Downregulation of epithelial DUOX1 in Chronic Obstructive Pulmonary Disease contributes to disease pathogenesis

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In-Press Preview

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Downregulation of Epithelial DUOX1 in Chronic Obstructive Pulmonary Disease

Contributes to Disease Pathogenesis

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COPD is a chronic respiratory disease characterized by small airway remodeling and alveolar emphysema due to environmental stresses such as cigarette smoking (CS). Oxidative stress is commonly implicated in COPD pathology, but recent findings suggest that one oxidant-producing NADPH oxidase homolog, dual oxidase 1 (DUOX1), is downregulated in the airways of COPD patients. We evaluated lung tissue sections from COPD patients for small airway epithelial DUOX1 protein expression, in association with measures of lung function and small airway and alveolar remodeling. We also addressed the impact of DUOX1 for lung tissue remodeling in mouse models of COPD. Small airway DUOX1 levels were decreased in advanced COPD, and correlated with loss of lung function and markers of emphysema and remodeling. Similarly, DUOX1 downregulation in correlation with extracellular matrix remodeling was observed in a genetic model of COPD, transgenic SPC-TNF-α mice. Finally, development of subepithelial airway fibrosis in mice due to exposure to the CS-component acrolein, or alveolar emphysema induced by administration of elastase, were in both cases exacerbated in Duox1-deficient mice. Collectively, our studies highlight that downregulation of DUOX1 may be a contributing feature of COPD pathogenesis, likely related to impaired DUOX1-mediated innate injury responses involved in epithelial homeostasis.
Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic irreversible disease of the lungs characterized by airflow limitation due to destruction of the lung parenchyma (emphysema) and/or remodeling of the small airways (1,2). COPD is a major and growing global health problem that is predicted to be the third leading cause of death worldwide by 2030 (3). In susceptible individuals, environmental insults such as cigarette smoke (CS) are at the foundation of COPD pathogenesis, which is characterized by persistent inflammation and a protease/anti-protease imbalance, collectively contributing to alveolar destruction and airway remodeling (4). Importantly, small airway disease and emphysema development may mechanistically be linked, since CS-induced small airway inflammation may propagate to the alveolar septa, in turn destroying bronchiolar-alveolar attachments, and eventually proceed into lung parenchymal destruction (5).

While classically thought to be independent pathological manifestations of COPD, more recent evidence indicates that more emphysematous lungs tend to have fewer small airways (6). In fact, the disappearance of small airways, which begins in the early stages of COPD, is a dominant characteristic in all COPD patients and appears to precede emphysema development (7).

A well-documented aspect of COPD is the presence of an oxidant/antioxidant imbalance (8), believed to be caused by reactive oxygen species (ROS) present in tobacco smoke or produced during chronic inflammation. This imbalance is illustrated by increased irreversible oxidation of critical biological molecules (9), evidence of mitochondrial dysfunction, and aging-related impairment in antioxidant defense mechanisms (10), and thought to contribute to injury to critical cell constituents, lung cell dysfunction and lung function decline (11,12). Based on this premise, antioxidant treatment therapies have been advocated for COPD, with limited success. While small molecule thiol antioxidants (e.g. N-acetyl cysteine, erdosteine) have shown some clinical benefit, this likely relates to their mucolytic properties rather than their proposed antioxidant effects.
Supplementation with other small molecular antioxidants has not shown any benefit and may even have adverse effects (14).

Contrasting the general concept of oxidative stress, regulated biological production of ROS by e.g. NADPH oxidases (NOX) is increasingly implicated in diverse biological processes via so-called redox-based signaling (15). The NOX family of NADPH oxidases produce ROS (O$_2^-$ or H$_2$O$_2$) as their primary function to mediate critical physiological functions including host defense, cell proliferation or differentiation (16). While all seven NOX enzymes are all expressed in various cell types within the lung, relatively little is known with respect to their potential role in COPD pathology. Several reports indicate that NOX4 is upregulated in airway smooth muscle of COPD patients and correlates with disease severity (17,18). NOX2, primarily expressed in cells of the innate immune system, also appears to be increased in COPD, and some studies suggest that NOX2 contributes to experimental emphysema, although contrasting findings were reported as well and NOX2-deficiency may even promote spontaneous emphysema (19-21). Recently, a crucial role was reported for the NOX organizer protein NOXO1, which regulates the function of several isoforms, in CS-induced emphysema (21). In contrast to the general concept of increased involvement of NOX enzymes COPD pathology, recent studies indicate that the dual oxidases DUOX1, and to a lesser extent DUOX2, are downregulated within the bronchial epithelia of healthy smokers and patients with COPD (21-23). Both DUOX1 and DUOX2 are primarily expressed in airway and alveolar epithelia, with a proposed critical function in innate antimicrobial and antiviral host defense, with DUOX1 being particularly critical in innate airway epithelial wound responses to diverse non-microbial triggers (24,25). In this regard, DUOX1 downregulation during COPD may conceivably contribute to disease progression or exacerbation, due to a decline in regenerative capacity and host defense. In addition, downregulation of epithelial DUOX1 was recently shown to promote features of epithelial-mesenchymal transition (EMT) (26), which may also be relevant for small airway remodeling in COPD (27).
The present study aimed to address the importance of DUOX1 downregulation in small airway remodeling as well as emphysema development in COPD. We observed that downregulation of airway DUOX1 in COPD patients was strongly correlated with lung function loss, and with markers of small airway remodeling and destruction. Moreover, we provide evidence that DUOX1 deficiency leads to enhanced features of small airway remodeling and emphysema in experimental mouse models of COPD, suggesting that DUOX1 downregulation in COPD may actively contribute to disease pathogenesis.
Results

