burden present in the CF lung, the high concentrations of NE are likely to overwhelm the levels of MMP-14–processed monocyte chemoattractant protein antagonists. This is supported by evidence showing significant IL-8 levels present in CF bronchoalveolar lavage fluid (20).

Another potential target for serine proteases is the protease-activated receptor family, particularly protease-activated receptor-2. The serine proteases act, at least in part, via protease-activated receptor-2, although in human bronchial epithelial cells it has been reported that NE and cathepsin G can actually disarm protease-activated receptor-2, therefore preventing further activation by trypsin (25).

There is also interplay between NE and MMPs in inflammation. NE activates MMP-9 directly and indirectly by inactivating TIMP-1, the naturally occurring inhibitor of MMP-9 (Figure 2) (26, 27). Furthermore, NE may activate MMP-2 through a mechanism that requires MMP-14 expression (28). Therefore, a variety of proteases liberated from neutrophils or actively expressed

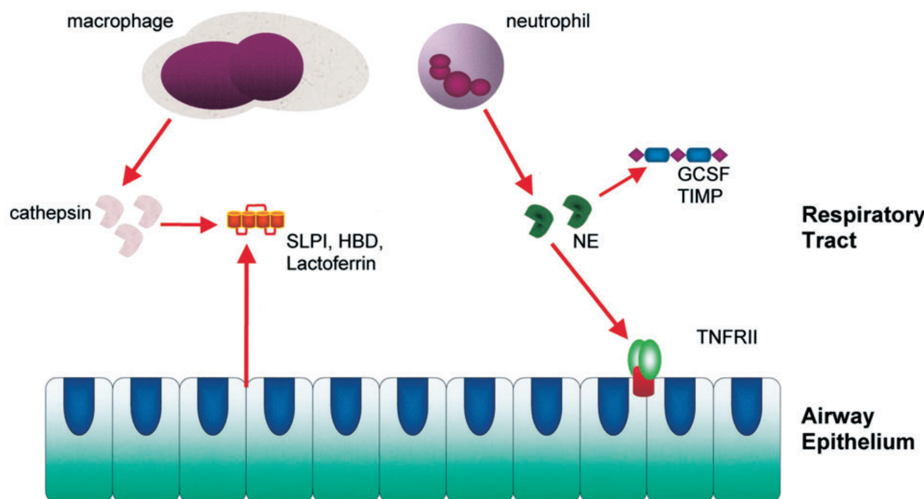

Figure 2. Protease-mediated cleavage of proteins involved in the innate immune response. NE can cleave various soluble (granulocyte colony-stimulating factor [GCSF] and tissue inhibitor of metalloproteases [TIMP]) and surface-bound proteins (tumor necrosis factor receptor 2 [TNFRII]). Cleavage of TNFRII may diminish neutrophil responses to TNF, and cleavage of TIMP may result in prolonged MMP action on the respiratory tract. Release of cathepsins by macrophages can lead to degradation of key antimicrobial proteins, such as lactoferrin, secretory leukoprotease inhibitor (SLPI), and human β-defensins (HBD), which may have repercussions for the antimicrobial screen in diseases characterized by a cathepsin burden.

on epithelial cells may interact with each other, thereby perpetuating a cycle of inflammation.

Transmigration

There are conflicting data surrounding the role of proteases in neutrophil transmigration from the vasculature, particularly regarding the role of NE and MMP-9 in this process (Figure 1) (29–32). Most of the data generated have relied on the use of synthetic protease inhibitors to prove or disprove that NE and MMP-9 are involved in the transmigration process. However, the generation of NE knockout mice appears to have shed new light on this process, with results indicating that neutrophil transmigration by NE-deficient neutrophils can still occur in response to cytokine stimuli (IL-1 and tumor necrosis factor α [TNF-α]), but that zymosan-induced neutrophil adhesion and transmigration is suppressed in NE knockout mice (33). Therefore, NE appears to play a role in neutrophil migration in response to specific stimuli. Research is needed to show which of the various pathogenic stimuli present in the chronic infective lung may induce NE-dependent neutrophil transmigration.

