UC Davis

UC Davis Previously Published Works

Title

Role of Dual Oxidases in Ventilator-induced Lung Injury.

Permalink

https://escholarship.org/uc/item/57h3h3jz

Journal

American Journal of Respiratory Cell and Molecular Biology, 64(2)

ISSN

1044-1549

Authors

Cagle, Laura
Linderholm, Angela
Franzi, Lisa
et al.

Publication Date

2021-02-01

DOI

10.1165/rcmb.2020-0197oc

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Check for updates

ORIGINAL RESEARCH

Role of Dual Oxidases in Ventilator-induced Lung Injury

Laura Cagle[1], Angela Linderholm[1,2,3], Lisa Franzi[1], Sabrina Hoehne[1], Jerold Last[1], Nicholas Kenyon[1,2,3], Beate Illek[4], and Richart Harper[1,2,3]

[1]University of California Lung Center, University of California, Davis, Davis, California; [2]Division of Pulmonary, Critical Care, and Sleep Medicine, University of California, Davis, Sacramento, California; [3]Department of Veterans Affairs, Northern California Health Care System, Mather, California; and [4]UCSF Benioff Children's Hospital, Children's Hospital Oakland Research Institute, Oakland, California

ORCID IDs: 0000-0001-9926-7183 (L.C.); 0000-0003-4181-277X (L.F.); 0000-0002-0884-6268 (S.H.); 0000-0001-8839-4400 (N.K.); 0000-0001-8097-6465 (R.H.).

Abstract

Positive-pressure ventilation results in ventilator-induced lung injury, and few therapeutic modalities have been successful at limiting the degree of injury to the lungs. Understanding the primary drivers of ventilator-induced lung injury will aid in the development of specific treatments to ameliorate the progression of this syndrome. There are conflicting data for the role of neutrophils in acute respiratory distress syndrome pathogenesis. Here, we specifically examined the importance of neutrophils as a primary driver of ventilator-induced lung injury in a mouse model known to have impaired ability to recruit neutrophils in previous models of inflammation. We exposed *Duoxa*$^{+/+}$ and *Duoxa*$^{-/-}$ mice to low– or high–tidal volume ventilation with or without positive end-expiratory pressure (PEEP) and recruitment maneuvers for 4 hours. Absolute neutrophils in BAL fluid were significantly reduced in *Duoxa*$^{-/-}$ mice compared with *Duoxa*$^{+/+}$ mice (6.7 cells/μl; 16.4 cells/μl; $P = 0.003$), consistent with our hypothesis that neutrophil translocation across the capillary endothelium is reduced in the absence of DUOX1 or DUOX2 in response to ventilator-induced lung injury. Reduced lung neutrophilia was not associated with a reduction in overall lung injury in this study, suggesting that neutrophils do not play an important role in early features of acute lung injury. Surprisingly, *Duoxa*$^{-/-}$ mice exhibited significant hypoxemia, as measured by the arterial oxygen tension/fraction of inspired oxygen ratio and arterial oxygen content, which was out of proportion with that seen in the *Duoxa*$^{+/+}$ mice (141, 257, $P = 0.012$). These findings suggest a role for dual oxidases to limit physiologic impairment during early ventilator-induced lung injury.

Keywords: positive-pressure ventilation; dual oxidases; acute lung injury; NADPH oxidases

Positive-pressure ventilation is a life-saving modality that has been used extensively for decades, yet ventilator-induced lung injury (VILI) occurs commonly, and few therapeutic modalities have resulted in improved outcomes. The ARDSNet trial focused on the use of protective low–tidal volume (LTV) ventilation and lowered the mortality rate by 22% by using lower ventilator settings, yet injury from positive-pressure ventilation has persisted, and no further improvements in mortality have occurred in more than two decades (1, 2).

An association between neutrophil recruitment into the lungs and disease severity has been identified in patients with acute respiratory distress syndrome (ARDS), yet the role of neutrophils as effector cells or simply markers of disease severity remains to be determined (3–7). Select studies have determined that the elimination of airway neutrophilia is associated with reduced lung injury, yet increased airway neutrophilia is correlated with higher airway neutrophil counts. Grommes and Soehnlein evaluated the role of neutrophils in acute lung injury and concluded that the presence of neutrophils within the airways was not directly injurious, but that the neutrophil degranulation caused local injury, suggesting that regulation of neutrophil activity may be a possible therapeutic approach (3).

To address the association between airway injury and airway neutrophilia, we

(Received in original form May 16, 2020; accepted in final form October 28, 2020)

Supported by U.S. National Institutes of Health grants R01 105573, T32 HL007013, and T32 OD 011147, the Department of Veterans Affairs, and Cystic Fibrosis Foundation (ILLEK15P0).