Airway epithelial DUOX1 is suppressed in COPD patients and correlates with lung function

Following up on previous studies demonstrating that lung epithelial DUOX1 mRNA expression is attenuated in active smokers and in patients with COPD (22,23), we evaluated tissue sections from a previous study cohort of COPD patients and control subjects at Maastricht University (UM) (28) for protein expression of DUOX1 in the small airways. Control subjects and COPD patients were age-matched, but the ratio of current to ex-smokers did significantly differ and COPD patients had smoked more pack-years (Supplemental Table S1). As expected, DUOX1 protein was prominently expressed in the bronchial and small airway epithelium, and small airway DUOX1 expression was found to be reduced in tissue sections from COPD patients, especially in very severe (GOLD IV) COPD patients, compared to age-matched non-COPD controls (Figure 1A and B, with representative images of score 1-4 (Supplemental Figure S1). Adjustment for smoking status had no effect on the ANOVA results comparing control and GOLD stages (Supplemental Table S2). After correcting for age, sex, pack years and smoking status by ANCOVA analysis, small airway DUOX1 scoring revealed a clear trend towards significance (p=0.058) between GOLD IV patients (Mean ± SE: 1.582 ± 0.210) and controls (Mean ± SE: 2.487 ± 0.262). We also examined the overall relationship between DUOX1 scores and pack-years of smoking and found no relationship (r = -0.083, p = 0.635) (Supplemental Table S3). The GOLD IV COPD patients included in this study underwent lung volume reduction surgery (LVRS) because of severe emphysema (29), suggesting that DUOX1 downregulation may be associated with emphysema.

Correlation of epithelial DUOX1 staining scores with parameters of lung function indicated a striking positive association of DUOX1 score with spirometric parameters (FEV₁, FVC) as well as diffusing capacity (DLco, diffusing capacity for carbon monoxide; which indicates loss of alveolar surface area and capillary bed, impairing diffusion (30)), both based on inclusion of all subjects
(including controls, Figure 1C and Table 1) as well as COPD patients (GOLD II vs GOLD IV) alone (Table 1). We examined the correlations between DUOX1 score and %FEVI, %DLCO, %FVC and FEV1/FVC, after adjustment for covariates (smoking status, age, BMI), which had little impact on the results, although the correlation between DUOX1 score and DLCO was slightly attenuated (Supplemental Table S3). Since relationships of DUOX1 scores with %FEVI, %DLCO, %FVC and %FEV1/%FVC may not be linear, we also computed non-parametric Spearman correlation coefficients based on ranks rather than numerical values. The results were very similar to the Pearson correlations, indicating that the linearity assumption is reasonable. We also computed Pearson correlations using a logarithmic transformation of DUOX1 score. Again, the results were very similar to those based on the untransformed score (Supplemental Table S3).

Alterations in lung tissue content of elastin and collagen from these patients were previously published (28), showing that elastin was significantly decreased in COPD patients in both alveolar as well as small airway walls, whereas collagen was found to be increased in both alveolar and small airway walls. DUOX1 staining scores measured in the present study positively correlated with the critical remodeling marker elastin in the small airways, although no statistically significant correlation was observed with collagen (Table 1). Overall, these results imply that the gradual loss of DUOX1 in the small airways of COPD patients is associated with impaired lung function, emphysema and airway remodeling in these patients.

DUOX1 downregulation in mouse models of COPD is associated with increased remodeling.

We next investigated whether DUOX1 was similarly downregulated in mouse models of COPD. First, we evaluated lung tissues from SPC-TNFα mice, transgenic mice that constitutively overexpress tumor necrosis factor-α (TNF-α) under the surfactant protein C promoter (SP-C).
These mice develop chronic neutrophilic inflammation, airway remodeling (increased collagen deposition and elastin remodeling) and parenchymal alveolar destruction with increased expiratory static compliance, indicative of COPD development (28,31-33). Consistent with our findings in COPD patients, DUOX1 was downregulated in 6-month old SPC-TNFα mice compared to wildtype counterparts, indicated by a trend towards decreased Duox1 mRNA expression (p=0.09) (Figure 2A), and significantly decreased small airway DUOX1 protein levels (Figure 2B and C). We assessed elastin and collagen remodeling in the parenchyma and small airways of these mice, which showed reduced levels of parenchymal and small airway elastin in SPC-TNFα mice (Figure 3A,B and G,H) as well as increased collagen (Figure 3D,E and 2J,K). Furthermore, parenchymal elastin staining, but not small airway elastin levels, positively correlated with the DUOX1 score (Figure 3C and I). No significant correlation was observed between parenchymal and small airway collagen levels and DUOX1 staining score (Figure 3F and L). Collectively, these findings are consistent with observations in human COPD, showing an association between DUOX1 downregulation and elastin degradation.

