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The NADPH oxidase DUOX1 in chronic lung diseases

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The NADPH oxidase DUOX1 in chronic lung diseases

Oxidative stress paradox during aging

Caspar Schiffers

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The NADPH oxidase DUOX1 in chronic lung diseases

Oxidative stress paradox during aging

Dissertation

To obtain the degree of Doctor at Maastricht University

on the authority of the Rector Magnificus, Prof. Dr. Rianne M. Letschert,

in accordance with the decision of the Board of Deans,

to be defended in public on Tuesday, the 11th of May at 16:00 hours

by

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Chapter 1

General introduction and thesis outline

The aging lung

Aging is described as the irreversible, gradual, and time-dependent functional deterioration of all vital organs after the reproductive phase of life is complete (1). Aging is now recognized to be triggered by the lifelong accumulation of cellular damage, resulting in cellular dysfunction and increased susceptibility to diseases and eventually death. As humans age, various age-associated changes in the respiratory system appear as well, termed the lung function trajectories. The first phase of this trajectory is lung growth, which occurs from birth until adulthood, and is characterized by increases in lung volume, an increase in the number of alveoli, and increased capillary networks. Once adolescence (~25 years of age) is reached, these numbers remain stable, termed the plateau phase (2). Around the age of 30-40 (3), lung function starts to decline with increasing age, termed the decline phase, but this may be variable in every individual based on various exposures, such as cigarette smoke, or genetics (**Figure 1**). It is important to recognize that individual lung growth may vary, and abnormal lung growth early in life has been observed, which may also affect later phases of lung function trajectories (4, 5). This range of lung function trajectories is observed in both health and disease and may have significant clinical consequences (4, 5).

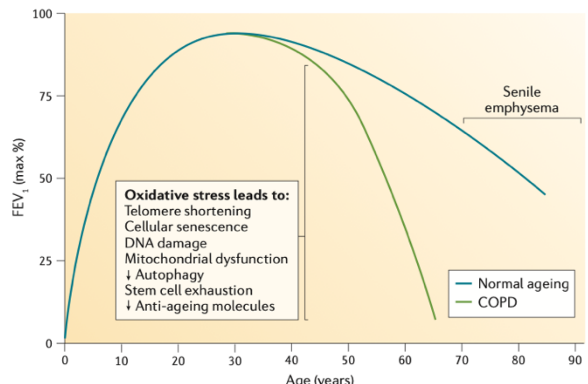

Figure 1: FEV1 declines with age, and accelerated lung aging in COPD. From *Barnes et al (2015) Nat Rev Dis Prim.*

The decline phase has various consequences for functional capacity in absence of underlying pathology and affects every individual, with early limitations only observed during exercise and later on during broader settings. Accordingly, with advancing age, the respiratory tract undergoes both structural and physiological changes, such as the loss of lung regeneration and pulmonary remodeling, which are largely associated with lung function decrease, (6). The aging lung is characterized by increased alveolar surface area (airspace enlargement) with concomitant decreases in lung elasticity. This loss in lung elasticity and airway enlargement result in increased FRC (functional residual capacity) and EELV (end-expiratory lung volume). Additionally, the FEV1/FVC (forced expiratory volume in one second, forced vital capacity) ratio, often used to diagnose chronic obstructive lung diseases, defined as the amount of air that can be forcibly exhaled following taking one's maximal inhalation,

decreases due to various factors including the loss of lung elasticity, airspace enlargement, and loss of e.g. respiratory muscle mass (2). Also termed the 'senile lung', these age-related structural lung changes are mainly attributed to an increase in the size of the alveolar space without any inflammation or alveolar wall destruction.

As mentioned before, aging is triggered by the lifelong accumulation of damage, resulting in cellular dysfunction, disease development, and ultimately, death. The respiratory system represents a critical interface with the external environment and is particularly susceptible to inhalation of damaging agents that are found in the external environment. As such, the lung is equipped with unique mechanisms to cope with these various inhaled gases, particles, as well as infectious hazards/stressors. These mechanisms, or responses, should be tightly controlled in order to preserve lung function while simultaneously shielding the lungs from such xenobiotic insults/injury. If these mechanisms are ineffective, environmental agents can induce subtle biochemical and physiological changes, but may also exacerbate existing and/or contribute to the development of age-related lung disease(s) in a more chronic setting (7). Indeed, the aging lung is believed to be more susceptible to these environmental insults as a result of ineffective defensive mechanisms, and may further aggravate injury-induced damage as well as aging (8, 9). While the senile lung is characterized by airspace enlargement without overt inflammation, such compromised and inappropriate responses to exogenous hazards in the aging lung may also result in chronic inflammation and alveolar wall destruction that may contribute to the development of e.g. emphysema (10, 11). However, the precise mechanisms behind the adverse effects of environmental factors on the aging lung remain incompletely understood.

Nevertheless, factors have been associated with these impaired responses during lung aging, including replicative and/or stress-induced cellular senescence (a stable cell cycle arrest), which is associated with a senescence-associated secretory phenotype (SASP), and immunosenescence, resulting in compromised and inappropriate cellular function and immune cell responses of e.g. innate and humoral immunity (12). The SASP is characterized by resident senescent cells that alter the cellular microenvironment through secreting factors that shift neighboring healthy proliferating cells into a more senescent- and pro-inflammatory state. In addition to damage-associated molecular patterns (DAMPs), the SASP may contribute to inflammaging/sterile inflammation observed during lung aging (13), which is characterized by pro-inflammatory cytokine release and chronic low-grade inflammation in the absence of an immunological threat (14). While associated with aging, the SASP has likely evolved as a mechanism to maintain homeostasis through senescent cell clearance,

progenitor cell repopulation, and wound healing and tissue repair (12, 15, 16), and has also been shown to counter early-life tumerogenesis (17).

The hallmarks of aging and age-related lung diseases

Lopez-Otín established the nine hallmarks of aging, elucidating the cell-autonomous and non-autonomous pathways involved in aging (**Figure 2**) (18). These hallmarks are as follows: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. In relation to these hallmarks, dysregulation of the extracellular matrix is a crucial modifier of cell-autonomous changes and functions (19). The origin of these various hallmarks is undoubtedly complex, and is likely to involve a combination of underlying processes that eventually result in aging, and the individual contribution of each hallmark to individual chronic lung diseases may vary. This is one of the captivating questions in science, and as such, theories of aging that speculate on this have appeared over the decades. Importantly, lung aging is associated with increased susceptibility to pulmonary disease development in the elderly (65 years of age), since the incidence of lung diseases such as chronic obstructive pulmonary disease (COPD), lung cancer, and idiopathic pulmonary fibrosis (IPF) increase with advancing age (20). Indeed, both IPF and COPD are now being referred to as diseases of accelerated lung aging (Figure 1) (21). Accelerated lung aging is recognized by accelerated or aggravated events of lung aging such as defective resolution of inflammation by environmental toxins, e.g. cigarette smoke, or other pollutants. Another chronic lung disease is asthma, but this disease is not characterized by accelerated lung

Figure 2: The hallmarks of aging. From *Lopez-Otín (2013) Cell.*

aging events. However, the increased aging of the population indicates that asthma prevalence may be underdiagnosed in older people (termed asthma in the elderly) and is associated with more comorbidities and higher mortality (22). The pathophysiological mechanisms are also less defined in these patients, but may be related to lung aging (23). While these chronic lung diseases all have lung aging as a common denominator, mechanisms underlying these age-related lung diseases are noticeably different. The importance of these mechanisms are related to the hallmarks of aging (18, 19). Conversely, while differential mechanisms are involved in such age-related lung diseases, a common denominator, namely oxidative stress, which is associated with elevated levels of reactive oxygen species (ROS) and/or impaired antioxidant defenses, applies to each of these, and will be discussed in a later section (24).

COPD

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 (**Figure 3**, panel C, the alveolar architecture has been destroyed resulting in emphysema) (25, 26), resulting in dyspnea (shortness of breath). Small airway disease and emphysema development are mechanistically related, since small airway inflammation may propagate to the alveolar septa, in turn destroying bronchiolar-alveolar attachments, and eventually proceed into lung parenchymal destruction (27-29).

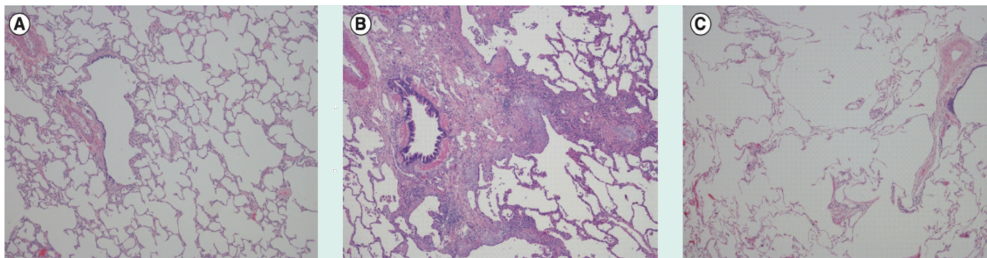

Figure 3: Histopathology of A) normal B) IPF C) COPD lung. *From Kinnula et al. (2009) Exp. Rev. Prot.*

Another histopathological feature often observed in COPD patients is seen in the vasculature with increased thickness of the arterioles, resulting in pulmonary hypertension as an additional complication of COPD (30). Various genetic factors have been established as risk factors for COPD, including the alpha1-antitrypsin (A1AT) gene (31).

Environmental insults, such as cigarette smoke (CS), are at the foundation of COPD pathogenesis, and are associated with persistent inflammation and a protease/anti-protease

imbalance (32, 33). The combination of exposure to these hazards such as smoking and related inflammation leads to lung tissue damage, abnormal tissue remodeling and tissue fibrosis (34). This is furthermore associated with thickening of (large and small) airways through subepithelial fibrosis and mucus plugging, and a related reduction of the airway lumen and may also induce emphysema through alveolar wall destruction and loss of alveolar surface area.

COPD has been associated with age-related lung function trajectories. While some adult COPD patients may display accelerated age-related lung function decline following normal lung growth, others show evidence of abnormal lung growth with normal age-related lung function decline (4, 5). As mentioned previously, the ageing lung is characterized by loss of elasticity, enlargement of alveoli as well as low grade inflammation, but not by destruction of the alveolar walls, which underlies emphysema in COPD. Cellular senescence is also observed during lung aging and may suggest a predisposition to COPD development. As such, examining and understanding the underlying molecular mechanisms involved in normal lung ageing may help to understand how tobacco smoke and other oxidative stressors may accelerate lung ageing and result in COPD development. **Figure 4** highlights the involved hallmarks of aging in COPD. Briefly, there is evidence for epigenetic alterations (in particular the dysregulation of HDACs (histone deacetylase)) together with loss of proteostasis (regulation of protein biogenesis, folding, trafficking and degradation), mitochondrial dysfunction, and cellular senescence in COPD pathogenesis (18, 19). Additional hallmarks such as altered intercellular communication (e.g. adaptive immune responses), and abnormal ECM (extracellular matrix) turnover and deposition, further contribute to COPD pathogenesis.

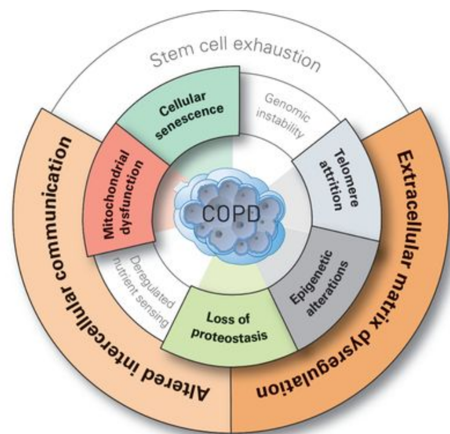

Figure 4: Hallmarks of aging in COPD. *From Meiners et al. (2015) ERJ.*

Asthma in the elderly, and Asthma-COPD overlap syndrome

Asthma-COPD overlap syndrome (ACOS) and asthma in the elderly, are clinically distinguished chronic lung diseases from COPD (35, 36). However, aging hallmarks for

asthma in the elderly and ACOS have not been established yet, but they may likely be related to the hallmarks established in COPD. Asthma is recognized by reversible airflow obstruction, bronchial hyperresponsiveness and airway inflammation (37), and includes symptoms such as wheezing, frequent cough, or shortness of breath. The airway epithelium and submucosa are critically involved in asthma pathogenesis and are strongly associated with airway remodeling (38). While asthma patients are commonly hyperresponsive to allergens such as house dust mite, a subgroup of asthmatic patients are not characterized by allergy (non-atopic). Based on age, gender, atopy, lung function, obesity and severity asthma can be classified into endotypes. These include early-onset asthma, which is typically characterized by allergic sensitization and exacerbations (Th2 high), and adult-onset asthma, which is often non-allergic and non-atopic (Th2 low) and associated with obesity, smoking, and other factors (39-41). While allergic asthma is more prevalent in children and young adults, the incidence in the elderly is growing and is associated with high mortality. The pathogenesis of asthma in the elderly seems to be characterized by more frequent, irreversible airway obstruction, eosinophilia, non-type 2 driven and less by an atopic response, but data is scarce as research does not focus on this asthmatic aging population (23, 42). Symptoms of asthma are heterogeneous and often under-diagnosed in the elderly due to similarities and overlap with other chronic pulmonary diseases, e.g. COPD (43). ACOS is recognized by features of both asthma and COPD, and is associated with persistent airway obstruction (44). While differences between asthma in the elderly and ACOS are poorly understood, a study found that ACOS displays lower diffusing capacity (Dlco), elevated neutrophil and IL-6 levels, worsened lung function and more comorbidities (45). Additionally, ACOS is associated with worse prognosis compared to patients suffering only from COPD, driven largely by increased risk of progression of their lung disease(s) and respiratory mortality (46).

Idiopathic Pulmonary Fibrosis (IPF)

IPF is the most common form of idiopathic interstitial lung diseases, affecting approximately 5 million people worldwide (47). It is characterized by scarring of the lung, specifically the interstitial tissue, resulting in destruction of the lung architecture (**Figure 3**, panel B, replacement of normal alveolar architecture by matrix and occurrence of fibroblastic foci), and a progressive and irreversible loss of lung function (48). This results in a stiffer and less elastic lung (decreased lung compliance), resulting in symptoms such as shortness of breath and a dry cough, ultimately leading to death from respiratory failure. At the cellular level, IPF

is recognized by impaired epithelial regeneration following epithelial injury and alveolar epithelial cell death (49). It is recognized by a pro-fibrotic environment, as a result of active sites of fibrogenesis (fibroblast foci) and myofibroblasts proliferation (and collagen deposition), as well as chronic low-grade inflammation (49). Risk factors for the development of IPF may include genetic mutations (e.g. alveolar stability, telomere biology) (50), but environmental compounds (smoking, asbestos) are also known triggers of IPF (51). Nevertheless, aging confers the greatest risk for IPF, beyond any known environmental or genetic risk factor, and has as such led to the notion IPF may represent a form of accelerated lung aging (52, 53). Overall, substantial evidence supports the view that two key cell populations, epithelial cells and fibroblasts, exhibit strong features of ageing in IPF. These include cell-autonomous alterations (**Figure 5**), such as genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, or cellular senescence (18, 19). Moreover, altered intercellular communication and stem cell exhaustion are potential hallmarks of lung fibrosis. Strong evidence further suggests that ECM dysregulation is a prominent ageing feature with significant pathological consequence, driven by impaired cell-matrix interactions.

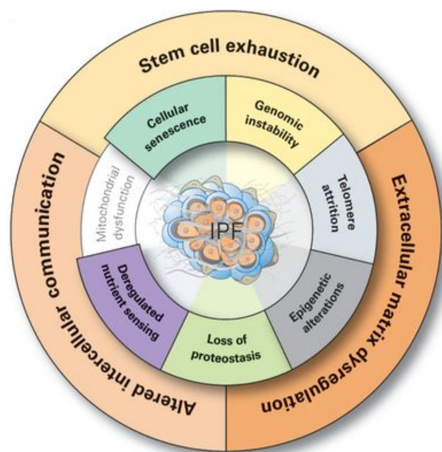

Figure 5: Hallmarks of aging in IPF. *From Meiners et al. (2015) ERJ.*

Reactive oxygen species (ROS) and the free radical theory of aging

Humans rely on essential and extremely complex networks of signaling events in order to coordinate cellular function in a vast variety of physiological processes, such as tissue homeostasis under normal conditions, as well as various repair mechanisms in response to tissue injury. Molecular oxygen (O_2) is required for cellular energy metabolism, which in turn maintains appropriate signaling and cellular homeostasis. Reactive oxygen species (ROS) are formed as a natural by-product of normal oxygen metabolism during such processes, with the majority of ROS deriving from the mitochondrial transport chain. ROS are classically considered toxic due to their ability to cause irreversible oxidative damage to lipids (lipid peroxidation), proteins (protein oxidation), and (mitochondrial) DNA (mtDNA) (54-56).

However, ROS may also be produced by enzymes during host defense mechanisms (e.g. phagocytosis) or as second messengers in signal transduction processes, reversibly controlling protein function by oxidation, which is also known as redox signaling (57). In response to potential ROS-induced (oxidative) damage, organisms have evolved protective mechanisms such as the production of several antioxidants, including the antioxidant enzymes glutathione (GSH), peroxiredoxins, thioredoxins, superoxide dismutase (SOD) and catalase (58). Under healthy conditions these endogenous antioxidant systems are capable of mitigating most of these ROS-induced damaging effects. However, in the case of oxidative stress, where ROS levels increase dramatically, there is an apparent imbalance between the production of these oxidants and the capacity of the cell to counteract the oxidant-induced damage by endogenous antioxidant production, resulting in cellular damage.
Theories on the role of free radicals, or ROS, in the context of aging of biological systems date back to 1956, when Denham Harman proposed the free radical theory of aging (FRTA). According to this proximate theory, aging is a consequence of free radical-induced cellular damage, which is produced during normal metabolism (59). A few decades later, he refined this theory, renaming it the mitochondrial free radical theory of aging (MFRTA), by including that mitochondrial dysfunction due to the accumulation of damage induced by ROS in turn induces the generation of even greater levels of ROS, eventually resulting in aging (60). Since 1956, numerous observations support this theory, whereas others seem to oppose it. In support there is a strong correlation between chronological age and the level of ROS generation and markers of oxidative stress, such as increased ROS levels (e.g. hydrogen peroxide; H_2O_2) and reduced levels of antioxidant activity (e.g. free/oxidized glutathione; GSH/GSSG)) (61-63). Moreover, several age-dependent diseases are associated with severe increases in oxidative stress (64). In line with this, numerous studies have shown that aggregation of mitochondrial damage may cause mtDNA mutations, and mtDNA is also more susceptible to ROS-induced damage (65), which play key roles in many chronic diseases associated with premature ageing, such as CKD (chronic kidney disease), type-2 diabetes and neurodegenerative diseases (66).
However, there are various observations that argue against the FRTA, of which a number are detailed below. First, there is a clear lack of correlation between levels of ROS and longevity in various species. An intriguing example of this is the naked mole rat, a rodent that displays relatively high levels of oxidative stress, elevated levels of oxidative damage and less robust repair mechanisms than their short-lived rodent counterparts (67, 68). Counterintuitive, the naked mole rat still maintains a superior health span which directly opposes the FRTA (69),

as they maintain normal activity, body composition, and reproductive and physiological functions with no obvious age-related increases in morbidity or mortality rate for over the majority of their lives. In addition, some mutant organisms associated with mitochondrial dysfunction (*C. Elegans* nuo-6 (NADH ubiquinone oxidoreductase 6) mutant, as well as Mclk1-mutant mice (mitochondrial 5-demethoxyubiquinone hydroxylase)) have a longer lifespan, which may in fact be due to increased levels of ROS (70, 71). Indeed, ROS signaling may extend lifespan through mitochondrial hormesis (mitohormesis), where relatively low levels of mitochondria-derived ROS may improve systemic defense mechanisms by inducing adaptive responses rather than cause damage (72). Furthermore, antioxidant supplementation to reduce oxidative stress has in various cases been shown to either have no effect on longevity (73) or to even be deleterious for longevity (74). In line, recent work has shown that genetic manipulation of 18 different genes of the antioxidant defense system showed no effect on lifespan in mice (except deletion of the Sod1 gene, which reduced mean and maximum lifespan). The study elegantly examined the contribution of these genes to lifespan by utilizing both knockout and transgenic mice (75, 76). Overall, these findings may suggest that oxidative damage does not play a dominant role in ageing, which is in direct contrast to the FRTA hypothesis.

The various oppositions to the FRTA have led to alternative theories of aging. A well-known example is the disposable soma theory by Kirkwood (77), which speculates that aging is a trade-off in the allocation of limited energy resources between maintenance and restoration of tissue homeostasis, and other traits needed for survival (e.g. reproduction). This theory is connected to the antagonistic pleiotropy theory of aging proposed in 1957 by Williams, as this theory explains that genes that confer a reproductive advantage early in life may have harmful effects later in life (78). Similar to the FRTA, one may assume that according to the disposable soma theory, long-lived species should be more resistant to oxidative damage due to superior tissue homeostasis (e.g. higher antioxidant defense(s), or lower levels of ROS, less cellular senescence) than short-lived species, which is simply not the case. These observations on ROS and aging, have also led to more recent theories of aging, such as the metabolic stability theory of aging (79). It derives partly from the observation that ROS have two kinds of effects on metabolic activity, since they may interact with DNA and RNA to impair cell function, but may also act as second messengers in signal transduction processes called redox signaling (64). This double-edged effect of ROS may imply that it is the ability of the cell to maintain stable ROS levels, rather than the total amount of ROS produced, that may play a critical role in maintaining the functional integrity of the cell and consequently,

the rate of aging of the organism. Overall, the question of why we age is unquestionably complex, and it may not be feasible to be explained by one single theory.

Aging: impaired adaptive antioxidant responses

The cellular defense against oxidative or electrophilic stress under normal physiological conditions is activation of endogenous antioxidant defenses, such as the Nrf2 (nuclear factor erythroid 2-related factor 2)-antioxidant response element signaling pathway. This pathway regulates the expression of genes (and translation into proteins) that are crucially involved in the detoxification and elimination of reactive oxidants and electrophilic agents through conjugative reactions and by enhancing cellular antioxidant capacity (80). Aging is associated with the loss of such antioxidant defense mechanisms, which are also observed in age-related lung diseases, and may in part underlie observed oxidative stress. Nrf2 was shown to be basally increased in bronchial epithelial cells with increasing age, which was postulated to manage the increased steady-state levels of oxidation (81). However, in response to oxidative stress, the inducibility of Nrf2-mediated antioxidant responses were compromised, and similar Nrf2 impairments were observed in mice (82). Furthermore, a recent study found that hydrogen peroxide metabolizing enzymes, such as catalase or peroxiredoxins, exhibit significant negative correlations with age (83). Additionally, in the age-related disease IPF, overexpression of glutaredoxin-1 (Grx1) rescued bleomycin- or transforming growth factor beta (TGF-β)-induced lung fibrosis in aged animals, further highlighting the importance of functional endogenous antioxidant defense mechanisms against oxidative stress (84). In contrast, other studies addressing antioxidant function in aging mice showed that constitutive overexpression of the antioxidant thioredoxin (Trx) 1 showed no life-extending effects in the later part of life (>22-25 months) and was even associated with enhanced tumor development at that age. This was explained through the suppression of the apoptosis signal-regulating kinase 1 (ASK1) pathway, thereby contributing to anti-apoptotic events (85). In addition, the authors conducted similar experiments in mice overexpressing Trx in both the cytosol (Trx1) as well as the mitochondria (Trx2) (86). Strikingly, they observed a significantly shorter lifespan compared to wildtype (WT) mice, and increased incidence as well as severity of lymphoma, suggesting that overexpression of Trx in both the cytosol and mitochondria resulted in deleterious effects on aging and accelerated the development of age-related diseases, especially cancer. Thus, while some endogenous antioxidants have been shown to be important in promoting longevity and healthy aging, others contradict such effects.

A brief overview of redox signaling

Cysteine oxidation

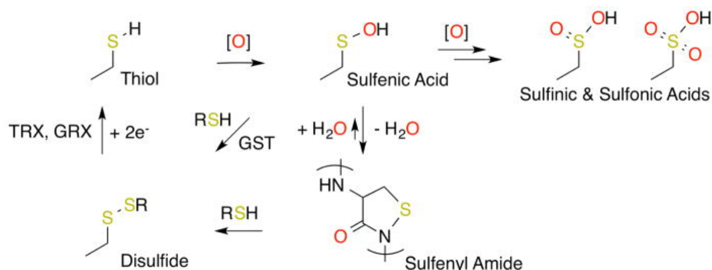

Figure 6: Cysteine intermediates involved in ROS-mediated redox signaling. From *Heppner et al (2017) Arch Biochem Biophys.*

Hydrogen peroxide (H_2O_2) and other oxidants (e.g. superoxide) can oxidize thiol (-SH) groups on cysteine residues within redox-sensitive proteins, thereby affecting protein function and cellular signaling. Upon oxidation of a thiolate anion by H_2O_2, a sulfenic acid (-SOH) is formed, which may affect the signaling function of the protein by altering its confirmation (**Figure 6**), as well as protein activity if the oxidized cysteine is located in its catalytic domain (87). When two or more cysteine residues within the same protein are oxidized, they may form an intramolecular disulfide bridge, thereby altering the confirmation and thus activity of the protein. A protein sulfenic acid can also react with glutathione (GSH), forming a gluthathione protein disulfide (GSSG), a process that is called protein-S-glutathionylation. Furthermore, a sulfenic acid can be reduced to thiolate anions by the antioxidants Trx and glutaredoxin (Grx), thereby changing the confirmation of the protein back to its original state (-SH). Alternatively, a protein sulfenic acid can also be further oxidized in the presence of high H_2O_2 concentration, to form sulfinic (-SO2H) or sulfonic acid (-SO3H) in an irreversible reaction. As such, first-degree oxidation of cysteine residues within proteins may serve as a reversible signal transduction mechanism, whereas sulfinic and sulfonic acid formation is irreversible and results in permanent protein damage.

Sources of ROS

The surface area of the lung is approximately 80–100 m^2, breathing 10.000–20.000 L of air per day, which signifies the highly susceptibility of the lung to its external environment. As a result, our lungs are exposed to various inhaled hazards that are found in the external environment, such as air pollution (smog, diesel) and particulates, infectious agents (viruses, bacteria), as well as cigarette smoke. These hazards may contribute to oxidative stress in the lung (88). For example, our lung immune cells respond to infectious agents by producing

large quantities of ROS from endogenous sources, e.g. myeloperoxidases (MPO), NADPH oxidases (NOX) and mitochondrial sources, such as superoxide ($O_2^{-}\cdot$), nitric oxide (NO), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl). While these reactive products are produced to destroy invading organisms and induce appropriate signaling pathways to restore cellular homeostasis, residual tissue damage may occur. Additionally, various sources of air pollution generate particulate matter (PM) that are well-known to induce lung injury and inflammation (89). Lastly, cigarette smoke, which is a major risk factor for age-related chronic lung diseases, contains radical molecules such as peroxides, hydroxyl radicals, and H_2O_2, as well as various electrophiles and aldehydes that may contribute to redox stress (90), and are involved in lung cellular injury (91).
Oxygen (O_2) is consumed in various metabolic reactions throughout various organelles of the cell, such as the mitochondria, the ER, and peroxisomes (**Figure 7**).

Mitochondria

Mitochondria are considered the dominant energy producer of the cell, since the mitochondrial electron transport chain is the main source of ATP in mammalian cells, and consumes 90% of cellular oxygen consumption and is a major source for cellular ROS production (92, 93), including $O_2^{-}\cdot$, the hydroxyl radical (OH·) and H_2O_2 (57).

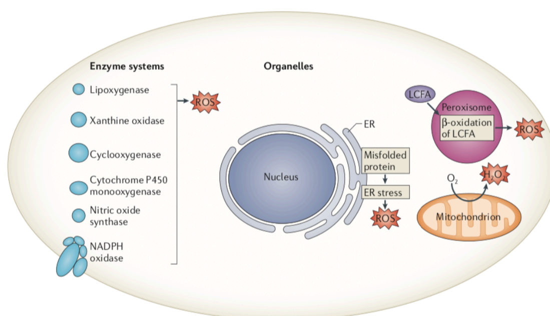

Figure 7: Enzyme sources of ROS. *From Holström,Finkel et al. (2014) Nat Rev Mol Cell Biol.*

Peroxisomes

De Duve and Baudhuin were the first to describe the respiratory pathway in peroxisomes (94), in which electrons removed from various metabolites reduce O_2 to H_2O_2 by various oxidases (e.g. uric acid oxidase), which is subsequently further reduced to H_2O by e.g. catalase or peroxidases that are also present in peroxisomes. The β-oxidation of fatty acids is the major metabolic process producing H_2O_2 in the peroxisomes.

Endoplasmatic Reticulum (ER)

The endoplasmic reticulum is an organelle consisting out of a network of tubules through which secretory and transmembrane proteins enter unfolded and after ER-mediated folding

mechanisms exit as either folded or misfolded proteins. While folded proteins are directed either toward other cell organelles, misfolded proteins are marked for degradation. Interestingly, the redox environment at the ER regulates the protein folding machinery (95). Protein disulfide isomerase (PDI) is an essential enzyme expressed in the ER that catalyzes formation of disulfide bonds between cysteine residues within proteins as they fold. During chaperone-assisted disulfide bond formation between polypeptide chain substrates of a protein, two electrons are provided to the cysteine residue within the PDI active site, and this transfer of electrons results in the production of ROS since oxygen serves as the terminal electron acceptor (96). In addition, NADPH oxidase enzymes, NOX4 specifically, is found at the ER and further contributes to ER-associated ROS generation (97).

NADPH oxidase (NOX) family

NADPH oxidase (NOX) enzymes participate in a wide range of biological processes, including chemical host defense, wound healing, and hormone synthesis (98). The first example of deliberate generation of ROS in mammalian cells was that of the phagocytic NOX (Phox, now NOX2), mainly found in neutrophils and macrophages, that catalyzes the respiratory burst (99). Since then, other NOX enzymes have been discovered, and are now considered 'professional' generators of ROS through the deliberate production of these ROS for cell signaling transduction, also known as redox signaling (100). Redox signaling by NOXes is carried out through initial post-translational regulation of protein function through cysteine oxidation of a target protein thiol (-SH), thereby forming a sulfenic acid (-SOH) (101, 102). Additionally, NOXes may induce oxidative PTM of other protein residues including tyrosine or methionine residues (103).

The family consists out of seven genes in humans (NOX1-5, and DUOX1/2) and six in mice (lacking Nox5), which are constitutively expressed and/or inducible by a wide variety of agents that include various growth factors, cytokines, shear stress, as well as agonists of T cell receptors (TCR) and G protein-coupled receptors (GPCR) (104, 105). NOX enzymes are classified into groups based on the presence of certain domains in addition to the gp91phox domain. NOX1-4 all contain the flavocytochrome catalytic moiety and require p22phox for enzyme stability and/or function. NOX5, in addition to comprising of the basic flavocytochrome catalytic moiety, contains an amino-terminal calmodulin-like domain that contains four calcium binding EF-hand structures. Lastly, the dual oxidase NOX enzymes, or DUOX, contain not only an NADPH-oxidase domain but additionally include a domain that is homologous to heme-containing peroxidases such as myeloperoxidase and lactoperoxidase

(LPO). These peroxidases catalyze the oxidation of (in)organic substrates by H_2O_2, thereby resulting in the formation of e.g. hypothiocyanite or hypochlorous acid, which exert bactericidal/antimicrobial activity (106). All NADPH oxidase catalytic components contain the C-terminal intracellular domain (with the NADPH and FAD-binding sites) and six transmembrane segments anchoring two heme groups, that are required for electron transfer from the cytosol to oxygen. DUOX enzymes contain an additional transmembrane α-helix that is linked at its N-terminus to the peroxidase-like domain (105).

NOX enzymes in the respiratory system

Specific isoforms of these NOX enzymes are expressed over a wide variety of cells within the lung (**Table 1**) and have been shown to be crucial in maintenance of cellular homeostasis.

Table 1: Human NADPH oxidase isoforms: the normalized expression (NX) in the lung according to the RNA Consensus dataset (from The Human Protein Atlas (107-109)), lung expression profiles, as well as the ROS they produce.

	Normalized Expression	Lung cell expression (110)	ROS production
NOX1	0.2	Endothelium Immune cells	$O_2^{\bullet -}$
NOX2/CYBB	49.4	Endothelium Immune cells Alveolar and airway epithelium	$O_2^{\bullet -}$
NOX3/gp91phox	0.1	Airway endothelium	$O_2^{\bullet -}$
NOX4	1.2	Smooth muscle and endothelium Myofibroblasts Immune cells Alveolar and airway epithelium	$O_2^{\bullet -}$ H_2O_2
NOX5	0.6	N.A.	$O_2^{\bullet -}$
DUOX1	48.7	Alveolar and airway epithelium	H_2O_2
DUOX2	1.2	Alveolar and airway epithelium	H_2O_2

Importantly, some NOX isoforms are thought to be more constitutively expressed (e.g. NOX2, DUOX1), whereas others are more inducible (e.g. DUOX2, NOX4), and may be partially reflected in the normalized expression data (obtained from The Human Protein Atlas) from **Table 1**. While various NOX enzymes have important homeostatic functions, NOX-derived

ROS have also been shown to be implicated in the pathology of various (age-related) diseases. Conceivably, these pathological roles of some NOX enzymes may be explained by the antagonistic pleiotropy hypothesis (111).

NADPH Oxidase 2 (NOX2)

NOX2 is most abundant in phagocytes, but also found in various lung cells, including those of the vascular system, fibroblasts, and epithelial cells (Table 1). The best studied function of NOX2 is its involvement in microbial killing by phagocytes (112), but NOX2 has also been implicated in regulation of endothelial inflammation (113) as well as virus-induced epithelial inflammation (114, 115).

Dual Oxidase 1 (DUOX1)

As mentioned earlier, dual oxidase enzymes (DUOX) have an additional transmembrane helix, an extracellular (EC) N-terminal domain with peroxidase homology, and two EF-hands within their first intracellular loop (105) (**Figure 8**). DUOX1, a member of the larger NADPH oxidase family, is primarily expressed in both alveolar and bronchial lung epithelial cells, produces the ROS hydrogen peroxide in response to calcium-dependent activation. In addition to its roles in hormone synthesis and anti-microbial and anti-viral host defense functions, it is involved in redox signaling and has been shown to be important in innate immune responses following injury or pathogen exposure by initiating type 2 immunity, which involves calcium-dependent activation of DUOX1 and downstream oxidation and activation of tyrosine kinases Src and the epidermal growth factor receptor (EGFR) (116, 117). DUOX1 is the main DUOX isoform at the airway epithelial surface that produces hydrogen peroxide in response to exogenous stimuli (e.g. allergens, ATP (118)) and bacterial stimuli (e.g. lipopolysaccharide (LPS), flagellin (119)). While LPS can activate DUOX1, airway epithelial antiviral as well as innate immune responses following such triggers seem to be mediated mainly by DUOX2 (120, 121). However, DUOX1 is strongly implicated in wound healing, the production of innate alarmins and type 2 immunity in response to protease allergens or other injurious triggers through redox signaling (117, 122).

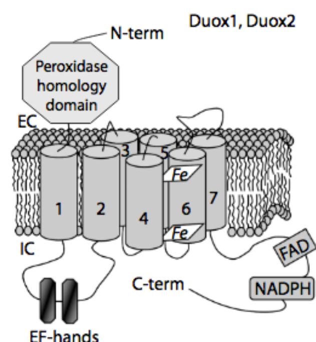

Figure 8: Structure of the NADPH oxidase DUOX1. *From Rada, Leto et al (2008) Contrib. Microbiol.*

Airway epithelial DUOX1, Th2 immunity, and epithelial repair/regeneration

The airway epithelium is recognized as the first barrier between the external environment and internal lung tissue (123). Cytokines of the IL-1 family, including the interleukins IL-1α and IL-33, are central mediators of innate immunity. They are constitutively expressed in epithelial cells (in the nuclei or cytoplasm) and are released following epithelial cell stimulation or injury. These IL-1 cytokines serve as innate alarmins, since they alert the immune system to induce appropriate responses (124, 125). IL-33 induces downstream activation of type 2 cytokine release (IL-5 and IL-13) and the release of various growth factors (e.g. amphiregulin, AREG) within various immune cells such as type 2 innate lymphoid cells (ILC2s) and other Th2 cells. Therefore, IL-33 has an important role in the resolution of inflammation and repair of tissue damage, due to the ability of IL-33 to target ST2-positive Treg (T regulatory cells) and ILC2 cells (124), as well as AREG-mediated restoration of epithelial integrity and homeostasis during infections of the lungs (126, 127). IL-5 and IL-13 may also drive further chemotaxis of various immune cells, including macrophages and eosinophils, that induce epithelial cell proliferation and restoration of barrier function following injury, and moreover promote epithelial stem cell differentiation into various specialized cell types, including mucus-secreting goblet cells (125). IL-4 and IL-13 may also activate fibroblasts, resulting in the secretion of collagens, as well as increased fibroblast contractility, which supports wound closure. Furthermore, macrophages play a role in the regulation of type 2 responses. Resident tissue repair macrophages provide key growth factors, which both aid in wound repair and promote fibrogenesis, whereas anti-inflammatory macrophages can dampen this response by providing IL-10 and transforming growth factor β (TGFβ), which promote regulatory T cell differentiation (128).

The immune cells, in association with fibroblasts and epithelial cells, determine whether type 2 responses result in successful wound healing, resolution of inflammation, and tissue regeneration, or progressive fibrosis and tissue pathology (**Figure 9**).

