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Matrix Metalloproteinases in Emphysema

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Abstract

Several studies have implicated a causative role for specific matrix metalloproteinases (MMPs) in the development and progression of cigarette smoke-induced chronic obstructive pulmonary disease (COPD) and its severe sequela, emphysema. However, the precise function of any given MMP in emphysema remains an unanswered question. Emphysema results from the degradation of alveolar elastin – among other possible mechanisms – a process that is often thought to be caused by elastolytic proteinases made by macrophages. In this article, we discuss the data suggesting, supporting, or refuting causative roles of macrophage-derived MMPs, with a focus on MMPs-7, -9, -10, -12, and -28, in both the human disease and mouse models of emphysema. Findings from experimental models suggest that some MMPs, such as MMP-12, may directly breakdown elastin, whereas others, particularly MMP-10 and MMP-28, promote the development of emphysema by influencing the proteolytic and inflammatory activities of macrophages.

Lung Structure.

The organization of the lung can be broken down into two physiologically distinct compartments: conducting airways and alveoli. The conducting airways begin as contiguous extensions from the carina, the bifurcated distal end of the trachea, and branch into progressively narrower tubes – from cartilage-ringed 1°, 2°, and 3° bronchi to terminal bronchioles – that spread throughout the organ to move inhaled air to and expired gasses from the distal lung. In addition to being conduits for air movement, the conducting airways play critical roles in innate defense, particle clearance, and regeneration. Obstruction of conducting airways due to excess mucus or ingrowth of fibrotic or inflamed tissue leads to severe outcomes as in cystic fibrosis, chronic bronchitis, and bronchiolitis obliterans syndrome (BOS), a devastating complication that can develop during rejection of transplanted lungs.

At the distal end of the terminal bronchioles are pulmonary lobules, which are grape-like clusters comprised of numerous spherical alveoli. An alveolus is basically an extracellular

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matrix (ECM) scaffold supporting the respiratory epithelium and vascular endothelium. The inner epithelial lining is comprised of type I and II pneumocytes. Type II cells are surfactant-producing, cuboid epithelium and the precursor of type I cells. They make up 80% of the alveolar epithelium yet occupy only 20% of its area. Type I cells are flattened epithelium that rest on 80% of the area of the inner alveolar wall. As for all epithelium, type I and II cells adhere to a basement membrane of their own making.

Alveolar Interstitial Compartment.

Within the narrow interstitial spaces between adjacent alveoli are capillaries, cells (pericytes and fibroblasts), and an elastin/collagen ECM. The capillary-alveolar interface – referred to as the respiratory unit – is structured for efficient gas exchange. The cellular components of respiratory units are limited to thin, non-nuclear areas of type I and endothelial cells, and the basement membranes of both cell types are fused. The alveolar wall is strengthened by elastic and type I and III collagen fibers. Elastic fibers are deposited primarily within the area of the alveolar duct, which is the transition from the terminal bronchiole to the alveolus. Collagen fibers, which are slightly more abundant than elastin fibers, are found throughout the alveolar interstitium [1,2]. Collagen fibers confer tensile strength, whereas the elastic fibers provide recoil of the alveolar during respiration, a critical physiologic function.

Alveolar Destruction.

Though optimal for gas exchange, the delicate structure of the respiratory unit makes it susceptible to injury and destruction. The destruction of alveolar ECM scaffold leads to a merging of residual tissue and generation of larger air sacs. Expulsion of air from the larger sacs is slowed, resulting in airflow obstruction and markedly impaired respiration. In some diseases, such as interstitial lung diseases, alveolar destruction occurs secondarily to other manifestations. For example, the infiltration of fibrotic cells and tissues results in destruction of alveolar septa and dilation of distal airspaces. In other conditions, such as emphysema and lymphangioleiomyomatosis (LAM), a progressive, often fatal diffuse cystic lung disease of women [3], destruction of the alveolar ECM is thought as a primary, though not necessarily the sole causative event. Other mechanisms, such as apoptosis of capillary endothelial cells and autoimmunity [4–9], may contribute to alveolar destruction. Still, degradation of structural ECM would need to occur for collapse of the distal lung air spaces. In this article, we discuss the role of proteinases, with an emphasis on macrophage-derived matrix metalloproteinases (MMPs), in the etiology of emphysema and the progressive destruction of alveolar ECM.

Emphysema and Elastin Degradation

COPD and Emphysema.

Chronic obstructive pulmonary disease (COPD), a leading cause of morbidity and mortality worldwide, comprises two distinct disorders: chronic bronchitis of the upper (conducting) airways and emphysema. Cigarette smoking is the leading cause of COPD [10–12], with air pollution also being a contributor. Chronic bronchitis is characterized by a chronic productive cough that may precede or follow development of airflow obstruction.

Emphysema, which develops in about 20% of smokers, is the permanent enlargement of airspaces distal to the terminal bronchioles (i.e., alveoli) and is a disease of the mature lung. Alveolar expansion is associated with some fetal or neonatal conditions, such as bronchopulmonary dysplasia. However, the causative mechanisms in these early diseases are likely the result of impaired or defective developmental processes involved in alveologenesis rather than the destruction of fully formed alveolar components. In contrast, emphysema develops slowly – after about 20–30 pack-years of continual smoking – in the presence of persistent low-grade inflammation. Overall, an abundance of human and animal data indicate that degradation of alveolar ECM, and specifically elastin, is a critical causative event in emphysema.

Genome Wide Associations Studies (GWAS) in Obstructive Lung Disease.