We additionally utilized a mouse model of chronic exposure to acrolein, a major bioactive component of cigarette smoke (CS) (34), which was previously shown to induce pulmonary injury and inflammation, mucus hypersecretion, as well as airflow limitation (35,36). Consistent with observations of reduced DUOX1 mRNA expression in the airways of smokers (22), chronic exposure of C57BL/6J mice to acrolein (5 ppm; 4 hrs/day, for 2 weeks) resulted in reduced lung DUOX1 protein (Supplemental Figure S2A) and Duox1 mRNA expression (Supplemental Figure S2B). Acrolein exposure also enhanced production of TGF-β1 (Supplemental Figure S2C), a profibrotic growth factor that is thought to contribute to features of EMT and subepithelial fibrosis in COPD (37-39). To address the potential impact of Duox1 downregulation for such EMT features, we isolated mouse tracheal epithelial cells (MTEC) from C57BL/6J mice and exposed them to either acrolein or TGF-β, over a 2-week period. Indeed, both acrolein and TGF-β1 caused
a downregulation of *Duox1* mRNA levels (**Supplemental Figure S2D**), and acrolein exposure resulted in a dose-dependent loss of E-cadherin, with a concomitant gain in vimentin (**Supplemental Figure S2E**). Moreover, acrolein-induced alterations in E-cadherin and vimentin were enhanced in MTECs from *Duox1*−/− mice, indicating that *Duox1* deletion by acrolein may contribute to EMT. In agreement with the effects of acrolein, *DUOX1* mRNA was also downregulated upon 24-hr exposure of primary bronchial epithelial cells (PBECs) to freshly prepared cigarette smoke extract (CSE) (**Supplemental Figure S2F**).

Chronic exposure of acrolein to mice also resulted in features indicative of enhanced peribronchiolar fibrosis (40), as illustrated by subepithelial collagen deposition (**Figure 4A** and **B**) and enhanced α-smooth muscle actin staining (**Figure 4C** and **D**). Importantly, these features of peribronchiolar fibrosis were enhanced in *Duox1*−/− mice, with acrolein-induced subepithelial collagen deposition being significant only in *Duox1*−/− mice and acrolein-induced peribronchial α-smooth muscle actin levels being significantly exacerbated in *Duox1*−/− mice. We examined lung tissue mRNA expression of several markers of inflammation and airway remodeling (**Supplemental Figure S3**). Acrolein exposure tended to increase *Il6, Il13* and *Cxcl1* (murine KC) mRNA levels (albeit non-significantly) and significantly increased *Mmp9* expression. However, no significant differences were observed between WT and *Duox1*−/− mice. Collectively, these various findings suggest that lung DUOX1 expression is reduced in several mouse models of COPD, and our observations of acrolein-induced EMT and peribronchiolar fibrosis suggest that loss of DUOX1 may sensitize airways to these important hallmarks of COPD.

**Duox1 deficiency enhances elastase-induced emphysema in mice**

To further explore a potential role for DUOX1 suppression in the development of COPD, we examined emphysema development in response to airway instillation of porcine pancreatic elastase (PPE), in age-matched WT and *Duox1*−/− C57BL/6NJ mice. As expected (41,42), PPE
exposure induced development of alveolar emphysema, as measured by increased alveolar airspace enlargement (Figure 5A and B). Importantly, elastase-induced airspace enlargement in this model was significantly worsened in Duox1−/− mice (Figure 5A and B), suggesting that the absence of DUOX1 increases susceptibility to elastase-induced emphysema. We observed reduced elastin levels in the remaining parenchymal tissue (Figure 6A and B) and small airways (Figure 6C and D) in response to elastase instillation in both WT and Duox1−/− mice. PPE-induced loss of parenchymal elastin appeared to be worsened in Duox1−/− mice (Figure 6B), but this was not statistically significant, and elastin degradation within the small airways was similar in WT and Duox1−/− mice (Figure 6D). Analysis of picrosirius red staining indicated tendencies toward increased in small airway collagen in response to elastase, but this was not statistically significant in either WT (p=0.1064) or Duox1−/− (p=0.6932) mice (Supplemental Figure S4A). No significant increases were observed in parenchymal collagen levels in response to PPE (Supplemental Figure S4B). To gain further mechanistic insight, we evaluated lung tissue mRNA expression of extracellular matrix proteins (e.g. Collagen 1a1, elastin) and markers of inflammation and remodeling. While several of these markers (Col1a1, Eln, Mmp12, Il13, Cxcl1) were significantly increased in elastase-exposed mice, no significant differences were observed between WT and Duox1−/− mice (Supplemental Figure S5). Since neutrophil infiltration plays an important role in emphysema development (43), we examined neutrophil activation by measuring intracellular and extracellular activity of the neutrophil granule protein myeloperoxidase (MPO) (44). Extracellular MPO activity levels in lung tissues from elastase-exposed Duox1−/− mice were significantly increased compared to corresponding WT mice, while intracellular MPO activity levels were unaffected (Figure 6E and F), suggesting that Duox1 deficiency promotes neutrophil activation and degranulation in this model of elastase-induced emphysema. Finally, based on previous findings implicating DUOX1 in airway production of amphiregulin (Areg), an important growth factor that contributes to epithelial regeneration after injury (45), we hypothesized that Duox1 deficiency may lead to impaired Areg production in this model. Lung tissue Areg protein levels
were similarly elevated in response to PPE in both WT and *Duox1*−/− mice (Supplemental Figure S6), even though lung tissue *Areg* mRNA tended to be suppressed in *Duox1*−/− mice treated with PPE compared to PBS controls (p=0.0563).
Discussion