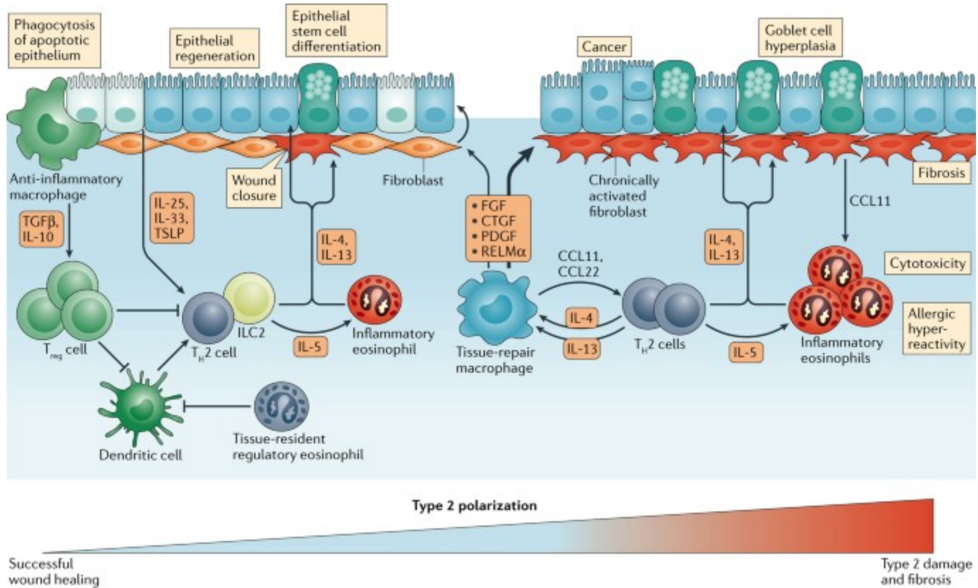

Figure 9: IL-33 signaling in type 2 immunity, wound healing and epithelial regeneration. From Gieseck III et al. (2018) *Nat. Rev. Imm.*

A role for DUOX1 in these type 2 immune responses was observed upon stimulation of airway epithelial cells with injurious triggers. Following exposure, ATP is rapidly released, which subsequently activates type 2 purinoceptor (P2YR2) signalling in airway epithelial cells, resulting in increases in intracellular calcium (Ca^{2+}) through Ca^{2+} influx (**Figure 10**). Other mechanisms leading to increased intracellular calcium levels may include activation of voltage-gated Ca^{2+} channels or transient receptor potential (TRP) channels, or through release from internal Ca^{2+} stores (by activation of G-protein coupled receptors, such as P2YR2). Collectively, these mechanisms were shown to be necessary for DUOX1 activation and subsequent DUOX1-mediated cysteine oxidation and activation of Src and EGFR, and downstream secretion of IL-33 and related cytokines, thereby initiating type 2 immune responses (117). These pathways are

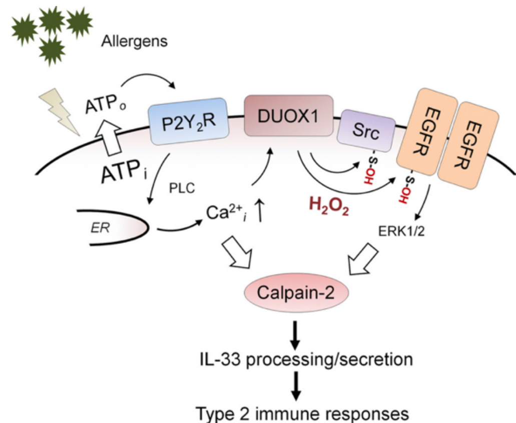

Figure 10: DUOX1-dependent innate injury responses following protease allergen exposure. From *Hristova et al (2016). J Allergy Clin Immunol*

similarly activated upon other forms of injury (e.g. exposure to cigarette smoke, or physical epithelial cell injury), resulting in regenerative responses through redox signaling events. While these type 2 immune responses normally resolve inflammation and result in successive wound healing and regeneration, chronic activation of these type 2 immune responses, as well as over-proliferation and differentiation at the wound site, can result in goblet cell hyperplasia, which induces excess mucus production. Excessive damaged epithelial cells also secrete alarmins (IL-33, IL-1α), which can augment type 2 responses by increasing the expression of IL-4, IL-5 and IL-13. Furthermore, excessive inflammatory eosinophilia can result in cytotoxicity, allergic hyperreactivity and fibrosis, and excessive/chronic type 2 signaling in fibroblasts may result in exaggerated deposition of extracellular matrix, ultimately leading to tissue scarring and fibrosis.

Similar to the notion that type 2 responses may induce beneficial responses as well as pathogenic features, DUOX1 has been shown to be upregulated in asthma, where it may contribute to exacerbated Th2 responses in response to e.g. allergens (116). On the other hand, DUOX1 has been shown to be suppressed in the airway epithelium in chronic lung diseases such as COPD and lung cancer (129, 130), where it may contribute to epithelial regeneration following injurious insults, importantly cigarette smoke.

Oxidative stress vs. oxidative signaling in lung aging, and age-related lung disease

While ROS have on one hand been shown to play key signaling roles in the lungs, they may also induce oxidative damage during settings of oxidative stress. These may affect numerous cellular processes, including DNA stability, cellular senescence, apoptosis, and ECM remodeling (101, 131). As such, the endogenous oxidant-antioxidant systems have significant physiologic roles in maintaining cell homeostasis and adaptation to environmental stress. The plausible double-edged function of ROS is explained through hormesis, which describes that any process may exhibit a biphasic response to an increasing amount of stimulus. In other words, ROS may elicit favorable biological response through redox signaling that promote homeostasis (‘eustress’) at low to intermediate levels, whereas excessive or deficient ROS levels may be detrimental, termed distress (**Figure 11**) (132). Similarly, the gradual ROS response hypothesis proposes that cells can tolerate age-dependent insults/damage through activation of protective ROS-dependent stress responses (133). However, the gradual increase of age-related damage increases these protective ROS responses, and may eventually render these protective responses uncapable of preventing the increasing amounts of damage, even though they are still being activated.

Consequently, these ROS, meant for promoting protective responses, may start to contribute to cell toxicity instead of protecting against age-related damage. Evidence of the ROS-generating NADPH oxidase family in oxidative signaling and/or oxidative stress during aging and in age-related chronic lung diseases are highlighted below.

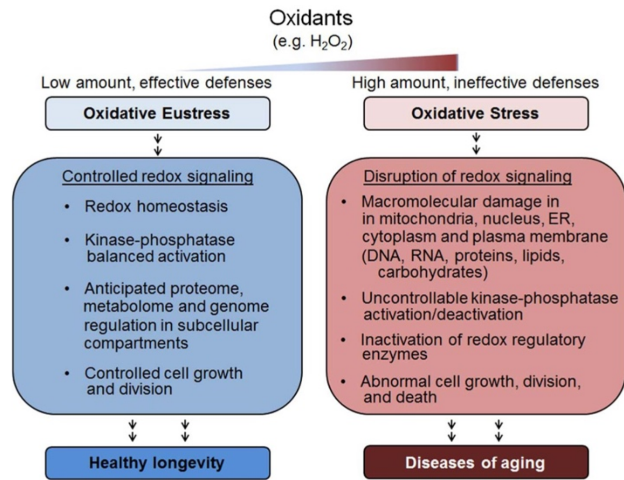

Figure 11: Oxidative stress theory of aging. *From Jones et al. (2017) Clinical Science.*

Aging

Indeed, there are various indications of oxidative (dis)stress during aging (and in age-related diseases). For example, nuclear and mitochondrial DNA damage through oxidation are significantly increased in all major tissues in aged organisms, including mice and rats (134) as well as humans (135, 136), and more specifically, in lungs (137, 138). Furthermore, substantially higher levels of DNA damage and lipid peroxidation products (e.g. lipid peroxides and MDA) have been observed in the lungs of aged organisms when compared with young counterparts (137, 138). DNA damage as well as lipid peroxidation in humans was further elevated in smokers, highlighting the significance of cigarette smoke (CS) in oxidative damage (137). Additionally, aging is recognized by various post-translational modifications (PTMs), including oxidative PTMs such as cysteine oxidation, that may also be involved in oxidative eustress. These modifications are observed in a large variety of proteins, including changes in structural proteins, enzymes, and proteins involved in signal transduction pathways (139, 140). A recent study explored the effects of age on tissue cysteine oxidation networks in mice, including the lungs (141), and is known to be targeted by the NADPH oxidase family (e.g. (142)). In contrast to the predominant understanding that non-specific protein oxidation increases with age, a loss of specific redox-regulated sites with age was observed in this study. Remarkably, the subcellular location of secreted proteins was less oxidized in the lung, which may be related to decreased cellular protein secretion in the aged lung. Moreover, aged tissues, including the lung, exhibit distinct age- and tissue-specific redox-regulated clusters reflecting a fundamentally reprogramed redox

signaling landscape in the aged lung (141). This is intriguing, because such reprogramming of redox signaling by ROS through e.g. NOX enzymes, may potentially regulate various hallmarks of aging, e.g. by controlling intercellular communication, mitochondrial (dys)function, proteostasis, or cellular senescence, and may also impact age-related chronic lung disease development.

To date, relatively little is known about NOXes in ageing. It is widely accepted that NOX4 activity is upregulated in VSMCs (vascular smooth muscle cells) with increasing age (143), as well as in the lung (83). Furthermore, one paper demonstrated that specifically senescent lung fibroblasts display increased levels of NOX4 through epigenetic histone modifications (144). Furthermore, NOX4 was demonstrated to be increased in whole lung tissues from aged mice in conjunction with elevated ROS levels in the bronchoalveolar lavage fluid (BALF), both in uninjured state as well as post-ALI (acute lung injury) induction by LPS or VILI (ventilator-induced lung injury) (145). These elevated levels of ROS and NOX4 increased LPS-induced endothelial cell permeability, which was associated with increased acute lung injury (ALI) severity. In contrast, a recent study found that whole lung *NOX4* mRNA levels were drastically downregulated with age, and that NOX4 deficiency did not impact lifespan (146). Additionally, while not studied in the context of aging, other studies found that NOX4 in the vasculature may be protective by promoting vasodilation and angiogenesis, and reducing blood pressure, and that upregulation of this NOX isoform may have potential therapeutic benefit in preventing vascular disease (147-149). Additionally, the loss of NOX2 in peripheral mononuclear cells with increasing age in humans was shown to be responsible for age-related dysfunction of immunosuppressive T cells (CD8+CCR7+ Tregs), thereby promoting pro-inflammatory responses that may increase susceptibility for the development of chronic inflammatory diseases (150). A related study demonstrated that NOX2 deficiency, through impaired development of Th17/Treg cells, spontaneously induced arthritis development in mice, of which the severity proportionally increased with age (151). Furthermore, deficiency of NADPH oxidase organizer 1 (NOXO1), which allows constitutive NOX1 expression, resulted in longer lifespan in mice, which may be achieved through better DNA repair capacity in NoxO1-deficient mice (152). In contrast to a role of NOX1, NOX2 and NOX4, no studies have addressed how (lung) DUOX1 is affected with age. However, an exceptionally intriguing finding is that of blistered-3 (BLI-3/Ce-Duox1), an NADPH oxidase isoform expressed in the nematode *C. elegans*, that shares 30% of its amino acid sequence to human DUOX1 (153). Emerging evidence suggests that the ROS generated by BLI-3 may act as a signaling molecule through redox signaling to promote oxidative stress resistance by

inducing Nrf2 and related antioxidant gene transcription (154-156). This finding also relates to longevity, since C. elegans that carry a dysfunctional BLI-3 mutation (either in the BLI-3 peroxidase domain or NADPH oxidase domain) are extremely short-lived. These findings may suggest a role for NOX enzymes in various hallmarks of ageing in the lung, such as genomic (in)stability, epigenetic alterations, (altered) intercellular communication and cellular (and immuno)senescence. However, the lack of data as well as conflicting findings regarding up- or downregulation of a given NOX enzyme in the lung make it difficult to elucidate the specific role of such a loss/gain of NOX enzyme expression and/or function on each of these hallmarks.

COPD

The age-related lung disease COPD has been strongly linked to increases in oxidative stress for some decades (32, 157), but the first evidence was described in the 1960s, where individuals with alpha-1-antitrypsin deficiency, a protein that is typically inactivated by oxidants, developed emphysema early, especially if the individual was a cigarette smoker (158). Indeed, the major risk factor in the development of this disease is smoking. Chronic exposure to cigarette smoke (or e.g. combustion products of biomass fuels (159) have been linked to marked increased levels of oxidative and carbonyl stress in the lungs (160, 161) that affect many of the hallmarks of aging (e.g. senescence, genomic instability, epigenetics), resulting in 'accelerated' aging of the lungs that underlies COPD pathogenesis (21). One study observed that in the airway epithelium specifically, aging and smoking were associated with enhanced oxidative stress and contributed to telomeric DNA damage foci, thereby inducing cellular senescence and the development of lung emphysema (162). Characteristic markers of oxidative stress in COPD include elevated concentrations of nitrotyrosine and various lipid peroxidation products (163), and during acute exacerbations in COPD alveolar macrophages produce elevated levels of ROS (164). Moreover, exhaled breath condensates from COPD patients show increased concentrations of e.g. H_2O_2 myeloperoxidase (MPO) and various markers of oxidative tissue damage (163).
With respect to oxidant-generating enzymes, specifically NADPH oxidases, there are various observations that link NOX enzymes to COPD pathology. NOX2-positive inflammatory cells are increased in COPD, and a contributive role of macrophage NOX2 in elastase-induced emphysema has been found (165). Additionally, elevated levels of NOX4 in airway smooth muscle in COPD were observed (166), which correlated with disease severity (167), and may be associated with pulmonary hypertension (168). Finally, in an animal model of emphysema

(TLR4 deficiency), endothelial NOX3 was increased, which contributed to elastolytic activity and oxidant generation (169). In contrast to a contributing role for NOXes in COPD pathogenesis, genetic deletion of NOX2 in mice aggravated CS-induced emphysema associated with increased inflammation, suggesting that NOX2 is protective in this process (170). Furthermore, brushings of tracheal and bronchial epithelium, as well as laser capture microdissection of bronchial epithelium, revealed DUOX1 suppression in patients with COPD and in healthy smokers without COPD compared to age-matched control subjects, implying that decreased airway epithelial expression of DUOX1 is associated with smoking and COPD (129, 171).

IPF

Oxidative stress has been implicated as a key mediator in the pathogenesis of IPF. Indeed, IPF patients display increased biomarker levels in exhaled breath condensate, such as the aforementioned H_2O_2 as well as 8-isoprostane (172), and lung tissue from these patients also demonstrate signatures of chronic oxidative (mitochondrial) damage (173). In IPF, the prevailing impression is that redox changes within the lung may induce a perpetuating pro-fibrotic tissue microenvironment by mediating cellular homeostasis (e.g. repair responses (174)). The most studied NADPH oxidase in this context is NOX4, and has been associated with such pro-fibrotic tissue environment (175-177). More specifically, a critical role for the NOX4 isoform in tissue repair functions of myofibroblasts and fibrogenesis was found. NOX4-dependent ROS production is TGF-β1 dependent (and this was unique to NOX4), a cytokine known to be overexpressed in fibrotic disease, which promotes fibroblast migration and induces a pro-fibrotic myofibroblast phenotype (e.g. differentiation, ECM deposition (175)). Accordingly, inhibition of NOX4 by siRNA or pharmacologic strategies alleviated the development of fibrosis in mice (177). The aging hallmark cellular senescence has been strongly implicated in IPF pathology, specifically in myofibroblasts. As such, it has been increasingly recognized as a therapeutic target for IPF (178). Indeed, senescent myofibroblasts accumulate in fibrotic foci in the lungs of IPF patients, with elevated NOX4 expression, resulting in an impaired Nrf2-mediated antioxidant response capacity (179). Furthermore, they found that in young mice, lung fibrosis was reversible since myofibroblasts exhibit apoptosis susceptibility thereby enabling resolution. However, in aged mice, myofibroblasts display cellular senescence and an apoptosis-resistant phenotype that impair the resolution of fibrosis. Interestingly, pharmacological or genetic targeting of NOX4, resulting in loss of NOX4 activity, induced a reversal of age-associated persistent fibrosis

and improved survival in aged mice with established fibrosis. Therefore, age-associated redox-imbalance, mediated by dysregulation of NOX4, may promote pro-fibrotic myofibroblast phenotypes, senescence, and persistent fibrosis in aging.
In addition to myofibroblasts, lung alveolar type 2 cells (AT2) display increased expression of NOX4 in IPF (176), and BALF neutrophils of IPF patients exhibit elevated expression levels of p47phox and p67phox (180). NOX4 knockdown (181) or genetic ablation of the p47phox subunit of NOX2 (182) was protective against development of bleomycin-induced pulmonary fibrosis. While p47phox deficiency was associated with lower collagen deposition and an elevated protease/anti-protease (MMP-9 (Matrix metallopeptidase 9)/TIMP-1 (tissue inhibitor of metalloproteinases 1)) imbalance in the lungs, NOX4 deficiency was associated with lower alveolar epithelial cell apoptosis. Similarly, inhibition of NOX4 in rodents resulted in attenuation of a fibrotic response, such as those involved in extracellular matrix components (e.g. collagen 1a1) and fibrotic remodeling (e.g. TGF-β1) (177). With respect to immune cells, a recent NOX4 has been demonstrated to induce profibrotic polarization of lung macrophages. NOX4 knockdown in macrophages was associated with decreased ECM deposition and protection from asbestos-induced pulmonary fibrosis, through mitochondrial biogenesis and ROS production (183). Interestingly, a similar role for NOX2 in macrophages has been found in emphysema (165). Moreover, a very recent role for DUOX1 in IPF was observed, as a study found that DUOX1 was upregulated in fibroblasts specifically in a model of bleomycin-induced pulmonary fibrosis, and that DUOX1-deficiency alleviated fibrosis development. They attributed this phenotype to the ability of DUOX1-derived H_2O_2 to augment the profibrotic TGF-β1/Smad pathway in fibroblasts, thereby promoting fibrogenesis (184).

Asthma

Oxidative stress and NOX enzymes appear to participate in asthma pathology. Asthmatic children show elevated biomarkers of oxidative stress in their blood, and inhaled corticosteroids (that relieve asthma symptoms) reduced *NOX2* mRNA expression in circulating leukocytes (185). Furthermore, the NOX2 inhibitor apocynin decreased H_2O_2 levels in exhaled breath condensates of asthmatic patients (186). Moreover, in a rodent model of OVA-induced acute allergic asthma, the NOX2 inhibitor ebselen improved inflammatory responses (187). Another study observed that in an OVA-induced allergic asthma model, increased expression of lung NOX1, 2, 3 and NOX4 were found, as well as enhanced oxidative damage markers, that were ameliorated using the anti-inflammatory

drug artesunate through Nrf2 activation (188). Conflicting these findings, NOX2 may also have protective roles in asthma, as NOX2-deficiency in mice with OVA-induced allergic airways disease aggravated inflammatory responses and airway hyperreactivity (AHR) compared to WT mice (189). Furthermore, airway smooth muscle cells that were isolated from biopsies from asthma patients showed increased *NOX4* mRNA levels, increased oxidative DNA damage along with increased ROS production, which were inhibited using NOX4 siRNA, diphenylene iodonium (DPI), or apocynin (190). Furthermore, ciliary beat frequency was impaired in bronchial epithelial cells isolated from neutrophilic asthma patients, which was associated with increased NOX4 expression, highlighting a role for NOX4 in ciliary dysfunction in asthma (191), and this dysfunction has also been shown to be impaired further during aging (192). In addition to NOX4, epithelial DUOX1 is induced by the Th2 cytokines IL-4 and IL-13, which are commonly elevated within asthmatic airways (193). Indeed, enhanced expression of *DUOX1* mRNA and protein are observed in isolated nasal or bronchial epithelial cells from allergic asthma patients (117, 191) and in animal models of house dust mite (HDM)-induced allergic airway inflammation (117). Furthermore, increased DUOX1 (and to a lesser extent DUOX2) has also been observed in the nasal mucosa of patients with chronic sinusitis (194).

Aims and outline of the thesis

An imbalance between oxidants and antioxidants has been implicated in various lung diseases, of which many are related to aging. Regardless of whether ROS may be the cause or a consequence of aging, there is little doubt that ROS play a critical role in the hallmarks of aging, and in age-related lung diseases. Rather than the classical view in which ROS (and ROS-generating molecules) are inherently damaging and contribute to disease development, DUOX1 may actually be crucial for cellular homeostasis through redox signaling, and DUOX1 is lost in age-related chronic lung diseases such as COPD. Since the current challenge is to improve our understanding of how redox imbalance, aging, and age-related lung disease are intertwined, the overall aim of this thesis is to explore the importance of DUOX1 during aging, and age-related lung diseases. The main focus was to study the role of the ROS-generating enzyme, DUOX1, both in vitro as well as in vivo utilizing models of airway epithelial injury responses, models of aging, and models of chronic lung diseases (COPD, fibrosis). Following this introduction and overall aim of the thesis, in Chapter 2 we examine the importance of the Ca2+ channel TRPV1 in DUOX1-mediated innate airway responses to allergen proteases or other injurious triggers. Our findings implicate that TRPV1 is critically involved in DUOX1-mediated responses, and may lead to new avenues for future therapeutic interventions in e.g. asthma. Chapter 4 explores the impact of aging on the various NADPH oxidases in the mouse lung. We observed that DUOX1 is the NOX isoform that is dramatically lost with increasing age. We furthermore show that in mice and in isolated mouse tracheal epithelial cells, age-related DUOX1 loss is responsible for diminished innate epithelial injury responses, and that these responses could be rescued by DNA demethylation. Lastly, we provide evidence that DUOX1 loss (utilizing *Duox1*-deficient mice) accelerates age-related senile emphysema development. Chapter 3 describes the involvement of DUOX1 in the age-related chronic lung disease COPD. Our results show that loss of airway epithelial DUOX1 is observed in COPD patients as well as in mouse models of COPD, and is associated with worsened lung function, emphysema, and airway remodeling, each of which are critical features in COPD pathology. Chapter 5 explored the effect of DUOX1-deficiency during aging on the pathogenesis of chronic lung diseases, including asthma, COPD, and IPF. Finally, the results as well as the implications and limitations of our findings are discussed in Chapter 6.

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Chapter 2

The transient receptor potential channel vanilloid 1 is critical in innate airway epithelial responses to protease allergens

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Abstract

The airway epithelium plays a critical role in innate responses to airborne allergens by secreting IL-1 family cytokines such as interleukin (IL)-1α and IL-33, as alarmins that subsequently orchestrate appropriate immune responses. Previous studies revealed that epithelial IL-33 secretion by allergens such as *A. alternaria* (ALT) or house dust mite (HDM) involves Ca^{2+}-dependent signaling, via initial activation of ATP-stimulated type 2 purinoceptors (P2YR2) and subsequent activation of the NADPH oxidase DUOX1. We sought to identify proximal mechanisms by which epithelial cells sense these allergens, and here highlight the importance of protease-activated receptors (PAR2) and transient receptor potential (TRP) Ca^{2+} channels such as TRP Vanilloid 1 (TRPV1) in these responses. Combined studies of primary human nasal and mouse tracheal epithelial cells, as well as immortalized human bronchial epithelial cells (HBE1), indicated the importance of both PAR2 and TRPV1 in IL-33 secretion by both ALT and HDM, based on both pharmacological and genetic approaches. TRPV1 was also critically involved in allergen-induced ATP release, activation of DUOX1, and redox-dependent activation of EGFR. Moreover, genetic deletion of TRPV1 dramatically attenuated allergen-induced IL-33 secretion and subsequent type 2 responses in mice in vivo. TRPV1 not only contributed to ATP release and P2YR2 signaling, but was also critical in downstream innate responses to ATP, indicating potentiating effects of P2YR2 on TRPV1 activation. In aggregate, our studies illustrate a complex relationship between various receptor types, including PAR2 and P2YR2, in epithelial responses to asthma-relevant airborne allergens and highlight the central importance of TRPV1 in such responses.

Introduction

The respiratory epithelium forms a protective barrier in the lung and plays a critical role in innate airway responses to environmental pathogens and airborne allergens (1, 2). An important component of innate epithelial responses to various environmental triggers is the rapid release of the interleukin (IL)-1 family member IL-33, normally expressed within the nucleus of epithelia, as an alarmin to induce appropriate immune responses by stimulating IL-33-specific receptors (IL1RL1/ST2) on various effector cells, such as T lymphocytes, innate lymphoid cells (ILC2), and mast cells (3-5). Such IL-33-dependent type 2 immune responses contribute to tissue regenerative responses and maintenance of homeostasis, but excessive IL-33 release and activation of type 2 inflammation has also been implicated in chronic lung pathologies, such as allergic inflammation and pulmonary fibrosis (6-8). The mechanisms by which different external triggers promote epithelial IL-33 release are still incompletely understood. While this is typically thought to involve passive IL-33 release upon epithelial necrosis, recent studies indicated the importance of signaling events independent of necrosis in epithelial IL-33 release by asthma-relevant allergens such as *A. alternata* (ALT) or *D. pteronyssinus* (house dust mite, HDM), which involve initial release of ATP as a cellular damage signal, and Ca^{2+} signaling due to activation of purinergic receptors on the epithelial surface (3, 9). Previous studies by our group demonstrated that allergen-induced IL-33 release requires the Ca^{2+}-sensitive NADPH oxidase DUOX1, which is primarily expressed within the respiratory epithelium and generates H_2O_2 to activate the tyrosine kinases Src and epidermal growth factor receptor (EGFR), thereby promoting processing and non-classical secretion of IL-33 (9).
The proximal mechanisms by which allergens such as ALT or HDM are sensed by airway epithelial cells or other innate immune cells are not well established, but serine or cysteine proteinases associated with these allergens, such as Der p 1, are thought to play a major role (10-12). Allergen-derived proteases can act on epithelial cells in different ways, including direct disruption of junctional proteins, production of toll-like receptor 4 (TLR4) ligands, and activation of cell surface protease-activated receptors (PAR), G protein-coupled receptors that comprise four members (PAR1, PAR2, PAR3 and PAR4). Among these, PAR2 has been most strongly implicated in epithelial responses to protease allergens including HDM and ALT (13-15). PAR2 is also the main isoform that is enhanced in asthmatic airways (16), and is involved in allergen-induced loss of epithelial integrity, allergen sensitization, and induction of Th2-polarizing cytokines (17, 18). Notwithstanding these various findings, the direct

importance of PAR receptors in allergen-induced epithelial IL-33 secretion has not yet been established.

Although PAR2 has been implicated in allergen-induced epithelial responses, it is not fully responsible for allergen-induced ATP release and Ca^{2+} signaling, and other Ca^{2+} channels have been implicated (15, 19). An important class of proteins that mediates intracellular Ca^{2+} increase in response to diverse stimuli comprises the transient receptor potential (TRP) Ca2+ channels, which are Ca^{2+}-permeable, non-selective cation channels that sense a variety of chemical and physical stimuli. Diverse TRP channels have been implicated in airway responses to various environmental triggers (e.g. tobacco smoke), and in various lung pathologies (20, 21). While typically thought to be confined to nociceptive neurons, several TRP channels, particularly members of the TRP vanilloid (TRPV) subfamily such as TRPV1 and TRPV4, are also found within the bronchial epithelium (20), and non-neuronal TRPV1 has been shown to be significantly upregulated in the airway epithelium of asthmatics (22) and in nasal polyps from patients with chronic rhinosinusitis (23). Population-based epidemiological studies revealed the involvement of TRPV1 in symptoms typically associated with asthma (24), and TRPV1 was found to contribute to airway hyperresponsiveness (AHR) and airway inflammation in mouse models of asthma (25, 26). Intriguingly, G-protein-coupled receptors such as PAR2 can dramatically sensitize TRPV1 responses during pain responses (27, 28), and TRPV1 was found to contribute to PAR2-induced hypersensitivity of dorsal root ganglion (DRG) neurons (29). However, the involvement of TRP channels in innate airway epithelial responses to allergens, and IL-33 secretion in particular, has to date not been addressed.

The present studies were undertaken to investigate the importance of PAR receptors and TRP channels in innate responses to asthma-relevant airborne allergens HDM and ALT, in studies with primary human nasal epithelial cells and murine tracheal epithelial cells. Our findings demonstrate that PAR2 and TRPV1 both critically contribute to allergen-induced ATP release, as an initial damage signal that promotes activation of DUOX1 and subsequent redox-dependent EGFR activation and IL-33 secretion. Moreover, TRPV1 was also critical for IL-33 secretion and subsequent type 2 inflammation after in vivo allergen challenge, and contributed to ATP-mediated IL-33 secretion, indicating potentiating effects of P2YR2 on TRPV1 activation. Collectively, our findings illustrate a central role of TRPV1 in innate epithelial responses to protease allergens, which acts in concert with PAR2 and P2YR2 to promote epithelial IL-33 secretion and subsequent activation of type 2 responses.

Materials and Methods

Cell culture and treatments

Primary nasal epithelial (HNE) cells were collected from healthy volunteers and patients with allergic rhinitis, and cultured as described previously (9). Immortalized human bronchial epithelial (HBE1) cells were cultured as described previously (9). Mouse tracheal epithelial (MTE) cells were isolated from wildtype C57BL/6J mice, BALB/c mice, or *Trpv1*-deficient mice (B6.129X1-Trpv1$^{tm1Jul/J)}$ and cultured as described previously (9). Prior to treatment with indicated stimuli or inhibitors, cells were incubated overnight in EGF-free complete media to suppress basal EGFR activity. Cells were pre-treated with various inhibitors for 30 minutes and stimulated with extracts of HDM (*D. pteronyssinus*; Greer Laboratories, Lot. #213051) or *A. alternaria* (Greer Laboratories, Lot. #217252) or other reagents of interest for either 10 minutes or 2 hours at 37°C and 5% CO_2. Alternatively, cells were transfected with human small interfering RNA (siRNA; Dharmacon, Supplemental Table 1) using DharmaFECT1 transfection reagent (Thermo Scientific), prior to treatment. Conditioned culture media were collected for cytokine analysis and cell extracts were prepared for mRNA or protein extraction for RT-PCR or Western blot analysis, respectively. Further experimental details are described in Supplemental Methods.

In vivo animal studies

C57BL/6J or *Trpv1*$^{-/-}$ mice (B6.129X1-Trpv1$^{tm1Jul/J}$; originally obtained from Jackson Laboratories and kindly provided by George Wellman, Department of Pharmacology, University of Vermont) aged 8–12 weeks were subjected to brief isoflurane anesthesia and administration of 50 µL of 1 µg/mL HDM extract (Greer Laboratories; Lot #218862, 1.27 endotoxin units/mg) or 50 µl PBS vehicle control, via the oropharyngeal route, and bronchoalveolar lavage (BAL) and lung tissues were collected 1 or 6 hours after challenge. In separate experiments, wildtype C57BL/6NJ mice and *Duox1*$^{-/-}$ mice (originally provided by Miklos Geiszt (9) and backcrossed to C57BL/6NJ background) were subjected to oropharyngeal instillation of capsaicin (30 µg/kg bodyweight in 50 µl PBS/0.5% EtOH; Sigma M2028) or vehicle control under isoflurane anaesthesia. After instillation, mice were maintained under 1% isoflurane anasthesia for a total duration of 1 hour, after which mice were sacrificed, and BAL and lung tissues were collected. All animal procedures were reviewed and approved by the Animal Care and Use Committee of the University of Vermont.

Biochemical assays

Cell culture supernatants or BAL fluids were analyzed for IL-33, KC/IL-8, IL-13, IL-5, IL-1α using DuoSet ELISAs (R&D Systems) according to the manufacturer's instructions. Extracellular H_2O_2 production was determined by lactoperoxidase-catalyzed crosslinking of o-tyrosine, which was analyzed by HPLC and fluorescence detection (9). ATP release into the medium was examined using a luciferase/luciferin bioluminescence ATP determination assay, according to the manufacturer's instructions (Molecular Probes). Activation of EGFR signaling was assessed by Western blot analysis of phospho-EGFR.

Statistical analysis

All quantitative data are presented as the mean ± SE. Statistical differences between groups were analyzed using 1- or 2-way ANOVA with Bonferroni post-hoc analysis in GraphPad Prism (version 7.0; GraphPad Software, La Jolla, CA). P values < 0.05 were considered significant.

Results

Allergen-induced IL-33 secretion from HNE cells involves protease activity and PAR2 activation

We used primary human nasal epithelial (HNE) cells from four healthy volunteers to assess mechanisms of innate responses to two common asthma-relevant allergens, A. alternata (ALT) or house dust mite (HDM), which both possessed detectable protease activity (**Fig. 1A**). Apical stimulation of HNE cells in Transwell inserts with either ALT or HDM resulted in rapid apical IL-33 release, which was not observed in the presence of either PMSF or antipain (**Fig. 1B**), two inhibitors of serine proteases. Since proteases can induce epithelial responses by activating PAR receptors (13), we evaluated mRNA expression of various PAR isoforms in HNE cells, and observed that HNE cells primarily express PAR2 and to a lesser extent PAR1 (Fig. S1). Accordingly, allergen-induced IL-33 secretion was not observed in the presence of the PAR2 inhibitor FSLLRY-NH2 (**Fig. 1B**). Moreover, IL-33 secretion could also be evoked by direct stimulation with PAR2 agonist peptides (SLIGLR-NH2 and f-LIGRLO-NH2), but not by agonists of PAR1 (thrombin and TFLLR-NH2) (**Fig. 1C**). Similarly, direct activation of PAR2, but not PAR1, promoted HNE cell production of extracellular H_2O_2, reflecting DUOX1 activation which was recently implicated in allergen-induced IL-33 secretion (9). Hence protease allergens evoke epithelial IL-33 secretion largely by stimulating PAR2 and subsequent activation of DUOX1.

Allergen-induced epithelial IL-33 secretion involves TRPV1

Airway epithelial cells may also express various TRP Ca^{2+} channels, which could be involved in allergen-induced Ca^{2+} signaling and DUOX1 activation. Therefore, we sought to address the mRNA expression of TRP channels isoforms in HNE cells, which revealed expression of both *TRPV1* and *TRPV4* (**Fig. S1**). We used various TRP channel inhibitors to address the potential involvement of TRPV1/4 in allergen-induced responses, which demonstrated that IL-33 release by ALT or HDM appeared to be suppressed by two TRPV1 inhibitors, JNJ17203212 and capsazepine, but not by the TRPV4 inhibitor RN1734 (**Fig. 1D**). Moreover, direct stimulation of HNE cells with agonists of TRPV1 (capsaicin or allyl isothiocyanate, although the latter also activates TRPA1) could evoke IL-33 secretion, as well as H_2O_2 production (reflecting DUOX1 activation; capsaicin was found to interfere with the H_2O_2 assay), whereas agonists of TRPV4 (GSK1016790A) or TRP isoform ankyrin 1 (TRPA1,

acrolein) were much less effective (**Fig. 1E**). Hence, allergen-induced H_2O_2 production and IL-33 secretion by HNE cells involves the contribution of TRP channels, and primarily TRPV1.

Activation of PAR2 and TRPV1 promotes ATP release and P2YR2-dependent signaling

Previous studies implicated epithelial ATP release and P2YR2 activation in allergen-induced IL-33 secretion (3, 9). We therefore examined whether activation of PAR2 or TRPV1 could evoke apical ATP release from HNE cells and act through P2YR2-dependent mechanisms. Indeed, direct activation of PAR2 (but not PAR1) or TRPV1 was capable of evoking rapid ATP release, comparable to stimulation with HDM or ALT (**Fig. 2A**). While measured extracellular ATP levels in cell culture media were in the nM range, concentrations near the cell surface are likely higher and sufficient to activate purinoceptors such as P2YR2 (30). The importance of P2YR2 in allergen-induced responses in HNE cells was confirmed by inhibition of both H_2O_2 production and IL-33 secretion by the P2YR inhibitor suramin (**Fig. 2B**), consistent with previous findings (3, 9). These findings suggest that protease allergens induce epithelial ATP release by a concerted mechanism involving initial activation of both PAR2 and TRPV1 (20, 27, 28), subsequently resulting in activation of P2YR2, DUOX1-dependent H_2O_2 production (9), and IL-33 secretion. Follow up studies were performed in HBE1 cells to determine the importance of P2YR2 in TRPV1/4-mediated responses, using siRNA silencing of P2YR2. As expected (3, 9), IL-33 secretion in response to allergens or to exogenous ATP was attenuated after silencing of P2YR2 (**Fig. 2C**). Similarly, P2YR2 silencing also attenuated IL-33 secretion in response to the TRPV1 agonist capsaicin or the TRPV4 agonist GSK1016790A, confirming that TRPV1/4-induced IL-33 secretion requires P2YR2-dependent DUOX1 activation.

TRPV1 activation is crucial for allergen-induced DUOX1 activation and EGFR signaling

To further elucidate the importance of TRPV1 in allergen-induced responses, we performed complementary studies with HBE1 cells and mouse tracheal epithelial (MTE) cells. Western blot analysis indicated the presence of TRPV1 protein in immortalized human bronchial epithelial HBE1 cells (**Fig. S1**). Consistent with our findings in HNE cells (Fig. 1), HDM-induced IL-33 secretion from HBE1 cells as well as MTE cells from either C57BL/6J mice or BALB/c mice could in each case be attenuated by inhibitors of TRPV1 (JNJ17203212 or capsazepine) or mimicked by direct stimulation of TRPV1 (capsaicin, AITC) as well as TRPV4 (GSK1016790A) (**Fig. 3A** and **B** and **Fig. S2**). Based on our previous studies indicating that innate epithelial responses to protease allergens involve redox-dependent activation of the

tyrosine kinases Src and EGFR (9), we addressed whether TRPV1 was also involved in allergen-induced activation of these signaling pathways. Indeed, pharmacological inhibition of TRPV1 strongly attenuated allergen- or ATP-induced activation of EGFR in HBE1 cells (**Fig. 3C** and **D**) as well as MTE cells (**Fig. S3**), and agonists of TRPV1 and TRPV4 were capable of inducing EGFR activation (**Fig. 3C-E** and **Fig. S3**). Because of the potential lack of specificity of pharmacological TRPV inhibitors, we also utilized siRNA silencing to assess the specific involvement of TRPV1 in allergen responses (**Fig. S1** and **S5B**). Indeed, IL-33 release induced by allergens, ATP, and the TRPV1 activator capsaicin, was markedly inhibited after TRPV1 siRNA (**Fig. 3F**). Interestingly, IL-33 secretion by GSK1016790A, was not affected by TRPV1 siRNA (**Fig. 3F**), illustrating the specificity of this agonist for TRPV4 and not TRPV1. Similar inhibitory effects of TRPV1 siRNA were also apparent with respect to H_2O_2 production by these triggers (**Fig. 3G**). Collectively, these findings indicate that allergen-induced DUOX1 activation, EGFR activation, and IL-33 secretion primarily involves TRPV1.