Surveying the genome to identify common single nucleotide polymorphisms (SNPs) linked to human traits provides a window into the genetic underpinnings of complex diseases. In the past decade, a number of GWAS have investigated the association between SNPs and obstructive lung disease, which encompasses emphysema [13–16]. While these efforts have identified several genetic variants linked to COPD, no ECM constituent or member of the MMP family has achieved genome-wide significance (typically defined as association P -value $< 5 \times 10^{-8}$). A limitation of standard GWAS methods is their reliance on strict, SNP-based statistical thresholds to minimize false positivity that consequently profoundly curtail the number of associations deemed significant. To overcome these shortcomings and building on the premise that genes do not exert their influence in isolation but rather cooperate in modular networks [17,18], we applied a pathway-based analysis to a large GWAS of subjects with airflow obstruction. This approach provided a comprehensive overview of the genetic programs associated with COPD, and identified multiple biological modules implicated in obstructive lung disease [19]. One prominent set of pathways mapped to ECM processes and subsequent network analysis of members of this module identified MMP-10 (see discussion below) as a key network hub and putative driver of ECM remodeling in COPD (Fig. 1).

ECM Target: Elastin or Collagen?

The progressive alveolar damage that defines emphysema is due to degradation of selective ECM components that likely results from excessive, unregulated, and prolonged proteolysis. The abundance of the fibrillar collagens in the alveolar wall and their role in the structural integrity of the alveolar sphere would seemingly make collagenolysis a reasonable culprit in the progression of emphysema. However, evidence in support of collagen breakdown as a causative process in emphysema is lacking. In fact, just the opposite seems to be true. Quantitative assessment of ECM amounts in areas of active emphysema in lungs from transplant recipients revealed that the content of fibrillar collagens is actually increased compared to the levels in comparable areas in non-emphysematous lungs [1,2,20]. Similarly, although limited collagen breakdown is evident soon after the onset of cigarette smoke exposure in guinea pigs, the relative volume of lung collagen increases during longer-term exposures [21]. The increase in collagen deposition at sites alveolar damage could indicate a reparative process. Although ectopic expression of human collagenase-1 (MMP-1; which is not in the rodent genome; Table 1) in mouse lung causes emphysema-like damage [22], it

was not shown if MMP-1 was expressed at sites of alveolar expansion or was acting as a collagenase. Similarly, though MMP-1 levels are elevated in bronchoalveolar lavage (BAL) of patients with emphysema, compared to smokers without disease, the levels did not correlate with disease severity or progression [23]. The accumulation of collagen at sites of emphysematous damage indicates that collagenases act in other compartments or are involved in processes unrelated to collagenolysis.

Overall, and has been summarized in more detail by Houghton [24], the breakdown of elastic fibers in the alveolar wall appears to be a critical and essential causative process in the development and progression of emphysema. Furthermore, specific elastin peptides generated by matrix degradation augment emphysema progression by acting as chemotactic agents that stimulate the influx of macrophages [25–27]. Ultrastructurally, elastic fibers are disorganized and fragmented in emphysematous lungs [28], and elevated levels of desmosine and isodesmosine, lysine crosslinks that are unique to elastin, are present in the blood and urine of smokers with emphysema [29]. Instillation of elastases, but not collagenases, into the lungs of experimental animals causes emphysema further implicating elastolytic enzymes in human disease [30–32].

The bulk of elastic fibers, which are abundant in blood vessels, lung, skin, and elastic cartilage, are deposited during late fetal and neonatal growth with essentially no renewed production during life in healthy, uninjured tissues, including lung [33,34]. During elastogenesis, tropoelastin, the monomeric precursor protein, is secreted and assembled on microfibrillar (fibulins, fibrillins) scaffolds. Via the activity of lysyl oxidase, the ϵ -amino groups of tropoelastin lysines residues are deaminated resulting in extensive covalent crosslinking of the monomers. The resultant polymeric fibers, which confer elasticity via energy-free repulsion of water from the hydrophobic domains, are highly resistant to proteolysis.

Because of these properties, elastic fibers are long-lived, a property that was best described in human lung. Shapiro et al. extracted elastin from cadaveric lung samples of people (all non-smokers) who were born from late 19th to mid-20th century and who had died at various ages. To assess elastin age and turnover rate, they quantified the racemization of L-enantiomers of aspartate to D-Asp – which accumulates at a rate of about 0.1% per year – and compared ¹⁴C levels in the fibers to the environmental levels through much of the 20th century. Due to atmospheric testing of nuclear weapons, ¹⁴C levels rose markedly in the 1950s then plummeted after the Test Ban Treaty was signed in 1963 thereby providing, in essence, a natural pulse-chase experiment. Their data demonstrated clearly that the elastin in human lungs is only deposited during the first 18 years of life [35]. In other words, the elastin laid down during your growth years is the elastin you will take to the grave. But elastin can be destroyed, and the loss of alveolar elastin in emphysema is a consequence of proteolytic destruction. Consequently, critical goals in emphysema research are to understand how to reboot alveolar formation (which we do not discuss here) and to find the causative cell and the destructive elastase(s) it makes. Knowing the specific proteinases causing elastolysis could lead to targeted therapies to reduce alveolar damage and loss of lung function in patients with COPD. Studies to date have largely pointed to specific

metalloproteinases made by macrophages, and below we summarize the key findings, discuss uncertainties, and propose new areas for investigation.

Macrophages in Emphysema

Macrophages have been long thought to be the culprit cells in emphysema [11,12,36,37]. Not only do they produce essentially all of the suspected destructive proteinases, but the number of macrophages is about 10-fold higher in the lungs of smokers than non-smokers and emphysema develops coincident with increased macrophage influx in both human smokers and in several mouse models [38–48]. In mouse models of cigarette smoke-induced emphysema, impaired macrophage influx is associated with protection from disease development [27,46]. Despite these compelling relationships, the exact role of macrophages in COPD remains to be identified, an issue that is further confounded by the functional heterogeneity of macrophages.

Macrophage Heterogeneity.