Oxidative stress is often implicated in the pathogenesis of COPD, but findings on the involvement of NADPH oxidases (NOX) in experimental models of COPD are variable and sometimes even contradicting (17,19-21). Our present findings extend intriguing previous observations that the primary epithelial NOX isoform DUOX1 is in fact downregulated within the airways of subjects with COPD (22,23), and demonstrate a gradual loss of small airway DUOX1 protein expression in COPD patients in correlation with lung function decline and extracellular matrix remodeling and emphysema. One limitation of our studies is that we not able to perform stereology according to published guidelines (46), since we did not have serial sections available. Although DUOX1 was significantly downregulated in patients with severe COPD (GOLD IV), it was not significantly reduced in patients with moderate COPD (GOLD II), which may suggest that DUOX1 status declines gradually as COPD progresses and may be a symptom of COPD pathology rather than a causative factor. In line with this argument, we observed that TGF-β, a feature of COPD pathology, can suppress DUOX1 within the airway. Alternatively, since DUOX1 downregulation may also be a result from smoking (22,23), it is also plausible that DUOX1 downregulation due to smoking may contribute to COPD progression, or that lowered airway DUOX1 status at the onset of COPD development may actually enhance its progression. In support of this latter suggestion, we provide evidence that Duox1 deficiency can worsen disease outcomes in 2 distinct mouse models that reflect different pathological hallmarks of COPD, ie. small airway subepithelial fibrosis and alveolar airspace enlargement. These observations would therefore suggest that the gradual loss of DUOX1 in human COPD, potentially as a result of smoking (22), may be a contributing factor in COPD development and its progression. Unfortunately, our present studies were based on only current or former smokers, and were not sufficiently powered to reveal a significant impact of smoking status on DUOX1. Previous studies have documented suppression of airway DUOX1 in active smokers compared to never smokers (22), consistent with our present findings using
CSE or acrolein, but it is unclear whether this also persists in former smokers. Also, other studies have suggested that CSE exposure may actually enhance DUOX1 (47), hence the precise relationship between smoking status and history and airway DUOX1 is complex. However, the fact that correlations between DUOX1 staining scores and lung function parameters were largely independent of smoking status (Figure 1B) would suggest that DUOX1 downregulation is associated with COPD severity and not with smoking history.

Outside the thyroid, DUOX1 is primarily expressed at mucosal surfaces, including the airway, and is thought to participate in oxidative mucosal host defense, analogous to the antimicrobial function of phagocyte oxidase. More recent studies demonstrated that DUOX1 contributes to innate epithelial and epidermal wound responses through redox-dependent activation of various cellular signaling pathways, and thereby contributes to maintenance of epithelial integrity (24). Downregulation of DUOX1 in COPD would therefore be expected to impair such innate lung injury responses and thereby result in impaired epithelial regenerative capacity. Of note, while our analysis of DUOX1 was largely based on analysis of small airways, DUOX1 is also present in the alveolar type II cells (48), where it likely plays similar roles in alveolar innate host defense and epithelial injury responses. Although our tissue stainings did not allow us to accurately quantify DUOX1 protein expression in the alveolar epithelium, we suspect that our observation of reduced small airway DUOX1 expression in COPD may also extend to similar DUOX1 downregulation in the alveolar epithelium of these patients. As a result, innate alveolar host defense and/or regenerative capacity may be diminished, and lead to emphysema development in COPD.

Our recent studies have suggested that DUOX1 silencing, as is observed in many lung cancers, can lead to epithelial reprogramming with features of EMT (26), which may also be relevant for small airway remodeling in COPD (27). Indeed, chronic exposure to CS, which may be the primary cause of COPD, is well-known to promote EMT features (27,49) and subsequent extracellular matrix remodeling and related thickening of the small airways (38,40), and may also potentially
result in impaired alveolar re-epithelialization (50). In our present studies we show that acrolein, a major CS-component, can similarly induce EMT features and small airway remodeling in mice, and that this was associated with Duox1 downregulation and, more importantly, enhanced by Duox1 deficiency. Thus, DUOX1 suppression during COPD may contribute to disease pathogenesis by enhancing EMT features and related airway remodeling.