TRPV1 potentiates P2YR2-mediated responses in airway epithelial cells

One unanticipated finding in our studies was the fact that TRPV1 inhibition and siRNA silencing also dramatically suppressed IL-33 release, H_2O_2 production, and EGFR activation in response to exogenous ATP (**Fig. 3**, **Fig. S2** and **S3**). While ATP is a ligand for P2YR2, high doses of exogenous ATP could potentially activate cells by P2YR-independent mechanisms, and we therefore evaluated IL-33 secretion by lower concentrations of ATP, and demonstrated that ATP induced IL-33 secretion at concentrations as low as 10 μM (which is relatively selective for P2YR), prevented by TRPV1 silencing in each case (**Fig. S4**). Of note, TRPV1 siRNA also modestly reduced P2YR2 mRNA expression (**Fig. S5**). In aggregate, these findings indicate that TRPV1 is not only involved in allergen-induced ATP release and downstream P2YR2-dependent signaling, but also critically contributes to ATP-dependent signaling in cooperation with P2YR2.

TRPV1 is critical for innate airway epithelial responses to HDM

To more firmly address the importance of TRPV1 in allergen-induced innate responses, we performed studies with *Trpv1*-deficient mice. We verified that TRPV1-deficiency did not affect expression levels of DUOX1, P2YR2, or TRPV4 (**Fig. S6**). Studies using isolated MTE cells from either wild-type or *Trpv1*-deficient mice confirmed that TRPV1 was critical for

acute ATP- or HDM-induced production of IL-33 as well other innate cytokines, such as IL-1α, or the murine IL-8 homolog KC (although ATP stimulation did not significantly increase KC production in this case) (**Fig. 4A-C**). Next, WT and *Trpv1*$^{-/-}$ mice were subjected to acute in vivo airway challenge with HDM (50 μg/ml) and lung lavage fluids were collected at various time points for analyses of IL-33 and related type 2 immune responses. As expected, HDM challenge resulted in rapid and transient production of IL-33 (**Fig. 5A**), IL-1α (**Fig. 5B**), amphiregulin (Areg) (**Fig. 5C**) and KC (**Fig. 5D**), which peaked at 1 hour after challenge and in most cases declined after 6 hrs. Each of these responses was significantly attenuated in *Trpv1*-deficient mice (**Fig. 5A-D**). In addition, HDM challenge induced delayed airway production of IL-5 and IL-13 (**Fig. 5E** and **F**), most likely as a result of initial IL-33 secretion, which was also attenuated in *Trpv1*-deficient mice. Finally, to address the involvement of DUOX1 in these TRPV1-mediated airway responses, WT or *Duox1*$^{-/-}$ mice were subjected to acute airway challenge with the TRPV1 agonist capsaicin, which was found to similarly evoke significant acute airway production of IL-33 and KC, and tended to increase IL-1α, responses that were largely attenuated in *Duox1*$^{-/-}$ mice (**Fig. 6A-C**). Combined with earlier studies indicating the importance of epithelial DUOX1 in production of these various cytokines by allergens (9), these results suggest a critical role for epithelial TRPV1 in DUOX1-mediated innate airway responses to airborne allergens such as HDM, particularly in IL-33 secretion and subsequent activation of type 2 immune responses.

TRPV1 and P2YR2 expression are increased in HNE cells from asthmatic subjects

We obtained HNE cells from both healthy controls and subjects with allergic asthma and rhinitis (9) and addressed expression of various genes addressed in this study. Notably, mRNA expression of both *P2YR2* and *TRPV1* were significantly enhanced in HNE cells from asthmatic subjects compared to non-asthmatic controls, whereas expression of PAR1/2 also tended to be increased (**Fig. 7**). This indicates that enhanced expression of these receptors may contribute to amplified type 2 immune responses to allergens or other exacerbating triggers in these subjects.

Discussion

Interleukin 33 (IL-33), a member of the IL-1 family, has received extensive recent interest as an epithelial-derived alarmin that mediates type 2 immune responses to various allergens, and because of its important role in type 2 asthma (31, 32). The mechanisms by which IL-33 is released from the epithelium during e.g. allergen challenge or exacerbations has remained somewhat enigmatic (4). Although IL-33 can be released passively from epithelial cells as an active cytokine due to necrosis, emerging studies indicate that various asthma-relevant allergens can promote active IL-33 secretion from airway epithelia due to initial release of ATP and activation of purinoceptor-dependent signaling pathways, which involve the NADPH oxidase DUOX1, redox-dependent activation of tyrosine kinases such as Src and EGFR, and activation of calpains (3, 9). The upstream mechanisms by which allergens initiate these responses have remained largely unclear. However, airway epithelial cells have been shown to express many diverse receptor types (e.g. TLRs, dectin-1) that may recognize different components of common allergens such as HDM or ALT (33). Extending previous findings implicating allergen-associated proteases in airway responses (10, 12), our present studies highlight the critical importance of protease-activated receptors, particularly PAR2, in epithelial IL-33 secretion in response to either ALT or HDM, which is associated with initial ATP release and activation of DUOX1. Moreover, while previous studies have implicated additional Ca^{2+} channels in airway epithelial responses to these allergens, such as P2X7 receptors (19) or Ca^{2+} release-activated Ca^{2+} (CRAC) channels (15), our findings also demonstrate the critical importance of the transient receptor potential channel TRPV1, as a common mediator of allergen-induced activation of DUOX1 and IL-33 secretion.

While PAR2 receptors can be directly activated by allergen-derived proteases, it is not clear how these allergens activate TRPV1. In addition to capsaicin, several other chemical ligands from exogenous (e.g. resiniferatoxin) or endogenous sources (so-called "endovanilloids" including lipid-derived products such as anandamide), are thought to directly activate TRPV1 (34, 35). Interestingly, segmental allergen challenge of patients with allergic asthma was found to enhance local production of anandamide (36). TRPV1 can also be activated by physical stimuli such as heat (>42°C), alterations in membrane potential, low pH (<6), and by various inflammatory mediators, although in these latter cases it is not always clear to what extent TRPV1 activation is due to direct stimulation or sensitization by additional cellular pathways. Our attempts to unravel the relative role(s) of the various receptor types in innate allergen responses yielded the surprising observation that, while direct activation of both

PAR2 and TRPV1 evoked ATP release and subsequent purinoceptor activation, TRPV1 was also critical for IL-33 release in response to extracellular ATP. This latter finding is consistent with previous studies indicating that ATP stimulation of neuronal cells activate Ca2+ responses and nociception through combined activation of TRPV1 and P2YR2 (37), and with the general concept that GPCRs such as P2YR2 as well as PAR2 can potentiate or sensitize TRPV1 activation (29, 38). Hence, our findings imply that concerted or combined activation of PAR2, P2YR2 and TRPV1 by protease allergens such as HDM and ALT is necessary for optimal activation of Ca^{2+} signaling and DUOX1 activation, as a central mechanism of IL-33 secretion and production of related cytokines. The precise sequence of molecular events between these different receptor pathways is difficult to decipher, since they are subject to various reciprocal interactions. For example, Ca^{2+} signaling mechanisms can enhance ATP release (39), but ATP can also activate Ca^{2+} signaling by both P2YR2 and TRPV1. Moreover, since TRP channels including TRPV1 are also subject to redox alterations (40), it is possible that DUOX1 activation may also contribute to TRPV1 activation in a positive feedback mechanism. Nevertheless, the main novel aspect of our present studies is the apparent central role of TRPV1 in these innate airway epithelial responses by allergens or by ATP, as illustrated in **Fig. S7**. Our findings may not be unique to TRPV1, as TRPV4 is also expressed within the nasal or airway epithelium (e.g. **Fig. 7**), and may contribute to pulmonary inflammatory disease (41). Moreover, TRPV4 can also be sensitized by PAR2 receptor activation in e.g. DRG neurons, intensifying neurogenic inflammation and responses to various stimuli (42). Indeed, our studies suggest that TRPV4 activation can also induce DUOX1 activation and IL-33 secretion from airway epithelial cells, although TRPV1 appears to be the primary isoform involved in similar responses to allergens.

We did not specifically address what form(s) of IL-33 are being released from airway epithelial cells upon exposure to allergen proteases, although our previous studies indicate that IL-33 is secreted primarily in its cleaved 18-kDa form (9). Recent studies indicated that exogenous allergen cysteine proteases (in e.g. A. alternata), as well as endogenous calpains from damaged airway epithelial cells, can process full length IL-33 (33 kDa) to its cleaved form (18 kDa), to increase its cytokine activity (12, 43). Conversely, IL-33 is also subject to inactivation by oxidation of critical cysteine residues (44), which would suggest that, while DUOX1-dependent oxidative mechanisms are critical for initiating IL-33 secretion in response to allergen challenge, DUOX1 might also contribute to IL-33 inactivation by oxidation. Nevertheless, our findings indicate that PAR2-P2YR2-TRPV1-DUOX1 is critical for initial IL-33 alarmin activation, as indicated by subsequent type 2 responses ((9) and present study).

Various TRP channels, including TRPV1, have been implicated in the pathology of asthma or related allergic diseases (21, 23, 45), and a loss of function single nucleotide polymorphism within TRPV1 (I585V) has been associated with lower risk of childhood asthma (24). Some studies in animal models of allergic asthma have suggested a contribution of TRPV1 to type 2 inflammation and airway hyperresponsiveness (25, 26), but other reports indicate that TRPV1-deficiency does not affect allergic asthma (46) or may even enhance Th2-biased inflammation (47). The contribution of TRPV1 to asthma has been attributed primarily to its role in nociceptor responses in sensory neurons, which are critical in development of cough and bronchospasm or airways hyperactivity, and also in allergic inflammation through neuro-immune crosstalk (45, 48). In addition to its presence in sensory neurons, TRPV1 has also been detected in the bronchial epithelium of asthma patients (22), and our studies highlight a specific role of epithelial TRPV1 in allergen-induced IL-33 secretion as a major factor in activation of type 2 inflammation. Interestingly, the IL-33 receptor ST2 is also expressed in DRG neurons and was found to mediate itch responses in a model of poison ivy-induced allergic contact dermatitis (49). Thus, the contribution of TRPV1 in allergen-induced cough or bronchospasm in asthmatic subjects could conceivably also involve initial IL-33 secretion through TRPV1 activation within the airway epithelium. Future studies with epithelia-specific deletion of TRPV1 would be needed to address this possibility.

In conclusion, our present findings highlight the importance of PAR2-P2YR2-TRPV1-DUOX1-mediated responses in innate epithelial responses to protease allergens, particularly with respect to IL-33 secretion and activation of type 2 inflammation. We recognize that our studies were based on acute challenge with relatively high doses of either HDM or ALT that may not necessarily reflect realistic exposure conditions. Although it is well established that exposure to HDM in dust has been associated with asthma development (e.g. (50)), the actual levels of airway HDM exposure are likely much lower than those used in the present study. Similarly, while fungal infection can contribute to asthma severity or complications (51), local concentrations of fungal allergens are difficult to estimate. However, the observed levels of IL-33 secretion in our present studies are quite similar to those observed in nasal secretions of asthmatic subjects during exacerbations by RSV (52) or in BAL of children with severe asthma with fungal sensitization (53). Therefore, we believe that our findings are informative with respect to proximal mechanisms that may contribute to such responses in asthmatic subjects during e.g. exacerbations, and offer mechanistic insight into the molecular pathways involved in exacerbated IL-33 responses observed in these settings. Observations from previous studies (9) and our present findings (**Fig. 7**) indicating that

P2YR2, PAR2, TRPV1, and DUOX1 are all elevated in basal nasal epithelial cells from patients with allergic asthma or chronic rhinosinusitis, combined with recent observations that basal epithelial cells from patients with allergic chronic rhinosinusitis display features of allergic inflammatory “memory” (54), would suggest that such elevations in PAR2-P2YR2-TRPV1-DUOX1 in the airways of these subjects may underlie increased risk for IL-33 activation and exacerbations (53, 55). In light of the current interest in developing biologics against IL-33 and related epithelial cytokines in treating allergic disease (5), our study indicates that selective targeting of upstream factors in this pathway, such as TRPV1, may also deserve consideration.

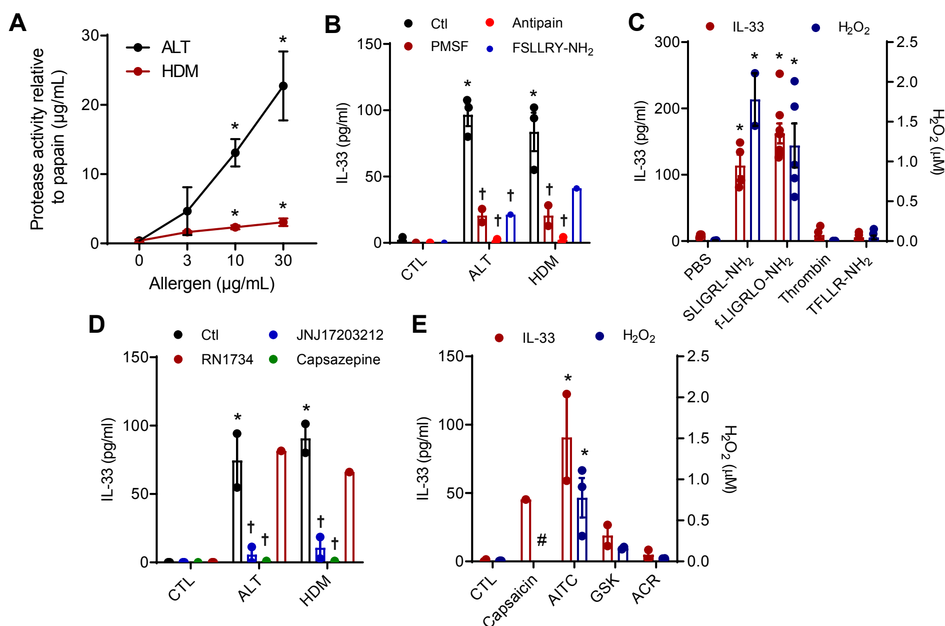

Figure 1. Protease allergens induce IL-33 release from nasal epithelial (HNE) cells via PAR2 and TRPV1. (A) Assessment of protease activity in allergen extracts, relative to papain (µg/mL, mean ± SE, n=2-6) after exposure to 0, 3, 10 and 30 ug/mL allergen (ALT or HDM, 50 µg/mL). *: $p < 0.05$ versus no allergen by one-way ANOVA. (B) Inhibition of allergen-induced IL-33 release from HNE cells by the serine protease inhibitors PMSF (100 µM) or antipain (50 µg/ml) or by the PAR2 inhibitor FSLLRY-NH2 (10 µM). (C) IL-33 release and extracellular H_2O_2 production induced by agonists of PAR2 (SLIGRL-NH2; 50 µM, f-LIGRLO-NH2; 5 µM), but not by agonists of PAR1 (thrombin; 30 nM, TFLLR-NH2; 50 µM). *: $p < 0.05$ versus PBS control by one-way ANOVA. (D) Inhibition of allergen-induced IL-33 release by the TRPV1 inhibitors JNJ17203212 (1 µM) or capsazepine (10 µM), but not by the TRPV4 inhibitor RN1734 (10 µM). (E) Stimulation of IL-33 release or H_2O_2 production by TRP channel agonists, capsaicin (10 µM), allyl isothiocyanate (AITC, 100 µM), GSK1016790A (10 nM) or acrolein (30 µM). *: $p < 0.05$ versus control by one-way ANOVA. Data represent mean ± SE from independent studies from 2-4 different donors. *: $p < 0.05$ versus control treatment. #H_2O_2 production by capsaicin could not be assessed due to its interference with the H_2O_2 assay.

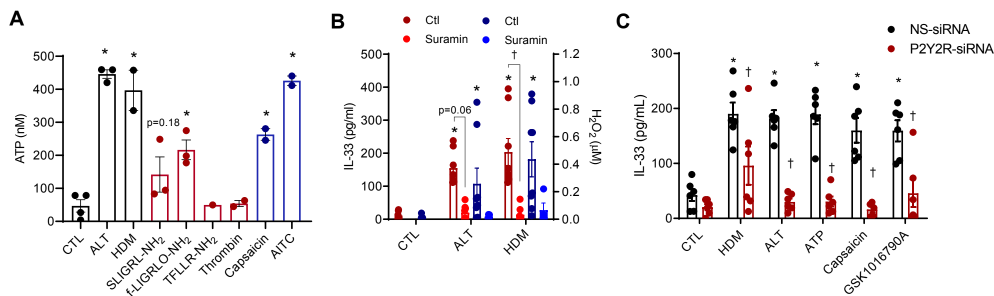

Figure 2. Importance of P2YR2 and DUOX1 in innate allergen responses. (A) Effects of protease allergens (black), PAR agonists (red) or TRPV1 agonists (blue) on extracellular ATP release from HNE cells after 15 min stimulation. *: $p < 0.05$ versus control by one-way ANOVA. (B) Inhibition of allergen-induced H_2O_2 production and IL-33 secretion from HNE cells by the P2YR inhibitor suramin (100 μM). Data represent mean ± SE from independent studies from 3-4 different donors. *: $p < 0.05$ versus control by one-way ANOVA † : $p < 0.05$ versus corresponding treatment without inhibitor by one-way ANOVA. (C) Measurement of IL-33 release from HBE1 cells (mean ± SE; n=5-6) following 2 hr stimulation with HDM (50 μg/mL), ALT (50 μg/mL) ATP (100 μM), the TRPV1 agonist capsaicin (10 μM) or the TRPV4 agonist GSK1016790 (10 nM), after siRNA silencing of P2YR2. *: $p < 0.05$ versus control treatment, † : $p < 0.05$ versus corresponding NS-siRNA treatment.

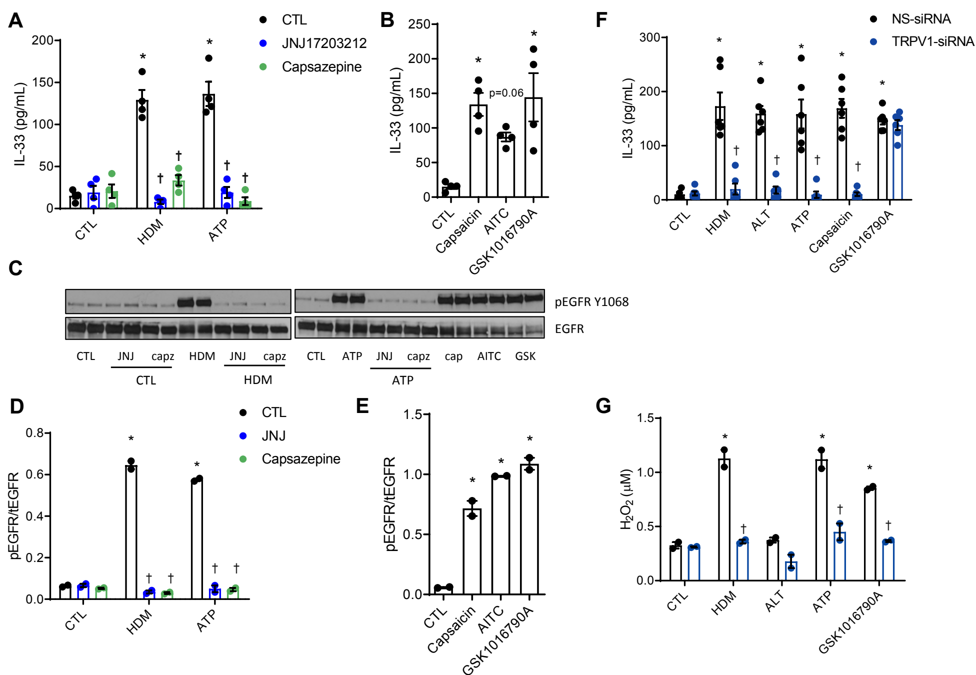

Figure 3 Involvement of TRPV1 in allergen-induced innate epithelial responses. (A) HBE1 cells were pre-treated with either the TRPV1 inhibitor JNJ17203212 (1 µM) or capsazepine (10 µM), and stimulated for 2 hours with HDM (50 µg/mL) or ATP (100 µM) for analysis of IL-33 secretion in the medium (mean±SE; n=4). (B) Activation of IL-33 release from HBE1 cells by 2 hr stimulation with TRPV1 agonists capsaicin (10 µM) or AITC (100 µM) or the TRPV4 agonist GSK1016790 (10 nM) (mean±SE; n=4). (C) Representative western blot analysis of HBE1 cell lysates for EGFR phosphorylation following 10-min stimulation with HDM (50 µg/mL) or ATP (100 µM), in the absence or presence of TRPV1 inhibitors JNJ17203212 (1 µM) or capsazepine (10 µM), or after stimulation with the TRPV1 agonist capsaicin (10 µM) or TRPV4 agonist GSK1016790 (10 nM). (D,E) Semi-quantitative analysis (mean±SE; n=4) of pEGFR/EGFR ratios normalized to HDM (D) or AITC (E), calculated using ImageJ software. Effect of TRPV1-siRNA on IL-33 secretion (F, mean±SE; n=6) or extracellular H_2O_2 production (G, mean±SE; n=2) upon stimulation with HDM (50 µg/mL), ALT (50 µg/mL), ATP (100 µM), the TRPV1 agonist capsaicin (10 µM), or the TRPV4 agonist GSK1016790 (10 nM). Note that H_2O_2 production by capsaicin stimulation could not be assessed due to its interference with H_2O_2 assay. *: $p < 0.05$ versus control treatment; † : $p < 0.05$ versus corresponding treatment without inhibitor or NS-siRNA treatment.

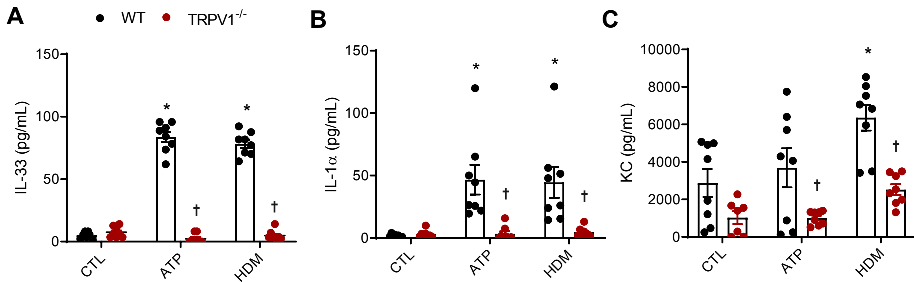

Figure 4. TRPV1-deficiency attenuates innate HDM responses. MTECs isolated from WT or *Trpv1*$^{-/-}$ mice were stimulated with HDM (50 µg/mL) or ATP (100 µM) for 2 hrs, and production of IL-33 (A), IL-1α (B), or KC (C) was measured in the medium by ELISA (mean±SE, n=8). *: $p < 0.05$ compared to control treatment; † : $p < 0.05$ compared to corresponding treatment in WT MTEC.

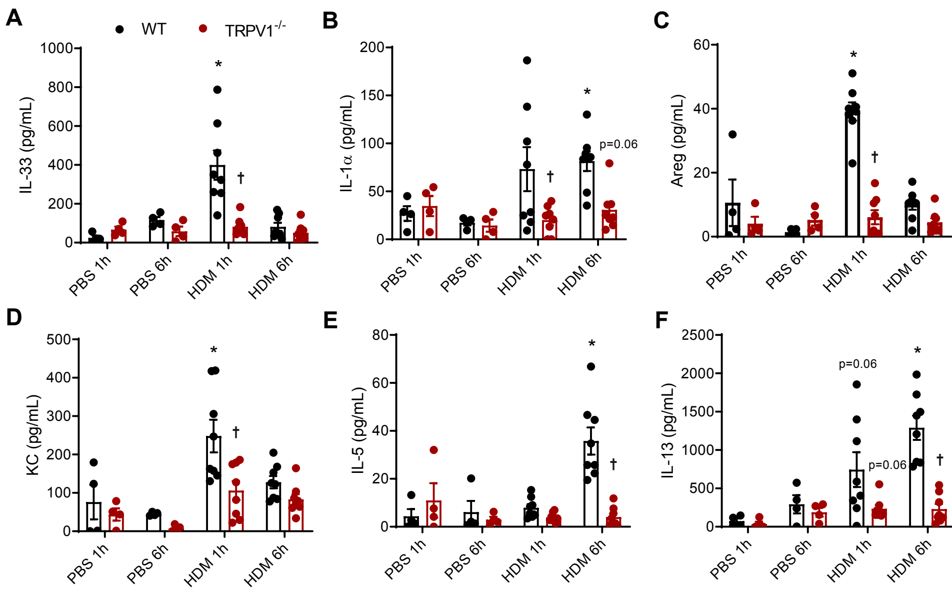

Figure 5. *Trpv1*-deficiency attenuates innate HDM responses and type 2 inflammation in vivo. Wildtype C57BL/6J (WT) and *Trpv1*$^{-/-}$ mice were challenged with HDM extract or vehicle control, and lung lavage was collected after 1 or 6 hours, for ELISA analysis of IL-33 (A), IL-1α (B), Areg (C) KC (D), IL-5 (E) or IL-13 (F) (mean and SE, n=4-8). *: $p < 0.05$ compared to corresponding PBS control; † : $p < 0.05$ compared to corresponding treatment in WT mice.

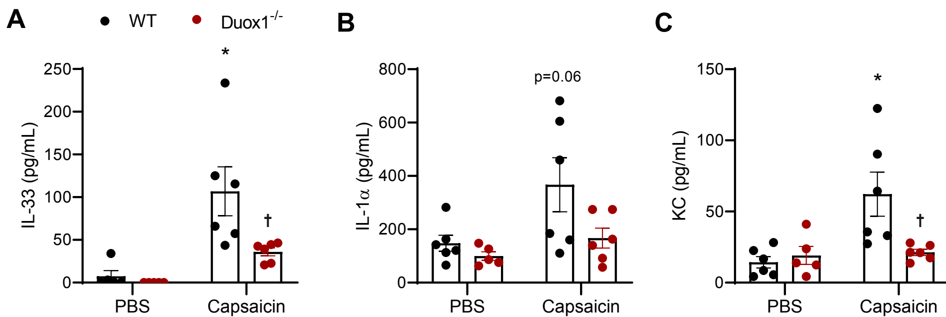

Figure 6. *Duox1*-deficiency attenuates innate cytokine responses in response to capsaicin. Wildtype C57BL/6NJ (WT) and *Duox1*$^{-/-}$ mice were challenged with capsaicin (30 μg/kg) and BAL was collected after 1 hr for ELISA analysis of IL-33 (A), IL-1α (B) or KC (C) (mean and SE, n=5-6). *: $p < 0.05$ compared to corresponding PBS control; † : $p < 0.05$ compared to corresponding treatment in WT mice.

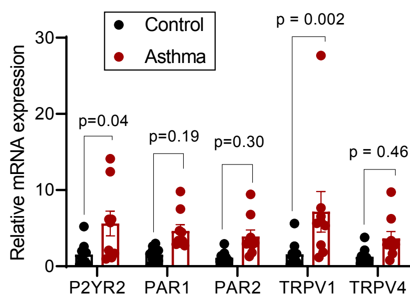

Figure 7. Enhanced mRNA expression of *P2YR2* and *TRPV1* in HNE cells isolated from asthmatic patients compared to controls. Relative mRNA expression of *P2YR2, PAR1, PAR2, TRPV1* and *TRPV4* in HNEs isolated from control (black) or asthmatic (purple) subjects. Data points represent the averages of duplicate analysis (mean±SE) in cells isolated from 10 control and 9 asthmatic donors. *$p < 0.05$ compared to control HNE subjects.

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Supplemental Information for:

The transient receptor potential channel vanilloid 1 (TRPV1) is critical in innate airway epithelial responses to protease allergens

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Supplemental Methods

Cell isolation and culture

Primary nasal epithelial cells (HNEs) were collected from healthy volunteers and patients with allergic rhinitis, by gentle stroking of the inferior turbinate surface with a Rhino-Probe curette (Arlington Scientific) and cultured as described previously (1). For experiments, HNEs were seeded on collagen-coated transwell inserts (Costar) and cultured for up to 4 days, until they reached a transepithelial resistance of >500 Ω.cm2. Culture media consisted out of a 1:1 mixture of bronchial epithelial cell basic medium and DMEM-H with SingleQuot supplements (Cambrex), bovine pituitary extracts (BPE, 13 mg/ml), bovine serum albumin (BSA, 1.5 µg/ml), and nystatin (20 units). Immortalized human bronchial epithelial (HBE1) cells were cultured as described previously (2, 3), and upon confluence seeded in 24-well tissue culture plates and grown in DMEM/F12 (Gibco, Cat. #11330-032), supplemented with 1% Penicillin/Streptomycin, cholera toxin, epidermal growth factor (EGF), insulin, transferrin, dexamethasone, BPE and BSA until confluent. Mouse tracheal epithelial cells (MTEC) were isolated from wildtype C57BL/6J mice, *Trpv1*-deficient mice (B6.129X1-Trpv1tm1Jul/J), or wildtype BALB/c mice, as described previously (4-6). In brief, after cannulating the trachea, a protease solution (0.1% protease Type 14 (Sigma) in minimal essential medium (MEM)) was injected through the cannula, trachea ends were sealed, and incubated overnight at 4 °C in MEM containing 3X Penicillin/Streptomycin. The next day, the trachea was flushed with media (containing 5% FBS, fetal bovine serum) to dislodge the cells. Subsequently, the cells were cultured in a collagen-coated T75 flask (Corning). When confluent, the MTECs were seeded in a 24-well collagen-coated tissue culture plate and grown in DMEM/F12 (Gibco, supplemented with L-glutamin, Penicillin/Streptomycin, cholera toxin, EGF, insulin, transferrin, dexamethasone and BPE).

In vivo allergen challenge

C57BL/6J or *Trpv1*$^{-/-}$ mice (B6.129X1-Trpv1tm1Jul/J; Jackson Laboratories) aged 8–12 weeks were subjected to brief isoflurane anesthesia and subsequent oropharyngeal (OP) administration of 50 µL of 1 µg/mL HDM extract (Greer Laboratories; Lot #218862, 1.27 endotoxin units/mg) or 50 µl PBS vehicle control, and upon sacrifice, 1 or 6 hours after PBS/HDM challenge, bronchoalveolar lavage (BAL) and lung tissues were collected. All animal procedures were reviewed and approved by the Animal Care and Use Committee of the University of Vermont.

In vivo instillation of capsaicin

Wildtype C57BL/6NJ mice and corresponding *Duox1*$^{-/-}$ mice (originally provided by Miklos Geiszt (4) and backcrossed to C57BL/6NJ background) were subjected to oropharyngeal (OP) instillation of capsaicin (30 µg/kg bodyweight in 50 µl PBS/0.5% EtOH; Sigma M2028) or 50 µl PBS/0.5% EtOH vehicle control, under isoflurane anaesthesia, and were maintained under 1% isoflurane anasthesia for a total duration of 1 hour, after which mice were sacrificed, and bronchoalveolar lavage (BAL) and lung tissues were collected. All animal procedures were reviewed and approved by the Animal Care and Use Committee of the University of Vermont.

Cell treatments

Prior to experimentation, cells were incubated overnight in EGF-free complete media to suppress basal EGFR activity. HNE cells were treated apically with the allergens HDM (D. pteronyssinus, house dust mite; 50 µg/mL) or ALT (*Alternaria alternata*; 50 µg/mL) for various times, depending on measured outcomes. Protease activity within these allergen extracts was measured using an EnzCheck® Protease Assay Kit (Thermo Fisher) and compared to Papain (Sigma). Alternatively, HNE cells were stimulated with agonists of PAR1 (thrombin; 30 nM, TFLLR-NH2; 50 µM) or PAR2 (SLIGRL-NH2; 50 µM, f-LIGRLO-NH2; 5 µM), or the TRP channel agonists capsaicin (10 µM), allyl isothiocyanate (AITC, 100 µM), GSK1016790A (10 nM) or acrolein (30 µM). In appropriate cases, cells were pretreated for 30 min with the P2YR antagonist suramin (100 µM), the serine protease inhibitors antipain (50 µg/mL) or PMSF (100 µM), the PAR inhibitor FSLLRY-NH2 (10 µM), the TRPV4 inhibitor RN1734 (10 µM), or the TRPV1 inhibitors JNJ17203212 (1 µM) and capsazepine (10 µM), prior to allergen stimulation.

HBE1 cells and MTECs were treated similarly with allergens, ATP (100 µM), PAR2 agonists (SLIGRL-NH2; 50 µM, f-LIGRLO-NH2; 5 µM), the TRPV1 agonists capsaicin (10 µM) and AITC (Allyl-isothiocyanate, 100 µM), or the TRPV4 agonist GSK1016790A (10 nM). Where indicated, cells were pre-treated with the TRPV1 antagonists JNJ17203212 (1 µM) and capsazepine (10 µM) for 30 minutes prior to stimulation.

siRNA silencing

HBE1 cells were transfected with human small interfering RNA (siRNA) targeting TRPV1, P2YR2, DUOX1 or non-specific RNA (Dharmacon, see Supplemental Table 1) at 100 nM using DharmaFECT1 transfection reagent (Thermoscientific). Following overnight transfection, medium was replaced with full DMEM/F12, and cells were grown for an additional 48 hours, after which cells were serum starved overnight. The next day, cells were stimulated with HDM (50 µg/mL), ALT (*Alternaria Alternata*, 50 µg/mL), ATP (100 µM), capsaicin (10 µM) or GSK1016790A (10 nM) for 15 minutes (H_2O_2 assay) or 2 hours (ELISA) at 37 °C.

qPCR analysis

Gene expression in HNE, MTE and HBE1 cells, as well as target gene knockdown (by siRNA) in HBE1 cells was verified by examining mRNA expression by real-time PCR. Target gene expression 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). The reaction used incubation at 37 °C for 50 minutes and was stopped by heating to 65 °C for 15 minutes. The real-time PCR reactions contained 0,5 µL cDNA, 5 µL iQ SYBR Green Supermix (Bio-Rad), 1 µL primer (Supplemental Table 2) in ddH2O (Invitrogen; 10 µM) and 3,5 µL ddH_2O. Amplification and detection were performed using the 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 minute and amplification at 72 °C for 30 seconds. Consequently, 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.

Western blot analysis

After stimulation, cells were lysed using Western solubilization buffer (WSB; 1% Triton; 50 mM HEPES, 250 mM NaCl, 10% Glycerol, 1.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl chloride, 1 mM EGTA, 2 mM Na_3VO_4, 10 µg/ml aprotinin and 10 µg leupeptin, pH 7.4). Next, samples were sonicated on ice, followed by centrifugation. Protein concentration was determined using the Bicinchoninic acid (BCA) Protein Assay Kit (Thermoscientific), according to manufacturer's instructions. To each sample 2x SRB (Sample Reducing Buffer, 0.125 M Tris-HCl, 4% SDS, 20% v/v Glycerol, 4.7 mM ß-mercaptoethanol and 0.02% Bromophenol blue in H_2O) was added (1:1) and consequently boiled at 100 °C for 5 minutes. Samples (15 µg) were loaded in a 10% polyacrylamide gel (Criterion TGX Precast gel, Bio-Rad) and run by gel electrophoresis. After protein transfer to nitrocellulose membranes, membranes were blocked in 5% instant non-fat dry milk blocking buffer (in TBST, 0.2 M Tris-Cl, 1.3 M NaCl, pH 7.6 in H_2O), and subsequently probed with antibodies targeting EGFR and Src (Cell Signaling; rabbit anti-pEGFR Tyr1068, rabbit anti-EGFR, 1:1000, rabbit anti-pSrc Tyr416, 1:1000; mouse anti-Src, 1:1000). HRP-conjugated anti-rabbit IgG (Cell Signaling) and anti-mouse IgG (Cell Signaling) were used for detection, and the blot was developed using the SuperSignal West Pico Chemiluminescent Substrate kit (Thermoscientific), followed by exposure to an X-ray film (Thermoscientific). Additionally, β-actin (1:1000; Cell Signaling) was used to ensure equal protein loading in all samples. ImageJ software was used to process the bands in order to evaluate the percentage of phosphorylated/total EGFR and Src compared to control.

ELISA

Murine or human cell culture supernatants or BAL fluids were analyzed for IL-33, KC (murine IL-8), IL-13, IL-5, IL-1α (pg/mL) by using DuoSet ELISAs (R&D Systems).

H_2O_2 assay

HNE or HBE1 cells were cultured as described above and placed in Hanks' balanced salt solution (HBSS, pH 7.4) for 15 min. After stimulation with appropriate stimuli for an additional 15 min, the conditioned media was collected, and mixed with an equal volume of L-tyrosine (1 mM) and lactoperoxidase (10 µg/ml) in PBS for 15 min. H_2O_2 production was measured as the transition of tyrosine to o,o'-dityrosine by peroxidase-catalyzed oxidation, which was quantified by fluorescence detection (excitation: 284 nm, emission: 410 nm) using high pressure liquid chromatography (HPLC), as described previously (2, 7).

ATP Luciferase Assay

ATP release into the apical medium was examined using a luciferase/luciferin bioluminescence ATP determination kit according to the manufacturer's instructions (Molecular Probes). The effects of various stimuli on extracellular ATP release in confluent HNE cells were addressed after 15 min stimulation. 100 µl media was collected, and subsequently analyzed with luciferase/luciferin in a Lumat LB 9507 luminometer (emission: 560 nm). The amount of ATP released was calculated using external ATP standards (1–100 nM). In these experiments, HNE cells were stimulated with the allergens HDM (50 µg/mL) or ALT (50 µg/mL), PAR activators PAR1 (thrombin; 30 nM, TFLLR-NH2; 50 µM), PAR2 (SLIGRL-NH2; 50 µM, f-LIGRLO-NH2; 5 µM) or TRPV1 activators capsaicin (10 µM), allyl isothiocyanate (AITC, 100 µM).

Statistical analysis

All quantitative data is reported as the mean ± SE from the indicated number (n). Statistical differences between samples were analyzed using 2-way ANOVA with Bonferroni post-hoc analysis in GraphPad Prism (version 7.0; GraphPad Software, La Jolla, CA). P-values < 0.05 were considered significant.