Innate Immunity

Proteolytic cleavage of pathogen recognition receptors. A key component of the innate cellular response to infection involves recognition of bacterial and viral antigens by Toll-like receptors (TLRs) present on many cell types. This group of transmembrane proteins recognizes bacterial and viral structures, leading to activation of signal transduction pathways and expression of proinflammatory cytokines (34). Proteolytic cleavage of TLRs and associated proteins may have consequences for the innate immune defenses of the respiratory tract. We have previously shown that NE incubation with human bronchial epithelial cells results in increased IL-8 expression, degradation of IL-1 receptor–associated kinase-1, and decreased TLR4 expression, indicating that proteolysis of TLR4 may initiate a sequence of events culminating in increased IL-8 expression by these cells (35). NE cleavage of TLR4 may result in a conformational change in the structure, resulting in its activation, or NE may liberate a cell surface–bound ligand of TLR4, leading to activation. It has also been demonstrated that cleavage of CD14 by NE on fibroblasts and monocytes results in decreased responsiveness to lipopolysaccharide in terms of TNF-α and IL-8 release (36, 37). Increased NE activity may have the effect of downregulating TLR4 and CD14 to the extent that activation of these pathogen recognition receptors by their cognate bacterial ligands is diminished, resulting in a decreased inflammatory response by macrophages and epithelium.

Cleavage of Soluble Proteins on the Respiratory Tract

Antiproteases. Members of the serine, MMP, and elastolytic cathepsin families are capable of degrading a wide variety of soluble proteins present on the respiratory tract. Serine proteases can cleave and inactivate protease inhibitors such as TIMP-1 and cystatin C, again highlighting interaction between proteases, because serine protease inactivation of MMP and cysteine protease inhibitors increases the likelihood of free MMP and cathepsin activity (27, 38). Likewise, a large number of MMPs (MMP-1, MMP-3, MMP7, MMP-8, MMP-9, and MMP-12) can cleave and inactivate α_1-antitrypsin, thereby allowing NE, cathepsin G, and proteinase 3 to remain active (39–43). This may be particularly important in the context of neutrophil activation, where release of both serine proteases and MMPs results in local inactivation of α_1-antitrypsin by MMPs, thus allowing NE to exert important microbicidal activity, as discussed later.

Host defense proteins. Serine proteases can cleave and inactivate other important antiinflammatory/proinflammatory stimuli, including surfactant D, flagellin, and annexin I (44–46). MMPs are involved in liberating growth factors from extracellular protein, such as occurs when MMP-3 and MMP-13 cleave the proteoglycan perlecan to liberate fibroblast growth factor and MMPs cleave decorin to liberate transforming growth factor β (47, 48). Likewise, insulinlike growth factor binding protein is cleaved to generate active insulinlike growth factor ligand (49). MMPs can also process and activate cytokines, including pro–IL-1β and pro–TNF-α, suggesting an indirect role in initiating inflammation cascades (50, 51). NE has been shown to cleave a number of innate immune proteins, including granulocyte colony-stimulating factor and its receptor and TNF receptor II (Figure 2) (52, 53). NE cleavage of TNF receptor II on neutrophils may play a role in the regulation of subsequent neutrophil functions, such as oxidant generation and adhesion.

Members of the elastolytic cathepsin family—cathepsins B, L, and S—are present and active in CF bronchoalveolar lavage fluid, and cathepsin L has been identified in emphysema bronchoalveolar lavage fluid. Cathepsins B, L, and S are capable of degrading small-molecular-weight peptides, such as β-defensins and secretory leukoprotease inhibitor, which are largely resistant to the actions of other serine and matrix metalloproteases (Figure 2) (54, 55). A recent study has shown that cathepsins can also rapidly inactivate lactoferrin, a major iron-binding protein of the airways (Figure 2) (56). This has consequences for the CF airways, because lactoferrin is the only known innate protein that can inhibit the formation of *Pseudomonas* biofilms. High cathepsin activity in *Pseudomonas*-positive sputa correlates with relatively decreased levels of lactoferrin. In addition, *Pseudomonas*-positive, cathepsin-rich/lactoferrin-deficient sputa are less efficient at preventing *Pseudomonas* biofilm formation than *Pseudomonas*-negative sputa.

The presence of secreted cathepsins in the lung may be necessary to regulate the activities of these cationic proteins and peptides; this process may become dysregulated in the chronic infective lung disease situation, thus compromising the antiprotease/antiinflammatory screen and thereby perpetuating inflammation and infection. We will now address the role of proteases in infection resolution and discuss how these activities become dysregulated in chronic infective lung disease.

INFECTION

Bacterial Killing

NE has been shown to be directly important in clearing gram-negative bacteria from the lung (Figure 3). In NE knockout mice, neutrophils migrate normally to sites of infection, but are unable to kill bacteria (57). In addition, NE knockout mice infected with gram-negative bacteria are more susceptible than their normal littermates to sepsis and death after intraperitoneal infection. Cathepsin-G knockout mice have survival rates similar to those of wild-type mice after challenges with various gram-positive and gram-negative bacteria, suggesting that NE may compensate for cathepsin G (58). NE can also exert indirect effects on bacterial killing by activating epithelial cells to upregulate expression of human β-defensin 2, a potent antimicrobial agent (59). More recently, NE has been identified as a component of neutrophil extracellular traps that are secreted from neutrophils as part of a chromatin-protein complex, capable of binding and killing gram-positive and gram-negative bacteria (60).