Author Contributions: L.C. designed and performed the experiments, analyzed the data, and wrote the manuscript. A.L., L.F., performed the experiments, provided critical evaluation of the data, and edited the manuscript. S.H. performed the experiments and edited the manuscript. B.I. designed experiments, provided critical evaluation of the data, and edited the manuscript. J.L. provided critical evaluation of the data and edited the manuscript. N.K and R.H. conceptualized the project, designed experiments, provided critical evaluation of the data, and edited the manuscript.

Correspondence and requests for reprints should be addressed to Richart Harper, M.D., 451 Health Science Drive, Suite 6521, Davis, CA 95616. E-mail: rwharper@ucdavis.edu.

This article has a data supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

Am J Respir Cell Mol Biol Vol 64, Iss 2, pp 208–215, Feb 2021
Copyright © 2021 by the American Thoracic Society
Originally Published in Press as DOI: 10.1165/rcmb.2020-0197OC on November 30, 2020
Internet address: www.atsjournals.org

examined VILI in a *Duoxa*$^{-/-}$ mouse model shown to have impaired neutrophil recruitment (8). Dual oxidases (DUOX1 and DUOX2) are members of the membrane-bound nicotinamide adenine dinucleotide phosphate oxidase family that reside on the epithelial surface of the lung and are integral in airway epithelial integrity and airway epithelial signaling during various inflammatory responses (9). DUOX appears to play a critical role in granulocyte recruitment, yet the mechanism is not yet fully understood (8, 9). Based on these observations, we hypothesized that *Duoxa*$^{-/-}$ mice would have reduced lung neutrophilia in a ventilator-induced acute lung injury model. resulting in less lung injury compared with *Duoxa*$^{+/+}$ mice (10).

A portion of this study was presented at the American Thoracic Society Symposium in 2018 (11).

Methods

Animals

Duoxa$^{+/+}$ mice (129S6/SvEvTac) were obtained from Taconic Biosciences. *Duoxa*$^{-/-}$ mice were generated as previously described (12). *Duoxa*$^{-/-}$ mice are phenotypically hypothyroid; they are treated with subcutaneous injections of 40 ng L-T_4/g body weight from birth to weaning and then supplemented with L-Thyroxine (Sigma) in the drinking water (9). *Duoxa*$^{+/+}$ mice were imported and housed in our facility for 1–6 weeks before study enrollment, with *Duoxa*$^{-/-}$ mice living in our facility from birth up to 15 weeks before study enrollment. Procedures with the mice were performed in accordance with an approved Institutional Animal Care and Use Committee protocol.

Ventilator Experiment Definitions

Mice were exposed to either protective (LTV; tidal volume of 5 ml/kg; positive end-expiratory pressure of 4 cm H_2O; respiratory rate of 170 breaths/min; recruitment maneuvers of 20 cm H_2O; inspiratory hold for 10 s, 20 m) or injurious (high tidal volume [HTV]; tidal volume of 10 ml/kg; PEEP of 0; respiratory rate of 110 breaths/min; recruitment maneuvers) ventilation. Oxygenation Index and Oxygen Saturation Index were calculated using mean airway pressure (MAP) (cm H_2O) × fraction of inspired oxygen (F_{IO_2}) × 100 ÷ arterial oxygen tension (Pa_{O_2}) or MAP × F_{IO_2} × 100 ÷ Sp_{O_2}, respectively (13). Wild-type and knockout mice were divided into nonventilated ($NV^{-/-}$ and $NV^{+/+}$), protective ($LTV^{-/-}$ and $LTV^{+/+}$), and injurious ($HTV^{-/-}$ and $HTV^{+/+}$) groups.

Experimental Groups and Protocol

Duoxa$^{-/-}$ and *Duoxa*$^{+/+}$ mice were sedated as previously described (10). Positive-pressure ventilation was instituted for 4 hours using a MiniVENT (type 845; Harvard Apparatus). Mice were assigned to groups as reported in Table 1. F_{IO_2} was set at 40% by using an OxyDial (Starr Life Sciences Corporation). Mice were monitored as previously described and killed via carotid artery exsanguination (10). Arterial blood gas samples were collected via carotid arterial puncture and analyzed using a standard blood gas analyzer (ABL 815; Radiometer).

BAL Processing

BAL was performed using 1-ml aliquots of sterile PBS. Live and differential cell counts, protein and albumin concentrations, and cytokine and chemokine concentrations (Milliplex; Millipore Corporation) were evaluated as described in the data supplement. Concentrations that were reported as out of range or below the detection limit were calculated by using the equation detection limit/(square root of 2) to allow for statistical comparison.