Circulating monocytes differentiate into a variety of tissue macrophages and dendritic cells that play essential, yet distinct roles in both promoting and resolving inflammation and in facilitating tissue repair and contributing to its destruction [49]. That a single cell type can serve opposing functions may seem counterintuitive, but dramatic phenotypic changes occur when macrophages respond to local stimuli [49–54]. Based on patterns of gene and protein expression and function, macrophages are commonly classified as classically activated (M1) or alternatively activated (M2) cells, as well as sub-M2 types [49–51,54]. The M1 phenotype is induced by infection and pro-inflammatory T_H1 cytokines [53]. M1 macrophages are effective at killing bacteria and release pro-inflammatory cytokines, such as IL-1 β , IL-12, and TNF α . In contrast, the M2 phenotype is induced by T_H2 cytokines IL-4 and IL-13 and other factors [53,54]. M2 macrophages release anti-inflammatory factors, such as IL-10 and TGF β 1, are weakly microbicidal, and promote repair [53]. Although dividing macrophages into M1 vs. M2 classes oversimplifies the complex continuum of functional and reversible states that these immune cells exist in *in vivo* [55–57], this lexicon provides an accessible description of the range of macrophage states.

M2-biased Macrophages in Emphysema.

Macrophages that function early in inflammation are distinct from those that function late in inflammation or in a persistent inflammatory response, such as long-term smoke exposure [54,58–65]. Whereas acute injury biases macrophages toward an M1 phenotype [54], long-term cigarette smoking promotes expansion of M2 macrophages [66]. Depletion of macrophages in the early phases of wound repair significantly impairs scar formation [67,68], whereas depletion of macrophages during later stages leads to an inability to resolve scars [62,69]. Hence, early phase macrophages, which are predominately M1-biased, contribute to ECM deposition, likely by producing profibrotic cytokines that promote the activation of resident fibroblasts and pericytes into ECM-producing myofibroblasts [54,58–61,70–74]. During the later resolution phase, macrophages tend to be remodeling-competent, M2-biased macrophages that clear excess scar deposition [60,72,75]. In acute models, depletion studies have indicated that M1-based macrophages are potentially

harmful, whereas as M2 macrophages are beneficial. Although far from being fully understood, the ability to degrade ECM appears to be – not surprisingly – the responsibility of macrophages and, in particular, M2 macrophages [60,62,76–80]. However, in chronic inflammatory settings, such as the airspace milieu of cigarette smokers, M2-biased macrophages [66] promote persistent ECM remodeling, potentially causing more harm than good.

ECM Degradation and Suspected Proteinases

Because destruction of elastin is the prominent characteristic feature of emphysema [11,20,28,81], extracellular proteinases with ability to degrade elastin are considered to be key drivers in this disease [11,37,39,82,83]. Regardless of their class, any proteinase that can degrade or cleave elastic fibers – which is determined *in vitro* – is an elastase, and some proteinases, such as MMP-12 (Table 1) and the serine proteinases neutrophil elastase (NE) and pancreatic elastase, carry this descriptor in their name (regardless if elastin breakdown is a physiologic function of the enzyme). The focus on elastases in emphysema arose from three broad observations. One, as discussed, elastin is degraded in emphysema, thereby implicating the action of an elastase. Two, emphysema develops spontaneously in people with $\alpha 1$ -antitrypsin (A1AT; Serpin A1) deficiency between ages 20–50 [84]. A1AT is a broad-acting serpin, a family of proteins that silence the activity of serine proteinases. Without sufficient A1AT, the unchecked activity of an enzyme, such as NE, would be allowed to chip away at alveolar elastin causing alveolar expansion. The development of emphysema in A1AT deficiency, which may be more linked to NE than to an MMP [85,86], led to the proteinase-antiproteinase imbalance concept of disease causation [82,87]. Three, as stated, instillation of elastases causes elastin degradation and emphysema in animals [30–32].

Metallo-elastases Made by Macrophages.

Although extracellular elastases are found in the metallo, serine, cysteine, and aspartate proteinase families, findings with both human samples and mouse models have largely implicated metalloproteinases in the breakdown of elastin in emphysema. For example, macrophages in the BAL of smokers with COPD have much more elastolytic activity than macrophages from healthy smokers and nonsmokers, and with the use of class-specific inhibitors, this increased ability to degrade elastin was largely due to metalloproteinase activity [39,88]. In line with these findings, several human and animal studies have linked many MMPs, particularly macrophage-derived MMPs, in COPD pathogenesis [12,39,89–97]. In addition, a broad-acting metalloproteinase inhibitor significantly blunted emphysema development in guinea pigs exposed to cigarette smoke [93]. In these studies, MMPs were speculated to contribute to disease progression by promoting inflammation and/or directly degrading ECM, most often elastin. However, as we discuss below, the precise function of any given MMP in COPD remains unclear. In addition, of the few specific MMPs thus far tested in genetically-defined mouse models of cigarette-smoke-induced emphysema (Table 1), most, if not all appear to function by influencing macrophage activation rather than directly acting to degrade elastin.

Furthermore, it is likely that members of other proteinase families, particularly cysteine proteinases, contribute to tissue destruction in emphysema [94]. Indeed, although Weiss and coworkers determined that much of the elastolytic activity of mouse and human macrophages is due to metalloproteinases – with human MMP-7 being the most potent metalloelastase – they also demonstrated an important contribution by secreted cysteine proteinases [98,99]. Although we focus on MMPs in this article, the importance of cysteine proteinases, some of which are potent elastases, as well other protease families [100], cannot be overlooked [94,101]. Several properties of cysteine proteinases indicate they could contribute to ECM destruction in lung disease. Although primarily lysosomal enzymes, some cysteine proteinases are secreted from macrophages [102] and active at neutral pH [103,104]. In addition, cysteine proteinases, such cathepsins S and K, efficiently degrade ECM proteins, including elastin [105]. The levels of cathepsin L and S and cystatin C, a natural inhibitor of cysteine proteinase, are elevated in lungs and plasma of smokers with emphysema [106–108]. Because they are produced and released by macrophages and are potent elastases, cysteine proteinases may play a critical role in emphysema, as they have been proposed to do in aneurysms [109], another disease characterized by destruction of elastin. Hence, the functions of secreted cysteine proteinases in smoke-induced lung disease provide many relevant opportunities for investigation.