Apoptosis and Phagocytosis

Inflammation resolution is normally achieved by the removal of dying apoptotic neutrophils from the respiratory tract by macrophages. This process involves recognition of apoptotic signature proteins by cell recognition receptors on the macrophage,

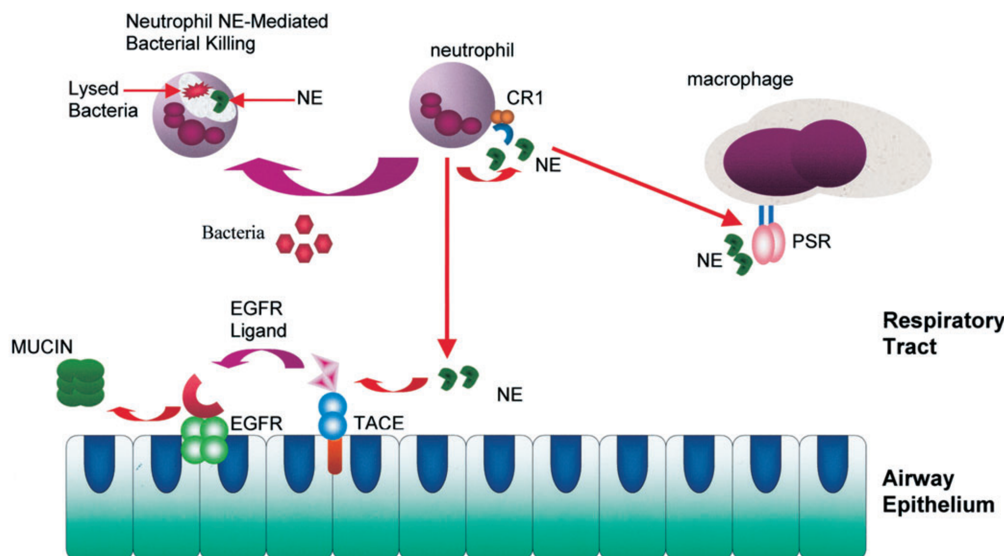

Figure 3. Role of proteases in infection. NE plays a key role by directly killing bacteria and, possibly as a result of an NE burden, inhibiting phagocytosis of bacteria by cleaving CR1 on neutrophils and phagocytosis of apoptotic neutrophils by cleaving the phosphatidylserine receptor (PSR) on macrophages. NE can also activate TNF-α–converting enzyme (TACE), thereby leading to epidermal growth factor receptor (EGFR) ligand liberation and activation of EGFR, resulting in mucin production.

such as the phosphatidylserine receptor, CD36, and various other receptors. However, in the presence of NE activity, such as occurs in the CF respiratory tract, there is decreased apoptotic cell removal. This occurs when NE cleaves the phosphatidylserine receptor on macrophages and thereby impairs the normal macrophage–apoptotic neutrophil interaction (Figure 3) (61). As a result, apoptotic neutrophil clearance is decreased. This has consequences for inflammation resolution, resulting in decreased production of transforming growth factor β, a suppressor of proinflammatory cytokine production, and increased release of proinflammatory intracellular contents from postapoptotic neutrophils. Phagocytosis of apoptotic neutrophils results in increased transforming growth factor–β production, which serves to prevent production of proinflammatory cytokines as part of the inflammation resolution response. To add to the inflammatory burden in CF, NE has also been shown to inhibit neutrophil phagocytosis of *Pseudomonas aeruginosa* by cleaving the complement receptor, CR1, and the opsonic C3 fragment, C3bi, creating an "opsonin receptor mismatch" (Figure 3) (62, 63). In contrast to the pivotal role NE plays in bacterial killing, it would appear that there is a balance between excessive extracellular NE activity (e.g., that which occurs in chronic infective processes such as CF and chronic obstructive pulmonary disease) and normal resolution of infection. In such circumstances, excessive NE activity may impair normal phagocytosis mechanisms and thus have repercussions for NE-mediated intracellular bacterial killing.