To gain additional mechanistic insight into the impact of Duox1 deficiency on acrolein-induced small airway remodeling as well as elastase-mediated airspace enlargement, we surveyed potential alterations in various markers of inflammation or remodeling that have previously been linked to DUOX1 in the context of innate airway injury responses or wound healing. These efforts unfortunately did not yield conclusive mechanistic insights, but in some cases showed surprising outcomes. For example, the matrix metalloproteinase Mmp-9, which was previously implicated in DUOX1-mediated epithelial wound responses (51) and has also been implicated in COPD as part of the protease/antiprotease imbalance (52), was found to be upregulated in response to chronic acrolein exposure, but this was similar in both WT and Duox1-deficient mice. The EGFR ligand amphiregulin (Areg) is produced as a critical mediator of epithelial regeneration during injury (45) through a pathway that may involve DUOX1 (53), although its importance in COPD is not well established. Our findings of PPE-induced emphysema suggested that lung tissue Areg mRNA tended to be suppressed in PPE-exposed Duox1−/− mice compared controls, but PPE-induced increases in lung tissue Areg protein levels were similar both WT and Duox1−/− mice (Supplemental Figure S6). It is unclear how such increase in Areg is relevant for emphysema, but upregulation of Areg in the airway basal cells in smokers has also been associated with basal cell and mucus hyperplasia (54), important features of COPD. Indeed, we observed increases in Muc5ac mRNA in PPE-treated mice, as well as Il13, an important mediator of mucus metaplasia and remodeling. PPE-induced upregulation of Muc5ac appeared to be further increased in Duox1-deficient mice (Supplemental Figure S5), which was unexpected in light of our previous
observation that DUOX1 contributes to Muc5ac expression and mucus metaplasia in the context of allergic airway inflammation (53). These latter studies also indicated a critical role for DUOX1 in production of IL-13 during allergic inflammation, but Il13 induction in the context of PPE-induced emphysema was unaltered in Duox1-deficient mice. These various differences in the apparent relationships between DUOX1, MMP-9, IL-13, Areg, or MUC5AC in these different contexts may be related to their different cellular source(s) for these mediators in these different disease models, whereas DUOX1 is likely involved only in epithelia-specific responses. Cell-specific analyses by e.g. single-cell RNAseq would be required to more clearly dissect this. Intriguing recent studies demonstrated that IL-13 induction within the alveolar epithelium impairs self-renewal and differentiation properties of alveolar type 2 cells, which is likely relevant to alveolar remodeling and emphysema development (55).

Neutrophilic inflammation has been implicated in COPD pathology (56,57) and in elastase-induced emphysema (41,42), and previous studies of allergic airways disease have linked DUOX1 to production of neutrophil chemokines (CXCL1) and neutrophil recruitment (53,58). However, we did not observe significant changes between WT and Duox1−/− mice with respect to overall neutrophil content during PPE-induced emphysema, based on intracellular MPO analysis, or with respect to induction of Cxcl1 mRNA. However, analysis of extracellular MPO activity, which likely reveals neutrophil activation and degranulation, showed an increase particularly in PPE-exposed Duox1-deficient mice. The relationship between DUOX1 and neutrophilia is undoubtedly complex and also context dependent. For example, in contrast to observations during allergic airway inflammation, DUOX1 was not found to affect CXCL1 production and neutrophil recruitment in response to e.g. LPS (59). Increased neutrophil degranulation in the context of Duox1 deficiency may enhance tissue destruction, due to secretion of neutrophil-derived proteases and/or MPO-catalyzed oxidative activation of MMPs (60) or inactivation of tissue inhibitors of MMPs (TIMPs) (61).
In summary, the current study highlights the potential importance of downregulation of airway (or alveolar) DUOX1 in the context of COPD, and indicates that it may be a contributing factor to COPD pathogenesis and progression. Although many questions remain with respect to the mechanisms involved, our observations suggest that DUOX1 downregulation can promote both small airway remodeling as well as alveolar airspace enlargement, which both could be related to altered epithelial biology and homeostasis. We did not address the mechanism(s) by which DUOX1 is downregulated during COPD, but suggest that one factor could be activation of TGFβ, a signaling pathway that is commonly activated by CS exposure and has been strongly linked to COPD (62,63). Alternatively, it is possible that epigenetic mechanisms, as seen in e.g. lung cancer, may also contribute to DUOX1 silencing in COPD (64). Lastly, our findings have important implications for the popular notion of antioxidant-based approaches as a potential treatment of COPD, as these could also impair beneficial DUOX1-mediated redox mechanisms that promote innate airway defense or epithelial homeostasis. Instead, targeted approaches to prevent DUOX1 downregulation or enhance its function in the context COPD might in fact be more beneficial in managing this devastating disease and would deserve further exploration.
Methods

Human study subjects and tissue collection

Lung tissues were obtained from the upper lobe subpleural area of 14 control, 16 GOLD II and 19 GOLD IV COPD patients at University Hospital Maastricht, as described previously by Eurlings et al. (28). Briefly, lung tissue sections of approximately 2 cm² were obtained from GOLD IV patients undergoing lung volume reduction surgery, and tissues from control and GOLD II patients were obtained as tumor-free tissues during resection of a solitary primary tumor. Other details regarding tissue collection, exclusion criteria, smoking history, and lung function analysis are described previously (28), and the same paraffin-embedded tissue sections were also used in the present study. Collection, storage and use of tissue and patient data occurred in accordance to the “Code for Proper Secondary Use of Human Tissue in the Netherlands”. The scientific board of the Maastricht Pathology Tissue Collection (MPTC) approved the use of materials for this study under MPTC 2009–22.

SPC-TNFα model

SPC-TNFα mice (n = 10, male and female), which are transgenic mice that exhibit chronic pulmonary inflammation resulting from overexpression of TNF-α in alveolar epithelial type II cells (TNF-α expression under the control of the promoter of surfactant protein C (SP-C; expressed by alveolar epithelium type II), were euthanized at 6-months of age, and lungs were harvested and paraffin embedded as previously described (32). Various readouts were compared between SPC-TNFα mice and age-matched male and female transgene negative littermates (WT, n = 10).