Supplemental Table 1: Dharmacon siRNA targets

siRNA target	Target sequence
TRPV1	GGAGACUAUUUCCGAGUUA UGACGAGCAUGUACAAUGA CAUCUAUGCCGUCAUGAUA CCCGAUAGCUCCUACAACA
P2YR2	UGCCGCUGCUGGUCUAUUA GGAAUGCGUCCACCACAUA CGACAGAACUGACAUGCAG CGAGAACACUAAGGACAUU
DUOX1	GCUAUGCAGAUGGCGUGUAtt UACACGCCAUCUGCAUAGCtg
Non-specific (NS)	UGGUUUACAUGUCGACUAA UGGUUUACAUGUUGUGUGA UGGUUUACAUGUUUUCUGA UGGUUUACAUGUUUUCCUA

Supplement Table 2: qPCR primers used in this study

Human/mouse	Primer	Forward/Reverse	Sequence
Human	*TRPV1*	F R	GGCTGTCTTCATCATCCTGCTGCT GTTCTTGCTCTCCTGTGCGATCTTGT
	TRPV4	F R	CTACGCTTCAGCCCTGGTCTC GCAGTTGGTCTGGTCCTCATTG
	P2YR2	F R	GCCAGTGTGAGGCTGTAACT AGCCAACTGGCTTTACAGTG
	P2YR6	F R	GTCTACCGCGAGAACTTCAA TGATCACCTTGGGCATAGTT
	DUOX1	F R	TTCACGCAGCTCTGTGTCAA AGGGACAGATCATATCCTGGCT
	DUOX2	F R	ACGCAGCTCTGTGTCAAAGGT TGATGAACGAGACTCGACAGC
	PAR1	F R	TGGAATAAGACAGAGACCTGCCCT GCTGACAGGCCTTGTTTGTGTGAA
	PAR2	F R	CTTTCTGTTCCCAGCCTTCCTC GCCCTCTTCCTTTTCTTCTCTGAGT
	GAPDH	F R	GAAGGCTGGGGCTCATTTG AGGCTGTTGTCATACTTCTCATGG
Mouse	*Duox1*	F R	ACCAGAACATTGCGATGTATGAG AGAAATGGACGGTATCCTGGA

	Duox2	F	GGCCTAAAGAAGAGGTTTGGCAAA
		R	GCCTCCGTGTACAGCCGGG
	Trpv1	F	CCACTGGTGTTGAGACGCC
		R	TCTGGGTCTTTGAACTCGCTG
	Trpv4	F	GACAACACCCGAGAGAACAC
		R	GGATGATGTGCTGAAAGACC
	Gapdh	F	CTGGAGAAACCTGCCAAGTA
		R	TGTTGCTGTAGCCGTATTCA

Supplemental Figures

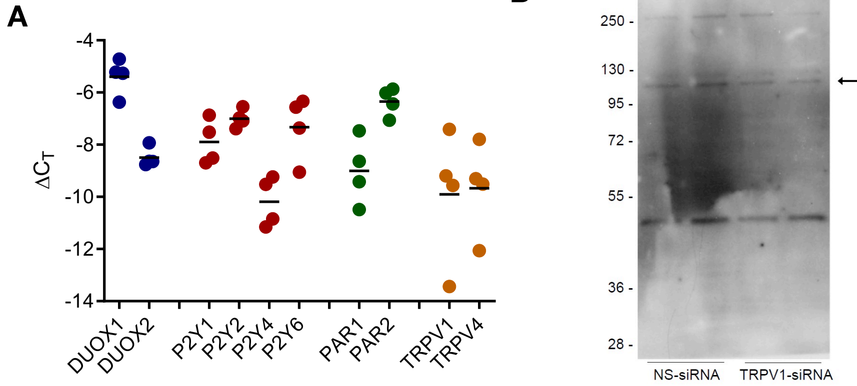

Figure S1. (A) mRNA expression levels of *DUOX1/2, P2Y1R, P2YR2, P2Y4R, P2Y6R, PAR1, PAR2, TRPV1* and *TRPV4* in HNE cells relative to *GAPDH*. Data points represent averages of duplicate analysis from cells from 4 different donors. (B) Western blot of HBE1 cell lysates using anti-TRPV1 (VR1 antibody (R130), sc-28759, Santa Cruz), indicating potential glycosylated TRPV1 band at about 110 kDa (see arrow), which was decreased after transfection with TRPV1-siRNA.

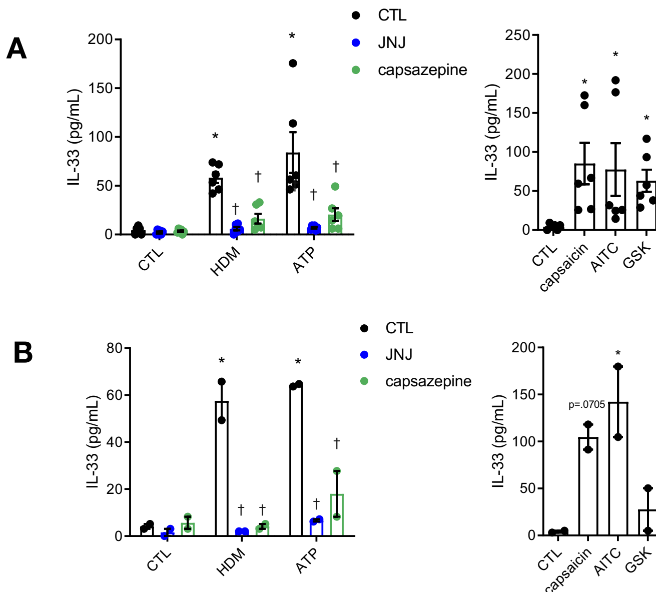

Figure S2. Role of TRPV1 in allergen-induced IL-33 secretion by MTEC from C57/BL6J or BALB/c mice. IL-33 release from MTECs isolated from C57/Bl6J (A) and BALB/c (B) mice following treatment with HDM or ATP (mean±SE, n=6 for C57/Bl6J, n=2 for BALB/c). Cells were either left untreated (CTL), or pre-treated with either JNJ17203212 (1 µM) or capsazepine (10 µM), and afterwards left untreated (CTL) or stimulated for 2 hours with HDM (50 µg/mL), ALT (50 µg/mL) or ATP (100 µM). Alternatively, IL-33 release (pg/mL) was measured after cells were left untreated (CTL) or stimulated with capsaicin (10 µM), AITC (100 µM) or GSK1016790 (10 nM) for 2 hours. *p<0.05 versus control treatment, †p<0.05 versus corresponding treatment without inhibitor.

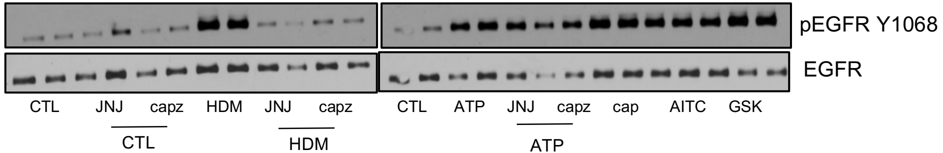

Figure S3. Role of TRPV1 in allergen-induced EGFR activation. Western blot levels of autophosphorylated EGFR (pEGFR at Y1068) and total EGFR in MTECs isolated from C57/Bl6J mice. Cells were either left untreated (CTL), or pre-treated with either JNJ17203212 (JNJ) or capsazepine (capz), and afterwards left untreated (CTL) or stimulated for 10 minutes with HDM, ALT, ATP. Alternatively, cells were stimulated with capsaicin (cap) or GSK1016790 (GSK).

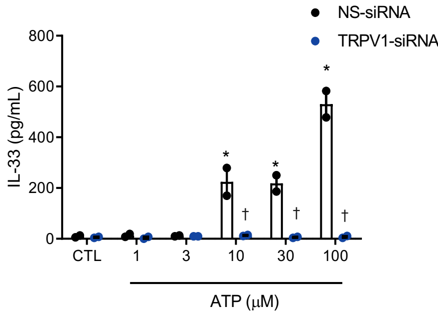

Figure S4. IL-33 release (pg/mL) in response to ATP at 1, 3, 10, 30 and 100 µM in NS- or TRPV1-siRNA transfected (mean±SE, n=2) HBE1 cells. *p < .05 versus NS-siRNA. †p < 0.05 versus corresponding NS-siRNA treatment.

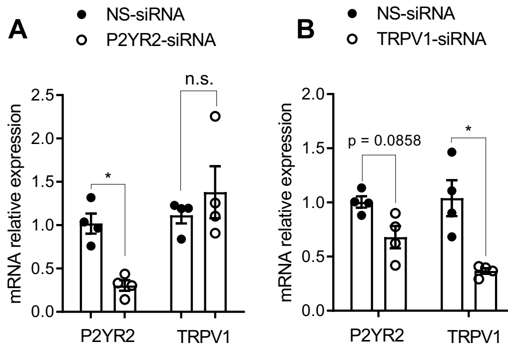

Figure S5. Effects of siRNA silencing of TRPV1 or P2YR2 in HBE1 cells. mRNA expression of *P2YR2* and *TRPV1* in NS-siRNA versus (A) TRPV1-siRNA transfected (mean±SE, n=4) or (B) P2YR2-siRNA transfected (mean±SE, n=4) HBE1 cells. *p < .05 versus NS-siRNA.

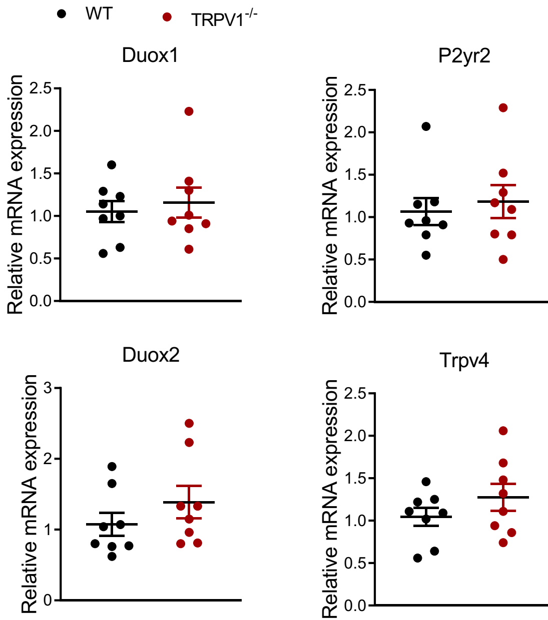

Figure S6. Effect of TRPV1-deficiency expression of *Duox1*, *Duox2*, *P2yr2* and *Trpv4*. Lung tissues from untreated WT or *Trpv1*$^{-/-}$ mice were evaluated for relative mRNA gene expression (mean±SE, n=8).

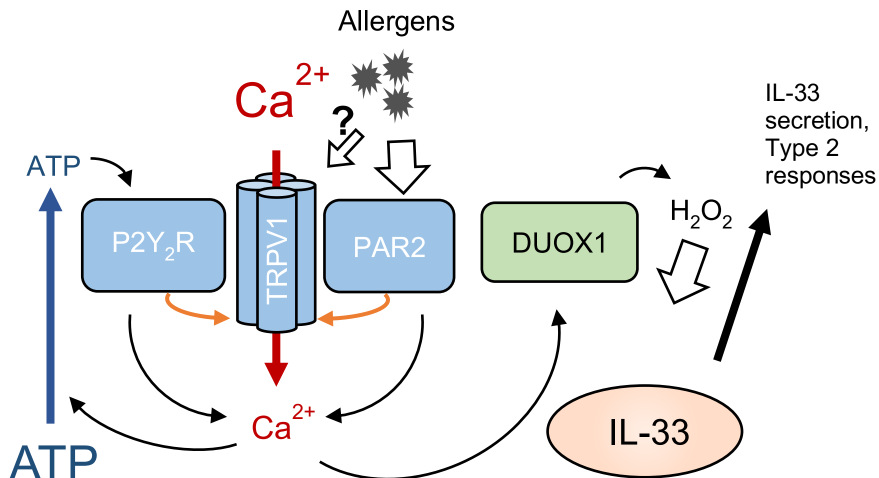

Figure S7. Schematic representation of proposed central role for TRPV1 in epithelial IL-33 secretion by protease allergens. Exposure to protease allergens can activate PAR2, resulting in Ca^{2+} increase and extracellular release of ATP, which subsequently activates P2YR2 signaling. Potential direct TRPV1 activation by allergen components (indicated by ?) or by allergen-induced production of endovanilloids also contributes to Ca^{2+} increase and ATP release. In turn, activation of PAR2 and P2YR2 can sensitize TRPV1 channel activation (illustrated by orange arrows). Collective Ca^{2+} signaling and ATP release and P2YR2 activation by these various mechanisms is necessary for DUOX1 activation and secretion of IL-33 and related cytokines.

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Chapter 3

Downregulation of epithelial DUOX1 in chronic obstructive pulmonary disease

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Abstract

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 found to be 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 (9-11), evidence of mitochondrial dysfunction, and aging-related impairment in antioxidant defense mechanisms (12), and thought to contribute to injury to critical cell constituents, lung cell dysfunction and lung function decline (13,14). 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 (14,15). Supplementation with other small molecular antioxidants has not shown any benefit and may even have adverse effects (16). 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 (17). The NOX family of NADPH oxidases produce ROS (O_2- or H_2O_2) as their primary function to mediate critical physiological functions including host defense, cell proliferation or differentiation (18). 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 (19-21). 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 (22-24). Recently, a crucial role was reported for the NOX organizer protein NOXO1, which regulates the function of several isoforms, in CS-induced emphysema (24). 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 (24-26). 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 (27-29). 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) (30), which may also be relevant for small airway remodeling in COPD (31).

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.

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. For detailed description on tissue collection, exclusion criteria, and other details, see Eurlings et al. (2014) (32). 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 approved the use of materials for this study under MPTC 2009–22.

SPC-TNFα model

SPC-TNFα mice (*n* = 10), which are transgenic mice that exhibit chronic pulmonary inflammation resulting from overexpression of TNF-α in alveolar epithelial type II cells (TNF- expression under the control of the promoter of surfactant protein C (SP-C; expressed by alveolar epithelium type II), were sacrificed at 6-months of age, and lungs were harvested and paraffin embedded as previously described (36). Various readouts were compared between SPC-TNFα mice and age-matched transgene negative littermates (WT, *n* = 10).

Acrolein model

Wildtype C57BL/6J mice aged 8-12 weeks, as well as *Duox1*$^{-/-}$ mice, originally generated on C5757BL/6J background and provided by Miklos Geiszt (68), were subjected to chronic acrolein exposure, as described previously (69). Briefly, mice were placed in a 2 L glass chamber and exposed to either 5 ppm (11.5 mg/m^3) 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 C57BL/6NJ mice and corresponding *Duox1*$^{-/-}$ mice (backcrossed to C57BL/6NJ 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 sacrificed,

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 (acrolein and PPE model) were collected upon completion of the indicated experiments, and left lung lobes were fixed in PFA and paraffin embedded for IHC. For MLI purposes, the lungs in the PPE model were fixed by tracheal instillation of 4% PFA at a pressure of 25 cm water for 20 minutes. Exclusively 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 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 GOLDIV COPD patients (4 μm thickness) were evaluated for the presence of the DUOX1 protein using a DUOX1 antibody (SantaCruz 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 2mm diameter) were scored for staining of DUOX1 (Small airway IHC score) based on a scoring scale of 1-4 in which 4 was considered the highest staining intensity, and a score of 1 the lowest staining intensity (minimal staining observed). Two independent researchers, blinded to the tissue identity, quantified the DUOX1 scoring. Lung tissue sections of the SPC-TNFα mice were similarly evaluated 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 (55).

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 (70), using the formula MLI = 2 * k * d * 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 (48). 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 (48).

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 obtained from 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 (71). 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 (72). In addition, MTECs were isolated from excised mouse tracheas from either WT mice or *Duox1*$^{-/-}$ mice (C57BL/6J)and cultured as previously described (73) 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 (**Table S2**) in ddH_2O (100 nM final) and 3.5 µL ddH_2O. 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 (74), 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. Correlations were analyzed by Pearson correlations. 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.

Results

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

Following up on previous studies demonstrating that epithelial DUOX1 mRNA expression is attenuated in active smokers and in patients with COPD (25,26), we evaluated tissue sections from a previous study cohort of COPD patients and control subjects at Maastricht University (UM) (32) 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 (**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**). 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). The GOLD IV COPD patients included in this study underwent lung volume reduction surgery (LVRS) because of severe emphysema (33), and DUOX1 downregulation may therefore 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; indicates loss of alveolar surface area and capillary bed, impairing diffusion (34)), 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**). Alterations in lung tissue content of elastin and collagen from these patients were previously published (32), 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 (32,35-37). Consistent with our findings in COPD patients, DUOX1 was downregulated in 6-months 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 2D,E** and **J,K**) as well as increased collagen (**Figure 2G,H** and **2M,N**). Furthermore, parenchymal elastin staining, but not small airway elastin levels, positively correlated with the DUOX1 score (**Figure 2F** and **L**). No significant correlation was observed between parenchymal and small airway collagen levels and DUOX1 staining score (**Figure 2I** and **O**). 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) (38), which was previously shown to induce pulmonary injury and inflammation, mucus hypersecretion, as well as airflow limitation (39,40). Consistent with observations of reduced DUOX1 mRNA expression in the airways of smokers (25), chronic exposure of C57BL/6J mice to acrolein (5 ppm; 4 hrs/day, for 2 weeks) resulted in reduced lung *Duox1* mRNA expression (**Figure S1A**). Acrolein exposure also enhanced production of TGF-β1 (Figure S1B), a profibrotic growth factor that is thought to contribute to features of EMT and subepithelial fibrosis in COPD (41-43). 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 (**Figure S1C**), and acrolein exposure resulted in a dose-dependent loss of E-

cadherin, with a concomitant gain in vimentin (**Figure S1D**). Moreover, acrolein-induced alterations in E-cadherin and vimentin were enhanced in MTECs from *Duox1*$^{-/-}$ mice (**Figure S1D**), 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) (**Figure S1E**).

Chronic exposure of acrolein to mice also resulted in features indicative of enhanced peribronchiolar fibrosis (44), as illustrated by subepithelial collagen deposition (**Figure 3A** and **B**) and enhanced α-smooth muscle actin staining (**Figure 3C** 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. 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 (**Figure S2**). Collectively, these various findings suggest that lung DUOX1 expression is reduced in several mouse models of COPD, and our findings of acrolein-induced EMT and peribronchiolar fibrosis indicate that loss of DUOX1 may sensitize airways 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 (45,46), PPE exposure induced development of alveolar emphysema, as measured by increased alveolar airspace enlargement (**Figure 4A** and **B**). Importantly, elastase-induced airspace enlargement in this model was significantly worsened in *Duox1*$^{-/-}$ mice (**Figure 4A** 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 4C** and **D**) and small airways (**Figure 4E** and **F**) 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 4D**), but this was not statistically significant, and elastin degradation within the small airways was similar in WT and *Duox1*$^{-/-}$ mice (**Figure 4F**).

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 (**Figure S4A**). No significant increases were observed in parenchymal collagen levels in response to PPE (**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 (**Figure S3**). Since neutrophil infiltration plays an important role in emphysema development (47), we examined neutrophil activation by measuring intracellular and extracellular activity of the neutrophil granule protein myeloperoxidase (MPO) (48). 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 4G** and **H**), 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 (49), we hypothesized that Duox1 deficiency may lead to impaired Areg production in this model. Indeed, lung tissue Areg protein levels were similarly elevated in response to PPE in both WT and *Duox1*$^{-/-}$ mice (**Figure S5**), even though lung tissue *Areg* mRNA tended to be suppressed in *Duox1*$^{-/-}$ mice treated with PPE compared to PBS controls (**Figure S5**; 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 (19,20,22-24). 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 (25,26), 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. Furthermore, based on various mouse models that reflect different pathological hallmarks of COPD, ie. small airway subepithelial fibrosis and alveolar airspace enlargement, we provide evidence that Duox1 deficiency can worsen some of these outcomes. These observations would therefore suggest that the gradual loss of DUOX1 in human COPD, potentially as a result of smoking (25), may be a contributing factor in COPD development and its progression.

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 (27). 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 (50), 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 (30), which is also relevant for small airway remodeling in COPD (31). Indeed, chronic exposure to CS, which may be the primary cause of COPD, is well-known to promote EMT features (31,51) and subsequent extracellular matrix remodeling and related thickening of the small airways (42,44), and potentially also resulting in impaired alveolar re-epithelialization (52). 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 address the potential mechanisms by which *Duox1*-deficiency may promote acrolein-induced small airway remodeling and elastase-mediated airspace enlargement, we evaluated 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 epithelial wound responses (53), has also been implicated in COPD as part of the protease/antiprotease imbalance (54), and was also found to be upregulated in our chronic acrolein model, although 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 (49) through a pathway that may involve DUOX1 (55), 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 (**Figure S5**). 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 (56), 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 (**Figure S3**), which is surprising as it contrasts our previous observation that DUOX1 contributes to *Muc5ac* expression and mucus metaplasia in the context of allergic airway inflammation (55). These latter studies also indicated a critical role for DUOX1 in production of IL-13, whereas IL-13 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 (57).

Neutrophilic inflammation has been implicated in COPD pathology (58,59) and elastase-induced emphysema (45,46), and previous studies of allergic airways disease have linked DUOX1 to production of neutrophil chemokines (KC) and neutrophil recruitment (55,60). We did not observe significant changes in overall neutrophil context during PPE-induced emphysema, based on intracellular MPO analysis, and increases in *Cxcl1* mRNA levels were similar in both WT and *Duox1*$^{-/-}$ mice. 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 KC production and neutrophil recruitment in response to e.g. LPS (61). 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 (62) or inactivation of tissue inhibitors of MMPs (TIMPs) (63).

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 (64-66). Alternatively, it is possible that epigenetic mechanisms, as seen in e.g. lung cancer, may also contribute to DUOX1 silencing in COPD (67). 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.

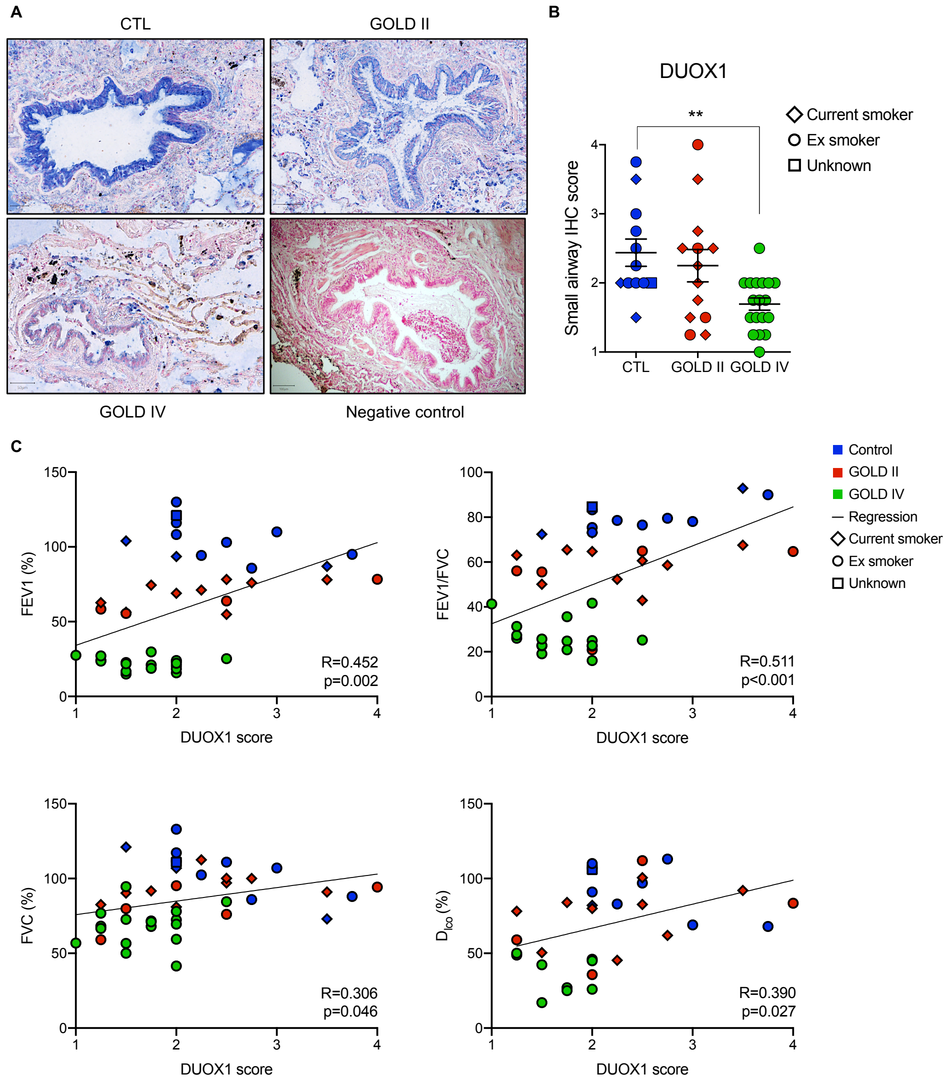

Figure 1: DUOX1 loss in small airways of COPD patients is associated with loss of lung function, emphysema, and airway remodelling. A) IHC for DUOX1 (blue) with nuclear fast red counterstaining (red) in lung tissue of a representative control, GOLD II and GOLD IV COPD patient. B) Small airway score of DUOX1 in control, GOLDII and GOLDIV COPD patients. Data shown as mean ± SE. C) Correlations (Pearson correlation, 2 tailed significance) between DUOX1 score and percentage (%) FEV1, FVC and DLCO, as well as the FEV1/FVC ratio in controls (blue), GOLD II (red), and GOLDIV (green) COPD patients. ** $p < 0.01$, by one-way ANOVA.

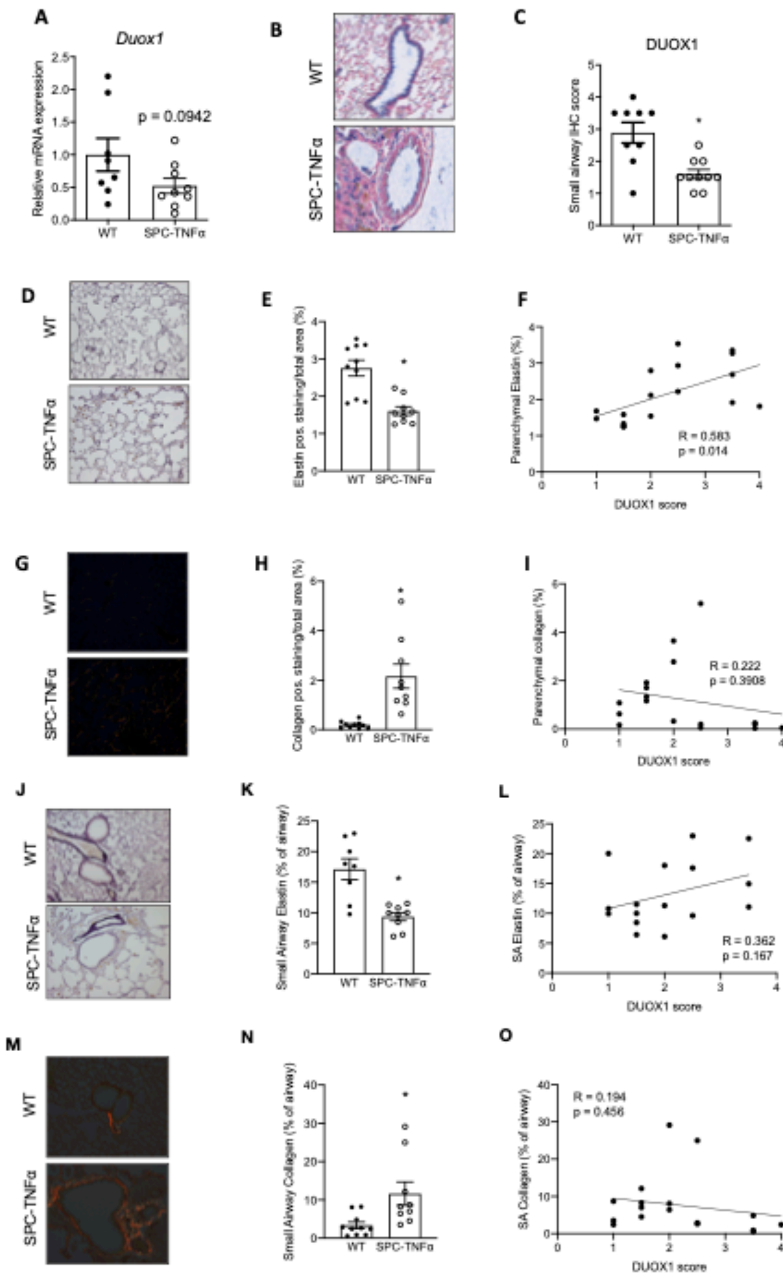

Figure 2: SPC-TNF-α mice display *Duox1* loss, which is associated with elastin remodelling. A) The relative mRNA expression (n=8-9 per group) of *Duox1* in 6-months old SPC-TNFα mice versus WT littermate controls controls. B) Representative alkaline phosphatase staining of DUOX1 in blue with corresponding small airway IHC score of airway epithelial DUOX1 in

6-months old SPC-TNFα mice compared to wildtype littermate controls (C). (D-O) Levels of parenchymal (% surface area) and small airway (% airway surface area) elastin and collagen in both 6-months old WT littermate controls and SPC-TNFα mice, subsequently quantified (n=8-10), and correlated to the small airway DUOX1 score. Data shown as mean ± SE. * $p < 0.05$, by 2-way ANOVA or 2-tailed unpaired *t*-test.

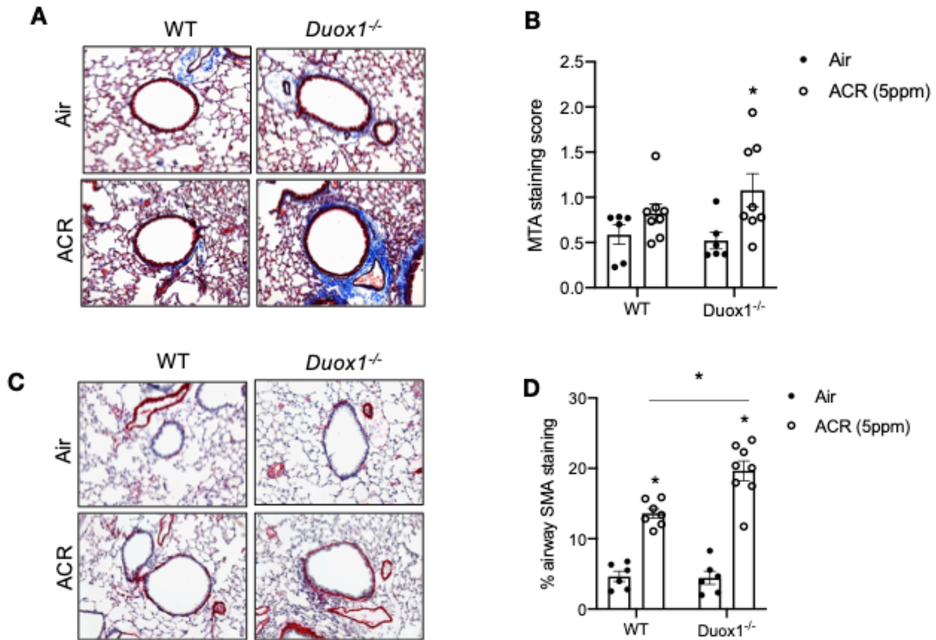

Figure 3: *Duox1* expression is reduced in a mouse model of chronic acrolein exposure, and *Duox1* deficiency sensitizes airways to the development of peribronchiolar fibrosis. C57BL/6J mice were exposed to acrolein (ACR) and analyzed for collagen by Masson's trichrome (A) or α-smooth muscle actin (C), including quantification of stainings (B and D, n=6-8). Data shown as mean ± SE. * $p < 0.05$, by 2-way ANOVA.

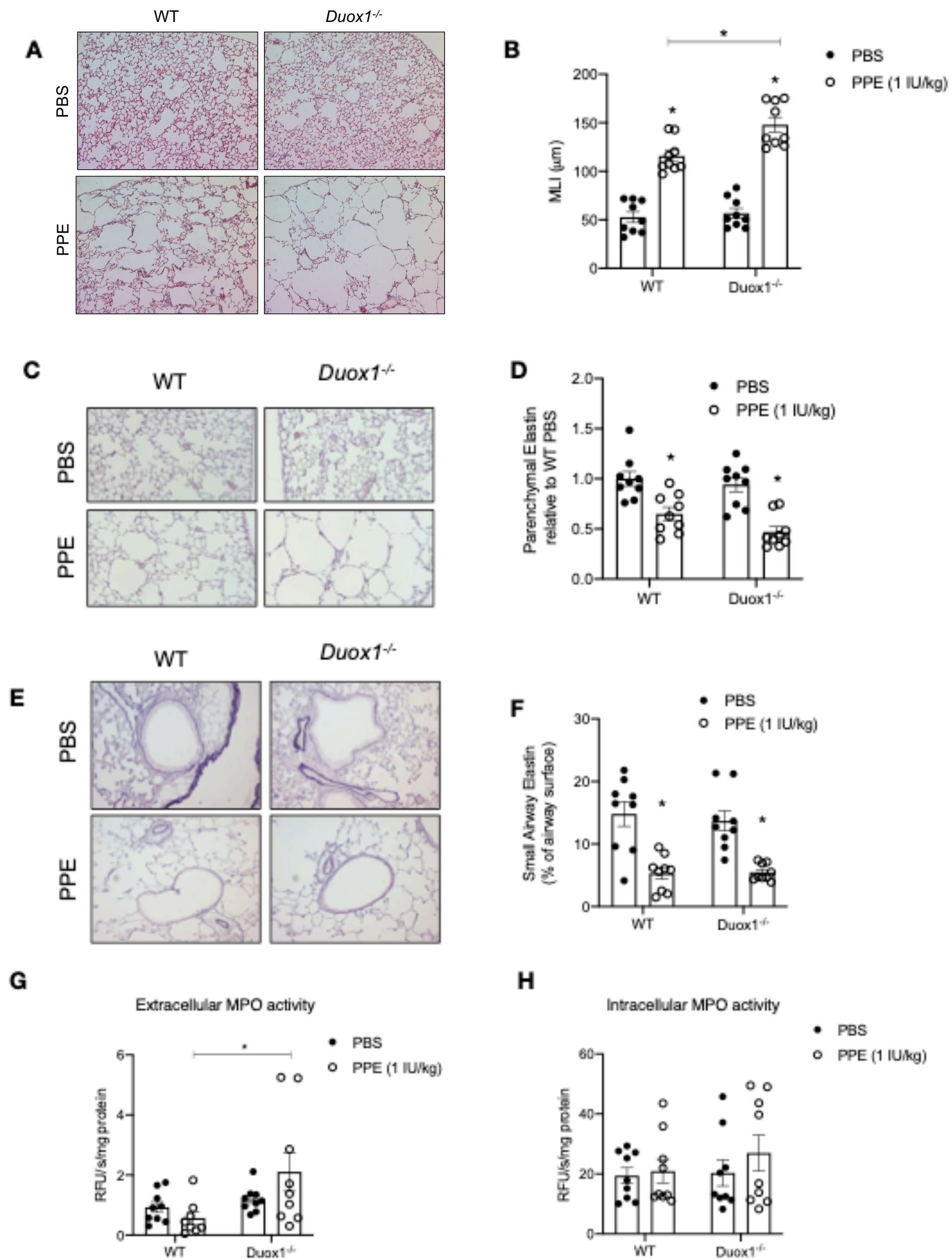

Figure 4: *Duox1* deficiency enhances development of elastase-induced emphysema through augmented neutrophil activation**.** A) Representative H&E stained images of alveolar enlargement in both WT and *Duox1*$^{-/-}$ mice. B) Alveolar mean linear intercept (MLI, µm) as a

measure of emphysema development in WT and *Duox1*$^{-/-}$ mice exposed to 50 μL porcine pancreatic elastase (PPE, or PBS control (n=9 per group). Parenchymal (C and D) and small airway (E and F) elastin levels in both WT and *Duox1*$^{-/-}$ mice in response to PPE or PBS control with Weigert's Resorcin Fuchsin staining. Extracelullar (G) and intracellular (H) myeloperoxidase activity in lung tissue homogenates of WT and *Duox1*$^{-/-}$ mice in response to PPE or PBS control. Data shown as mean ± SE. * 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

Correlations DUOX1	**All subjects (n=49)**		**COPD patients (n=35)**	
	R	p	R	p
FEV_1 (%)	**0.452**	**0.002** $	**0.524**	**0.002**
FVC (%)	**0.306**	**0.046** $	**0.492**	**0.005**
FEV_1/FVC	**0.511**	**0.000** $	**0.408**	**0.023**
DLCO (%)	**0.390**	**0.027** $	**0.501**	**0.018**
Elastin SA	**0.540**	**0.001**	**0.438**	**0.032**
Collagen SA	-0.256	0.127	-0.025	0.905
Elastin to Collagen SA	**0.428**	**0.012**	0.278	0.210

$: Data is graphically presented in Figure 1C. SA: Small

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Supplemental Information for:

Downregulation of Epithelial DUOX1 in Chronic Obstructive Pulmonary Disease Contributes to Disease Pathogenesis

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Supplemental Table 1:

Clinical characteristics of study subjects

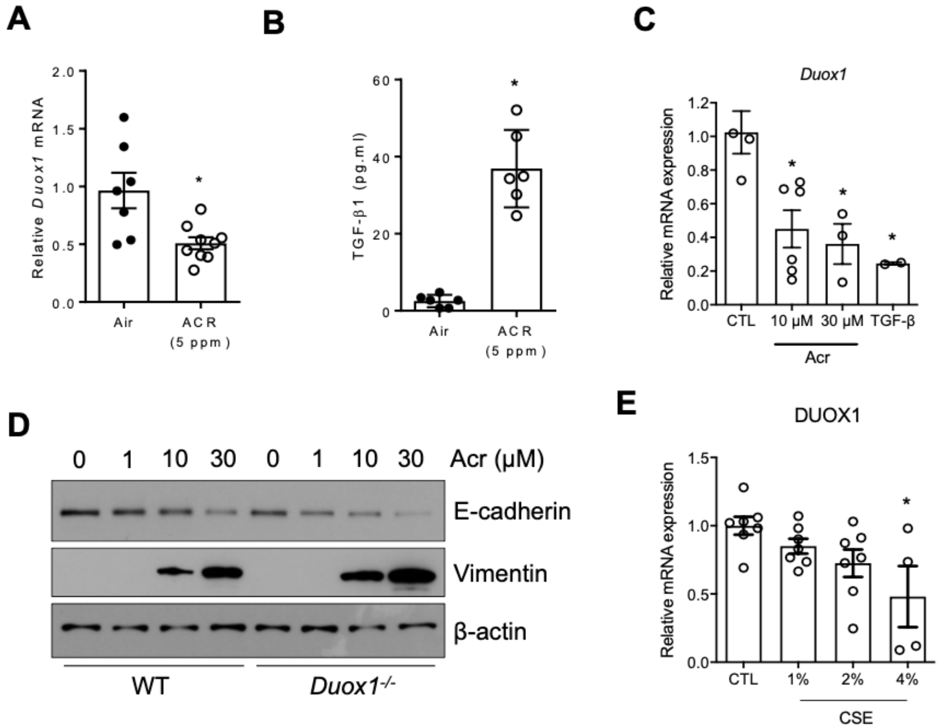
	Control	GOLD II	GOLD IV
Number	14	16	19
Age (yrs)	63 ± 6	65 ± 7	61 ± 7
Sex (M/F)	9/5	13 / 3 * $	12/7
Pack-years [a]	25 ± 6	46 ± 14 *	47 ± 29 *
Smoking status (current/ex)	4/9	11/5	0/19 * #
FEV1 (% predicted)	107 ± 15	67 ± 9 *	22 ± 4 * #
FEV1/FVC	80 ± 6	59 ± 9 *	26 ± 7 * #
Dlco (% predicted) [b]	95 ± 16	77 ± 18 *	35 ± 15 * #

*Data displayed as mean ± SD or ratio. * $p < 0.05$ compared to controls. # significant compared to GOLD II. $ significantly different compared to GOLD IV. [a]Values from 3 controls, 4 GOLD II and 2 GOLD IV patients missing. [b]Values from 2 controls, 1 GOLD II and 9 GOLD IV patients are missing. FEV1 and FEV1/FVC are measured post-bronchodilation*

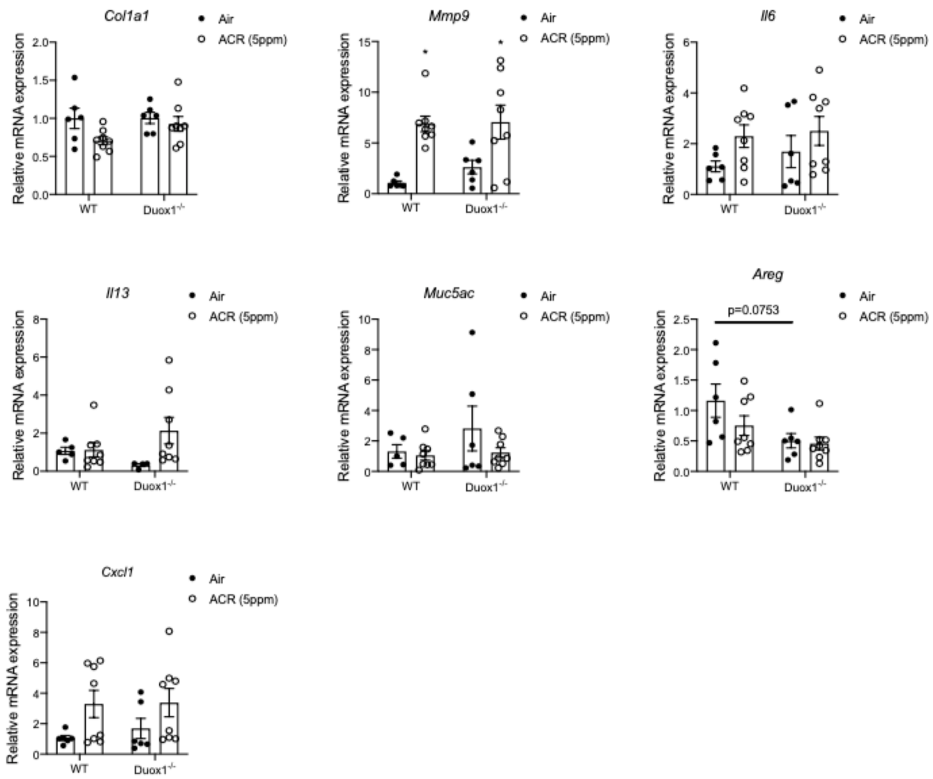
Supplemental Table 2: qPCR primers used in studies

Target gene	Forward/ Reverse primer	Primer sequence
DUOX1 (human)	F	TTCACGCAGCTCTGTGTCAA
	R	AGGGACAGATCATATCCTGGCT
RPL13A (human)	F	CCTGGAGGAGAAGAGGAAAGAGA
	R	TTGAGGACCTCTGTGTATTTGTCAA
Duox1	F	ACCAGAACATTGCGATGTATGAG
	R	AGAAATGGACGGTATCCTGGA
Gapdh	F	CTGGAGAAACCTGCCAAGTA

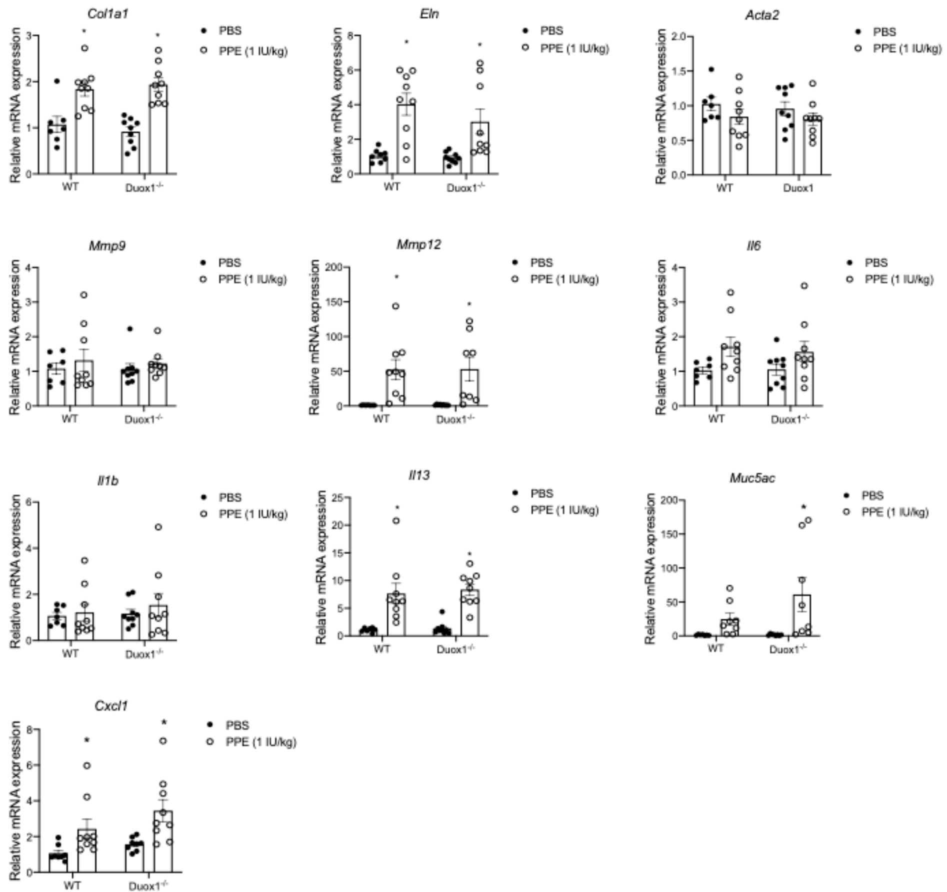
	R	TGTTGCTGTAGCCGTATTCA
Col1a1	F	CACCCTCAAGAGCCTGAGTC
	R	AGACGGCTGAGTAGGGAACA
Cxcl1	F	GTG AAT CAA GAC ATA GTT AAC C
	R	GTG AAT CAA GAC ATA GTT AAC C
Il6	F	AACGATGATGCACTTGCAGA
	R	GGAAATTGGGGTAGGAAGG
Areg	F	AAC GGT GTG GAG AAA AAT CC
	R	TTG TCC TCA GCT AGG CAA TG
Eln	F	TCCTGGAGCCACTCTTACAG
	R	CTCTCTCTCCCCAATTAGCC
Acta2	F	CGCTGTCAGGAACCCTGAGA
	R	CGAAGCCGGCCTTACAGA
Mmp9	F	CTCACTCACTGTGGTTGCTG
	R	TGGTTATCCTTCCTGGATCA
Mmp12	F	TTTCTTCCATATGGCCAAGC
	R	GGTCAAAGACAGCTGCATCA
Il1b	F	GCCCATCCTCTGTGACTCAT
	R	AGGCCACAGGTATTTTGTCG
Il13	F	CTACAGCTCCCTGGTTCTCT
	R	TTGCTCAGCTCCTCAATAAG
Tgfb	F	TGC TTT AGC TCC ACA GAG AA
	R	TGG TTG TAG AGG GCA AGG AC
Muc5ac	F	AGTCTCTCTCCGCTCCTCTCAAT
	R	CAGCCGAGAGGAGGGTTTGATCT



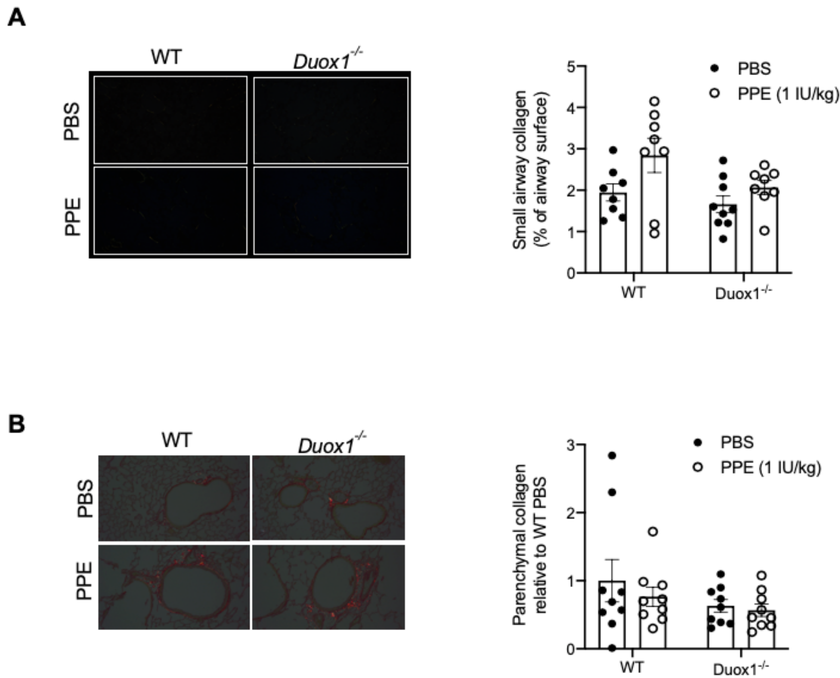
Supplemental Figure 1: *Duox1* expression is reduced in a mouse model of chronic acrolein exposure, in isolated mouse tracheal epithelial cells (mTEC) exposed to acrolein or TGF-β, and in primary bronchial epithelial cells (PBEC) exposed to cigarette smoke. A) lung tissue *Duox1* mRNA and B) BAL fluid TGF-β1 levels (n=6-9) in mice exposed to acrolein (5 ppm; 4 hrs/day, for 2 weeks) or air. C) Cultured mTECs from WT (and *Duox1*$^{-/-}$ mice in (D)) were exposed daily to acrolein (1 - 30 μM) or TGF-β (10 ng/ml) for 14 days and analyzed for *Duox1* mRNA or D) E-cadherin and vimentin by Western blot. E) DUOX1 mRNA expression in isolated PBECs from non-COPD patients 24 hours after treatment with 1,2 or 4% cigarette smoke extract (CSE); Values from 2-3 different donors. Data shown as mean ± SE. n=6-10; *: $p < 0.05$, by 1-way ANOVA, or 2-tailed unpaired *t*-test.



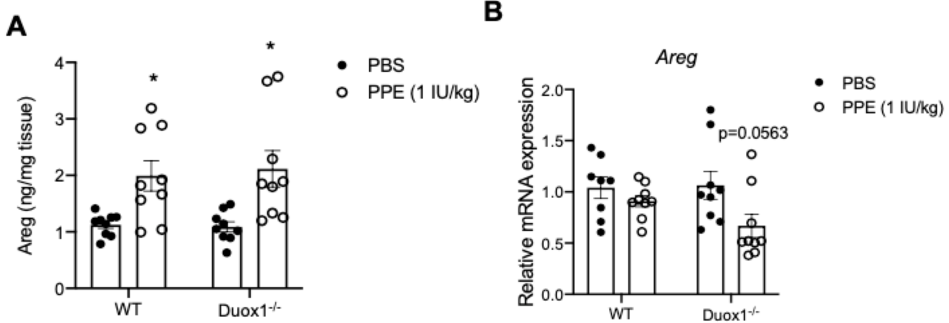
Supplemental Figure 2: mRNA analysis of various markers of matrix remodeling, tissue regeneration, and tissue inflammation in WT and *Duox1*$^{-/-}$ mice exposed to acrolein (5 ppm; 4 hrs/day, for 2 weeks) or air. Data shown as mean ± SE. n= 4-8. * $p < 0.05$, by 2-way ANOVA.



Supplemental Figure 3: mRNA analysis of various markers of matrix remodeling, tissue regeneration, and tissue inflammation in WT and *Duox1*$^{-/-}$ mice exposed to 50 μL porcine pancreatic elastase (PPE, 1 IU/kg bodyweight, 1 day/week, for 3 weeks) or PBS control. Data shown as mean ± SE. n=8-9; * $p < 0.05$, by 2-way ANOVA.



Supplemental Figure 4: Small airway and parenchymal collagen levels are unaltered in WT and *Duox1$^{-/-}$* mice exposed to porcine pancreatic elastase (PPE). Small airway (A) and parenchymal (B) collagen levels were determined in both WT and *Duox1$^{-/-}$* mice in response to porcine pancreatic elastase or PBS control with Picrosirius Red staining and quantification. Data shown as mean ± SE. n= 8-9.



Supplemental Figure 5: *Areg* mRNA is suppressed in *Duox1$^{-/-}$* deficient mice upon elastase exposure, but whole lung tissue levels are similarly increased in WT and *Duox1$^{-/-}$* mice. A) lung tissue Areg protein (ng/mg) and B) mRNA expression in WT and *Duox1$^{-/-}$* mice exposed to 50 μL porcine pancreatic elastase (PPE, 1 IU/kg bodyweight, 1 day/week, for 3 weeks) or PBS control. Data shown as mean ± SE. n=8-9; * $p < 0.05$, by 2-way ANOVA.

Chapter 4

Downregulation of DUOX1 function contributes to aging-related impairment of innate airway injury responses and accelerated senile emphysema

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Abstract

Aging is associated with a gradual loss of organ function due to increased cellular senescence, decreased regenerative capacity and impaired innate host defense. In the respiratory tract, these age-related phenomena contribute to gradual loss of lung function and increased susceptibility to chronic age-related respiratory diseases. It is widely appreciated that aging is characterized by impaired redox balance, which may largely be linked to mitochondrial dysfunction. However, it is less clear how aging impacts on the expression and function of NADPH oxidases (NOX) as critical mediators of redox-based signaling. Here, we demonstrate that normal aging of C57BL/6J mice resulted in markedly decreased lung epithelial expression of the NOX isozyme DUOX1, which was associated with impaired DUOX1-mediated acute airway injury responses mediated by redox-dependent activation of the epidermal growth factor receptor (EGFR) and the non-receptor tyrosine kinase Src. Moreover, we also show that DUOX1-deficiency accelerated age-related airspace enlargement and decline in lung function in C57BL/6J mice, although it did not significantly affect other features of lung aging. Intriguingly, observations of age-related DUOX1 downregulation and enhanced airspace enlargement due to DUOX1 deficiency in C57BL/6J mice, which lack a functional mitochondrial nicotinamide nucleotide transhydrogenase (NNT), were less dramatic in C57BL/6NJ mice with normal NNT function. Overall, our findings highlight the potential importance of DUOX1 downregulation for age-related impairment of lung function and innate airway injury responses, although this is strain-dependent and may involve interactions with other molecular features of aging such as metabolic alterations due to NNT deficiency.

Introduction

Aging affects most organisms and is characterized by irreversible, gradual, and time-dependent functional deterioration of all vital organs after the reproductive phase of life (1-3). With respect to the lung, its development continues until adolescence as indicated by continued increases in lung volume, number of alveoli, and expansion of the capillary networks (4), but lung function gradually declines thereafter due to both structural and functional changes, including impaired lung regeneration/repair responses, pulmonary remodelling, increased lung compliance (ability to stretch/expand) and a reduced lung elastic recoil (rebound following stretching) (5, 6). Underlying these structural and functional changes are well-known molecular hallmarks of aging, such as genomic instability, mitochondrial dysfunction, epigenetic alterations, cellular senescence, and altered intercellular communication (7). Lung aging also involves extracellular matrix dysregulation (8). Collectively, these hallmarks contribute to lung structural changes, airspace enlargement, age-related loss of lung function, and senile emphysema development (5, 9).
Oxidative stress has long been recognized to play an important role in (lung) aging and age-related chronic lung diseases, and is characterized by increased levels of reactive oxygen species (ROS), e.g. through smoking or various endogenous sources (e.g. inflammation), as well as impaired antioxidant defense mechanisms (10-13). Indeed, the free radical theory of aging, first proposed in the 1950s, describes ROS as detrimental species that contribute to accumulating irreversible oxidation of essential biological molecules, such as DNA, lipids and proteins, thereby resulting in irreversible chronic tissue injury and progressive functional decline (14). However, decades of research observations have led to evidence supporting and opposing this theory (15, 16). As such, ROS may not inherently be detrimental. Since the introduction of the FRTA, it has become clear that mitochondria are considered a major cellular source of ROS production since these species are generated as a byproduct during oxidative phosphorylation (17, 18). This has led to a revised mitochondrial theory of aging (19), which proposes that the age-related aggregation of mitochondrial oxidative damage is thought to contribute to (mitochondrial) DNA (mtDNA) mutations, resulting in a vicious cycle with further accumulation of damage (11). However, this theory remains controversial (20, 21), and it has in fact been observed that relatively low levels of mitochondria-derived ROS may improve systemic defense mechanisms by inducing adaptive responses and improving

lifespan (22-24), referred to as mitochondrial hormesis (mitohormesis). Further complicating this issue is the recent discovery of the enzymatic production of ROS by different NADPH oxidase (NOX) enzymes (25), which increased the recognition of hydrogen peroxide (H_2O_2) and other ROS molecules as important mediators in cytokine and growth factor signaling through redox signaling mechanisms (26).

Although various NOX enzymes are expressed in different lung cell types, relatively little is known with respect to age-related alteration of NOX function or potential contribution of NOX to lung aging. Analysis of RNAseq data indicates that lung NOX4 levels positively correlate with age (27), which may be relevant for proposed roles of NOX4 in age-related pulmonary fibrosis (28). Although NOX4 deficiency in mice was not found to result in increased longevity (29), deficiency of the NADPH oxidase organizer 1 (NOXO1), a component of functional NOX1, resulted in increased lifespan in mice, possibly related to enhanced DNA repair capacity (30). Conversely, studies in *C. elegans* suggest that its major NOX homolog Duox/BLI3 may actually prolong longevity, especially in the context of environmental stress (31, 32). In the mammalian lung, DUOX1 is primarily expressed in the bronchial and alveolar epithelium with a proposed function in innate mucosal host defense and epithelial injury responses to help maintain epithelial integrity (33-36). However, it is unclear whether DUOX1 function is altered during aging or impacts on age-related lung dysfunction.

In the present studies, we explored potential alterations in lung NOX expression during aging, which revealed a striking age-related downregulation of DUOX1 expression. We therefore explored the potential consequences of DUOX1 suppression for aging-related impairment of lung function and observed that DUOX1-dependent innate airway epithelial injury responses were dramatically suppressed with age and that DUOX1-deficiency enhances some molecular and functional features of lung aging. However, we also noted marked differences between different mouse strains with respect to age-related DUOX1 alterations and the impact of DUOX1 deficiency for age-related lung function changes.

Methods

GTEx RNAseq data

RNAseq data obtained from dbGaP (Accession phs000424.v7.p2, 06/2018) as part of the Genotype-Tissue Expression (GTEx) Project. GTEx is supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS. This dataset includes information on 56,202 transcripts from 11,688 samples derived from 53 tissue types and 714 human donors. Our analyses utilize data from 342 human lung samples. Quantified RNA values are reported as transcripts per million reads (TPM). For sample exclusion criteria, 85 lung samples were removed from the GTEx dataset prior to analysis based on accompanying data indicating the donor suffered from a chronic interstitial lung disease. For statistical analysis, Spearman correlation analysis was performed for each transcript as a function of donor age. Statistical analyses were completed using base R (v3.5.1) within RStudio (v1.1.453).

Cell models

MTE cells (MTECs) were isolated from tracheas of either young (2 months) or old (13-16 months) wild-type (WT) C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) using overnight incubation with 0.1% protease 14 (Sigma-Aldrich, St. Louis, MO). Thereafter, MTECs were cultured on a rat tail collagen I gel (BD Biosciences, San Jose, CA) in DMEM/F12 media (Invitrogen, Grand Island, NY) supplemented with 20 ng/mL cholera toxin (List Biological Laboratories, Campbell, CA), 10 ng/mL EGF (Calbiochem, San Diego, CA), 5 mg/mL insulin (Sigma), 5 mg/mL transferrin (Sigma), 100 nM dexamethasone (Sigma), 15 mg/mL bovine pituitary extract (Invitrogen), 2 mM L-glutamine, and 50 U/50 mg/mL Penicillin/Streptomycin (Pen/Strep) (Invitrogen), as described previously (81, 82). MTECs were used at passages 2 and 3. For experimentation, MTECs were seeded at $2x10^5$ cells/cm^2 in 24-well plates (BD Labware, Bedford, MA) and cultured for an additional 3–4 days. Prior to cell stimulation, cells were starved overnight in EGF-lacking DMEM/F12 medium and stimulated with extracts of *Dermatophagoides pteronyssinus* (50 μg/mL; house dust mite (HDM); Greer Laboratories; 1.27 endotoxin units/mg), ATP (100 μM Sigma, St Louis, Mo) or LPS (1 μg/mL from *Escherichia coli* 055:B5; Sigma; 1 mg) and conditioned media were collected at indicated times for cytokine analysis and other biochemical assays described below. Cell extracts were prepared for mRNA extraction and RT-PCR analysis, biochemical

assays, or protein analysis by means of SDS-PAGE and Western blotting. In appropriate experiments, MTECs were pre-incubated with the DNA methyltransferase inhibitor (DNMTi) 5-Aza-2'-deoxycitydine (aza, 1 μM; Sigma) daily, for a total of 72 hours.

Mouse studies

Young (2-3 months) and aged (12-16 months) C57BL/6J wildtype (WT) mice age-matched *Duox1*-deficient (*Duox1*$^{-/-}$) mice (originally generated using a retroviral-based gene-trapping method (Lexicon Pharmaceuticals) and backcrossed onto a C57BL/6J or C57BL/6NJ background (83)) were euthanized and lung tissues were analysed for various readouts, including *Duox1* mRNA and protein expression, airspace enlargement, and various indices of lung aging. C57BL/6J and C57BL/6NJ mice (Jackson Laboratories, Bar Harbor, ME), aged 2-3 months, 12-16 months (C57BL/6J) or 19-21 months (C57BL/6NJ), were subjected to brief isoflurane anaesthesia for oropharyngeal administration of extracts of HDM (1 μg/μL in PBS; 50 μL per mouse; Greer Laboratories, Lot 234400) or PBS control. Broncho alveolar lavage (BAL) and lung tissues were collected at 1 or 6 hrs after challenge for various analyses. All animal procedures were reviewed and approved by the Animal Care and Use Committee of the University of Vermont.

Measurement of pulmonary function

Young (2-3 months) and old (12-17 months) C57BL/6J or C57BL/6NJ WT mice, as well as age-matched C57BL/6NJ *Duox1*$^{-/-}$ mice on both backgrounds, were used in the pulmonary function tests. Mice were anesthetized with sodium pentobarbital (90 mg/kg) and tracheas were cannulated and connected to a computer-controlled small animal ventilator (FlexiVent; SCIREQ). Mice were paralyzed with pancuronium bromide (0.8 µg/kg i.p.), and depth of anaesthesia was monitored continuously with EKG. Following stabilization and assessment of lung volume history, PEEP (Positive end-expiratory pressure) measurements were performed and were controlled by submerging the expiratory limb from the ventilator into a water trap. Starting at the functional residual capacity (FRC) defined by the PEEP, the FlexiVent was programmed to deliver seven inspiratory volume steps for a total volume of 1.2 ml followed by seven expiratory steps, pausing at each step for 1 s. Plateau pressure (P) at each step was recorded and related to the total volume (V) delivered to produce a quasi-static PV curve at PEEP3. The mice were then again ventilated at a PEEP of 3 cm H_2O and were given a lethal dose of sodium pentobarbital (150 mg/kg) and cardiac arrest was confirmed with EKG. To prevent absorption atelectasis the ventilation continued with 100%

N_2 and ventilation rate was decreased to allow for timely tying of the trachea at end exhalation. The lungs and heart were removed *en bloc* and the heart and mediastinal tissue removed. Next, lung volume was measured according to the Archimedes principle similar to what was described previously but without fixing the lungs (84).

Histology and immunohistochemistry stainings

Left lung lobes from both young (2-3 months) and aging (12-16 months) WT and *Duox1*$^{-/-}$ C57BL/6J or C57BL/6NJ mice were fixed in PFA and paraffin embedded, and 5-μm sections were stained with haematoxylin and eosin (H&E) for quantification of airspace enlargement. Additionally, lung sections were evaluated for the presence of DUOX1 protein using DUOX1 (SC48858, 1:200) 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. Additionally, Weigert's Resorcin Fuchsin (according to Electron Microscopy Sciences protocol) and Picrosirius Red (PSR) stainings (85), were used for the quantification of elastin and for collagen, respectively. PSR and Weigert's Resorcin Fuchsin stainings were quantified using MetaMorph imaging software (Molecular Devices). Senescence-associated ß-galactosidase staining (Cell Signalling) was used to stain MTECs according to the manufacturer's instructions. Briefly, cells were incubated with the staining solution for at least overnight at 37 °C, 0% CO_2, and after sufficient staining was observed, images were taken. All images were taken utilizing the Olympus BX50 microscope.

Quantification of airspace enlargement

Enlargement of alveolar spaces was determined by quantifying the mean linear intercept (*L*m, chord length) using Stereo-Investigator software. Only sections without cutting artifacts, compression, or hilar structures were used in the analysis. The chord length (or mean linear intercept) L_m was measured by first placing 40 μm spacing between lines over the tissue sections, followed by counting points (P) hitting airspaces, of which intersections (I) with the alveolar surface were marked on the alveolar walls to estimate surface. The L_m was then calculated according to previous established methods (86, 87), using the formula $L_m = 2 * k * d * P/I$, 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 alveolar airspaces and *I* is number of intersections marked between the probe-lines and the surface of the alveoli.

Measurement of ATP Release

ATP release into the BAL fluid was monitored using a luciferase/luciferin bioluminescence ATP determination kit (Molecular Probes, Eugene, OR) according to the manufacturer's instruction and utilizing the Lumat LB 9507 luminometer (Oak Ridge, TN). The amount of ATP released was calculated using external ATP standards (1–1000 nM).

Total RNA Isolation and Reverse Transcription (RT)-PCR

Tissue RNA was extracted using TRIzol (Invitrogen) and the RNeasy Mini Kit (Qiagen) and reverse transcribed using M-MLV reverse transcriptase and Oligo(dT)12–16 primer. Total cell RNA was extracted using the GeneJET RNA Purification Kit (Thermofisher) and reverse transcribed using the iScript™ cDNA synthesis kit (Bio-Rad). qPCR was performed using SYBR Green PCR Supermix (Bio-Rad) with predesigned primers (**Table S1**) and normalized to GAPDH using the ΔΔCT method. A qPCR procedure was used with pre-incubation for 10 minutes at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds and amplification at 72 °C for 30 seconds. Consequently, a post-PCR melt curve was performed by a 0.11°C increment increase every second from 60°C to 95°C.

ELISA

Cell culture supernatants, whole lung lysates or BAL fluids from mice were analysed for IL-33, IL-1α, Cxcl1/KC, Areg, IL-5, IL-13, TNFα, IL-1β, and IL-6, using DuoSet ELISAs from R&D Systems.

Western blotting

Cell lysates or lung homogenates were prepared using Western solubilization buffer (50 mM HEPES, 250 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 10% glycerol, 1 mM EGTA, 1 mM PMSF, 2 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin; pH 7.4), and samples containing equal amounts of protein (measured using the BCA protein assay kit; Pierce) were separated on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with litibodies against EGFR, pEGFR Tyr1068, pSrc Tyr416, Src (all from Cell Signalling), β-actin (Sigma), cytokeratin 5 (KRT5) and alpha-smooth muscle actin (α-SMA, both Abcam), Collagen 1a1 (Phospho Solutions), p63 and E-cadherin (both Cell Signalling), CCSP (Millipore). Primarily antibodies were probed with secondary antibodies conjugated with HRP (Cell Signalling) and detected by chemiluminescence using SuperSignal™ West Pico or Femto (Thermo Scientific).

Analysis of protein cysteine oxidation

Cells or lung tissues were homogenized in deoxygenated Western solubilization buffer containing 1 mM DCP-Bio1 (Kerafast), 200 U/ml catalase (Worthington), and 10 mM *N*-ethylmaleimide (NEM; Sigma-Aldrich); incubated for 1 hour on ice; and centrifuged (5 minutes, 15,000 *g*) to remove debris. Excess DCP-Bio1 and NEM were removed by 6 successive washes with 20 mM Tris HCl (pH 7.4) on Amicon Ultra-0.5 Centrifugal Filter Devices (Millipore), and biotin-tagged proteins were subsequently purified using Neutravidin agarose beads (by overnight incubation with 50 μl of 50:50 slurry; Pierce), followed by successive washes with 1% SDS, 4 M urea, 1 M NaCl, and 100 mM ammonium bicarbonate (with and without 10 mM DTT) to remove nonspecific bound proteins (14, 63). Avidin-bound proteins were eluted from the beads using 2× reducing sample buffer containing β-mercaptoethanol (5 min; 90°C) and separated by SDS-PAGE for Western blot analysis. Whole cell lysates or tissue homogenates were analysed similarly as input controls.

Haplotaxis assay

MTECs isolated from young (2 months) and aging (13 months) mice (2 x 10^5 cells/insert) were plated in Boyden-like chambers containing 10-mm polycarbonate membrane inserts with a 8 μm pore size (Nunc International), and were precoated with 10 μg/ml fibronectin (Invitrogen) or the negative control (BSA; 10 mg/mL) for 2 hours. Following incubation for 6 h at 37°C to allow cell adhesion, media was refreshed, and cells were treated with ATP (100 μM) and incubated for 24 h at 37°C. Subsequently, non-migrated cells were removed from the membranes with a cotton swab. The remaining migrated cells were stained with crystal violet and extracted in 200 μL of 10% acetic acid (pH 4.5) for analysis of absorbance (A) at 540 nm. The resulting cell migration absorbance value was corrected for the absorbance of the negative control BSA.

DNA isolation, bisulfite conversion of DNA, PCR amplification and sequencing

DNA was isolated from lung tissue or MTECs using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. Following isolation, DNA was bisulfite converted using the EZ DNA Methylation Lightning kit (Zymo Research) according to manufacturer's instruction. PCR amplification of the mouse *Duox1* promoter (starting at chr2:122,139,282) was performed using primers (left: TTAGTTTTTAAGAGTTGGAATTTGG, right: TAAACCCAAACAAACTAAATCATTC) that amplified a 597 base pair product containing

a total of 30 CpGs, of which 19 were considered to be in a within the CpG island (250 base pairs total), and subsequently, DNA was sequenced.

Statistical analysis

All data is reported as the mean ± SEM from the indicated number. Statistical differences between groups were evaluated by means of 1-way or 2-way ANOVA with Bonferroni post-analysis using GraphPad Prism (version 7.0d; GraphPad Software, La Jolla, CA) and were considered significant at a p value of less than 0.05.

Results

Lung tissue Duox1 levels decrease with age

To address the effect of aging on NOX expression, we evaluated whole lung tissues from C57BL/6J mice of young (2-3 months) and more advanced age (12-16 months). Lung tissue mRNA levels of *Nox1*, *Nox2*, or *Nox3* were not significantly altered with age, and *Nox4* tended to increase slightly (**Fig. 1A**), as expected (27). Conversely, we observed a striking age-associated decrease in lung tissue mRNA levels of *Duox1* and, to a lesser extent, *Duox2* (**Fig. 1A**). Accordingly, we observed decreased bronchial Duox1 protein levels by immunohistochemistry (IHC) in lung tissues from aging (13 months) compared with young (2 months) mice (**Fig. 1B**). Similar age-related changes in DUOX1 were also observed in human lung tissues, based on RNA sequencing data obtained from the GTEx database (27). Spearman correlation between donor age and DUOX1 expression demonstrates significant negative age-dependence: *Rho* = -0.16, *p* = 0.0037 (red dots indicate female donors; black dots indicate male donors (**Fig. S1**).

Aging attenuates DUOX1-dependent innate airway epithelial injury responses

We previously reported that DUOX1 is critical for innate epithelial responses to injury or non-microbial triggers such as airborne allergens, which involve initial production of epithelial-derived mediators, such as the cytokines IL-33, IL-1α, and the murine IL-8 homolog KC, and the epidermal growth factor receptor (EGFR) ligand amphiregulin (Areg) (33, 34, 37). To address the relevance of observed DUOX1 downregulation with age, we assessed the impact of aging on these innate airway responses to airway challenge with house dust mite (HDM). Indeed, whereas acute airway HDM challenge induced significant production of IL-33, IL-1α, KC, and Areg into the BAL of young C57BL/6J mice, these responses were dramatically reduced in aging (13 months) mice (**Fig. 2A**). Since these HDM responses were previously found to depend on initial release of the damage signal ATP and activation of P2YR2 receptor (34, 38), we determined the potential effect of age on HDM-induced ATP release (**Fig. 2B**), or lung tissue *P2yr2* mRNA expression (**Fig. S2**). Both were similar in young versus old mice, indicating that the impaired innate airway responses due to age were a result of altered responses to ATP-induced signaling, e.g. due to downregulation of DUOX1. To more directly assess whether these age-related impaired innate airway responses are due to altered epithelial responses, we isolated tracheal epithelial cells (mTECs) from either young

or aged mice for in vitro stimulation with either HDM or exogenous ATP. Characterization of mTECs from young vs. old mice did not reveal significant differences in basal cell markers (p63, Krt5), the epithelial marker E-cadherin, or markers of epithelial remodeling (SMA, collagen 1α1), but mTECs from old mice displayed increased levels of CCSP (Club Cell Secretory Protein) (**Fig. S3A**) and increased senescence-associated beta-galactosidase staining (SA-β-gal) (**Fig. S3B**). We verified that *Duox1* mRNA expression was attenuated in mTECs from aged mice, whereas mRNA expression of *P2yr2* was similar in mTECs from young and aging mice (**Fig. 2C**). However, in contrast to *in vivo* findings, *Duox2* mRNA was slightly enhanced in mTECs from older mice. Consistent with our *in vivo* findings, innate cytokine responses to either HDM or ATP were dramatically attenuated in mTECs from aged mice compared to young mice (**Fig. 2D**). For comparison, acute production of KC from mTECs in response to bacterial LPS, previously shown to be DUOX1-independent (39, 40) was similar between young and aged mice (**Fig. S4**). Lastly, mTECs isolated from aged mice also displayed impaired ATP-dependent migratory capacity in a haplotaxis assay (**Fig. 2E**), which was previously shown to be DUOX1-dependent (36).

Our findings suggest that impaired epithelial responses due to aging are strongly related to decreased DUOX1 expression and/or function. To more directly test this, we assessed DUOX1-mediated redox-based activation of the non-receptor tyrosine kinase Src and EGFR (34), by analyzing cysteine oxidation (-SOH, sulfenylation) within these kinases. Consistent with reduced DUOX1 function in mTECs from aged mice, we observed that the extent of cysteine oxidation within Src and EGFR in response to ATP or HDM was markedly attenuated in mTECs from aged mice (**Fig. 2F**). Together, these results highlight that the loss of epithelial DUOX1 due to aging is associated with reduced DUOX1-dependent innate epithelial injury responses.