MMPs: Effectors of Macrophage Activation.

Although some specific MMPs have been shown to have causative roles in emphysema in mouse models (Table 1), as we discuss below, with the possible exception of MMP-12, apparently most MMPs do not contribute to alveolar damage by directly degrading ECM. Rather, some MMPs control the ability of macrophages to degrade elastin, but how they do so and what proteinases actually destroy alveolar ECM remain unknown. As their name implies, MMPs are thought to degrade ECM, a function that is indeed performed by some members [98,110–112]. However, matrix degradation is neither a shared nor predominant function of these proteinases. Indeed, numerous findings demonstrate that individual MMPs regulate specific immune processes, including macrophage activation [113–123]. For example, MMP-10, –12, and –28 either promote or restrict macrophage influx into lung [27,46,117,120], and MMP-8, –10, –13, and –28 all influence macrophage activation with different functional outcomes [80,119,120,123–125].

MMPs control immune functions typically by gain-of-function processing of non-ECM proteins, such as cytokines, chemokines, and surface proteins [116,126–130]. Furthermore, several MMPs can affect the activity of other MMPs, typically by removing the repressive prodomain [131–134]. Two other important concepts, both supported by many observations with gene-targeted mice [113,115,135], are that 1) individual MMPs perform specific, non-redundant functions with no evidence of functional compensation by other MMPs. 2) In normal processes, such as repair and immunity, MMPs typically serve a beneficial role. However, if their expression is prolonged, then their catalytic activity can lead to disease, a concept that is likely relevant to ECM destruction in emphysema. Because MMPs act on much more than ECM, they can contribute to alveolar destruction by directly degrading ECM or indirectly by shaping the proteolytic phenotype of macrophages [136–138], as may be the role of MMP-10 and –28.

Determining the Role of MMPs in Cigarette Smoke-induced Emphysema.

In this section, we outline a general approach for evaluating and determining the role for a specific MMP in emphysema. Other than the experimental model, the experimental strategy we discuss would be applicable to studying MMPs (or any proteinase) in a range of systems. We do not claim to be dogmatic. The approach we outline is an approach, not the only approach.

A reasonable first observation would be to determine if the MMP of interest is present and active in the human disease and elevated (or reduced) from its level in healthy tissue. These endpoints can be collected by measurement of mRNA, protein, and active proteinase, among other means. Though gelatin zymography is often used for MMP activity, this technique is more an assay of amount than activity. After all, the inactive zymogen (hence, the assay's name) is detected just as efficiently as the active proteinase. However, with internal standards, one can derive a relative ratio of pro-MMP:active MMP by comparing the levels of each form, which differ by about 10 kD for essentially all MMPs [132]. The main advantages of gelatin zymography are that it is very sensitive, easy to do, and works across species. The major limitation is that this assay detects essentially only MMP-2 and MMP-9. Other substrate zymograms (e.g., κ -elastin or casein), which can detect several other MMPs, are much less sensitive. Overall, determining mRNA levels combined with immunostaining or *in situ* hybridization to identify cell source and location (ideally, where the disease lesions are seen) provide a reasonable approach to associate a given MMP with disease progression. If specific antibodies are available for the MMP of interest, flow cytometric approaches are, of course, a powerful means to assign cell identity.

Once altered expression of an MMP in disease tissue is established, functional validation can be done in genetically-defined mouse models [139]. Mice with targeted deletion of a specific MMP are exposed to smoke of tobacco cigarettes, most often reference cigarettes manufactured by the Kentucky Research and Development Center, University of Kentucky. Typically, the exposure protocol is 4 cigarettes per day, 6 days per week for 4–6 months, thereby making this a labor-intensive experimental model. Exposures can be done with precise (and expensive) nose-only equipment [48] or with simpler (and cheaper) chambers in which mice are flooded with smoke [46]. Regardless of the apparatus, the mice receive a massive – yet subtoxic – dose of smoke and develop chronic macrophage-rich inflammation that leads to emphysema within 4–6 months. A potential shortcoming with this model is that the hefty smoke exposure could lead to lung injury that, in turn, could cause emphysema-like pathology.

By comparing disease extent (e.g., via morphometry of lung sections and pulmonary function) between litter-matched wildtype and MMP-null mice, one can determine a role in emphysema. By use of conditional-null mice, which has not yet been done for any MMP knock-out strain in smoking models, one can potentially derive a more refined conclusion on the critical cell source of the MMP. At this point, among the follow-up mechanistic question that could be addressed are whether the MMP functions in smoking-related lung via proteolysis and, if so, what is/are critical protein substrate(s).

MMPs have motifs away from the catalytic domain – called exosites – that are involved in their ability to bind other proteins, lipids, and carbohydrates, interactions that are likely critical for controlling specific enzyme:substrate interactions [132,140]. As MMPs lack inherent substrate specificity (unlike, e.g., coagulation cascade serine proteinases), their activity *in vivo* is likely limited to specific proteins by allosteric interactions that confine the proteinase to pericellular niches and, in turn, substrates. However, their ability to bind macromolecules provides MMPs the potential to control cellular activities without functioning as a proteinase, as has been shown for MMP-12 (see below). One way to determine if the MMP-dependent role in emphysema is a consequence of the enzyme's proteolytic activity is to recover the cell type-specific expression of the missing MMP (i.e., in the null background) with mutant proteins that possess or lack catalytic activity [139,141]. By substituting Gly for a conserved Val in the prodomain, proMMPs undergo efficient autolytic activation to their active form. In contrast, Ala substitution of the electrophile Glu renders a catalytically inactive MMP. Both mutations do not affect production, structure, and secretion of the enzyme.