Acrolein model
Wildtype male and female C57BL6/J mice aged 8-12 weeks, as well as age-matched male and female Duox1−/− mice, originally generated on C57BL6/J background and provided by Miklos Geiszt (65), were subjected to chronic acrolein exposure, as described previously (66). Briefly, mice were placed in a 2 L glass chamber and exposed to either 5 ppm (11.5 mg/m³) of acrolein vapor or control air, for 4 hrs/day, 5 days/week, for 2 weeks total. Mice were euthanized after the final exposure, and lung tissues were collected for analysis of various readouts.

Elastase (PPE) model

Wildtype male and female C57BL6/NJ mice aged 8-12 weeks and corresponding age-matched male and female Duox1−/− mice (backcrossed to C57BL6/NJ mice (Jackson Laboratories)) were subjected to oropharyngeal (OP) instillation of Porcine Pancreatic Elastase (PPE; 1 IU/kg bodyweight in 50 µl PBS; Elastin Products Company, EC134) or 50 µl PBS vehicle control, under brief isoflurane anaesthesia, which was repeated once a week for a total of 3 weeks. One week after the final instillation, mice were euthanized, and lung tissues were collected for analysis of mean linear intercept (MLI), as well as other outcomes.

Lung tissue fixation and immunohistochemistry (IHC)

Mouse lung tissues were collected upon completion of the indicated experiments, and left lung lobes were fixed in PFA and paraffin embedded for IHC. For MLI analysis, the lungs in the PPE model were fixed by tracheal instillation of 4% PFA at a pressure of 25 cm H₂O for 20 min. Only sections that displayed no cutting artifacts, compression, or hilar structures were used in the MLI analyses.

Five µm tissue sections were cut and stained with either H&E or Masson’s trichrome (MTA) using standardized protocols following deparaffinization. Additionally, fixed sections were
immunohistochemically stained for α-smooth muscle actin (Sigma-Aldrich, A2547, 1:8000),
detected using Vectastain Alkaline Phosphatase Universal, Vector Red (Vector Laboratories). For
elastin staining, slides were incubated for 20 min in Weigert's Resorcin-Fuchsin (Electron
Microscopy Sciences) at 60–70°C. Collagen was stained by incubation for 90 min in 0.1% Sirius
Red in saturated picric acid (Electron Microscopy Sciences).

Paraffin-embedded tissue sections from non-COPD control subjects and GOLD II and GOLD IV
COPD patients (4 μm thickness) were evaluated for the presence of the DUOX1 protein using a
DUOX1 antibody (Santa Cruz Biotechnology, SC48858, 1:500) and visualized utilizing a biotin-
conjugated secondary antibody (Dako, E0466), the Vectastain Peroxidase ABC Kit and Enzyme
Substrate (Vector Blue; Vector Laboratories), with Nuclear Fast Red counterstaining. Small to
medium size airways, defined as smaller than 2 mm diameter) were scored for staining of DUOX1
based on a scoring scale of 1-4 (Supplemental Figure S1), in which 4 was considered the highest
staining intensity, and a score of 1 the lowest staining intensity (minimal staining observed).
Negative staining controls were performed by omission of the primary antibody. Two independent
researchers, blinded to the tissue identity, quantified the DUOX1 scoring in 2-4 airways per tissue
section to obtain an average small airway DUOX1 staining score for each section, after which the
individual staining scores for each observer were averaged, thus obtained mean scores that are
asymptotically continuous and normally distributed. Lung tissue sections of the SPC-TNFα mice
were evaluated similarly for the presence of small airway Duox1 protein as described above
(antibody dilution 1:200 (SC48858)).

Stainings for elastin, collagen and α-SMA were quantified using MetaMorph imaging software
(Molecular Devices). MTA stainings were quantitatively scored as described previously (53).

Quantification of airspace enlargement
Enlargement of alveolar spaces was determined by quantifying the mean linear intercept (MLI) using Stereo-Investigator software (MBF Bioscience). For each lung, 4-5 images were analyzed, with a minimum of 50 measurements per image. Briefly, MLI was measured by first placing 40 μm spacing between lines over the tissue sections and consequently marking points (P) on the alveoli airspace to estimate volume, and intersections (I) are marked on the alveolar walls to estimate surface. The MLI was then calculated according to previous established methods (67), using the formula $MLI = 2 \times k \times d \times P/I$, in which $k$ is the length of line used to probe, $d$ is number of lines per point, $P$ is the number of points marked in the alveoli air-spaces and $I$ is number of intersections marked between the probe-lines and the surface of the alveoli.

Myeloperoxidase (MPO) assay

Myeloperoxidase activity in lung tissues was measured according to the step-by-step protocol (44). Briefly, lungs were placed in extraction buffer (0.32 M sucrose, 1 mM CaCl2, 10U/ml Heparin in HBSS) for 2 hours on ice to extract extracellular proteins. After incubation, the supernatant was transferred, precipitated, and resuspended in PBS (extracellular fraction). The lungs were then placed in CTAB buffer (50 mM cetyltrimethylammonium bromide in 50 mM potassium phosphate buffer at pH=6), and were subsequently homogenized, sonicated and freeze-thawed in liquid nitrogen. After centrifugation, supernatant was collected, representing the intracellular protein fraction. Following extraction of both intracellular and extracellular proteins, MPO was captured using MPO ELISA dilution buffer (Hycult) on anti-MPO antibody coated plates (Hycult) for 1 hour at room temperature. Assay wells were then washed, and MPO activity of antibody-captured MPO was assessed with ADHP according to protocol (44).