DUOX1 deficiency in mice enhances age-related senile emphysema

In light of previous findings linking DUOX1 with epithelial regenerative responses following injury (41), and the presence of DUOX1 in the alveolar epithelium (42), we speculated that the age-related decline in DUOX1 might also contribute to functional and morphological features of lung aging. To test this, we compared age-related alterations in lung tissue morphology and lung function between wild-type and *Duox1*$^{-/-}$ mice. As expected (43), we observed increased airspace enlargement in aged mice (12-16 months) compared with young animals, as indicated by an increased mean linear intercept (MLI) of the alveoli (**Fig. 3A**), and this was significantly enhanced in aged *Duox1*$^{-/-}$ mice compared to age-matched

WT controls. Aged *Duox1*$^{-/-}$ mice also displayed enhanced thoracic gas volume compared with age-matched WT counterparts (**Fig. 3B**). No such differences were observed in young *Duox1*$^{-/-}$ mice compared with WT counterparts, neither with respect to the alveolar MLI (**Fig. 3A**) or lung volume (data not shown). To address lung functional changes, we performed analysis of respiratory mechanics on young (2-3 months) WT C57BL/6J mice, as well as aged WT and *Duox1*$^{-/-}$ (12-16 months) mice using FlexiVent. Pressure-volume (P-V) loops, performed at a positive end-expiratory pressure (PEEP) of 3 cm H_2O, which demonstrated a left shift of the inspiration-expiration P-V loop in aged mice (**Fig. 3C,** *red*) compared with young mice (*black*), reflecting increased lung compliance (loss of static elastic recoil) indicative of age-related senile emphysema. In line with the MLI data, this left shift was further enhanced in aged *Duox1*$^{-/-}$ mice (*blue*) compared with age-matched WT controls. Peak pressure/volume ratios observed at maximal inspiration indicated significantly reduced elastance in aged *Duox1*$^{-/-}$ mice compared with WT mice (**Fig. 3D**), and calculated slopes of linear pressure-volume graphs of inspiration curves recorded at PEEP3 (**Fig. 3E**) were significantly increased in aged *Duox1*$^{-/-}$ mice compared with WT mice, further supporting increased lung compliance in aged *Duox1*$^{-/-}$ mice. Collectively, these findings indicate that *Duox1*-deficiency accelerates age-related airspace enlargement, decreases lung elastance and increases lung compliance, features indicative of accelerated development of senile emphysema.

Effect of DUOX1 deficiency on hallmarks of lung aging

Based on the observed accelerated age-associated functional decline in *Duox1*$^{-/-}$ C57BL/6J mice, we next addressed whether *Duox1*-deficiency enhances other common hallmarks of lung aging, such as parenchymal extracellular matrix remodeling (of elastin and collagen), markers of cellular senescence and expression of a senescence-associated secretory phenotype (SASP). However, *Duox1*-deficiency did not affect age-related increases in parenchymal collagen deposition (measured by Picrosirius Red staining; **Fig. 4A**) or decreases in parenchymal elastin content (measured by Weigert’s Resorcin Fuchsin; **Fig. 4B**). Interestingly, lung mRNA expression of *Col1a1* and *Col3a1* were reduced with age in both WT and *Duox1*$^{-/-}$ mice, but the profibrotic gene *Nox4* was significantly increased in aged *Duox1*$^{-/-}$ mice (**Fig. S5**).

As expected, aging was associated with increases in lung tissue mRNA levels of *p16ink4a* and *p19arf*, both common replicative senescence markers (44), which was further enhanced in the case of *p16ink4a* in aged *Duox1*$^{-/-}$ mice (**Fig. 4C**). Aging-related cellular senescence was also associated with increased lung tissue levels of the pro-inflammatory cytokines IL-6, IL-1β and TNFα (**Fig. 4D**), indicative of increased senescence-associated low-grade inflammation known as inflammaging (45-47), but this was not enhanced further in old *Duox1*$^{-/-}$ mice. Additionally, mRNA analysis of these cytokines revealed similar increases in lungs of aged WT and *Duox1*$^{-/-}$ mice (except *Il6 in Duox1*$^{-/-}$ mice; **Fig. S5**). Overall, these finding do not support a general role for *Duox1*-deficiency in enhancing cellular/molecular lung aging, although some features of aging (*Nox4, p16ink4a*) appear to be enhanced *Duox1*$^{-/-}$ mice.

Aging-related impact on DUOX1 and lung function are different in C57BL/6NJ mice compared to C57BL/6J mice

Our findings reported so far were based on studies of mice with a C57BL/6J background, obtained from Jackson Labs. These mice have a naturally occurring deletion of exons 7-11 of the *Nnt* gene, which encodes for a nicotinamide nucleotide transhydrogenase (NNT) that is important in regulating NAD and NADP(H) status. Indeed, loss of NNT has a significant impact on mitochondrial redox homeostasis (48, 49), cellular senescence (50), and may be a contributing factor in the different molecular signatures of aging across these mouse strains (51). Because of the importance of these metabolic pathways in maintaining redox balance and their role in aging (7), we performed similar studies in C57BL/6NJ mice (which do not contain the Nnt mutation) and *Duox1*$^{-/-}$ mice backcrossed onto the C57BL/6NJ background. Similar to our original findings in C57BL/6J mice, we again observed markedly impaired innate airway responses to HDM challenge in aged C57BL/6NJ mice (19-20 months), with respect to early production of IL-33, IL-1α, KC, and Areg into the BAL (**Fig. 5**), as well as subsequent type 2 cytokine responses (IL-5 and IL13; **Fig. 5**). However, and somewhat surprisingly, we did not observe age-related suppression of lung tissue *Duox1* mRNA in C57BL/6NJ mice (p=0.1095) (**Fig. 6A**), compared with original findings in C57BL/6J mice. Therefore, the observed impaired innate epithelial injury responses due to aging cannot be fully explained by changes in *Duox1* expression but may be related to age-related effects on other factors involved in DUOX1 activation or function. Moreover, advanced age showed no impact on *P2yr2* mRNA expression levels in these mice (**Fig. S6**). With respect to age-related

alveolar enlargement, this was also observed in aged C57BL/6NJ mice (**Fig. 6B**) but was not further enhanced by *Duox1*-deficiency. Analysis of lung function indicated increased lung compliance in aged C57BL/6NJ mice, similar to previous findings with C57BL/6J mice, and was in this case only marginally enhanced in aged *Duox1*$^{-/-}$ mice, although age-related decreases in elastance were significant only in *Duox1*$^{-/-}$ mice (**Fig. 6C** and **D**). Collectively, the apparent impact of *Duox1*-deficiency on age-related senile emphysema observed in our original studies with C57BL/6J mice is considerably less dramatic in C57BL/6NJ mice and may be related to enhanced *Duox1* downregulation due to altered metabolic status caused by Nnt deficiency in the former case.

Aging-related impairment of innate airway epithelial injury responses is associated with increased DNA methylation

Epigenetic changes such as altered DNA methylation are strongly implicated in age-related chronic lung diseases such as COPD and lung cancer (52-54). Moreover, downregulation of DUOX1 in the context of various epithelial cancers has been attributed to DNA hypermethylation at CpG islands within the DUOX1 promoter region (55-58). Therefore, we assessed whether aging-related impairment in lung function may be associated with DUOX1 promoter hypermethylation. First, we evaluated potential alterations in gene expression of DNA methyltransferases (DNMT; *Dnmt1*, *Dnmt2*, *Dnmt3a*, *Dnmt3b*) or Ten-eleven translocation methylcytosine dioxygenases (TET; *Tet1*, *Tet2*, *Tet3*) in lung tissues or mTECs from either young or aging C57BL/6J mice. Indeed, lung tissue mRNA expression of some DNMTs increased with age (**Fig. S5** for lung tissues**; Fig. S7** for mTECs), but overall expression of TETs was not affected. To test the potential importance of increased DNA methylation for age-related impairment of innate epithelial injury responses, we exposed mTECs from young or aging mice to the DNA methyltransferase inhibitor 5-Aza-2'-deoxycytidine (aza) for 72 hours prior to stimulation with HDM or ATP. Interestingly, aza treatment markedly enhanced HDM- or ATP-stimulated release of IL-33 and IL-1α in mTECs from aging mice, to levels similar to those observed in MTECs from young mice, indicating that inhibition of DNA methylation can restore these age-related impaired responses (**Fig. 7**). Aza treatment appeared to slightly enhance mRNA expression of *Duox1* and *Duox2* in mTECs from either young or old mice, but neither of these increases were statistically significant (**Fig. S7**).

Lastly, we attempted to determine whether aging enhances promoter hypermethylation within the DUOX1 promoter, using bisulfite conversion and sequencing. We evaluated the most abundant CpG island region with the DUOX1 promoter, even though it is less rich in CpG compared to the human DUOX1 gene. This region contained a defined CpG island of 250 base pairs harboring 19 CpG sites. Out of those CpG sites, 2 CpGs were found to be identically methylated in lungs or MTECs of both young and aging mice, whereas 17 CpGs remained identically unmethylated. Furthermore, treatment of MTECs isolated from both young and old mice with the DNA methyltransferase inhibitor aza did not demethylate these 2 CpGs. Overall, while we observed some evidence of increased DNA methylation in suppressing innate airway epithelial injury responses during aging, we could not fully attribute this to altered methylation of *Duox1*, and DNA methylation of other (yet unidentified) genes likely contributes to this outcome.

Discussion

Aging is generally recognized as a consequence of the lifelong accumulation of cellular damage, resulting in cellular dysfunction, thereby increasing susceptibility to diseases or death (59), and the free radical theory of aging or revised mitochondrial theory of aging have been countered by the concept of mitohormesis (22, 60), and are further complicated by the growing appreciation of NOX enzymes in many specialized and beneficial cell functions by ROS-mediated redox signaling. Yet, only a marginal number of studies have addressed how NOX enzymes impact on aging or, conversely, how aging affects NOX function or activity. Our present studies were based on observations of a striking downregulation of the NOX homolog DUOX1 within the lung during aging, findings that were corroborated by similar findings in human lung tissues. Moreover, this was accompanied by dramatically impaired innate airway responses in aging mice to non-microbial injurious triggers (such as the allergen HDM) that are mediated by DUOX1-dependent redox-based activation of signaling pathways (e.g. Src/EGFR), and involve epithelial secretion of alarmins and other mediators that are important for maintaining epithelial integrity (34-36). Furthermore, we also provide evidence that age-related loss of lung DUOX1 may contribute to age-related structural and functional alterations in the lung, potentially due to impaired innate DUOX1-dependent regenerative responses (2, 3), since these alterations were enhanced in mice that completely lack DUOX1.

Loss of lung DUOX1 and related impaired reparative responses were also associated with accelerated airspace enlargement, increased lung compliance, and senile emphysema development, which may contribute to the development and/or progression of age-related chronic respiratory diseases. Indeed, such DUOX1 downregulation has been shown in the airways of subjects with COPD (61-63). Additionally, cigarette smoke, the major driver of COPD pathology, induces DAMP release (including ATP) from airway epithelial cells (64), and impaired ATP-induced innate epithelial injury responses due to aging may contribute to decreased regenerative capacity in the lung epithelium and thereby to COPD pathology. Previous studies that addressed age-related lung respiratory system mechanics support our current findings in WT mice, as aged C57BL/6J mice displayed similar increased lung compliance, airspace enlargement, as well as alterations in parenchymal elastin and collagen content (43).

Type 2 immune responses are well known to support lung tissue reparative and regenerative responses (65). Indeed, IL-33 has an important role in the resolution of inflammation and repair of tissue damage through its interaction with regulatory T cells and type 2 innate

lymphoid cells (ILC2s) (66, 67), which may also be impaired during aging (68). Furthermore, IL-33 promotes tissue regeneration following parasitic infections by priming proper Th2 immunity (69), and by promoting ILC2-derived release of the epithelial growth factor Areg (70). As such, our current findings of impaired epithelial IL-33 and Areg release as a result of age-related DUOX1 loss suggest a mechanism by which airway epithelial crosstalk with ILC2/Tregs involved in epithelial regeneration is suppressed during aging.

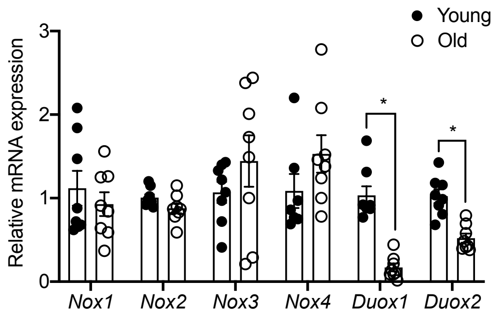
To explain the accelerated age-related emphysema development in *Duox1*$^{-/-}$ mice, we sought to address common underlying hallmarks of lung aging. While these features were largely similar in aged WT and *Duox1*$^{-/-}$ mice, our observation of increased replicative senescence markers (e.g. p16^{INK4a}) in aged *Duox1*$^{-/-}$ mice may be related to accelerated senile emphysema development. Indeed, deletion of cells expressing p19ARF, also encoded by the *CDKN2A* locus, was found to largely prevent aging-associated lung function decline and senile emphysema (71). While DUOX1 has not previously been linked to cellular senescence during lung aging, one study highlighted a contributing role for DUOX1 in DNA damage and senescence-related growth arrest in the thyroid (72).

With respect to the potential mechanism by which DUOX1 is downregulated during aging, we considered potential hypermethylation of the DUOX1 promoter, as observed previously in the context of lung cancer (73). However, while our bisulfite sequencing revealed methylation of some CpG sites in the Duox1 promoter, this was independent of age or aza treatment. Moreover, the mouse promoter region of Duox1 contains a relatively low percentage of GC sites compared to the human gene and may not fit the definition of a CpG island. Nevertheless, observations that inhibition of DNA methylation by aza can restore innate epithelial injury responses suggest the importance of increased DNA methylation of one or more factors (including perhaps DUOX1) involved in such impaired responses. Indeed, lung aging has been associated with increased transcriptional noise at the cellular level of most cell types, indicating impaired epigenetic control (74). For example, one study showed that aged mice display more irregular transcriptional responses upon immunological activation (75), indicating that the age-related impairment to upregulate appropriate key activation pathways (including epigenetic control) may in part explain the decrease of immune function observed in aged animals. Furthermore, it is notable that DNA methylation patterns can rapidly change in response to environmental stimuli (76), including HDM (77), raising the possibility that rapid changes in DNA methylation might play a role in innate immune responses.

One surprising outcome of our studies was that age-related DUOX1 downregulation was not observed in aged WT mice of the C57BL/6NJ strain in comparison to mice on the C57BL/6J background. The *NNT* gene mutation known to occur in C57BL/6J mice results in defective NNT function, and has been implicated in cardiovascular disease/hypertension (78) as well as lung viral infections (79). The loss of NNT function has been shown to induce mitochondrial dysfunction through HIF-1α and HDAC1-dependent pathways (49) and has been shown to increase cellular senescence (48-50). Therefore, our results of age-related DUOX1 loss in C57BL/6J mice may be related to the absence of the *NNT* gene and may suggest a link between mitochondrial (dys)function and the loss of DUOX1.

With respect to the potential impact of age-related DUOX1 downregulation on altered redox homeostasis during aging, a recent study explored the effects of age on tissue cysteine oxidation networks in mice, including the lungs (80). In contrast to the prevailing notion that non-specific protein oxidation increases with age, these studies highlighted distinct age- and tissue-specific redox-regulated clusters reflecting a fundamentally reprogrammed redox signaling landscape in the aged mouse, and demonstrated oxidation of some redox-regulated sites, e.g. in secreted proteins, actually declines with age. Such reprogramming of redox signaling during aging may be associated with altered NOX expression, including DUOX1, and contribute to various hallmarks of aging, e.g. by controlling intercellular communication, mitochondrial (dys)function or cellular senescence. In combination, these various alterations (altered redox status, cellular senescence, impaired reparative responses) may ultimately result in accelerated senile emphysema in *Duox1*$^{-/-}$ mice.

Overall, the current study highlights that during aging, lung DUOX1 levels decrease in both humans and mice, and thereby lead to age-related impairment of DUOX1-dependent roles in airway host defense and regenerative capacity thus accelerating age-related airspace enlargement and senile emphysema. We speculate that such alterations in DUOX1 may also be a predisposing factor in development of age-related chronic respiratory diseases.



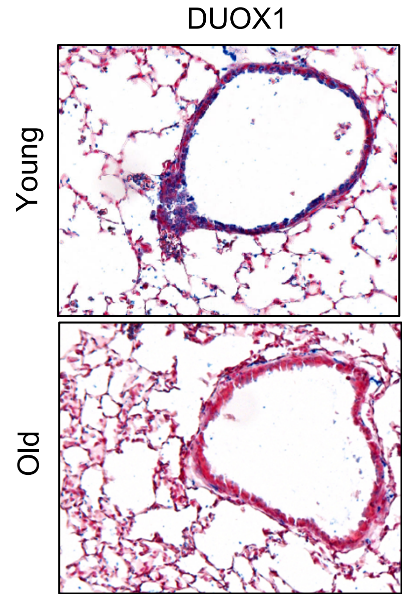
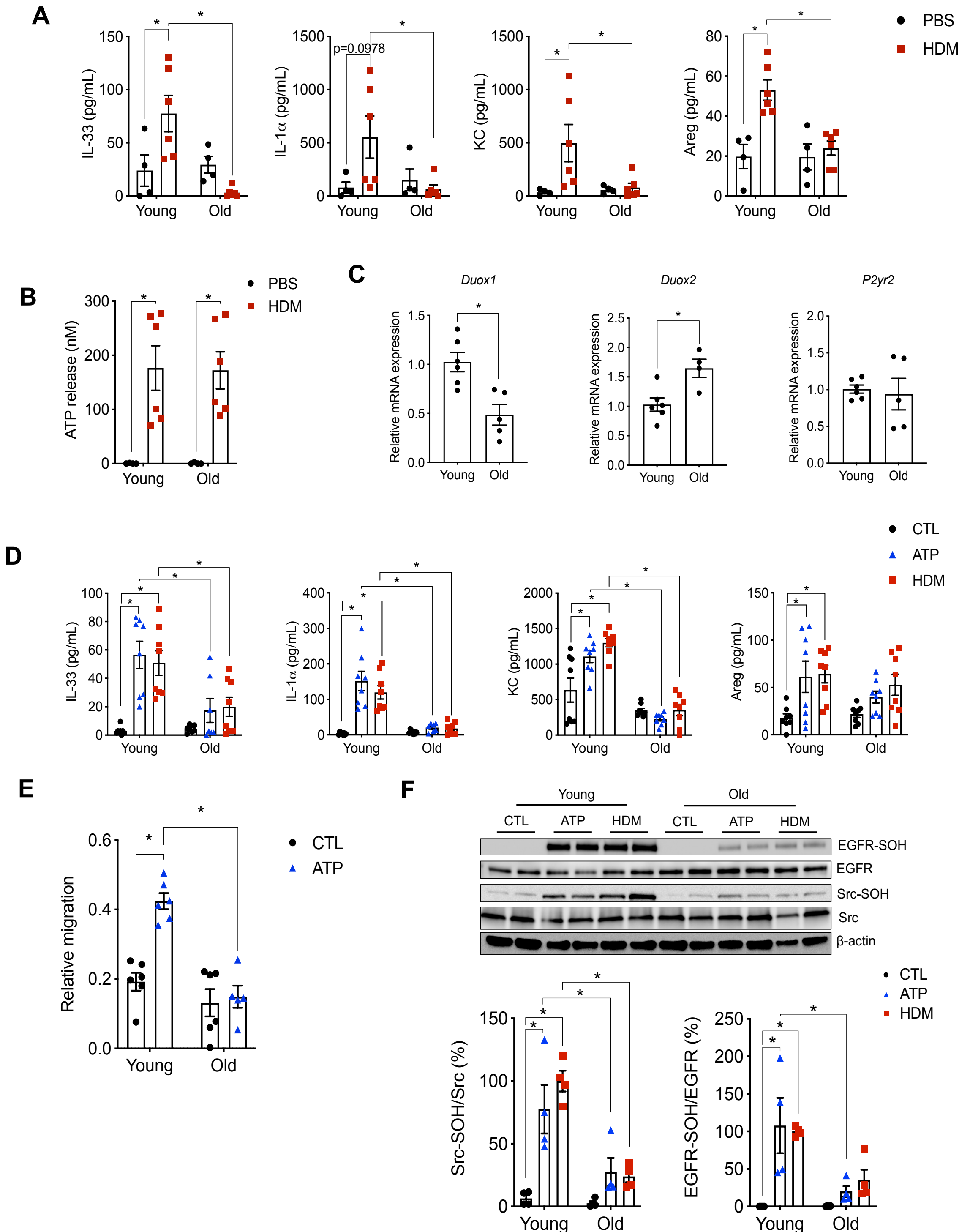


Figure 1: A) *Duox1* and *Duox2* mRNA decrease in aging mice C57BL/6J mice (n=8 per age group) (12-16 months) versus young mice (2-3 months, n=8 per group) **B**) Representative images of decreased airway DUOX1 (*blue*) staining in lung tissues from 13-month-old C57BL/6J wildtype mice versus 2 months old C57BL/6J wildtype mice. Data shown as mean ± SEM. *$p \leq 0.05$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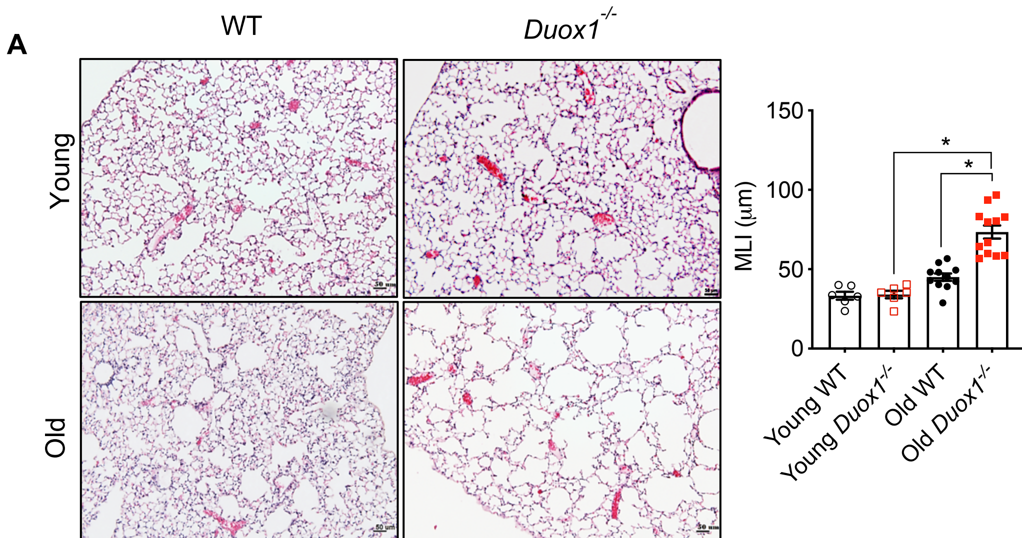
IL-33 (pg/mL)
IL-1α (pg/mL)
p=0.0978
KC (pg/mL)
Areg (pg/mL)
Young
Old
PBS
HDM
B
ATP release (nM)
C
Duox1
Duox2
P2yr2
Relative mRNA expression
D
CTL
ATP
E
Relative migration
F
EGFR-SOH
EGFR
Src-SOH
Src
β-actin
Src-SOH/Src (%)
EGFR-SOH/EGFR (%)

Figure 2: Allergen- or injury-induced innate responses are diminished with age. Young (2-3 months) or aging (12-16 months) C57BL/6J mice were challenged with HDM (1 μg/μL, 50 μL per mouse) or PBS (control) for 1 hour. **A)** IL-33, IL-1α, KC and Areg release into BAL as measured by ELISA (n=4-6 from 2 separate experiments). **B)** ATP release (nM) into BAL (n=4-6 from 2 separate experiments)**. C)** *Duox1*, *Duox2 and P2yr2* mRNA expression in isolated MTECs from young (2 months) and aging (13 months) C57BL/6J mice (n=4-6). **D)** IL-33, IL-1α, KC and Areg release (pg/mL) in conditioned media of isolated MTECs from 2 month and 13 months old mice after 2-hr stimulation with ATP (100 μM) or HDM (50 μg/mL) for 2 hours (n=8). **E)** Relative mTEC migration in haplotaxis assay (OD_{570}; n=5-6 from 2 separate experiments), following 24 hours ATP (100 μM) stimulation. **F)** Representative western blot analysis of sulfenylation of Src (Src-SOH) and EGFR (EGFR-SOH) in MTECs from young (2 months) or old (13-months) C57BL/6J mice after 10-min stimulation with HDM (50 μg/mL) or ATP (100 μM), based on DCP-Bio1 derivatization. Graphs represent quantification of relative sulfenylation by densitometry (normalized to HDM-stimulated MTECs from young mice) (n=4 from 2 separate experiments)**.** Data shown as mean ± SEM. *p ≤ 0.05



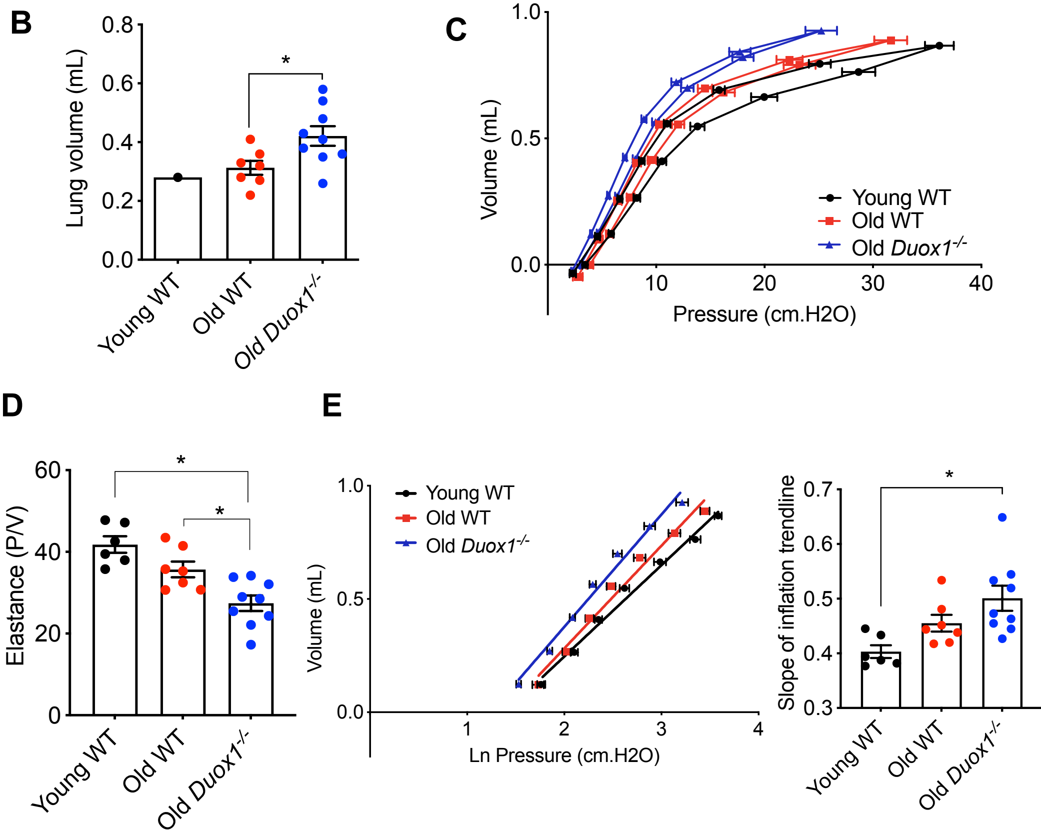
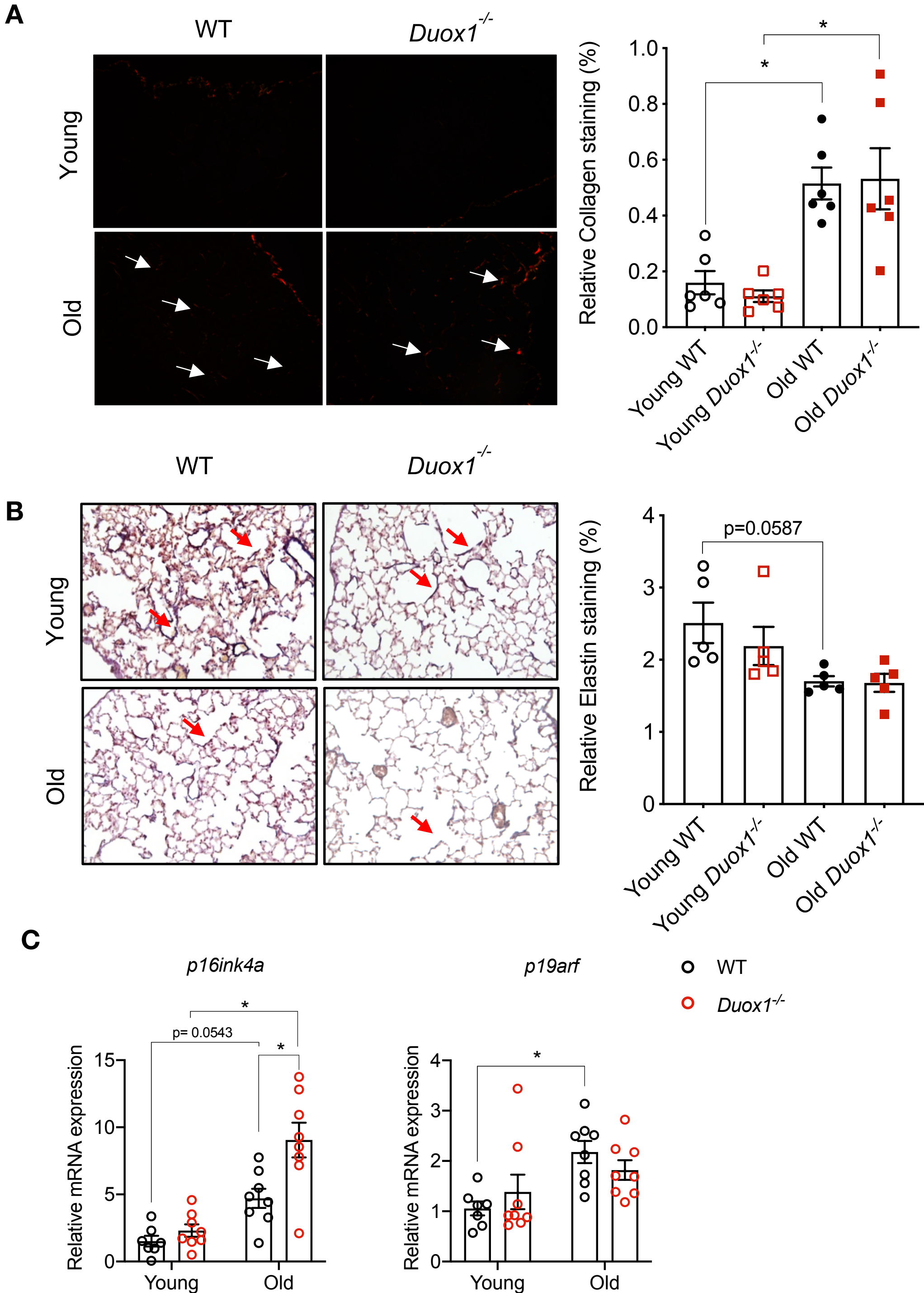


Figure 3: Aging *Duox1*$^{-/-}$ C57BL/6J mice display accelerated age-related airspace enlargement and lung function decline when compared to wildtype age-matched controls and young wildtype mice. **A)** Representative image of histology staining (H&E) of mouse lung tissue sections and mean linear intercept (μm) measurements (n=6-12 per group) in 2-3 months WT and *Duox1*$^{-/-}$ and 12-16 months old WT and *Duox1*$^{-/-}$ C57BL/6J mice. **B)** Total lung volume in young (2-3 months) WT and aging (12-16 months old) WT and *Duox1*$^{-/-}$ mice (n=1-10 per group). **C)** Pressure-Volume loop at PEEP 3 in young (2-3 months, n=6) WT C57BL/6J mice, aging WT (12-16 months old, n=7) and *Duox1*$^{-/-}$ (12-16 months old, n=9) C57BL/6J mice by FlexiVent. **D)** PEEP3 Elastance at peak pressure/volume (E=P/V). **E)** Slope of inspiration curve of the linearized pressure-volume curve at PEEP3. Data shown as mean ± SEM. *$p \leq 0.05$


A
WT
Duox1-/-
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Relative Collagen staining (%)
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Old Duox1-/-
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WT
Duox1-/-
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Relative Elastin staining (%)
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p=0.0587
Young WT
Young Duox1-/-
Old WT
Old Duox1-/-
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p16ink4a
p19arf
WT
Duox1-/-
Relative mRNA expression
15
10
5
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Relative mRNA expression
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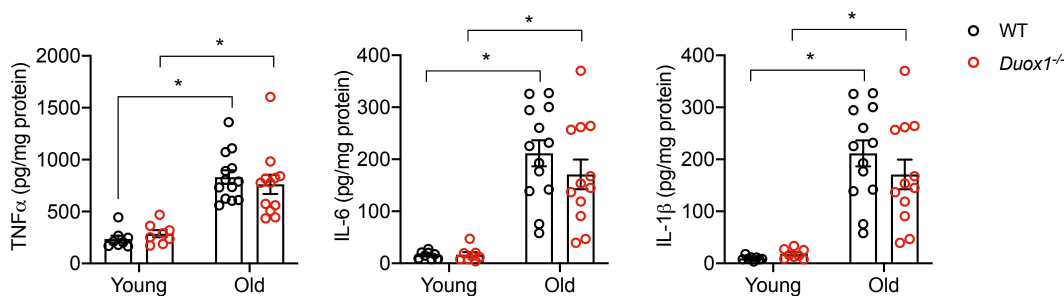

Figure 4: Features of lung aging are not accelerated in aging *Duox1*$^{-/-}$ mice when compared to aging WT mice. **A)** Picrosirius red staining (Relative collagen %; collagen positive staining/total staining area) of WT and *Duox1*$^{-/-}$ C57BL/6J mice at both 2-3 months and 12-16 months of age (n=6) with quantitative MetaMorph analysis. **B)** Weigert's Resorcin Fuchsin staining (Relative elastin %; Relative collagen %; collagen positive staining/total staining area) of WT and *Duox1*$^{-/-}$ C57Bl/6J mice at both 2-3 months and 12-16 months of age (n=5) with quantitative MetaMorph analysis. **C)** Whole lung tissues mRNA expression of *p16ink4a* and *p19arf* in 2-3 months and 12-16 months old wildtype and *Duox1*$^{-/-}$ C57Bl/6J mice (n=7-8). **D)** Lung tissue cytokine levels of TNF-α, IL-6 and IL-1β (pg/mg protein) in young (2-3 months) and old (12-16 months) wildtype and *Duox1*$^{-/-}$ C57Bl/6J mice (n= 5-8). Data shown as mean ± SEM. *p ≤ 0.05

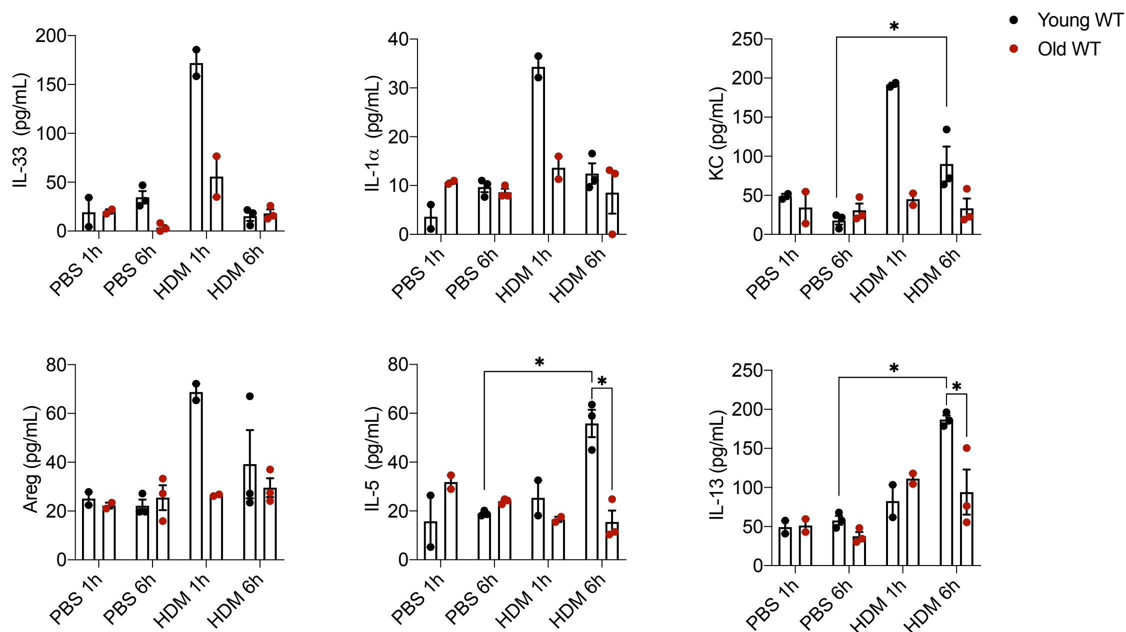

Figure 5: IL-33, IL-1α, KC, Areg, IL-5 and IL-13 release (pg/mL) in the bronchoalveolar lavage fluid as measured by ELISA in both young (2-3 months) and old (19-20 months) wildtype C57Bl/6NJ mice exposed to PBS or HDM for 1 or 6 hours (n=2-3). Data shown as mean ± SEM. * p ≤ 0.05