Histopathology

The left lung was fixed with 1% paraformaldehyde, sectioned, and stained with hematoxylin and eosin or Ly6G antibody (BD Pharmingen). An acute lung injury scoring system (14) was applied as described previously (10) and adjusted to reflect the lack of hyaline membranes present in ARDS-like lesions in mice (15). Further details are available in the data supplement.

Statistics

GraphPad Prism 8 (GraphPad Software, Inc.) was used for data analysis. Outliers were identified, normality was assessed, and data were evaluated by using a one-way ANOVA, Kruskal-Wallis test, Welch's *t* test, or Mann-Whitney test with a predetermined *P* value of <0.05 unless otherwise specified. Further details are outlined in the data supplement.

Results

Positive-Pressure Ventilation Period

Both *Duoxa*$^{-/-}$ mice and *Duoxa*$^{+/+}$ mice had a median age of 12 weeks (range, 8–15 wk and 9–15 wk, respectively). *Duoxa*$^{-/-}$ mice had a median weight of 21.9 g (range, 18–28 g), and *Duoxa*$^{+/+}$ mice had a median weight of 21.4 g (range, 19–25 g). No significant differences were noted between age and weights between groups. All mice survived to the endpoint of the experiment at 4 hours of positive-pressure ventilation. Respiratory rates were set for the two tidal volumes to maintain normocapnia (unpublished pilot data). In the protective ventilation group *Duoxa*$^{-/-}$ mice had a median Pa_{CO2} of 33 mm Hg, and *Duoxa*$^{+/+}$ mice had a median Pa_{CO2} of 39 mm Hg. *Duoxa*$^{-/-}$ and *Duoxa*$^{+/+}$ mice in the injurious ventilation group had a median Pa_{CO2} of 44 mm Hg and 42 mm Hg, respectively.

Histopathology

Overall, lung histology scores were not significantly different between the protective and injurious ventilation strategies in both the *Duoxa*$^{-/-}$ and *Duoxa*$^{+/+}$ groups. Lung histology scores were significantly elevated in the $LTV^{-/-}$ group (mean 0.58 ± 0.11) compared with the $LTV^{+/+}$ group (mean 0.41 ± 0.16; $P = 0.0151$), but no significant differences were noted in the $HTV^{-/-}$ group compared with the $HTV^{+/+}$ group (Figure 1A). Histology images showed evidence of proteinaceous debris as well as hemorrhage and neutrophilic influx in the interstitium, consistent with VILI (Figure 1B). Consistent with our hypothesis, Duoxa$^{-/-}$ mice had significantly lower neutrophil counts than Duoxa$^{+/+}$ mice in the alveolar compartment (mean 11% vs. 25%; $P = 0.0006$), perivascular compartment (mean 7% vs. 21%; $P = 0.003$), and peribronchiolar compartment (mean 11% vs. 23%; $P = 0.012$), but not in the vascular compartment (mean 14% versus 15%, $P = 0.718$) (Figures 1C–1E). No hyaline membrane formation was observed in any group, which was predicted based on the short experimental exposure (4 h).

Alveolar–Capillary Barrier

Other important components of acute lung injury (alveolar edema and pulmonary capillary congestion) were assessed by concentrations of BAL albumin. Ventilation

Table 1. Experimental Groups

	Nonventilated	Protective Ventilation	Injurious Ventilation
Duoxa$^{-/-}$	($n = 3$)	Tidal volume 5 ml/kg PEEP 4 cm H_2O Recruitment maneuvers 4 h ($n = 10$)	Tidal volume 10 ml/kg PEEP 0 cm H_2O Recruitment maneuvers 4 h ($n = 10$)
Duoxa$^{+/+}$	($n = 3$)	Tidal volume 5 ml/kg PEEP 4 cm H_2O Recruitment maneuvers 4 h ($n = 10$)	Tidal volume 10 ml/kg PEEP 0 cm H_2O Recruitment maneuvers 4 h ($n = 10$)

Definition of abbreviation: PEEP = positive end-expiratory pressure.
Mice were assigned to nonventilated, protective ventilation, and injurious ventilation groups.

in all animals demonstrated significant increases in albumin compared with nonventilated control animals (Figure 2). Albumin values in the HTV$^{-/-}$ group (mean 0.17 mg/ml) and HTV$^{+/+}$ group (0.21 mg/ml) were significantly higher compared with the nonventilated animals (NV$^{-/-}$ 0 mg/ml; $P = 0.018$ and NV$^{+/+}$ 0 mg/ml; $P = 0.001$). Albumin values were significantly higher in the HTV$^{+/+}$ group compared with the LTV$^{+/+}$ group (mean 0.21 mg/ml vs. 0.11 mg/ml; $P = 0.031$), but no differences were noted in the HTV$^{-/-}$ group compared with the LTV$^{-/-}$ group (mean 0.17 mg/ml vs. 0.14 mg/ml; $P > 0.999$).