Cell culture
Primary bronchial epithelial cells (PBECs), kindly provided by the Primary Lung Culture (PLUC) facility of the Maastricht University Medical Center (Maastricht, the Netherlands), were isolated from lung tissues resected during lobectomies or pneumonectomies of patients who underwent surgery for lung cancer. Collection, storage and use of tissue and patient data were performed in agreement with the "Code for Proper Secondary Use of Human Tissue in the Netherlands" (http://www.fmwv.nl). The scientific board of the MPTC approved the use of materials for this study under code MPTC2010-019. In addition, formal permission was obtained from the local Medical Ethic Committee code 2017-0087 and patients provided written informed consent to permit the use of the material for research. PBECs of three donors without known history of chronic lung disease were isolated and cultured as previously described (68). Upon confluence, cells were starved overnight, after which cells were exposed to varying concentrations of cigarette smoke extract (CSE; 1%, 2% or 4%) for 24 hours. 3R4F Research Cigarettes (University of Kentucky, Lexington, KY) were removed from their filters and CSE was prepared in HBSS as previously described (69).

In addition, primary mouse tracheal epithelial cells (MTECs) were isolated from excised mouse tracheas from either WT mice or Duox1−/− mice (C57Bl6/J) and cultured as previously described (25) and used for in vitro experiments.

ELISA

Cell culture supernatants or BAL fluids were analyzed for TGF-β and amphiregulin (Areg) using DuoSet ELISA’s (R&D Systems) according to the manufacturer’s instructions.

Western blot analysis
Cell lysates were prepared using Western solubilization buffer (50 mM HEPES, 250 mM NaCl, 1.5 mM MgCl₂, 1% Triton X-100, 10% glycerol, 1 mM EGTA, 1 mM PMSF, 2 mM Na₃VO₄, 10 μg/ml aprotinin, 10 μg/ml leupeptin; pH 7.4). Samples containing equal amounts of protein (BCA protein assay kit; Pierce) were separated on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with antibodies against Vimentin (#5741; 1:500; Cell Signaling); β-actin (A5316; 1:5.000; Sigma) or E-cadherin (#3195; 1:1000; Cell Signaling). Antibodies were probed with rabbit- or mouse-specific secondary antibodies (Cell Signaling) conjugated with HRP and detected by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

qPCR analysis

Target gene expression in lung tissues was analyzed by qPCR and normalized to GAPDH using the ddCT method. RNA was purified according to the GeneJET RNA Purification Kit (Thermoscientific); First-strand cDNA was synthesized from 1 μg purified RNA using an M-MLV Reverse Transcriptase Kit (Invitrogen). Real-time PCR (qPCR) reactions contained 0.5 μL cDNA, 5 μL iQ SYBR Green Supermix (Bio-Rad), 1 μL primer (Supplemental Table S4) in ddH₂O (100 nM final) and 3.5 μL ddH₂O. Amplification and detection were performed using a CFX96 Real-Time PCR Detection System (Bio-Rad). The following qPCR procedure was used: Pre-incubation for 3 minutes at 95°C, followed by 40 cycles of denaturation at 95°C for 5 seconds, annealing at 60°C for 1 min and amplification at 72 °C for 30 s. A post-PCR melt curve was performed at 95°C for 10 seconds, followed by a 0.5°C increment increase every 5 seconds from 65°C to 95°C. For the SPC-TNFα mouse model and human PBECs, RNA was purified using the High Pure RNA isolation kit (Roche), first-strand cDNA was synthesized from 1 μg purified RNA using the Transcriptor cDNA Synthesis Kit (Roche). qPCR reactions contained SensiMix SYBR Hi-ROX Kit (Quantace-Bioline, London, UK) with 300 nM primers and were performed in a 384-well MicroAmp
Optical 384-Well Reaction Plate (Applied Biosystems) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). The expression of the genes of interest was normalized with a correction factor derived by GeNorm (70), based on the expression RPL13A as reference gene.

Statistics

All quantitative data, unless specifically indicated, are presented as the mean ± SE. Statistical differences between groups were analyzed using 2-way ANOVA with Tukey post-hoc analysis in GraphPad Prism (version 8.3.0; GraphPad Software, La Jolla, CA). Patient characteristics data from the human COPD cohort are displayed as mean ± SD and were normally distributed. Basic characteristics were analyzed using ANOVA or Chi Square. DUOX1 staining in this cohort was analyzed using ANCOVA using age, sex, pack years and smoking status as covariates with Bonferroni post-hoc analysis. The number of current (12) and former (30) smokers provided 80% power to detect differences in DUOX1 due to smoking status of 0.7 or larger. Among current smokers, the study had 80% power to detect differences in DUOX1 staining scores between control and GOLD II patients ≥ 1.7, while among former smokers there was 80% power to detect differences ≥ 1.0 between control and GOLD II patients, and differences ≥ 0.7 between control and GOLD IV patients. Correlations between DUOX1 staining scores and other parameters were analyzed by Pearson correlations, with 2-tailed significance. P values < 0.05 were considered significant.