Mucin Production

Mucins are involved in binding and removing bacteria via the mucociliary ladder, a process by which bacteria are transported along the respiratory tract and then ingested (64). Two of the most prominent mucins, MUC2 and MUC5AC, are produced following ligand-dependent phosphorylation of the epidermal growth factor receptor (65). Transforming growth factor α, heparin binding epidermal growth factor, epidermal growth factor, and amphiregulin are among the ligands responsible for binding to and activating the epidermal growth factor receptor (Figure 3) (66). These ligands are synthesized as transmembrane proforms and are processed to the mature forms by a group of metalloproteases, members of the ADAM family of proteases, the most prominent of which are TNF-α–converting enzyme (ADAM17) and ADAM10. The gram-positive bacterial product, lipoteichoic acid, is known to activate ADAM10 (67), whereas a host of stimuli activate TNF-α–converting enzyme, including gram-negative bacteria, such as *Pseudomonas*, lipopolysaccharide, cigarette smoke, and, interestingly, NE (Figure 3) (68–70). The role of NE and TNF-α–converting enzyme in mucin production is most relevant in CF where there is an NE burden and mucus hypersecretion but may also be important in other chronic lung diseases, such as chronic obstructive pulmonary disease and asthma, where mucus hypersecretion is also a common feature. Another mucin, MUC1, plays a critical role in protecting mucosal epithelia from microbial and enzymatic attack. MUC1 shedding is controlled by TNF-α–converting enzyme, although recent evidence demonstrates that MUC1 can be shed by MMP-14 (71, 72). MUC1 has also been shown to act as an adhesion site for *Pseudomonas*, an interaction that is abolished by proteolytic cleavage by NE, suggesting a possible role for this protein in binding and removal of bacteria (73).

CONCLUSIONS

Three major classes of proteases—serine proteases, MMPs, and cysteinyl proteases—have been identified in the lung. They are associated traditionally with chronic lung disease and airway extracellular matrix destruction. However, evidence is accumulating to show that each protease family has a multitude of regulatory functions, which makes them of pivotal importance in inflammation, innate immunity, and infection. In chronic infective lung diseases, these processes become dysregulated because of extracellular protease activity, which leads ultimately to upregulation of proinflammatory mediators, increased recruitment of inflammatory cells to the lung, impaired phagocytosis, increased mucin production, and inactivation of important innate and antimicrobial proteins. This results in sustained inflammation and predisposition to infection. One way to treat such protease-mediated events in chronic infective lung disease is with antiprotease therapy, which neutralizes excessive extracellular protease activity without compromising the normal physiologic role of proteases. Antiprotease trials have been performed using α_1-antitrypsin and secretory leukoprotease inhibitor, both of which have successfully inhibited NE activity *in vivo* in CF and α_1-antitrypsin deficiency (74–76). Other inhibitors of serine proteases, MMPs, and cathepsins are being developed, including synthetic inhibitors to combat protease-induced lung destruction.

However, future research will also have to address why there is prolonged extracellular protease activity in chronic infective lung diseases (CF and chronic obstructive pulmonary disease) compared with those conditions characterized by a shorter duration of protease activity (pneumonia). In the cases of CF and chronic obstructive pulmonary disease, it may be that underlying genetic mutations in immune and nonimmune cells predispose toward greater release or expression of proteases. There is already some evidence for this hypothesis. α_1-Antitrypsin deficiency is characterized by a decreased level of circulating α_1-antitrypsin, which results in early-onset emphysema in affected individuals. It was originally believed that that this emphysema was caused by decreased α_1-antitrypsin in the respiratory tract, leading to unopposed and prolonged NE activity, as outlined in the quantum proteolysis hypothesis (77). However, recent evidence suggests that the Z variant of α_1-antitrypsin, when polymerized, may be proinflammatory, acting as an important chemoattractant for neutrophils in the α_1-antitrypsin–deficient lung and adding to the excessive neutrophil and NE burden (78, 79). In a similar fashion, CF epithelium produces elevated levels of IL-8 and decreased levels of IL-10 compared with non-CF epithelium, which may help explain the neutrophil/NE burden observed in the CF lung (80). Furthermore, CF neutrophils exhibit an inflammatory phenotype and on stimulation shed less L-selectin and secrete more oxidants and NE than neutrophils from individuals with non-CF bronchiectasis and control subjects (81–83). In this context, mutations facilitating an influx of inflammatory cells, which may themselves exhibit a dysregulated response, will almost certainly result in elevated extracellular protease activity that cannot be neutralized effectively. Because of the multiple protease activities present in these chronic infective lung diseases, it would be important to identify whether there is a key protease or proteases central to direct tissue destruction or activation of other proteases in the chronic infective lung. Under these circumstances, neutralization of one such protease with a specific antiprotease may be sufficient to lessen the overall protease burden in chronic infective lung disease without the need for inhibition of all proteases.

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