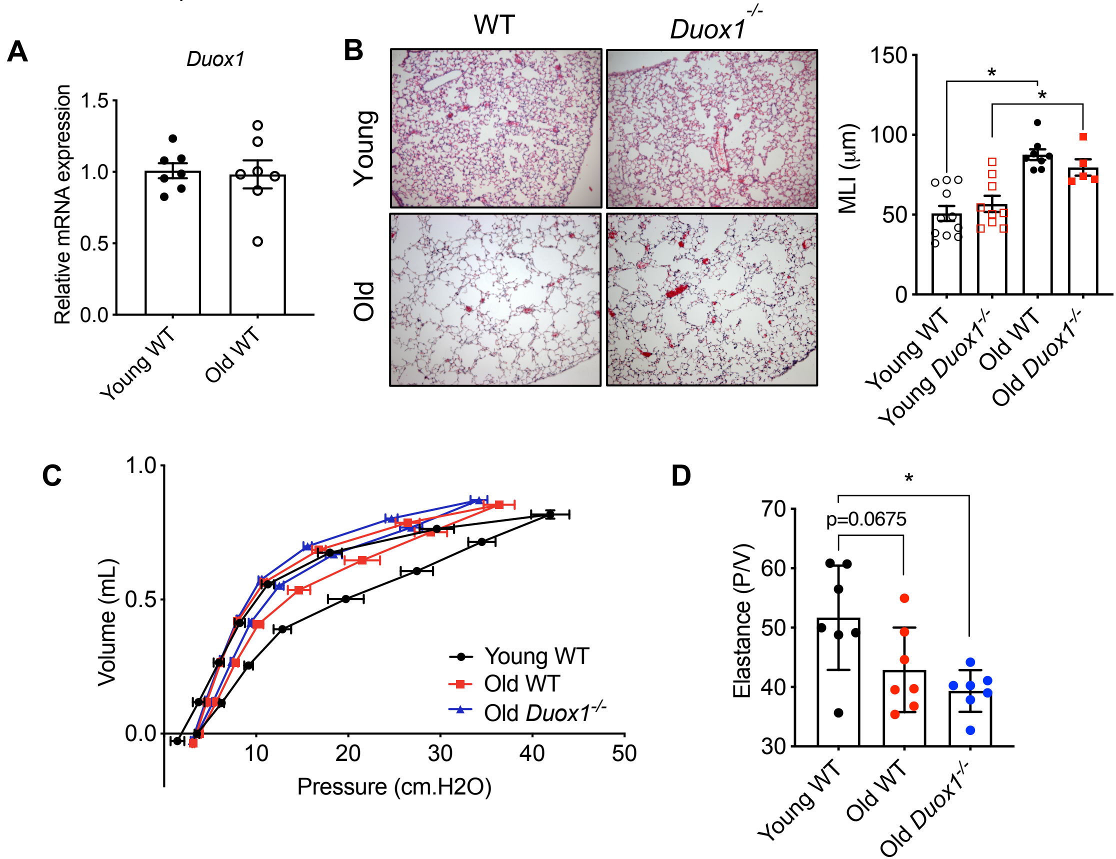

Figure 6: Age-related changes in *Duox1* mRNA, lung morphology and lung function in WT and *Duox1*$^{-/-}$ C57BL/6NJ mice. **A)** *Duox1* mRNA levels in young (2-3 months) and aging (15-17 months) mice (n=7 per group). **B)** Mean linear intercept (µm) measurements (n=5-11 mice per group) of young (2-3 months) and old (17-19 months) WT and *Duox1*$^{-/-}$ C57BL/6NJ mice with corresponding H&E images. **C)** Pressure-Volume loop at PEEP 3 in young (2-3 months) and aging (15-17 months) WT and *Duox1*$^{-/-}$ C57BL/6NJ mice (n=7 per group) by FlexiVent **D)** PEEP3 Elastance at peak pressure/volume (E=P/V). Data shown as mean ± SEM. * p ≤ 0.05

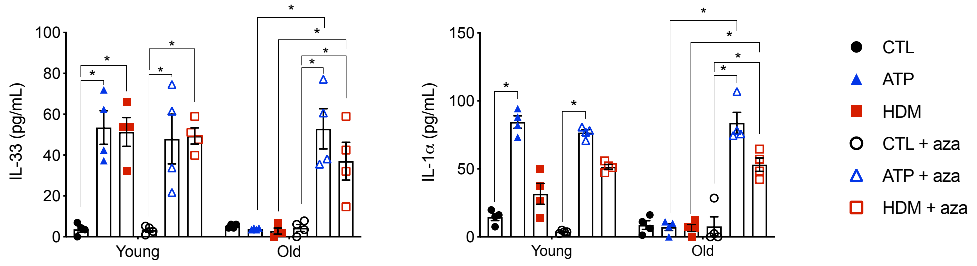

Figure 7: DNA methyltransferase inhibition restores innate responses to injury or allergen challenge in isolated MTECs. Cytokine release of both IL-33 and IL-1α (pg/mL) in MTECs from both 2 months and 13 months old C57Bl/6J WT mice with or without pre-treatment with the DNA methyltransferase inhibitor 5-Aza-2′-deoxycytidine (aza) and upon subsequent stimulation with HDM (50 μg/mL) or ATP (100 μM) for 2 hours (n=4). Data shown as mean ± SEM. *$p \leq 0.05$

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Supplemental Information for:

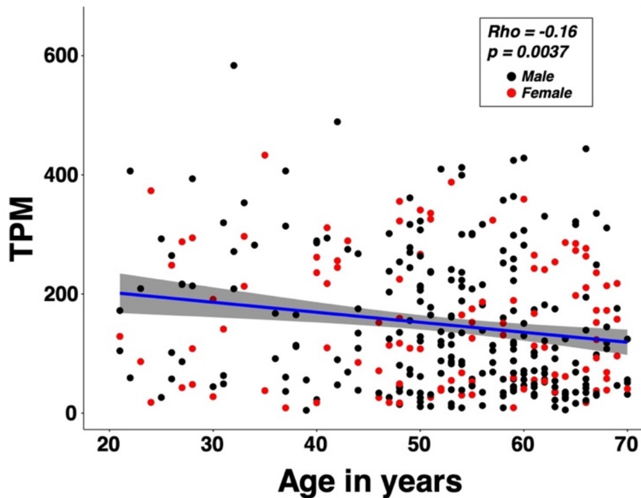
Downregulation of DUOX1 contributes to aging-related impairment of innate airway injury responses and accelerated senile emphysema

Caspar Schiffers, Christopher M. Dustin, Lennart K.A. Lundblad, Milena Hristova, Aida Habibovic, David J. Seward, Nirav Daphtary, Minara Aliyeva, Emiel F.M. Wouters, Niki L. Reynaert, and Albert van der Vliet

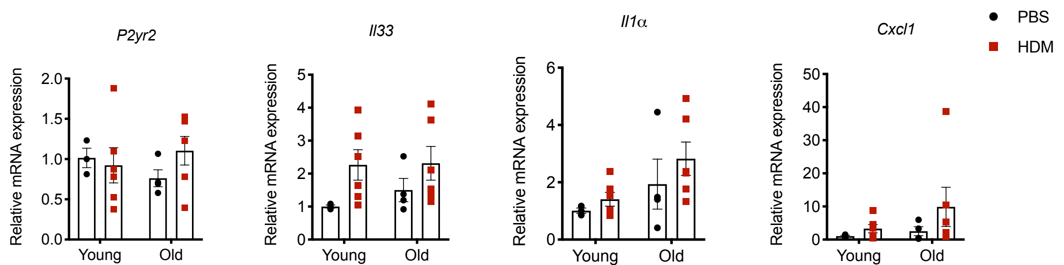
Supplemental Table 1: Mouse qPCR primers used in studies

Target gene	Forward/Reverse primer	Primer sequence
Duox1	Forward	ACCAGAACATTGCGATGTATGAG
	Reverse	AGAAATGGACGGTATCCTGGA
Duox2	Forward	GGCCTAAAGAAGAGGTTTGGCAAA
	Reverse	GCCTCCGTGTACAGCCGGG
P2yr2	Forward	CTGGAACCCTGGAATAGCACC
	Reverse	CACACCACGCCATAGGACA
Mmp9	Forward	CTCACTCACTGTGGTTGCTG
	Reverse	TGGTTATCCTTCCTGGATCA
Gapdh	Forward	CTGGAGAAACCTGCCAAGTA
	Reverse	TGTTGCTGTAGCCGTATTCA
Il33	Forward	GATGGGAAGAAGGTGATGGGTG
	Reverse	TTGTGAAGGACGAAGAAGGC
Il1a	Forward	CTTAAAGAACTGTTACAGTGAAAACG
	Reverse	TGGTCAATCGCAGAACTGTAGTCT
Cxcl1	Forward	TGGGATTCACCTCAAGAACA
	Reverse	GTGCCATCAGAGCAGTCTGT
p16ink4a	Forward	CCCAACGCCCCGAACT
	Reverse	GCAGAAGAGCTGCTACGTGAA
p19arf	Forward	TGAGGCTAGAGAGGATCTTGAGA
	Reverse	GCAGAAGAGCTGCTACGTGAA
Nox1	Forward	GCTGGATTTGAGAGCGTTGC
	Reverse	GGTGGTATCTAGGGCTATGCT
Nox2	Forward	GAATCAGCCTTAGTGTCACAGG
	Reverse	ATTCCGGTATGCGTCCAGC
Nox3	Forward	GCCTACGGGATAGCTGTCAA
	Reverse	GGACTGCAGATGGGTGACTC
Nox4	Forward	GAAGGGGTTAAACACCTCTGC
	Reverse	ATGCTCTGCTTAAACACAATCCT
Dnmt1	Forward	GAGGAAGGCTACCTGGCTAA
	Reverse	AGTGAGAGTGTGTGTTCCGT
Dnmt2	Forward	AGCAAGCCTCTTGCAGTAGA
	Reverse	AGGGAGGGGAGTGATAGATG
Dnmt3a	Forward	TAAGCTGGAGCTGCAAGAGT
	Reverse	ATTTCAGTGCACCACAGGAT
Dnmt3b	Forward	ACTTGGTGATTGGTGGAAGC
	Reverse	CCAGAAGAATGGACGGTTGT
Tet1	Forward	AAGAAGAGGAAATGCGAGGT
	Reverse	GGCCATTTACTGGTTTGTTG
Tet2	Forward	ATGCAGAGAGATGCCTTCAC
	Reverse	GCCGTGTAGCTGTAGATCGT
Tet3	Forward	TTGTGGAATCCTGTGAAGGT
	Reverse	AAGGGATCCCACAGTTTCTC
	Forward	CACCCTCAAGAGCCTGAGTC

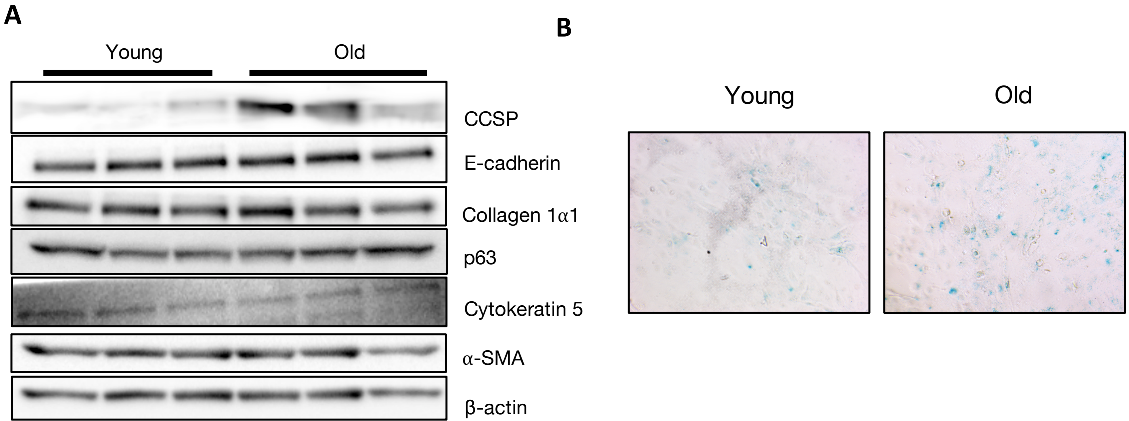
Col1a1	Reverse	AGACGGCTGAGTAGGGAACA
Col3a1	Forward	CCGAACTCAAGAGTGGAGAA
	Reverse	GGCCATAGCTGAACTGAAAA
Il6	Forward	AACGATGATGCACTTGCAGA
	Reverse	GGAAATTGGGGTAGGAAGG
Tnfa	Forward	CCCTCACGACGTGGGCTACAG
	Reverse	GCTACGACGTGGGCTACAG
Il1b	Forward	GCCCATCCTCTGTGACTCAT
	Reverse	AGGCCACAGGTATTTTGTCG



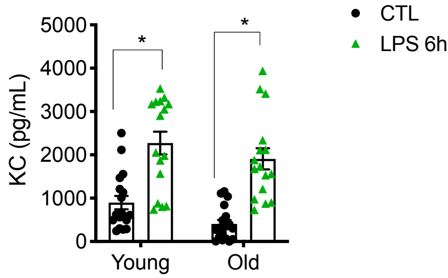
Supplement Figure 1: Human DUOX1 mRNA expression in TPM (Transcripts Per Kilobase Million). Data obtained from the GTEx dataset. Spearman correlation between donor age and DUOX1 expression demonstrated significant negative age-dependence: R=-0.16, p=0.0037. Red dots indicate female donors; black dots indicate male donors.



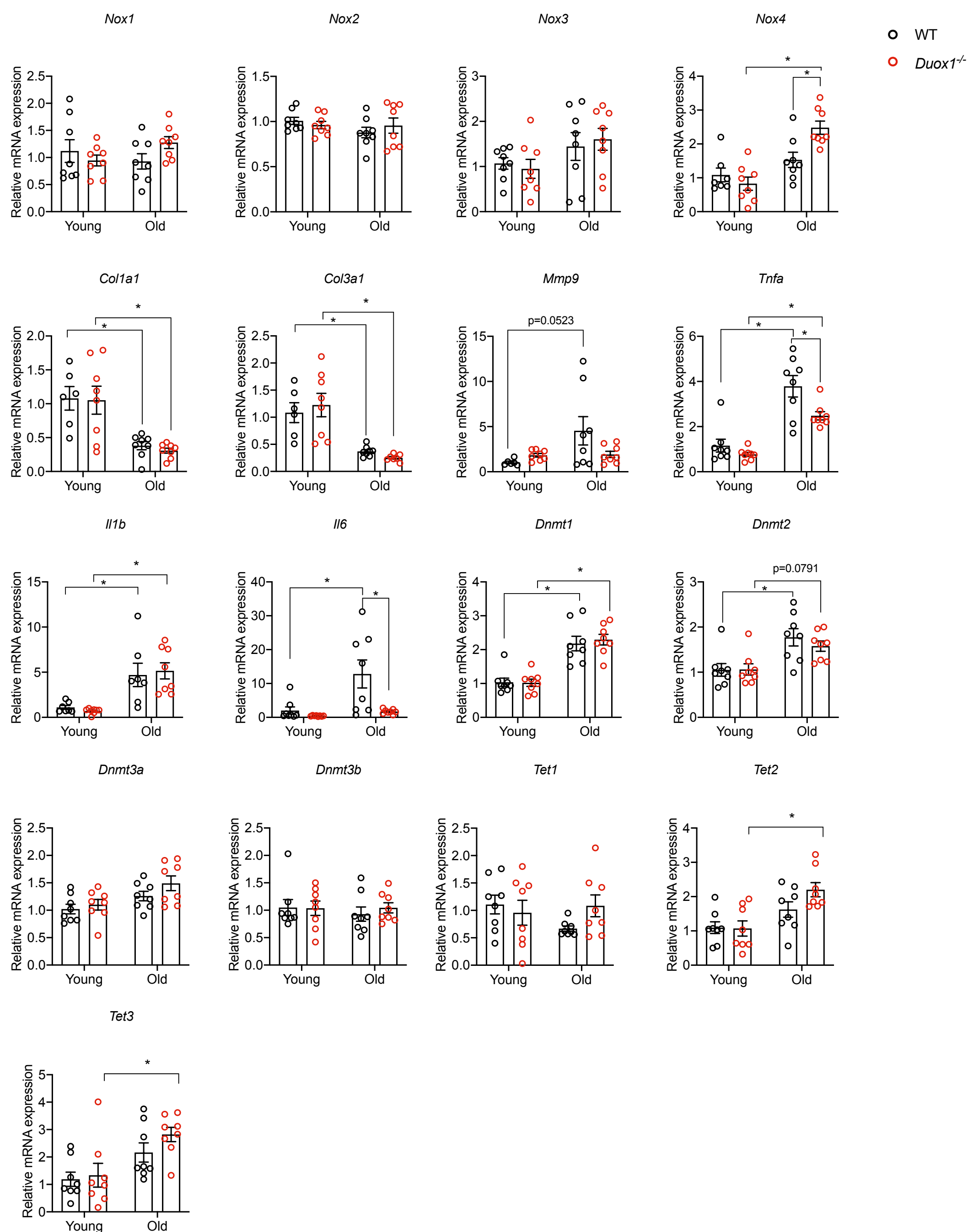
Supplemental Figure 2: mRNA expression data in lung tissues from both young and old wildtype C57Bl/6J mice exposed to PBS or HDM (n=3-6, from 2 separate experiments). Data shown as mean ± SEM. * $p \leq 0.05$



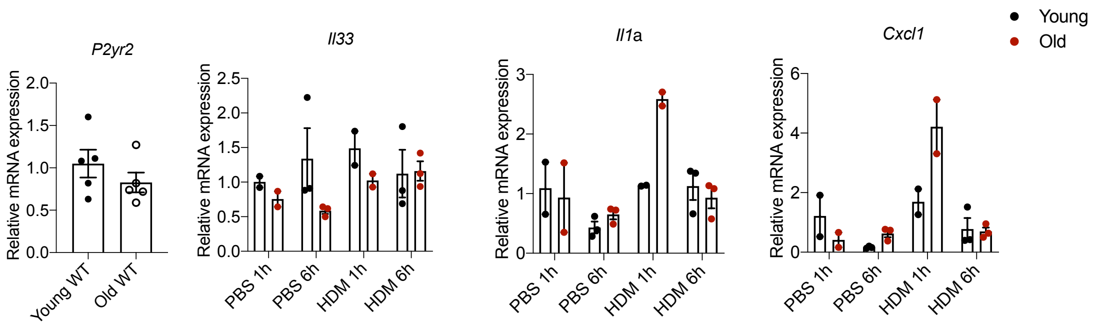
Supplement Figure 3: A) Cell characterization of MTECs isolated from both young (8 weeks) and aging (13 months) wildtype mice. B) Senescence-associated ß-galactosidase staining in MTECs isolated from young (8 weeks) and aging (13 months) C57Bl/6J mice.



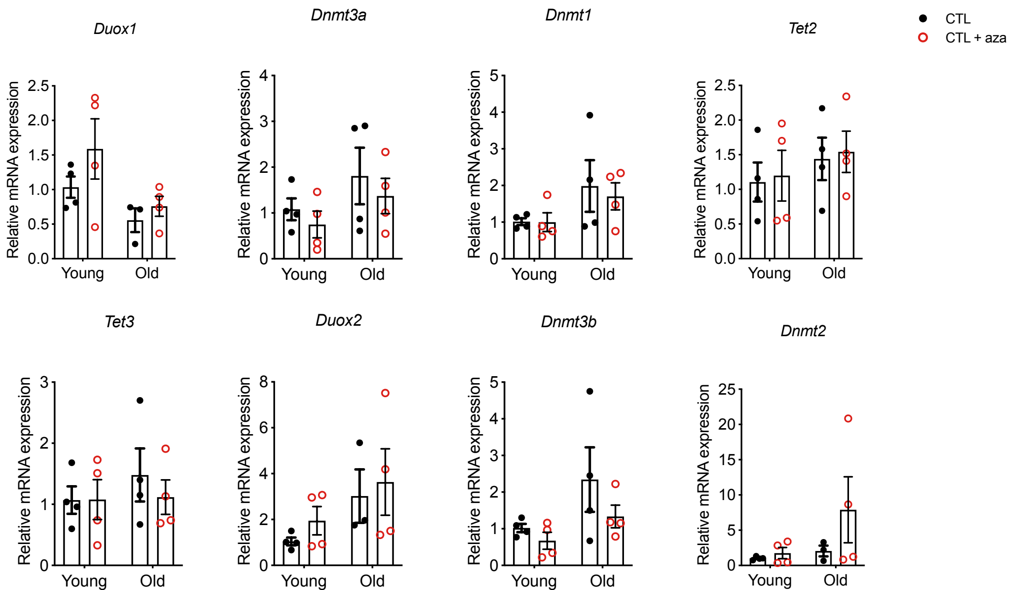
Supplement Figure 4: The release of KC (pg/mL) in both 8 weeks and 13 month old MTECs (from C57Bl/6J) after stimulation with LPS (1 ug/mL) or ATP (100 uM) for 6 hours (n= 10-18, 4 separate experiments). Data shown as mean ± SEM. *p ≤ 0.05



Supplement Figure 5: mRNA expression in lung tissues from both young (8-12 weeks) and old (12-14 months) wildtype and *Duox1*$^{-/-}$ C57Bl/6J mice (n=8 per group). Data shown as mean ± SEM. *p ≤ 0.05



Supplemental Figure 6: mRNA expression of P2yr2 and innate cytokines in lung tissues from both young (8-12 weeks) and old (19-20 months) wildtype C57Bl/6NJ mice. Data shown as mean ± SEM.



Supplement Figure 7: Relevant mRNA expression in isolated MTECs from both young and old wildtype C57Bl/6J mice following aza treatment (n=3-4). Data shown as mean ± SEM. *p ≤ 0.05.

Chapter 5

The contribution of aging and DUOX1 downregulation to the development of age-related chronic lung diseases

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Chapter 6

General Discussion

An apparent disbalance between oxidants and antioxidants has been implicated during lung aging and in the pathogenesis of various age-related chronic lung diseases. In apparent contrast to the free radical theory proposed by Denham Harman in the 1950s (1), in which reactive oxygen species (ROS) are defined as inherently detrimental, collective work presented in this thesis would suggest that the ROS-generating NADPH oxidase enzyme Dual Oxidase 1 (DUOX1) may in fact be crucial for maintaining airway epithelial cellular homeostasis through a process known as redox signaling. Since it is currently unknown how DUOX1 may affect lung aging and age-related chronic lung disease development, the overall aim of this thesis was to explore the role of DUOX1 in lung aging, and its role in the development and pathogenesis of age-related lung diseases COPD, IPF, and asthma (in the elderly).

Implications for aging

The report on Global Health and Aging by the National Institute of Aging (NIA) states that the world hit a demographic milestone, since people aged 65 years or older now outnumbers children under the age of 5 (2). The apparent aging of the population worldwide is largely driven by decreased fertility and increased life expectancy. Indeed, the number of people aged 65 or older is projected to grow from an estimated 524 million in 2010 to 1.5 billion in 2050. This has also led to a shift in causes of disease and death, and in the present, non-communicable diseases, including COPD, that more commonly affect adults and older people, impose the greatest burden on global health. The hallmarks of aging (3), which are cellular and molecular features that are considered to contribute to the aging process and together determine the aging phenotype, are observed in lung aging as well as age-related chronic lung diseases, such as COPD and IPF. These diseases are now considered to be diseases of accelerated lung aging (4, 5). ROS have been speculated to play an important role in aging and age-related chronic diseases have been associated with oxidative stress. As such, many theories have come forward including the well-known free radical theory of aging, but also more recent theories such as the disposable soma theory of aging. A better understanding of the underlying molecular roles of ROS in (the hallmarks of) lung aging, as well as in the pathophysiology of chronic lung diseases, will help to better understand how these species may be involved therein, and may lead to the development of novel target therapies of redox proteins to improve healthy aging and in (the prevention of) chronic lung diseases. These novel therapies are needed, since there is little evidence that current

antioxidant therapeutics (e.g. antioxidant supplementation) are clinically beneficial. As mentioned in the introduction, the first functional role attributed to NADPH oxidase (NOX) enzymes in mammalian cells was the deliberate ROS production by phagocytic NOX (NOX2), which catalyzes the respiratory burst that allows phagocytic cells to eliminate microbes (6). Since then, other NOX enzymes have been discovered, that deliberately produce ROS for similar roles in host defense, biosynthetic functions (hormone synthesis), and for cell signaling transduction that is also known as redox signaling (7). This thesis highlights that the NADPH oxidase DUOX1, which produces the ROS H_2O_2, is critically involved in airway epithelial innate responses to injury or by other exogenous hazards through Ca^{2+}-dependent signaling that involves the G-protein coupled receptors PAR2 and P2YR2, and the transient receptor potential channel TRPV1 (**Chapter 2**) (8). These airway epithelial responses, governed by DUOX1, are crucial for the reparative capacity at the site of injury. In addition, DUOX1 has previously been identified to play an important role in various biological processes, including host defense (9). Therefore, impairment of airway epithelial responses through either the functional impairment of DUOX1, or lower DUOX1 levels, may have implications for lung epithelial homeostasis.

Dysregulation of ROS-generating enzymes during aging and in lung pathology

The airway epithelium of the lungs forms the critical interface with the external environment as it is the first site of exposure to environmental factors (e.g. allergens, viruses, microbes or cigarette smoke), and therefore susceptible to inhalation of damaging agents that are found in the external environment (10). Our lungs have therefore developed unique mechanisms to preserve lung function while simultaneously protecting the lungs from such xenobiotic insults/injury. During aging however, the lungs are believed to be more susceptible to these environmental insults as a result of ineffective defensive mechanisms (e.g. repair pathways, resolving mechanisms), thus resulting in altered responses. Consequently, these insults induce exaggerated responses in the lungs resulting in biochemical and physiological changes, and prolonged exposure may contribute to aging (11, 12), and/or contribute to the development of age-related lung disease(s) or exacerbate existing disease (13). Based on the findings of this thesis that highlight the importance of the calcium channel TRPV1 in DUOX1-mediated innate responses (**Chapter 2**), airway epithelial DUOX1 plays a pivotal role in the regulation of such protective responses. In addition, our findings in **Chapter 4** explored the impact of aging on the various NOX enzymes in the lung and highlighted that DUOX1

(and to a lesser extent DUOX2) is the NOX isoform that declines with increasing age, in both mice and humans. We moreover established that the age-related loss of DUOX1 in the mouse lung, as well as in isolated mouse tracheal epithelial cells, impaired epithelial innate responses to injury, which suggests that it is the loss of an ROS-generating enzyme that is associated with diminished lung reparative responses. As such, ROS-generating enzymes cannot be generalized to contribute to aging or lung pathology, as they play important functions in cellular homeostasis. Hence, it is the dysregulation of these enzymes, either resulting in increased or decreased function, that likely contributes to aging or pathology, and age-related DUOX1 loss may thus contribute to lung aging and age-related lung disease development. Indeed, our findings in **Chapter 4** demonstrate that DUOX1-deficiency accelerates age-related senile emphysema development, implying that loss of DUOX1 (function) may accelerate lung aging. Still, the senile lung is characterized by airspace enlargement without overt inflammation. However, prolonged exposure of the lungs to the environment as we age, with a less capable defense mechanisms to fight these agents contained in the environment, may also increase susceptibility for chronic inflammation and alveolar wall destruction that may contribute to the development of e.g. emphysema (14, 15). Since DUOX1 is important for airway epithelial reparative responses to environmental agents, its age-related loss may also be involved in more chronic inflammatory diseases that are commonly associated with aging. One chronic lung disease that is associated with aging and particularly driven by environmental factors, with a prominent role for cigarette smoke, is COPD. Indeed, the observations in **Chapter 3** provide evidence that COPD is associated with loss of airway epithelial DUOX1, as well as in mouse models of COPD, and that this DUOX1 loss is associated with loss of lung function, pulmonary emphysema, and airway remodeling. Together, **Chapters 3** and **4** provide evidence that it is the loss of function, rather than a gain of function, of the ROS-generating enzyme DUOX1 that is associated with impaired type 2 immune responses and reparative capacity, and contributes to accelerated lung aging as well as chronic respiratory disease development and pathology.

The traditional idea that oxidative stress is underlying aging and age-related disease development was instigated by the free radical theory of aging (FRTA) and had been challenged over the last decades. In addition to previous findings which indicate that oxidative stress is not inherently contributing to aging, the current thesis further challenges the free radical theory of aging. As such, alternative theories, detailed in the introduction, have come forward. Some of the ROS-producing enzymes may follow the theory of antagonistic pleiotropy of aging (16), which is described as the phenomenon where genes

that are considered beneficial (contributing to fitness) in the earlier stages of life may actually be detrimental later in life. While that seems to be the case for some NOX enzymes (e.g. NOX4), this may not be true for all isoforms, importantly DUOX1, which is highlighted in this thesis. These results may thus imply that it is the disruption/reprogramming of redox signaling networks, which under healthy conditions mediate appropriate stress responses as well as aspects of cell maintenance and homeostasis, that may contribute to ageing hallmarks and age-related (lung) disease development. As aforementioned, this was elegantly illustrated in a recent publication that mapped the cysteine redox proteome, and observed a reprogrammed age-associated redox signaling landscape, which was applicable to various organs (e.g. lung, heart, liver) (17). The age-related loss of lung DUOX1 may thus affect the age-associated redox signaling landscape of the lungs, and future studies should be explored to improve our understanding of DUOX1 therein. As mentioned in **Chapter 1**, DUOX1 activation induces H_2O_2 production, which is able to target protein cysteine thiols, leading to its oxidation and formation of a sulfenic acid (e.g. Src, EGFR and protein-tyrosine phosphatase 1B (PTP1B) (18)). In a highly oxidative redox environment, DUOX1-derived H_2O_2 may result in further oxidation of a sulfenic acid intermediate, thereby forming a sulfinic or sulfonic acid. Through DUOX1-mediated sulfenic acid formation, DUOX1 may also indirectly contribute to the generation of protein-S-glutathionylation (18). Therefore, DUOX1 may alter protein functions and signaling pathways beyond those evidenced in the current thesis, and may thereby contribute to the redox signaling landscape of the lungs.

DUOX1 as an indicator of increased susceptibility to age-related lung disease development

COPD is a major global health issue that is predicted to be the third leading cause of death worldwide by 2030 (19), and current therapies that are limited to disease management by e.g. controlling symptoms, are inadequate. As such, it is of crucial importance to find novel targets for treatment that may better help COPD management and reduce exacerbations. Our studies detailed in **Chapter 3** are of significant relevance. Indeed, **Chapter 3** highlighted that in COPD patients, decreased lung DUOX1 levels are associated with loss of lung function, pulmonary emphysema, and airway remodeling. Therefore, these findings may suggest that lung DUOX1 could be used as a target for screening in individuals for susceptibility to accelerated lung aging or the development of chronic lung disease development, potentially by bronchoscopic brushings (e.g. (20)). In comparison to individuals

with relatively high levels of DUOX1, individuals with relatively low levels may be predisposed to age-related chronic lung disease development, in particular COPD patients. Of note, the studies performed in **Chapter 3** addressed DUOX1 levels in COPD patients who were either ex- or current smokers. While **Chapter 3** did find strong negative correlations of DUOX1 levels with disease severity, highlighting that it may have a contributory role in COPD pathogenesis, we cannot entirely rule out the possibility that loss of airway epithelial DUOX1 may in fact be a consequence of smoking or COPD. As evident from our human COPD patient studies in **Chapter 3**, GOLD IV COPD patients were ex-smokers, whereas GOLD II COPD patients and control subjects were a mix of current and ex-smokers. As such, it is plausible that the observed decrease in lung DUOX1 may in part be a result of smoking instead of COPD, as our mouse studies similarly show that DUOX1 can be suppressed by acrolein (a major aldehyde found in cigarette smoke) and primary airway epithelial cells from healthy controls show DUOX1 loss when exposed to cigarette smoke extract (CSE) (**Chapter 3**). In addition, one study suggested that both cigarette smoke and CSE may temporally and differentially impact DUOX1 expression (21). Whereas 6-weeks exposure to cigarette smoke-induced DUOX1 upregulation in the airways of mice, CSE treatment resulted in DUOX1 downregulation after 12 hours, upregulation at 48 hours, and again downregulation 72 hours after exposure in bronchial epithelial cells. These data demonstrate that DUOX1 downregulation through smoking is complex, and it would therefore be of additional interest to improve our understanding to what extent smoking status may impact DUOX1 levels in (COPD) patients through these potential screening methods. However, it is important to mention that the GOLDIV COPD patients underwent lung volume reduction surgery specifically because of extreme emphysema, which suggests that the observed decreases in lung DUOX1 is to some extent associated with emphysema. Further supporting this finding is the observed DUOX1 loss in the SPC-TNFα mouse model of COPD, which is a model that does not include cigarette smoke but displays chronic inflammation in the lungs similar to that observed in COPD patients. This may suggest that chronic inflammation may induce DUOX1 suppression independent of cigarette smoke.

We have established that there is a negative correlation between lung DUOX1 levels and increasing age in humans (**Chapter 4**). However, many individuals at more advanced age may display this DUOX1 loss to a much lesser extent or may even display increases. Previous studies highlight that DUOX1 is in fact upregulated in allergic asthma (and other allergic diseases such as allergic rhinitis and atopic dermatitis) contributing to Th2 immune responses and allergen sensitization. While evidence seems to favor the decline of allergen

sensitization with age, as children usually outgrow their asthma, the prevalence of allergic asthma in the elderly population with respiratory symptoms is substantial (22). Indeed, asthma in the elderly is increasing due to an aging society and has significant socioeconomic burden to the healthcare system. These patients also have higher medical needs and poorer outcomes than younger asthmatic patients due to frequent exacerbation and hospitalization (23). It is therefore of importance to better recognize and characterize pathology in these patients, to ultimately advance therapeutics for these patients that may prevent or better control disease, and thereby improve quality of life. From our studies, it is conceivable to visualize that patients displaying age-related DUOX1 loss may be at increased susceptibility to develop COPD, whereas patients that do not display DUOX1 loss with increasing age may be at increased risk to develop (allergic) asthma or idiopathic pulmonary fibrosis (IPF), although this is unquestionably more complex since epithelial DUOX1 loss may also promote EMT which has in turn been associated with IPF and COPD. **Chapter 5** demonstrates that in contrast to COPD, DUOX1 may be contributory to IPF pathogenesis, which has also been suggested by others (24), whereas a role for DUOX1 in allergic asthma in the elderly remains less clear. However, elderly patients with allergic asthma that display an age-related loss of lung DUOX1 may experience a decline in allergen sensitization that is commonly observed in the elderly. Whether DUOX1 may contribute to such asthma presentation is unclear from the present studies and remains to be explored. Of additional interest is the effects of smoking in asthma. Cigarette smoke may contribute to asthma development but can also suppress allergic airway inflammation (25), and smoking may also suppress DUOX1 (**Chapter 3**). It would therefore be of interest to address whether smoking may affect allergic asthma in the elderly, potentially through alterations in DUOX1. As mentioned in **Chapter 1**, some elderly patients may also present ACOS (asthma-COPD overlap syndrome) that is recognized by features of both asthma and COPD, and is associated with persistent airway obstruction and accelerated lung function decline (26). In these patients, a mechanism associated with DUOX1 loss may be involved, similar to which is observed in **Chapter 3**, which may contribute to disease pathogenesis. While it is tempting to speculate potential roles for DUOX1 in these diseases, it is important to recognize that much work remains to be done that may better define asthma in the elderly as well as ACOS. Of note, while not addressed in **Chapter 3** or **Chapter 4**, TRPV1, as evidenced in **Chapter 2**, may likely be altered with aging and/or in age-related chronic lung diseases as well. For example, our established pathway involving PAR2-P2YR2-TRPV1-DUOX1 in the airways may underlie

increased airway inflammation as well as risk for exacerbations exacerbations (27, 28), and such exacerbations have been associated with asthma in the elderly (29) and IPF (30).

Addressing the effect of age in disease models that have been associated with aging

Currently, most research utilizing disease models to explore the development or pathology of a disease, or testing drug efficacy, are conducted in animals of relatively young age. Importantly, many of the hallmarks of aging have not yet manifested in mice at that age. As such, there is a need to acknowledge the impact age may have on pathology and studies should be encouraged to incorporate models of aging in future studies of disease models. Therefore, new studies should aim to investigate disease development and/or progression in animals of both young and old(er) age to more fully and comprehensively address the disease model, which would increase translatability into human disease pathology as well. For example, in the case of the NOX enzymes, targeting these NOX enzymes at the appropriate age (e.g. when NOX up/downregulation is observed in humans) is crucially important. Additionally, it should be noted that the development of a disease in murine or other animal models are always accelerated when compared to that same disease in humans. As such, there is an inherently different approach regarding therapeutic intervention (e.g. timing, and duration) in animal models of disease versus human disease. Another consideration therein is that humans are usually treated once disease has manifested, whereas animal studies often use prophylactic treatments or knockout strategies before disease manifestation, that do not translate into human disease management. It is crucial to address important and challenging questions such as these that would allow us to better monitor whether age-associated oxidative damage in e.g. COPD may be either preventable, reversed, or whether further oxidative damage may be halted in the future, and apply therapeutic strategies accordingly. With respect to these therapeutics, there is a need for NOX isoform-specific, as well as cell-type specific NOX targeting by therapeutics, and future research focusing on these aspects should be encouraged. Simultaneously, such strategies would allow us to more specifically determine which NOXes (and localization thereof) are important in lung disease development and/or progression, and whether they are contributing to or may be a consequence of aging or age-related lung disease development.

Along similar lines, and highlighted in **Chapter 5**, models that address age-related diseases should be carefully considered. For example, we utilized PPE as a model to induce emphysema (which for example did not impact DUOX1 expression). Emphysema may also

be achieved by e.g. cigarette smoke (CS) exposure, which is recognized to have different pathogenic mechanisms involved (31) (and can suppress DUOX1 (**Chapter 3**)) and may thus result in differential disease outcomes.

Moreover, a subset of elderly asthmatics have a history of early-onset asthma, and present a clinically different asthma phenotype (e.g. more atopic disease) than elderly asthmatics with late-onset asthma (32). Mouse models addressing asthma in the elderly, centered around either early- or late-onset asthma, would thus require distinct mouse models. For example, mice may be sensitized at young age, and allergic airway disease induction may be addressed at later age through chronic allergen challenge.

Current strategies targeting oxidative stress in chronic lung diseases

The most widely used therapeutic that targets oxidative stress, or oxidative stress-induced damage, and prevent further oxidative damage, is antioxidant supplementation. Additionally, therapeutics that promote endogenous antioxidant responses (e.g. Nrf2 activators) are currently in use. The clinical relevance of such antioxidant agents (such as thiol molecules) are inconsistent and vague. Positive outcomes seem to be related to their mucolytic properties rather than their antioxidant activities (e.g. reduced exacerbations), whereas others have found that they have no benefit or may even have adverse effects (33-35). In light of the current thesis, these findings may not be too surprising, as the primary function of antioxidant supplementation is to prevent further oxidative damage by scavenging ROS, and may thus interfere with redox signaling events that require ROS (**Chapter 2, 3** and **4**) to maintain cellular homeostasis. Antioxidant therapies should be carefully considered, also considering the observation that COPD patients, where antioxidants are often suggested, are at increased risk for the development of lung carcinoma (36).