Study approval

All animal procedures conducted at the University of Vermont were reviewed and approved by the Animal Care and Use Committee of the University of Vermont. Animal procedures conducted
at Maastricht University were approved by the Institutional Animal Care Committee of Maastricht University, The Netherlands.
Author contributions

C.S. and C.v.d.W performed the analysis of human tissues and primary human cell cultures; C.S., R.A.B., A.H. and M.H. performed and analyzed the animal studies; S.L., C.M.D. and M.H. contributed to data analysis; N.L.R. and E.F.M.W. assisted with experimental design and analysis of human COPD studies; and P.M.V. assisted with statistical analyses. A.v.d.V. was responsible for the conception and overall supervision of the project, and the final version of the manuscript; C.S. wrote the draft of the manuscript, and all authors contributed to the planning, discussion and interpretation of experiments, and to the writing of the manuscript.
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1 References


Figure 1: DUOX1 loss in small airways of COPD patients is associated with loss of lung function, emphysema, and airway remodelling. (A) IHC for DUOX1 (blue) with nuclear fast red counterstaining (red) in lung tissue of a representative control, GOLD II and GOLD IV COPD patient (including a negative control; 100x magnification). (B) Small airway score of DUOX1 in control, GOLD II and GOLD IV COPD patients. Data shown as mean ± SE. ** p < 0.01, by one-way ANOVA. (C) Pearson correlations (2-tailed significance) between DUOX1 scores and %FEV1, %FVC, FEV1/FVC ratio and %DLCO, determined in (32), in all subjects studied. Color labels highlight controls (blue), GOLD II (red), and GOLD IV (green) COPD patients, and symbols shapes represent smoking status (current or former smokers).
Figure 2: Airway epithelial DUOX1 is decreased in SPC-TNF-α mice. (A) Relative Duox1 mRNA expression in lungs of 6 months-old SPC-TNFα mice and littermate controls (n=8-9 from 2 separate experiments; 2-tailed unpaired nonparametric t-test). (B,C) Representative staining of DUOX1 protein (in blue; 400x magnification) with corresponding small airway IHC score of airway epithelial DUOX1 in SPC-TNFα mice compared to wildtype littermate controls (n=9-10). Data shown as mean ± SE. * p < 0.05, by 2-tailed unpaired t-test.
**Figure 3:** Airway epithelial DUOX1 loss in SPC-TNF-α mice is associated with elastin remodelling.

(A-L) IHC analysis (200x magnification) and quantification (% surface area) of elastin and collagen in parenchyma (A-F) and small airways (G-L) in 6-month-old WT and SPC-TNFα mice. Quantified stainings (n=9-10) were correlated to small airway DUOX1 scores. Data shown as mean ± SE. *p < 0.05, by 2-tailed unpaired t-test.
**Figure 4**: *Duox1* deficiency sensitizes airways to acrolein-induced peribronchiolar fibrosis.

C57BL/6J mice were exposed to acrolein (ACR) and analyzed (200x magnification) for collagen by Masson’s trichrome (A,B) or α-smooth muscle actin (C,D). Quantification of staining was based on 6-8 mice per group from 2 separate experiments, and shown as mean ± SE. * p < 0.05, by 2-way ANOVA.
Figure 5: Duox1 deficiency enhances development of elastase-induced emphysema. WT and Duox1−/− mice were exposed to 50 µL porcine pancreatic elastase (PPE) or PBS control, and lung tissues were analysed by H&E staining (100x magnification) for alveolar enlargement (A), with corresponding (B) calculation of alveolar mean linear intercept (MLI, µm) as a measure of emphysema. Data shown as mean ± SE; n=9 per group, from 2 separate experiments. * p < 0.05 by 2-way ANOVA.
Figure 6: Development of elastase-induced emphysema is associated with decreased alveolar and small airway elastin levels, and Duox1 deficiency enhances elastase-induced extracellular myeloperoxidase (MPO) activity. Analysis of parenchymal (A,B) and small airway (C,D) elastin levels (200x magnification) in both WT and Duox1−/− mice in response to PPE or PBS control with Weigert’s Resorcin Fuchsin staining. Analysis of extracellular (E) and intracellular (F) MPO activity in lung tissue homogenates. Data shown as mean ± SE; n=9 per group, from 2 separate experiments. * p < 0.05 by 2-way ANOVA.
Tables

Table 1: Correlations of DUOX1 staining score with lung function parameters and markers of extracellular matrix remodelling in the small airways

<table>
<thead>
<tr>
<th>Correlations DUOX1</th>
<th>All subjects (n=49)</th>
<th>COPD patients (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>p</td>
</tr>
<tr>
<td>FEV1 (%)</td>
<td>0.452</td>
<td>0.002 *</td>
</tr>
<tr>
<td>FVC (%)</td>
<td>0.306</td>
<td>0.046 *</td>
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<tr>
<td>FEV1/FVC</td>
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<td>0.000 *</td>
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<td>DLCO (%)</td>
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<td>0.027 *</td>
</tr>
<tr>
<td>Elastin SA</td>
<td>0.540</td>
<td>0.001</td>
</tr>
<tr>
<td>Collagen SA</td>
<td>-0.256</td>
<td>0.127</td>
</tr>
<tr>
<td>Elastin to Collagen SA</td>
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<td>0.012</td>
</tr>
</tbody>
</table>

*: Data is graphically presented in Figure 1C. SA: Small Airway epithelium