Alveolar Neutrophils and Inflammatory Mediators

BAL total leukocyte counts (Table 2) were similar across all groups, with no significant differences using a Kruskal-Wallis test and Dunn's multiple comparisons test. Similar to our lung histology findings, neutrophil percentages were significantly lower in the *Duoxa*$^{-/-}$ mice compared with the *Duoxa*$^{+/+}$ mice after injurious ventilation (7% vs. 18%; $P = 0.003$) (Figure 3A). Absolute neutrophil counts were significantly lower in the *Duoxa*$^{-/-}$ mice compared with the *Duoxa*$^{+/+}$ mice after injurious ventilation as well (6.7 cells/μl vs. 16.4 cells/μl; $P = 0.003$) (Figure 3B).

To determine whether differences in BAL neutrophils were due to changes in bronchoalveolar cytokines/chemokine concentrations, we measured various common neutrophil chemoattractants in BAL supernatants (Figure 3C). Paradoxically, the murine IL-8 homolog MIP-2 was higher in the *Duoxa*$^{-/-}$ injurious ventilation group compared with the *Duoxa*$^{+/+}$ injurious ventilation group (36.9 pg/ml vs. 5.2 pg/ml; $P = 0.002$) (Figure 3D). No differences were noted when evaluating the other IL-8 homologs CXCL1/KC (KC) (54.1 pg/ml vs. 70.3 pg/ml; $P = 0.082$) or CXCL5/LIX (LIX) (2.8 pg/ml vs. 2.8 pg/ml; $P = 0.489$) in the