As mentioned in the introduction, one intriguing finding is the potential link between Nrf2 and NADPH oxidases, in which NOX-derived ROS may act as a redox signal to maintain homeostasis. While the homeostatic capacity progressively declines during aging, it is maintained in long-lived animals to promote healthy aging. In *C. elegans*, ROS generated by dual oxidases (BLI-3 in C. *Elegans*) are important in promoting pathogen infection (37), oxidative stress resistance and longevity through sulfenylation of IRE-1 at the ER, and downstream Nrf1,2,3 activation (SKN-1 in *C. elegans*) upon an oxidative insults (38, 39). Interestingly, this pathway is well conserved in the human liver cancer cell line HepG2 cell line (39). This cell line also expresses DUOX1, and epigenetic silencing of DUOX1 in various human liver cancer cell lines, including HepG2, promoted tumorigenesis, highlighting its

potential role as a tumor suppressor (40). Overall, appropriately controlling NADPH oxidase activity for local and physiological redox signaling to maintain cellular homeostasis, through e.g. Nrf2, might be a therapeutic strategy to promote healthy aging.

It is difficult to predict how such treatment strategies may be applicable to the aging population in absence of lung pathology. Nrf2 activation has been shown to be generally impaired during aging, and potential approaches restoring Nrf2 function, such as those of pharmaceutical, (epi)genetic, and dietary (e.g. caloric restriction) origin may thus have potential benefits (41, 42). With respect to age-related lung pathologies, evidence seems to follow the concept that there is an acceleration in some of the hallmarks of aging. Thus, there may be value in preventing the acceleration of various hallmarks that induce age-related lung pathology through increased endogenous antioxidant responses (Nrf2 activators), thereby promoting 'healthy' aging of the lung, which may potentially increase lifespan (e.g. in mice (43), *Drosophila* (44)). Indeed, therapeutics targeting Nrf2 in age-related chronic lung diseases, such as sulforaphane and quercetin, have shown beneficial effects (e.g. reduced inflammation, ECM remodeling and oxidative stress, improved antibacterial defenses) in both COPD and IPF patients, as well as mouse models thereof (45-50).

DUOX1 as a therapeutic target

It is important to emphasize that the various therapeutics that increase antioxidant defenses are often unspecific, inefficient, and importantly, may also impair beneficial redox signaling. Therefore, another field of therapeutic intervention may be to target ROS-generating molecules, such as NADPH oxidases, e.g. DUOX1 (**Chapter 3** and **4**) directly, or indirectly through other proteins involved in its activation (e.g. TRPV1, **Chapter 2**). In the case of insufficient redox signaling (e.g. **Chapter 3**), interventions could be proposed that may promote NOX activation, whereas during oxidative stress, inhibition strategies would be favorable. For example, targeted approaches to prevent DUOX1 downregulation or enhance its function in the context COPD may be beneficial in managing this devastating disease and would deserve further exploration.
Because of the apparent role of NADPH oxidases in oxidative stress, they have been the target of therapeutic intervention for a while. Two of the older compounds that have been widely used as NOX inhibitors are diphenyleneidonium (DPI) and apocynin (51). DPI is a general inhibitor that shows no selectivity within the NOX isoforms. In contrast, apocynin is not a direct inhibitor of NOXes and probably inhibits by through oxidant scavenging. A major

concern is the specific roles of individual NOX enzymes in various biological and pathological contexts and has rendered these non-selective inhibitors unsatisfactory. As such, various efforts are made for the development of isoform-specific inhibitors of NOX enzymes. For DUOX1, there are currently no specific therapeutic targets as screening efforts do not address efficacy against it (52). In the case of e.g. asthma in the elderly or IPF, it may be favorable to inhibit DUOX1 activity to alleviate (age-related) asthma symptoms and exacerbations, or pulmonary fibrosis, respectively. In this context, TRPV1 could be a likely candidate for intervention as it contributes to DUOX1 activation (**Chapter 2**), but this may have effects independent of its role in DUOX1 activation and is therefore less desirable. However, since this thesis suggest important protective responses for DUOX1 through redox signaling, and DUOX1 is reduced during aging and in COPD, it may also be of interest to develop therapeutic activators of DUOX1 (or indirect, through e.g. TRPV1 activation in airway epithelial cells specifically) that may increase protein function or prevent downregulation. Such methods may include airway epithelial treatment with demethylation agents, as DUOX1 has previously been shown to be hypermethylated in e.g. cancer (53), or IL-13, which induces epithelial DUOX1 (54). However, these interventions are not selective and therefore inadequate. To our knowledge, no specific therapeutic activator of DUOX1, or any NADPH oxidase for that matter, exists. For DUOX1, PKA (protein kinase A)-mediated phosphorylation on Ser955 of DUOX1 has been shown to induce DUOX1-dependent H_2O_2 (55), highlighting that strategies affecting DUOX1 phosphorylation may be of therapeutic interest. Furthermore, membrane lipids (e.g. arachidonic acid) may alter NOX activity through conformational changes to e.g. p47phox (56, 57), suggesting that modifications to membrane lipid profiles could be a potential future strategy to modulate NOX activity. Successful design of such therapeutic activators or inhibitors that are specific for the NADPH oxidase isoforms will also allow us to improve research to better define the specific role(s) of these enzymes in health and disease, including research focusing on aging and age-related lung diseases.

Future directions

The current thesis addressed the role of DUOX1 in lung aging as well as in the development and pathogenesis of age-related lung diseases. Our approaches to elucidate the role for DUOX1 therein utilized both young and aging WT mice, as well as young and aging mice with a global deletion of DUOX1. Although we confirmed our findings in isolated primary airway epithelial cells of young and old mice, highlighting the importance of airway epithelial

DUOX1, DUOX1 is expressed outside of the lung. Therefore, we cannot conclude that our findings implicating the importance of DUOX1 in e.g. senile emphysema development or reparative immune responses, originate exclusively from lung DUOX1, or more specifically, from DUOX1 in the airway epithelium. Therefore, to more specifically elucidate to what extent airway epithelial DUOX1 is responsible for such mechanisms, we would have to conduct our current studies using transgenic mice with specific airway epithelial DUOX1 deletion (e.g. CCSP-directed Cre recombinase-mediated ablation of DUOX1). Our studies addressed mainly bronchial/tracheal expression of DUOX1 in relation to aging and reparative responses. However, given that DUOX1 is also expressed in the alveolar epithelium (alveolar type 2 cells, AT2), *in vitro* studies should be conducted in isolated AT2 cells from young and old WT and *Duox1*-deficient mice, to establish the role for alveolar DUOX1 herein. We could also explore alveolar organoid cultures derived from young versus old mice, or young and old WT versus young and old *Duox1*-deficient mice, to establish alveolar growth, differentiation, and responses to e.g. injurious triggers. Subsequently, alveolar organoids could be used to address whether DUOX1 during aging, through its potential effect on alveolar epithelial regeneration and differentiation, may be significant for alveolar remodeling, lung injury and age-related emphysema development. Interestingly, the interleukin IL-13 was recently found to impair division and differentiation of alveolar epithelial cells following injury (58), indicating that IL-13 impacts self-renewal and differentiation properties of alveolar type 2 cells. Since we observed age-related DUOX1 loss with concomitant impaired reparative responses (including IL-13 release), it would warrant further studies using these alveolosphere models (59) to shed light on how DUOX1 may affect alveolar biology.

Moreover, the current thesis was not successful in elucidating how airway epithelial DUOX1 is suppressed with age, as promoter hypermethylation did not seem to be involved (**Chapter 4**), unlike a role of promoter hypermethylation in human DUOX1 (53) and may also speak to differential regulation of DUOX1 in mouse versus human. Other mechanisms that may be involved, but not currently addressed in this thesis, may be the age-related suppression of DUOXA1, the required maturation factor for DUOX1 (60), Furthermore, DUOX1 expression may be regulated by Th2 cytokines IL-4 or IL-13 (54) (potentially related to STAT6 activation (61)), and these mechanisms may also be impacted with age. Future studies could be conducted where the lungs of young and old WT or *Duox1*-deficient C57BL/6J mice are analyzed for cysteine proteomics, or other proteomic approaches, to examine which (redox-sensitive) proteins targeted by DUOX1 are involved, and to explore the impact of DUOX1 on the oxidation status of these protein targets (e.g. reversible or irreversible cysteine oxidation).

Furthermore, our finding in **Chapter 4** where treatment of primary epithelial cells isolated from old mice with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (aza) restored innate reparative responses deserves further attention. Follow-up studies should be performed *in vivo*, e.g. by using acute HDM challenge after treatment of young and old mice with aza and determine whether this similarly restores innate cytokine responses. Subsequently, we could perform (single-cell) DNA methylome sequencing studies on lung from aging mice, or epithelial cells from aging mice, following treatment with aza, to determine how the methylome is affected with age and which factors (including perhaps DUOX1) are involved in age-related impaired responses (and may be involved in restoration of such responses following aza treatment). Furthermore, we did not address whether *Duox1*-deficient mice, in comparison to WT mice, may display decreased longevity, as is the case for the Bli-3-deficiency in *C. elegans*. Addressing such a question would increase our knowledge as it may indicate that DUOX1 may promote oxidative stress resistance, and thus longevity.

As **Chapter 5** indicates, C57BL/6NJ mice were used in our age-related lung disease models, which do not display age-related *Duox1* loss. As such, our results of age-related *Duox1* loss in C57BL/6J mice may potentially be related to the absence of the *NNT* gene and may suggest that the altered metabolic status due to NNT deficiency may regulate DUOX1. Of note, alterations in metabolic status due to NNT deficiency may potentially induce deregulated nutrient sensing, e.g. by affecting the insulin and IGF-1 (Insulin-like growth factor 1) signaling pathway which is also involved in aging (62). Furthermore, the mitochondrial dysfunction arising through NNT deficiency would likely affect cellular bioenergetics, cell death, and inflammation, which are also known to contribute to aging. However, while such perturbations of metabolic status due to NNT deficiency are intriguing, this is outside the scope of the current thesis. As to how such metabolic alterations may affect DUOX1 levels is currently unknown, but we could further expand on this by addressing the link between NNT function and lung DUOX1 levels, by modulating its activity or by e.g. siRNA approaches, which may also help us elucidate how age-related DUOX1 loss occurs. Furthermore, future studies should also aim to address the age-related chronic lung disease models (**Chapter 5**) in mice of the C57BL/6J strain, where age-related DUOX1 loss was observed. Since these mice more accurately reflect age-related DUOX1 loss in humans, studies performed in these mice may allow us to better elucidate the role for airway epithelial DUOX1 in chronic lung disease development during aging.

Conclusion

Reactive oxygen species (ROS) play an important role in aging. Understanding the underlying age-related redox modifications, that are commonly associated with oxidative stress during aging, would allow us to develop new therapeutic targets or identify biomarkers, to better control these age-related redox alterations, which may improve healthy aging or disease management. Importantly, reactive oxygen species (ROS) have been shown to control cellular homeostasis, e.g. through its contribution in wide-ranging processes (e.g. growth factor signaling, immune responses) through redox signaling, of which the NADPH oxidases (NOX) are a prominent example. However, the role of ROS generated by these NOXes has been largely overlooked in aging, as mitochondria are commonly considered the predominant source of ROS relevant for the aging process. The work in this thesis demonstrates that aging disrupts redox regulation in the lung which affects lung aging and age-related chronic lung disease development. More specifically, we show novel evidence highlighting that the airway epithelial NOX dual oxidase 1, or DUOX1, governs airway epithelial homeostasis during aging. With increasing age, lung DUOX1 levels gradually decrease, and associated DUOX1-mediated reparative responses to injury, as highlighted in this thesis, are dramatically lost with age. Furthermore, the age-related loss of DUOX1 during aging may predispose for senile emphysema development, and may also contribute to the development of COPD, a disease of accelerated lung aging. On the other hand, preliminary evidence in this thesis suggests that DUOX1 may contribute to late-onset allergic asthma and IPF. This thesis discussed that lung, and specifically airway epithelial DUOX1 loss, could be an indicator of increased susceptibility to accelerated aging and age-related lung disease development. Screening for lung DUOX1 levels in individuals of more advanced age, as well as in COPD patients, and restoring airway epithelial DUOX1 function, although such treatment options currently do not exist, would be of interest to tailor more personalized treatment. In conclusion, and in contrast to the free radical theory of aging, the NOX enzyme DUOX1 is a redox protein involved in maintaining lung homeostasis. Alterations of such NOX enzymes in the lung should be considered in the clinic as they may underlie aging, and susceptibility to age-related disease development, and may in the future provide more tailored treatment options that may contribute to healthy aging, improved clinical symptoms and improved quality of life.

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Summary

Samenvatting

Summary

The results described in this thesis highlight that during aging, loss of airway epithelial dual oxidase 1, or DUOX1, is associated with impaired reparative capacity of the lungs and accelerates senile emphysema development, and additionally highlight that DUOX1 loss may increase susceptibility to age-related chronic lung disease development, importantly COPD. **The general aim of this thesis was to explore whether DUOX1 function is altered during aging, and whether such alterations may contribute to age-related lung diseases.** The thesis introduction and overall aim of this thesis are described in **Chapter 1**. **Chapter 2** examined the importance of the Ca^{2+} channel TRPV1 in DUOX1-mediated innate airway responses to allergen proteases or other injurious triggers that are crucial for airway epithelial repair. Our findings implicate that TRPV1 is critically involved in DUOX1- mediated responses, and may lead to new avenues for future therapeutic interventions in e.g. asthma. In **Chapter 3**, we established that in COPD, there is a loss of airway epithelial DUOX1, which is associated with worsened lung function, emphysema, and airway remodeling, each of which are critical features in COPD pathology. In **Chapter 4** we determined the impact of aging on the various NADPH oxidases in the lung. We observed that DUOX1 is the NOX isoform that is dramatically lost with increasing age. We furthermore show that in mice and in isolated mouse tracheal epithelial cells, age-related DUOX1 loss is responsible for diminished innate epithelial injury responses, and that these responses could be rescued by DNA demethylation. Lastly, we provide evidence that the loss of DUOX1 accelerates age-related senile emphysema development. While the studies in **Chapter 5** are preliminary, they explored the effect of aging, and DUOX1-deficiency during aging, on the pathogenesis of chronic lung diseases, including asthma (in the elderly), COPD, and IPF. While aging did not impact on overall COPD or IPF pathogenesis, it was associated with impaired inflammation, airway remodeling, and mucous metaplasia in a model of allergic airways disease. While DUOX1 did not further impact on elastase-induced experimental emphysema during aging, it did contribute to type 2 and type 17 immune responses in allergic airways disease. Moreover, DUOX1 during aging contributed to fibrosis, based on bleomycin- induced hydroxyproline levels. In **Chapter 6**, the main findings are summarized and elaborately discussed including the implications and limitations of our findings. In conclusion, this thesis contributes to a better understanding of how the redox environment, with a specific focus on the NADPH oxidase DUOX1, is involved in aging and age-related lung diseases.

The results herein demonstrate the importance of DUOX1 during aging, as the loss of airway epithelial DUOX1 during aging is associated with senile emphysema development and airway epithelial loss of reparative capacity. Furthermore, in the age-related chronic lung disease COPD, observed loss of small airway DUOX1 is associated with loss of lung function, emphysema, and airway remodeling. Furthermore, this thesis increases the knowledge on how the redox environment is altered during aging, and in age-related chronic lung diseases, through these novel findings indicating the importance of airway epithelial DUOX1. Our results moreover indicate the need for research to acknowledge the importance of aging in age-related lung disease development, and should encourage futures studies to incorporate age in studies that may address such questions. Furthermore, our results also indicate that lung DUOX1 levels could be targeted through e.g. inhalation strategies in the aging population, to achieve more tailored and personalized treatment.

Samenvatting

De resultaten die in dit proefschrift zijn beschreven, benadrukken dat het verlies van dual oxidase 1, of DUOX1, in het luchtwegepitheel tijdens veroudering geassocieerd is met een verminderde herstelcapaciteit van de longen en een versnelling in de ontwikkeling van seniel emfyseem. Daarnaast benadrukken deze resultaten dat verlies van DUOX1 de gevoeligheid voor leeftijdsgerelateerde ontwikkeling van chronische longziekten, met name COPD, kan verhogen. **Het algemene doel van dit proefschrift was om te onderzoeken of DUOX1-functie tijdens veroudering verandert, en of dergelijke veranderingen kunnen bijdragen aan leeftijdsgebonden longziekten.** De introductie van dit proefschrift en het algemene doel van dit proefschrift worden verder toegelicht in **Hoofdstuk 1**. **Hoofdstuk 2** onderzocht het belang van het calcium kanaal TRPV1 in DUOX1-gemedieerde aangeboren luchtwegreacties op allergeenproteasen of andere schadelijke triggers die cruciaal zijn voor het herstel van het epitheel van de luchtwegen. Onze bevindingen impliceren dat TRPV1 kritisch betrokken is in door DUOX1 gemedieerde reacties, en deze bevindingen kunnen mogelijk leiden tot nieuwe wegen voor toekomstige therapeutische interventies in bijvoorbeeld astma. In **Hoofdstuk 3** hebben we vastgesteld dat er bij COPD sprake is van verlies van DUOX1 in het luchtwegepitheel, wat geassocieerd is met een verslechterde longfunctie, emfyseem en luchtwegremodellering, dat stuk voor stuk cruciale kenmerken zijn in COPD-pathologie. In **Hoofdstuk 4** hebben we de impact van veroudering op de verschillende NADPH-oxidases in de long bepaald. We hebben vastgesteld dat DUOX1 de

NOX-isovorm is die dramatisch verloren gaat in de long tijdens veroudering. We laten verder zien dat bij muizen en in geïsoleerde muis tracheale epitheelcellen het leeftijdsgebonden DUOX1-verlies verantwoordelijk is voor verminderde aangeboren epitheliale reacties, en dat deze reacties kunnen worden gered door DNA-demethylering. Ten slotte leveren we bewijs dat het verlies van DUOX1 de ontwikkeling van leeftijdsgebonden seniel emfyseem versnelt. Hoewel de studies in **Hoofdstuk 5** preliminair zijn, was het doel van deze studies om het effect van veroudering en DUOX1-deficiëntie tijdens veroudering op de pathogenese van chronische longziekten, waaronder astma (bij ouderen), COPD en IPF, te bestuderen. Hoewel veroudering geen invloed had op de algehele pathogenese van COPD of IPF in muizen, was veroudering wel geassocieerd met verminderde ontsteking, en remodellering van de luchtwegen en mucus metaplasie in een model van allergische luchtwegaandoeningen. Hoewel DUOX1 geen verdere invloed had op door elastase geïnduceerd experimenteel emfyseem tijdens veroudering, droeg het wel bij aan type 2 en type 17 immuunresponsen bij allergische luchtwegaandoeningen. Bovendien droeg DUOX1 tijdens veroudering bij aan fibrose, gebaseerd op bleomycine-geïnduceerde hydroxyproline levels. In **Hoofdstuk 6** worden de belangrijkste bevindingen samengevat en uitvoerig besproken, inclusief de implicaties en beperkingen van onze bevindingen. Concluderend draagt dit proefschrift bij aan een beter begrip van hoe de redoxomgeving, met een specifieke focus op de NADPH oxidase DUOX1, betrokken is bij veroudering en leeftijdsgebonden longziekten. De resultaten hierin tonen het belang van DUOX1 tijdens veroudering aan, aangezien het verlies van luchtwegepitheel DUOX1 tijdens veroudering geassocieerd is met de ontwikkeling van seniel emfyseem en het verlies van herstellend vermogen van het luchtwegepitheel. Bovendien wordt bij de leeftijdsgebonden chronische longziekte COPD het waargenomen verlies van DUOX1 in de kleine luchtwegen geassocieerd met verlies van longfunctie, emfyseem en remodellering van de luchtwegen. Bovendien vergroot dit proefschrift de kennis over hoe de redox-omgeving verandert tijdens veroudering en bij leeftijdsgerelateerde chronische longziekten. Onze resultaten geven bovendien aan dat er drang is naar onderzoek die het belang van veroudering bij de ontwikkeling van leeftijdsgerelateerde longziekten erkent, en toekomstige studies moeten dan ook worden aanmoedigd om leeftijd te includeren in studies die dergelijke vragen willen beantwoorden. Bovendien geven onze resultaten ook aan dat DUOX1-niveaus in de long kunnen worden gescreend alsook getarget (bijvoorbeeld door inhalatiestrategieën) bij de vergrijzende bevolking, om zo tot een meer op maat gemaakte en gepersonaliseerde behandeling te bereiken.

Impact

Background

The elderly population is growing rapidly worldwide, and will reach previously unprecedented levels, largely through decreased fertility and increased life expectancy (1). Indeed, the number of individuals aged 65 or older is predicted to grow from about 524 million in 2010 to nearly 1.5 billion in 2050. At the beginning of the 20th century, major health threats were infectious and parasitic diseases (e.g. pneumonia, influenza, tuberculosis), also termed communicable diseases (2). However, the advances in modern medicine over the last 200 years have greatly shifted the diseases causing major mortality, and at present, non-communicable diseases (e.g. cancer, heart disease, diabetes, chronic respiratory disease) are the leading cause of mortality. These non-communicable diseases more frequently affect adults and the elderly population, and impose the greatest (economic) burden on global health (3). Thus, instead of healthy aging of the elderly population, the aging population currently experiences many years of living with these disabilities. It is estimated that about 100.000 people die from age-related causes every day (4), of which pulmonary diseases, such as acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (IPF), account for roughly 7.3 million deaths, which equals 14% of all deaths, worldwide yearly, and largely affects the elderly population (5). In addition, the elderly population is the most at-risk group affected by aforementioned communicable diseases through increased susceptibility to (respiratory) infections, a relevant issue that is made very clear and relevant with the COVID-19 pandemic (6). As the elderly population is increasing, novel therapeutics are required for these non-communicable diseases that improve quality of life and may result in healthy aging (growing old without age-related disabilities), which would also significantly reduce economic and healthcare burden. However, to establish such therapeutics for pulmonary diseases commonly associated with aging, it is critical to understand the fundamental causes and the underlying mechanisms of lung aging and age-related chronic lung diseases.

Research

Oxidants, also termed free radicals or reactive oxygen species (ROS), are compounds generated by metabolism of oxygen, and are found within the environment due to air pollution but are also produced within our bodies due to oxygen metabolism. These ROS may cause harm to cells if their levels become too high (oxidative stress) (7,8). To protect against oxidative stress, our body is equipped with defense mechanisms (antioxidants) that may keep these oxidants at levels where they are not damaging by scavenging them. A

theory regarding ROS and aging was already described in the 1950's, where it was proposed that the lifelong accumulation of damage due to ROS may cause aging and age-related chronic disease development (9). Furthermore, during aging, there is an apparent imbalance between oxidants and antioxidants in favor of oxidants, resulting in oxidative stress, and this has been shown in various age-related lung diseases, such as COPD (7,10). At (relatively low) levels, these oxidants can actually perform important signaling functions, also termed redox signaling (11). One well-recognized function of ROS is the involvement of ROS in host defense, as these species are essential for the removal of pathogens such as bacteria, or cell debris (termed phagocytosis). The phagocyte (cells that perform phagocytosis) NADPH oxidase (NOX), or NOX2, is widely appreciated as a critical component of antimicrobial host defense through the production of ROS (12). Additional NOX enzymes have been discovered in non-phagocytic cells that appear to possess diverse redox signaling functions such as the involvement in cell proliferation, differentiation, and in regulation of gene expression. Several of these NOX enzymes are also expressed in the lungs, where they participate in such redox signaling events following bacterial or viral infection or environmental stress. Specifically, the airway epithelial cells express the NOX enzyme dual oxidase 1, or DUOX1. Upon activation, DUOX1 functions through the production of regulated levels of hydrogen peroxide (H2O2), that has been shown to have important roles for epithelial repair processes following injury (13,14). As such, DUOX1 is be an enzyme that, rather than causing damage to our cells through the production of oxidants, actually has important protective functions. As such, the important contribution of NOX enzymes in e.g. epithelial and inflammatory cell signaling as well as host defense may explain why antioxidant supplementation does not improve lifespan, and is often ineffective during disease presentation (15,16), as scavenging of these ROS species generated by NOX enzymes that are important in various biological functions could be detrimental. In light of this concept, the aim of this thesis was to explore whether DUOX1 function is altered during aging, and whether such alterations may contribute to age-related lung diseases.

Interestingly, we observed that DUOX1 expression and activity was dramatically lost with age in the lungs (or more specifically, airway epithelial cells) of mice as well as in humans, and that this resulted in impaired immune responses and decreased repair capacity of lung epithelial cells in response to injurious challenges often encountered in the environment. Furthermore, we observed that lifelong absence of DUOX1 in mice enhanced some aspects of lung aging, as these mice developed accelerated age-related degenerative changes in the lungs, with enlargement of the alveolar walls, also termed senile emphysema. Furthermore,

we observed that in the age-related chronic lung disease COPD, DUOX1 protein levels are also suppressed, which was observed in both human lung tissues from COPD patients and in mouse models of COPD. The level of lung DUOX1 positively correlated with lung function in these COPD patients, indicating that low levels of DUOX1 are associated with worse lung function. Furthermore, we observed that lifelong absence of DUOX1 in mice worsened experimental emphysema development in a mouse model, which is characterized by alveolar enlargement through alveolar wall destruction, a major hallmark of COPD. Additionally, we found that DUOX1 suppression in COPD may be in part explained by smoking, which is a major risk factor for chronic lung disease development. Because of our observation of decreased DUOX1 during aging, we also sought to explore how aging and DUOX1 impact on other age-associated chronic lung diseases such as asthma in the elderly and idiopathic pulmonary fibrosis. Although results from these studies are preliminary, we observed that aging does not significantly affect the pathogenesis of experimentally-induced pulmonary fibrosis using administration of the chemotherapeutic agent bleomycin. Moreover, we found that DUOX1 is actually enhanced during experimental pulmonary fibrosis, and that lifelong absence of DUOX1 appeared to minimize some critical features of IPF pathogenesis. We also observed that aging leads to decreased features of experimentally-induced allergic asthma in mice induced by repeated exposure to house dust mite allergen, with less inflammation, remodeling of the airways, and mucous overproduction. While DUOX1 strongly contributed to these features of allergic asthma in young mice, it contributed less strongly to allergic inflammation in the elderly mice and appeared to alter the type of inflammation observed.

The findings obtained from the research conducted in this thesis are of scientific relevance, as knowledge gained from the current studies may contribute to the scientific field of lung aging and age-related chronic lung disease. Furthermore, they may also contribute to social sectors such as healthcare and pharmaceutical companies, and may support potential solutions to social challenges such as the worldwide aging population. First of all, our findings challenge the the overall perception that ROS contribute to aging and age-related lung disease, and may additionally warrant caution for antioxidant supplementation, which is thought to be beneficial during aging and in age-related diseases. As previously mentioned, people should be informed about the potential detrimental effects of antioxidants (as they may prevent beneficial signaling functions of NOX enzymes such as DUOX1 as described in this thesis) during aging and in chronic diseases, and may even be discouraged from such supplementation strategies. Moreover, our research may open up avenues to try

and unravel how modulation of DUOX1 during aging may limit and/or delay lung aging and age-related lung disease development. Future studies could explore if restoring DUOX1 (meaning restoring DUOX1 levels/activity) within airway epithelium of elderly mice (or human subjects) could potentially protect against the loss of repair responses and senile emphysema as a result of aging, or whether this may protect against the development of features of emphysema/COPD in mouse models of COPD. Although this thesis focused on the importance of the NOX enzyme DUOX1 during aging, our work may also provoke research to further address the importance of how age-related changes in the redox balance may contribute to (lung) aging and age-related disease development. For example, the question of how aging may affect other NOX enzymes, or other cellular sources of ROS in the cell, may be addressed in future studies. Vice versa, whether these ROS-generating sources may be involved in aging, and age-related pathology, either by progressing or slowing aging, are questions that are just as important. To this end, we have prepared a concise literature review/perspective, that will focus on these sources of ROS and will highlight the scientific evidence for these sources in lung aging and age-related lung pathologies. Additionally, this perspective will also aim to re-emphasize the importance of addressing these critical questions mentioned above. Our results may also be of relevance to healthcare, pharmaceutical companies, and to the critical issue of a worldwide aging population with reduced quality of life due to disabling conditions. In the future, lung DUOX1 levels (or hypermethylation status of the DUOX1 promoter) could be envisioned as a screening tool in individuals at middle age (~45-65 years of age) to monitor for susceptibility to age-related chronic lung disease development. In individuals where relatively low levels of airway epithelial DUOX1 would be observed (in healthy individuals of middle age, or e.g. COPD patients), strategies that would restore lung DUOX1 function/expression would be of interest to tailor more personalized treatment. These strategies may ultimately increase quality of life in the elderly by concomitantly reducing disabling conditions and improving lung health, thereby contributing to healthy aging. Ultimately, the goal is to improve lung health in the elderly, as lung health is associated with good health (such as increased metabolic rate, activity, and physical performance), which will also significantly reduce the healthcare costs (17). However, treatment options targeting DUOX1 in the elderly currently do not exist and additionally, these strategies would have to be non-invasive. As such, to fully realize such strategies, therapeutics have to be developed to allow for such non-invasive treatment, and would likely involve inhalation strategies.

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List of publications

Presentations

Grants/Awards

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Caspar Schiffers, Christopher M. Dustin, Lennart K. Lundblad, Milena Hristova, Aida Habibovic, David J. Seward, Nirav Daphtary, Minara Aliyeva, Emiel F.M. Wouters, Niki L. Reynaert and Albert van der Vliet. *Downregulation of DUOX1 function contributes to aging-related impairment of innate airway injury responses and accelerated senile emphysema.* Accepted in AJP: Lung Cellular and Molecular Physiology. 2021.

Cheryl van de Wetering, Evan Elko, Marijn Berg, **Caspar Schiffers**, Maarten van den Berge, Martijn Nawijn, Emiel F.M. Wouters, Yvonne M.W. Janssen-Heininger and Niki L. Reynaert. *Glutathione S-transferases and their implications in the lung diseases asthma and Chronic Obstructive Pulmonary Disease: early life susceptibility?* Accepted in Redox Biology. 2021.

Christopher M. Dustin, Aida Habibovic, Milena Hristova, **Caspar Schiffers**, Miao-Chong Joy Lin, Robert A. Bauer, David E. Heppner, Nirav Daphtary, Minara Aliyeva, and Albert van der Vliet. *Redox-dependent activation of Src kinase mediates epithelial IL-33 production and signaling during acute airway allergen challenge.* Accepted in the Journal of Immunology. 2021.

Caspar Schiffers, Cheryl van de Wetering, Robert A. Bauer, Aida Habibovic, Milena Hristova, Christopher M. Dustin, Sara Lambrichts, Emiel F.M. Wouters, Niki L. Reynaert and Albert van der Vliet. *Downregulation of Epithelial Dual Oxidase 1 (DUOX1) in Chronic Obstructive Pulmonary Disease Contributes to Disease Pathogenesis.* JCI Insight. 2020.

Caspar Schiffers, Milena Hristova, Aida Habibovic, Christopher M. Dustin, Karamatullah Danyal, Emiel F.M. Wouters, Niki L. Reynaert and Albert van *der Vliet*. *The transient receptor potential channel vanilloid 1 (TRPV1) is critical in innate airway epithelial responses to protease allergens*. Am J Respir Cell Mol Biol. 2020 Mar 17.

Andrew C Little, Milena Hristova, Loes van Lith, **Caspar Schiffers**, Christopher M Dustin, Aida Habibovic, Karamatullah Danyal, David E Heppner, Miao-Chong J Lin, Jos van der Velden, Yvonne M Janssen-Heininger, Albert van der Vliet. *Dysregulated Redox Regulation Contributes to Nuclear EGFR Localization and Pathogenicity in Lung Cancer. Sci Rep. 2019 Mar 19;9(1):4844.*

Christoper M. Dustin, Milena Hristova, **Caspar Schiffers**, Albert van der Vliet. *Proteomic Methods to Evaluate NOX-Mediated Redox Signaling. Methods Mol Biol. 2019;1982:497-515.*

Submitted/in preparation

Caspar Schiffers, Emiel F.M. Wouters, Niki L. Reynaert, Albert van der Vliet. *Redox Stress in Age-related Chronic Lung Disease: Roles of NOX Enzymes and Implications for Antioxidant Strategies.* In preparation.

Conference abstracts and presentations

Caspar Schiffers, Milena Hristova, Christopher M. Dustin, Aida Habibovic, Lennart K.A. Lundblad, David J. Seward, Emiel F.M. Wouters, Niki L. Reynaert and Albert van der Vliet. *Downregulation of DUOX1 contributes to aging-related impairment of innate airway injury responses and accelerated senile emphysema development. SFRBM Conference 202*0. Poster Presentation

Caspar Schiffers,, *Cheryl van de Wetering, Christopher M. Dustin, Milena Hristova, Aida Habibovic, Emiel F.M. Wouters, Niki L. Reynaert and Albert van der Vliet. Downregulation of Epithelial Dual Oxidase 1 (DUOX1) in Chronic Obstructive Pulmonary Disease Contributes to Disease Pathogenesis. SFRBM Conference 2019, Las Vegas, NV, USA.* Poster presentation

Caspar Schiffers, Milena Hristova, Christopher M. Dustin, Aida Habibovic, Lennart K.A. Lundblad, David J. Seward, Niki L. Reynaert, Emiel F.M. Wouters and Albert van der Vliet. *Downregulation of Lung DUOX1 During Aging Attenuates Innate Epithelial Injury Responses and May Predispose Development of COPD*. American Thoracic Society International Conference 2019, Dallas, TX, USA. Oral presentation (Abstract was nominated)

Caspar Schiffers, Christopher M. Dustin, Milena Hristova, Aida Habibovic, Niki Reynaert, Emiel F.M. Wouters, and Albert van der Vliet*. The Transient Receptor Potential Vanilloid 1 (TRPV1) channel as a critical factor in allergen-induced innate immune responses in the airway epithelium* Society for Free Radical Biology and Medicine, Chicago, IL, USA. Oral presentation (Abstract was nominated)

Caspar Schiffers, Christopher M. Dustin, Lennart Lundblad, Milena Hristova, Aida Habibovic, Niki Reynaert, Emiel F.M. Wouters, David J. Seward, and Albert van der Vliet*. Downregulation of lung DUOX1 during aging attenuates innate epithelial injury responses and may promote development of pulmonary emphysema.* Gordon Research Conference, Federation of American Societies For Experimental Biology (FASEB), Olean, NY, USA. Oral presentation (Abstract was nominated)

Caspar Schiffers, Christopher M. Dustin, Lennart Lundblad, Milena Hristova, Aida Habibovic, Niki Reynaert, Emiel F.M. Wouters, David J. Seward, and Albert van der Vliet*. Downregulation of lung DUOX1 during aging attenuates innate epithelial injury responses and may promote development of pulmonary emphysema.* Gordon Research Conference, NOX Family NADPH Oxidases, Les Diablerets, Switzerland. Poster Presentation (Albert van der Vliet presented)

Caspar Schiffers, Christopher M. Dustin, Lennart Lundblad, Milena Hristova, Aida Habibovic, Niki Reynaert, Emiel F.M. Wouters, David J. Seward, and Albert van der Vliet*. Progressive loss of dual oxidase 1 (DUOX1) contributes to impaired airway epithelial wound*

responses in the aging lung. Society for Free Radical Biology and Medicine, Baltimore, MD, USA. Oral presentation (Abstract was nominated)

Caspar Schiffers, Emiel Wouters, Ramon Langen, Judith Ceelen, Albert van der Vliet and Niki Reynaert. *Protective oxidants in COPD: is DUOX1 a crucial player?* NUTRIM Symposium, Maastricht University, Maastricht, the Netherlands. Poster Presentation

Caspar Schiffers, Niki Reynaert, Emiel F.M. Wouters and Albert van der Vliet. *Protective oxidants in COPD: is DUOX1 a crucial player?* Wetenschapsdag NRS 8th Young Investigator Symposium, Netherlands Respiratory Society, Amsterdam, the Netherlands. Oral Presentation

Caspar Schiffers, Milena Hristova, Aida Habibovic and Albert van der Vliet. *Unraveling the role of the Transient Receptor Potential Cation Channel Subfamily V Member 1 (TRPV1) in airway epithelial signaling in asthma.* De Week van de Longen 2016, Ermelo, the Netherlands. Poster Presentation

Caspar Schiffers, Emiel Wouters, Ramon Langen, Judith Ceelen, Albert van der Vliet and Niki Reynaert. *Protective oxidants in COPD: is DUOX1 a crucial player?* Wetenschapsdag Longziekten, Maastricht University, Maastricht, the Netherlands. Oral Presentation

Grants/awards

2020	**Young Investigator Travel Grant**, from the Netherlands Respiratory Society (NRS), for attendance of the GRC 2020 conference (GRC was cancelled due to COVID-19).
2019	**Young Investigator Award**, at the SFRBM 2019 Conference
2018	**Young Investigator Award**, at the SFRBM 2018 Conference
2018	**Short Talk/Abstract Award**, at the FASEB 2018 Conference on The Lung Epithelium in Health and Disease
2014	**Freemover grant**, to support Senior Practical Training at the University of Vermont, Burlington, Vermont, USA, from Maastricht University

Curriculum Vitae

Caspar Hubertus Joseph Schiffers was born on the 15th of June 1991 in Kerkrade, the Netherlands. Following graduation in high school (VWO) in 2010, he studied the bachelor Biomedical Sciences at Maastricht University. After graduating in 2013, he started the Master program Biomedical Sciences with a specialization in Clinical Molecular Sciences (CMS) at the Translational University of Limburg (tUL).

During his Master, he first worked on a project elucidating the role of antibiotics on the composition of the gut microbiota in relation to insulin sensitivity. For his Master thesis, he studied the effects of the calcium channel TRPV1 in the context of redox-dependent innate airway epithelial responses to injury at the University of Vermont, for which he received a travel grant. After graduation in 2015, he started as a PhD student at the Department of Respiratory Medicine and School of Nutrition and Translational Research in Metabolism (NUTRIM) at Maastricht University Medical Center+, in collaboration with the department of Pathology and Laboratory Medicine at the University of Vermont. His PhD project aimed to address the role of the NADPH oxidase DUOX1 during aging and in chronic lung diseases, and conducted the studies presented in this thesis under supervision of Prof Dr. Albert van der Vliet, Prof. Dr. Emiel Wouters and Dr. Niki Reynaert. He also supervised several students, attended several courses and international conferences, and received several awards for his presentations. Caspar will continue his scientific career as a Research Scientist for the LEAD study (Lung, heart, social, body) at the Ludwig Boltzmann Institute in Vienna, Austria.