To understand how a proteinase functions, one needs to identify its protein substrate(s); however, reaching this goal is not straightforward [113]. Many presumed MMP substrates were “identified” by incubating purified proteinase with a given protein – most often an ECM protein – under ideal conditions. A major shortcoming with this approach is that shows what an MMP can do, not what it does *in vivo*. Thus, other approaches are needed to uncover true, physiologic substrates. Deduction has been the most successful approach to finding candidate substrates for specific MMPs. For example, overt phenotypes in null-out mice have pointed to candidates, such as excess of collagen deposition in *Mmp14*^{-/-} mice (type I collagen) [110] and maintained cell-cell junctions (E-cadherin) in injured epithelium of and reduced apoptosis (FasL) in *Mmp7*^{-/-} mice [142,143], among others [121,127,144]. As MMPs bind substrates via exosites, these interactions can be exploited to design affinity approaches to find binding partners [145]. More recently, proteomics has emerged as a powerful analytical tool to identify substrates of specific proteinases [146–153]. A basic strategy would be to use quantitative subtractive proteomics to compare the cell surface and secreted/shed proteomes between samples with or without MMP activity.

An issue with conventional proteomics is that MMP-cleaved substrates are further digested by trypsin, used in sample preparation for mass spectrometry (MS) analysis, and these peptides are not directly identified by post-MS data acquisition computational analysis. Because *in silico* trypsin digest databases are based on the intact protein, these neopeptides are “invisible” but can be found by directed post-acquisition analysis. However, this issue can be exploited. Because proteolysis generates a new N-terminus, these ends can be enriched by depleting known trypsin peptides by differential isotopic labeling approaches, which also allows identification of the P1' amino acid in the cleavage site [152,154].

As it is likely that multiple proteins will be identified via a subtractive proteomics approach, a hierarchical strategy can be used to trim and validate candidates. As the candidate substrates must be a secreted, transmembrane, or membrane-associated protein, proteins from other cellular compartments can be eliminated as false-positives. Furthermore, in emphysema a focus can be placed on proteins with a known or potential role in

inflammation or ECM metabolism, especially if the protein is known to be shed from the cell surface. In addition, candidate substrates must meet the following biologic/biochemical criteria: i) *In vivo*, the substrate should be shed/degraded in wildtypes but not (or accumulate) in MMP-null mice. ii) *In vivo*, the putative substrate and the MMP should be expressed by the same cells at the same time. iii) The substrate cleavage site *in vivo* must match that mapped in defined and cell-based *in vitro* degradation assays. If these three criteria are met, then a variety of loss- and gain-of-function approaches can be applied to assess if the substrate cleavage mediates expected disease outcomes. A particular powerful validation strategy is to over-express MMP-resistant substrate (mutation of the cleavage site) in wildtype mice/cells and demonstrate that they behave like MMP-null cells.

Below we discuss findings that have implicated (MMP-10, -12, and -28) or rejected (MMP-9) roles for specific MMPs in smoke-induced emphysema. These examples have largely followed the general experimental approach discussed above (but not necessarily in the order we outlined). That is, these enzymes were found to be elevated in lungs of smokers with COPD, and a role in emphysema was demonstrated or refuted using genetically-defined mice exposed chronic to cigarette smoke.

MMP-12: Macrophage Metalloelastase.

Compared to healthy and former smokers and nonsmokers, MMP-12 levels are about 4–10-fold elevated in BAL from smokers with COPD [95], suggesting role in emphysema. On the other hand, a promoter SNP in the *MMP12* gene – of unknown functional impact – is associated with reduced risk for developing COPD in smokers [96]. In the lab, an abundance of experimental data have compellingly established a role for MMP-12 in the development of emphysema in mice. Near the end of the last century, Shapiro and coworkers demonstrated that MMP-12 is required for the development of emphysema in mice exposed to cigarette smoke [46]. Since then, data from several, distinct models have convincingly demonstrate that macrophage-derived MMP-12 is required for alveolar failure in mice, a topic that was thoroughly discussed in a recent review [24]. For example, spontaneous emphysema develops at about the same age and rate in adult mice lacking the $\alpha_v\beta_6$ integrin (*Itgb6*^{-/-}) on lung epithelial cells [155] or the cytochrome b-245 beta chain (*Cybb*^{-/-}), a phagocyte-specific component of NADPH oxidase, in macrophages [47]. That these similar phenotypes manifest after lung development is complete and normal in both strains indicates that the alveolar expansion occurred via a mechanism relevant to emphysema. In both studies, crossing the *Itgb6*^{-/-} or *Cybb*^{-/-} mice with *Mmp12*^{-/-} mice prevented the development of emphysema. Furthermore, in both studies, the targeted gene product had an effect on moderating MMP-12 production or activity. In the absence of $\alpha_v\beta_6$, altered TGF β 1 signaling markedly stimulated *Mmp12* expression by macrophages [155], and impaired oxidase generation in *Cybb*^{-/-} macrophages eliminated a cell-autonomous means to silence MMP-12 activity [47].

Overall, an abundance of data demonstrates a critical role for MMP-12 in the development of smoke-induced emphysema in mice, and the high levels of this MMP lungs of smokers with COPD implicates it in the human disease as well. But is MMP-12 causing emphysema by directly degrading alveolar elastin? Mouse macrophages in culture require MMP-12 to

degrade elastin [156], and the level of elastin fragments – a reasonable surrogate endpoint of elastin degradation – are reduced in the BAL of smoke-exposed *Mmp12*^{-/-} mice [27]. Despite these compelling data, direct elastolysis by MMP-12 was not demonstrated, and MMP-12 released by macrophages is not effective at degrading elastin [98].

MMP-12 could impact elastin degradation by affecting macrophage behavior, which is apparently how both MMP-10 and MMP-28 function in emphysema (below). Macrophage influx into the lungs of smoke-exposed mice is dependent on MMP-12 [46], a process that has been linked to MMP-12-dependent elastin degradation. Houghton et al. [27] reported that MMP-12 is needed for the generation of a macrophage chemotactic activity in the smoke-exposed lung, which they identified as a 6-amino acid fragment of elastin. Interestingly, years before, Senior and coworkers established that this same elastin fragment – VGVAPG – is a potent macrophage chemoattractant [26,157,158]. Though these data provide additional evidence that MMP-12 functions as an elastase, they also can be interpreted as showing that this proteinase affects macrophage activation. Indeed, in studies on helminth-mediated fibrosis, Wynn and coworkers concluded that MMP-12 impacts the pro-inflammatory activity of macrophages and their ability to express other MMPs with ECM-degrading activity [159].

MMP-12 could influence lung disease independent of its proteolytic activity. In virus-infected cells, including macrophages, secreted MMP-12 is internalized (by an unknown mechanism) and transported into the nucleus where it modulates the expression of genes that code for putative substrates [160]. A consequence of nuclear import of MMP-12 protein is enhanced NFκB signaling, a pro-inflammatory process that is relevant to emphysema [161]. Furthermore, the non-catalytic C-terminus of MMP-12 has potent antibacterial activity [162]. The role of these non-proteolytic functions in destructive lung disease remains unknown.

The findings with MMP-10 and MMP-28 further question how MMP-12 functions in emphysema. As we discuss below, both *Mmp10*^{-/-} and *Mmp28*^{-/-} mice are protected from the development of emphysema due to long-term cigarette smoke exposure. However, protection in either strain is not associated with marked changes in MMP-12 expression. Compared to the levels in wildtype animals or cells, *Mmp12* mRNA levels are not altered in smoke-exposed *Mmp28*^{-/-} mice [48] or in M1-biased *Mmp10*^{-/-} macrophages [120]. Thus, MMP-12 is apparently necessary but not sufficient for the development of emphysema. As discussed, both MMP-10 and MMP-28 appear to promote the development of emphysema by controlling distinct programs in macrophages (Fig. 2).

MMP-10: Stromelysin-2.

In both humans and mice, MMP10 is not expressed in developing or adult tissues, including lung [120,163]. However, in response to injury or infection, MMP10 is induced by macrophages and to a lesser extent by epithelial cells [80,120]. The widespread expression of MMP10 among tissues and insults suggests that this MMP serves critical roles in the host response to environmental insults. In models of lung infection, skin injury, and colitis, MMP-10 moderates inflammation by promoting the transition from M1 to M2 macrophages [80,120,125]. In addition, MMP-10 is required for M2 macrophages to efficiently degrade

fibrillar collagen [80]. Because it cannot cleave fibrillar collagens [164], MMP-10 may control the expression or activation of macrophage-derived collagenase. Indeed, the lack of collagenase activity in *Mmp10*^{-/-} macrophages was due to reduced expression of MMP-13 (collagenase-3). Thus, MMP-10 appears to have important roles in controlling macrophage activation, including the induction of ECM-degradation programs in M2-biased cells (Fig. 2).

As stated, the GWAS studies discussed above identified *MMP10* has a highly connected hub within the ECM module (Fig. 1) suggesting that this proteinase is a relevant disease target. In support this idea, MMP-10 is produced by macrophages from human smokers with emphysema [165] and, quite important, is one of two genes whose expression within areas of active emphysema progression correlates with reduced lung function in smokers [166]. In addition, the levels of MMP-10 protein in BAL correlates with more severe forms of emphysema in human smokers [167]. Overall, the human data with MMP-10 are among the more compelling in linking a specific MMP to the emphysema, and this linkage has been validated in mice. Using a model of chronic (6 mo) exposure to cigarette smoke, we found that *Mmp10*^{-/-} mice are resistant to the development of emphysema [19].

These mouse findings complement well the human data and indicate that macrophage MMP-10 contributes to the progression of emphysema. Although MMP-10 does have elastase activity *in vitro* (Table 1), it is not a particularly potent elastase [168]. Furthermore, compared to other MMPs, MMP-10 is expressed at low levels by macrophages, about 1,000-fold less than MMP-12 [120], suggesting that insufficient enzyme is made for a meaningful or direct impact on elastin breakdown. Rather, as discussed above, MMP-10 appears to control macrophage activation and promotes the ECM-degrading activity of M2 macrophages [80]. Thus, in a chronic setting like the smoke-exposed lung, MMP-10-driven ECM remodeling by macrophages could be a key regulatory event in the pathogenesis of emphysema.

MMP-28: Epilysin.

MMP-28 is the last identified member of the MMP family. In both humans and mice, *MMP28* and *Mmp28* mRNAs are constitutively expressed at high levels in the epithelium of many tissues, particularly in the lung [169–171], observations that led to it being named epilysin. We reported that monocytes and macrophages also express MMP-28 and that it functions to restrain macrophage recruitment to tissue and modulates macrophage polarization, promoting a less inflammatory, more reparative phenotype (i.e., M2-biased) [117,119,172]. MMP-28 protein levels are increased in human COPD lung tissue, and chronic cigarette-smoke exposure to mice led to an increase in *Mmp28* mRNA levels in alveolar macrophages and lung tissue [48]. These recent expression data suggest that MMP-28 has a role in emphysema pathogenesis. In fact, we found that *Mmp28*^{-/-} mice were protected from emphysema development due to chronic cigarette-smoke exposure model indicating that MMP-28 contributes to disease pathogenesis.

MMP-28 is not an elastase (Table 1) and does not appear to function as a matrix-degrading proteinase [169,173]. Reduced airspace enlargement in *Mmp28*^{-/-} mice could be due to an effect on inflammation. Indeed, *Mmp28*^{-/-} mice had reduced numbers of lymphocytes,

neutrophils, and alveolar macrophages in the lung compared to smoke-exposed wildtype mice. Whole lung transcriptomics revealed that MMP-28 was associated with stimulation of chemokine expression. These findings suggest that MMP-28 promotes a chronic inflammatory response to cigarette smoke involved in emphysema pathogenesis (Fig. 2). However, the specific contributions of epithelial and macrophage MMP-28 to emphysema development and its mode of action (i.e., its proteolytic substrates) have yet to be determined.

MMP-9: Gelatinase B.

MMP-9 is an elastase [174], is produced by both human and mouse macrophages [175], and is present in the lungs of patients with COPD and emphysema [90,175], making it a reasonable suspect proteinase. In addition, a SNP in the *MMP9* promoter, which leads to enhanced transcription and is linked to the severity of atherosclerosis [176], associates with the risk of developing smoke-induced emphysema [177]. Though these varied observations would lead one to propose that MMP-9 contributes to alveolar damage in emphysema, other data – both human and mice – indicate that it does not. *Mmp9*^{-/-} mice are not protected from developing emphysema-like alveolar damage in response to LPS-mediated inflammation [178] and, quite important, develop the same degree of cigarette smoke-induced inflammation and alveolar damage as wildtypes [97]. In humans, MMP-9 levels in blood do not track with emphysema progression or severity [23] and the levels of macrophage *MMP9* mRNA in lung do not correlate with markers of ongoing lung damage and do not differ between regions with or without emphysema [97].

In contrast, other mouse models have suggested a causative role for MMP-9 in emphysema. Transgenic over-expression of human MMP-9 in macrophages leads to spontaneous emphysema in adult mice [179]; however, a caveat with this type of experimental strategy is that it shows what a proteinase can do, not what it does do. In a transgenic model of IL-13-induced lung inflammation, MMP-9 deficiency protected against alveolar expansion [180]. However, in this model, in which IL-13 is over-expressed by airway epithelial cells, MMP-9 could influence other IL-13-dependent processes that, in turn, affect alveolar remodeling. Overall, we agree with the conclusion of Atkinson et al. [97] that “*specific inhibition of MMP-9 is unlikely to be an effective therapy for cigarette smoke-induced emphysema*”. In other words, MMP-9 has little to no role in ECM breakdown in emphysema (Table 1).

MMP-7: Matrilysin.

Compared to healthy controls, elevated levels of MMP-7 are detected in blood of smokers with COPD and correlated with reduced lung function [181,182], and a polymorphism in the *MMP7* gene correlates closely with COPD risk [183]. However, unlike MMP-10, it has not yet been assessed if MMP-7 is produced at sites of tissue destruction in human lungs, but this should be examined. As stated, MMP12 released by macrophages is not that effective at degrading elastin, but human MMP-7 is a potent elastase [98]. Although MMP7 is expressed by mucosal epithelium and macrophages in human, in mice it is produced only by epithelial cells [184]. The development and extent of cigarette smoke-induced emphysema does not differ between wildtype and *Mmp7*^{-/-} mice (WCP, unpublished observations), but these negative data may reflect that the proteinase is not produced by the critical cell type in this

model, i.e., macrophages. But even if MMP-7 was expressed by mouse macrophage, it may not have a role in emphysema in this animal model. Unlike the human proteinase, mouse MMP-7 is not an elastase [184]. Thus, an appropriate experiment would be to express human MMP-7 in mouse macrophages, an approach well justified by the human data linking MMP-7 to emphysema.

Other Metalloproteinases.

As we discussed above, airspace enlargement that arises during development may be mechanistically distinct from the development of emphysema in adult lungs as a consequence of long-term cigarette smoke exposure. That said, understanding mechanisms of spontaneous airspace enlargement could shed light on processes relevant to onset or progression of emphysema. However, with the exception of MMP-14, lung developmental phenotypes have not been reported for mice lacking specific MMPs. *Mmp14*^{-/-} mice have reduced alveolar area and number of alveolar pores (pores of Kohn) and increase airspaces, a phenotype that resembles emphysema [185]. MMP-14 has been linked to the activation of pro-MMP-2 [132], but these defects are not phenocopied in *Mmp2*^{-/-} mice [185]. The *Mmp14*^{-/-} alveolar phenotypes were not associated with abnormalities in the deposition of collagen and elastin or with increased inflammation, thereby setting MMP-14-deficiency (at least in young mice) apart from what occurs in smoke-exposed adult lungs. As global silencing of *Mmp14* leads to perinatal lethality [110,186], a role for MMP-14 in smoke-induced lung disease in mature mice has not yet been assessed.

Tissue inhibitor of metalloproteinases 3 (TIMP-3), which is prominent in the lung [187], can silence the activity of several MMPs and other metalloproteinase, such as ADAMs and ADAMTSs [188]. *Timp3*^{-/-} mice have impaired branching morphogenesis *in utero* [189] and, after birth, develop spontaneous airspace enlargement and impaired lung function that are evident 2 weeks after birth [190,191]. These conditions progress with time and are associated with reduced collagen deposition, increased collagenolysis and metalloproteinase activity, but with no increase in inflammation [190]. Though the lack of an effect on inflammation and the increase in collagenase activity do not gel with what is typically seen in emphysema, the progressive development of emphysema-like lesions in aged *Timp3*^{-/-} mice suggests that unchecked metalloproteinases produced by resident cells could contribute to disease pathogenesis. Indeed, mutations in *TIMP3* associate strongly with the development of spontaneous and severe emphysema in two families with Sorsby fundus dystrophy [192]. Thus, further research into a role for TIMP-3 in emphysema is warranted.

Other MMPs and other classes of metalloproteinases could function in COPD and lung destruction. For example, increased MMP-13 (collagenase-3) levels are associated with the development of spontaneous airspace enlargement in mice lacking α 1,6-fucosyltransferase [193], but an association with disease in humans is lacking. Though MMP-2 levels are increased in human COPD specimens [194], a functional role for MMP-2 in emphysema has not been studied. As discussed elsewhere [195], ADAMTSs are emerging as a class of metalloproteinases with relevant roles in ECM degradation, yet their role in lung disease is unknown. A PubMed search with the string “emphysema and ADAMTS” returned no hits.

Thus, in addition to cysteine proteinases, assessing the role of specific ADAMTSs in destructive lung disease would be a worthy area of exploration.

Summary

Based on the reported findings discussed above, MMP-10, -12, and -28 each appear to have distinct, non-overlapping roles in the development of cigarette smoke-induced emphysema. Each is necessary for alveolar destruction and likely act via distinct mechanisms. Furthermore, other MMPs, particularly MMP-7, may serve critical roles in disease pathogenesis and, hence, are worthy of further investigation. Although much data indicates that MMP-12 functions to directly degrade elastin, other observations, especially the protection against the development of emphysema in *Mmp10*^{-/-} and *Mmp28*^{-/-} mice who are replete with MMP-12, suggest that this proteinase functions elsewhere. One possibility is that these three MMPs control separate pathways of macrophage activation but that each converge on common cellular programs regulating the ability of these cells to degrade ECM, as shown for MMP-10. Hence, a goal of our ongoing research program is to identify the substrates that MMP-10 and MMP-28 degrades or sheds. We speculate the substrates will be surface proteins on macrophages whose cleavage will influence the phenotype and behavior of these effector leukocytes. Such studies may identify factors that activate pathways that can be targeted for modulating the pathobiology of COPD, reveal novel MMP substrates promoting emphysema pathogenesis beyond elastolysis, and open the door to new therapeutic targets. Furthermore, additional insights into function can be gleaned by use of integrative approaches to merge large-scale human population genetics (GWAS, whole-genome sequencing) with lung tissue-specific assays (gene expression, proteomics, metabolomics) and validation using animal models and cell-specific *in vitro* studies.

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Highlights

Several studies have implicated a causative role for specific matrix metalloproteinases (MMPs) in the development and progression of cigarette smoke-induced chronic obstructive pulmonary disease (COPD) and its severe sequela, emphysema. However, the precise function of any given MMP in emphysema remains an unanswered question. In this article, the authors discuss findings supporting or refuting the roles for specific MMPs in the development of destructive lung disease.

Enriched Biological Modules in Airflow Obstruction GWAS

Figure 1. Graphical overview of enriched biological modules associated with obstructive lung disease.

This diagram was constructed based on gene set enrichment analysis of a large-scale GWAS of subjects with airflow obstruction. Each depicted module is an aggregate of multiple highly enriched and functionally related pathways (false discovery rate < 0.001). For select modules, a few representative pathways have been labeled (e.g., TGF- β signaling, Ceramide pathway, NOS signaling) for illustrative purposes. Note the diversity of biological processes associated with COPD, spanning developmental programs, immunity, proliferation, apoptosis and extracellular matrix (ECM). Deeper exploration of the ECM module using

gene product interaction network analysis identified MMP10 as the most interconnected node and a potential orchestrator of tissue remodeling in obstructive lung disease. Other ECM network genes included collagens, laminins, fibrillins and fibulins. NOS, nitric oxide synthase; TGF- β , transforming growth factor beta; EGF, epidermal growth factor; PDGF, platelet-derived growth factor. Modified from [19].

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Figure 2. Diverse Role of Macrophage MMPs in Emphysema.

Both MMP-10 and MMP-28 have causative roles in the development of cigarette smoke-induced emphysema. These two MMPs promote the conversion of M1-biased macrophages to M2-biased cells but distinct functional consequences. Whereas MMP-10 influences the proteolytic potential of macrophages, MMP-28 shapes the pro-inflammatory activity of these cells. In contrast, other MMPs, such as MMP-12 and possibly MMP-7 (in human macrophages), may directly degrade alveolar elastin, likely along with other macrophage enzymes, such as specific cysteine proteinases.

Table I.**Roles of Macrophage MMPs in Emphysema**

Macrophage MMPs¹		Human	Mouse	Is an Elastase	Role in Emphysema²	Ref
MMP-1	Collagenase-1	✓			May Contribute ³	[22]
MMP-7	Matrilysin	✓		✓ ⁴	Maybe	
MMP-8	Collagenase-2	✓	✓		Not tested	
MMP-9	Gelatinase B	✓	✓	✓	Little to No Role	[97]
MMP-10	Stromelysin-2	✓	✓	✓	Contributes	[19]
MMP-12	Macrophage metalloelastase	✓	✓	✓	Contributes	[46] ⁵
MMP-13	Collagenase-3	✓	✓		Not tested	
MMP-14	MT1-MMP	✓	✓		Not tested	
MMP-28	Epilysin	✓	✓		Contributes	[48]

¹This is not a complete list of the MMPs that are or can be expressed by human and mouse macrophages. E.g., our publically available datasets of mouse macrophage transcriptomics [120] reveal expression of additional MMPs (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=epkvmssmvhmrlmf&acc=GSE78175>).

²As determined by mouse models of long-term exposure to cigarette smoke; except for MMP-1.

³As discussed in the text, ectopic expression of human MMP-1 in mice led to spontaneous emphysema; however, the recombinant proteinase was not shown to be expressed by host (mouse) macrophages.

⁴Human MMP-7 is a potent elastase; mouse MMP-7, which is not expressed by mouse macrophages, has no elastolytic activity.

⁵Among many others; see text.