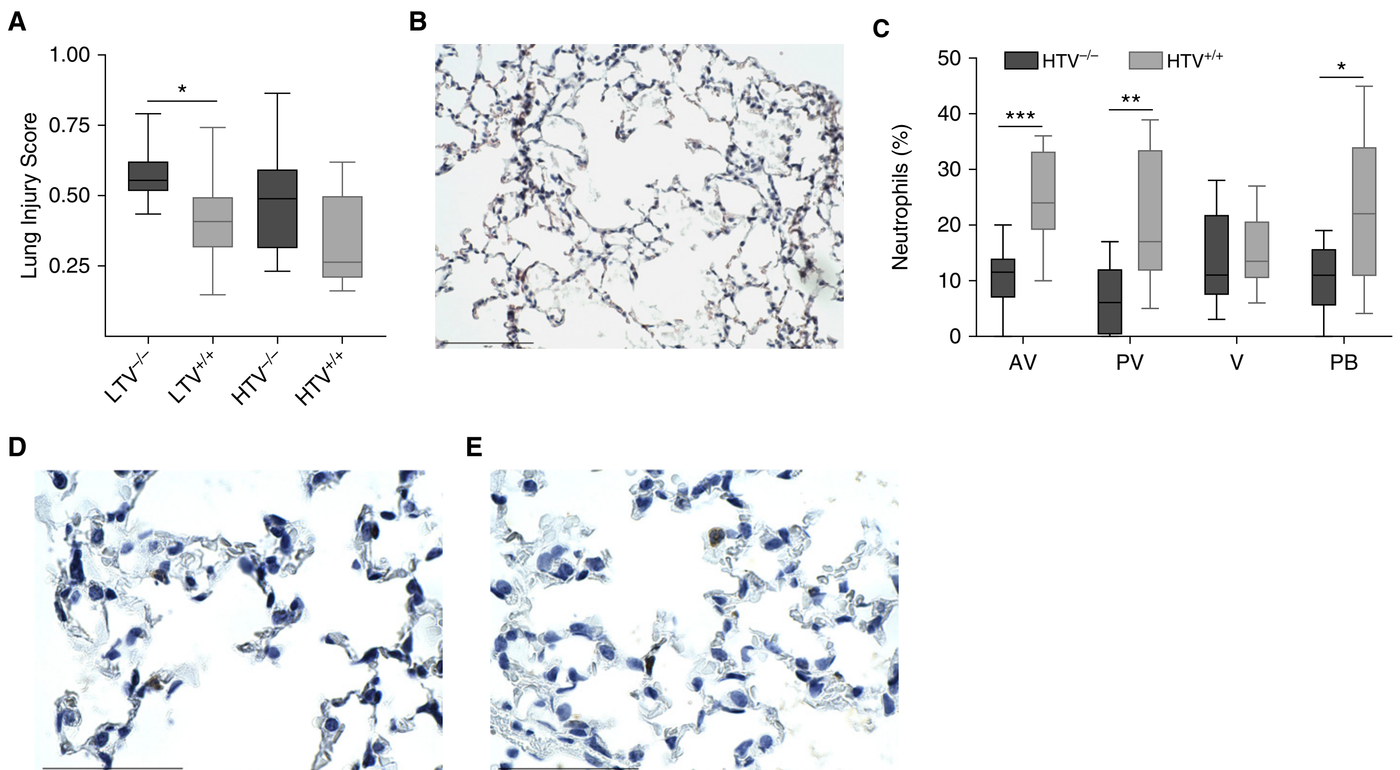

Figure 1. Differential neutrophil recruitment in *Duoxa*$^{-/-}$ and *Duoxa*$^{+/+}$ mice after ventilator-induced lung injury. (*A*) Lung injury scores in LTV and HTV groups of *Duoxa*$^{-/-}$ and *Duoxa*$^{+/+}$mice. (*B*) Lung histopathology (hematoxylin and eosin stain. Scale bar, 100 μm. (*C*) Neutrophil counts (percentage of total cells) in AV, PV, V, and PB compartments from lung sections stained with rat anti-Ly6G (αLy6G) antibody. (*D* and *E*) Immunohistochemical staining (αLy6G) in HTV$^{-/-}$ (*D*) and HTV$^{+/+}$ mice (*E*). Data are shown as median values with 95% confidence intervals. $n = 10$ per group. Scale bar, 100 μm. *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$. AV = alveolar; HTV = high tidal volume; LTV = low tidal volume; PB = peribronchiolar; PV = perivascular; V = vascular.

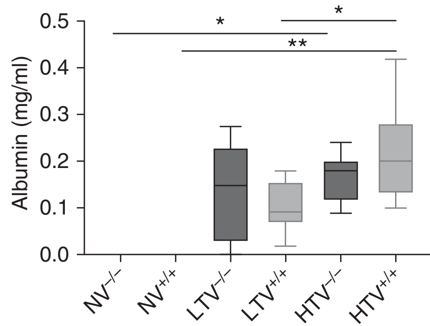

Figure 2. Ventilation increased leak of albumin into the alveolar compartment. Albumin concentrations in BAL fluid recovered from NV, LTV, and HTV groups of *Duoxa*$^{-/-}$ and *Duoxa*$^{+/+}$mice. Data are shown as median values with 95% confidence intervals. $n = 10$ per group. $^{*}P \leqslant 0.05$ and $^{**}P \leqslant 0.01$. NV = nonventilated.

HTV$^{-/-}$ group versus HTV$^{+/+}$ group. This discordance between neutrophil recruitment and MIP-2 concentrations suggests that MIP-2–derived neutrophil recruitment is impaired in the *Duoxa*$^{-/-}$ mice. Differential cytokine expression of multiple cytokines, including PAF (platelet-activating factor) (1.1 pg/ml vs. 0.9 pg/ml; $P = 0.006$), was seen in the HTV$^{-/-}$ group compared with the HTV$^{+/+}$ group (Table E1 in the data supplement). The mechanisms to explain these cytokine differences remain unclear.

Pulmonary Function

Duoxa$^{-/-}$ and *Duoxa*$^{+/+}$ mice were ventilated in their respective groups based on V_T settings adjusted according to individual body weight. Figure 4A shows that all animals met their tidal volumes goals (LTV$^{-/-}$ 5 ml/kg, LTV$^{+/+}$ 5 ml/kg, HTV$^{-/-}$ 10 ml/kg, and HTV$^{+/+}$ 10 ml/kg; $P = 0.776$). MAP varied between *Duoxa*$^{-/-}$ and *Duoxa*$^{+/+}$ groups in their respective ventilation groups as depicted in Figure 4B, with the median MAP of 4.5 cm H_2O in the *Duoxa*$^{-/-}$ protective ventilation group and of 5 cm H_2O in the *Duoxa*$^{+/+}$ protective ventilation group ($P < 0.0001$). The MAPs for the *Duoxa*$^{-/-}$ injurious ventilation group and the *Duoxa*$^{+/+}$ injurious ventilation group were 7.1 cm H_2O and 6.9 cm H_2O, respectively ($P = 0.019$).

Physiologic dysfunction was based on the amount of hypoxemia in each group. Sp_{O_2} was lower in the high-V_T groups compared with the low-V_T groups, consistent with the induction of lung injury in our model, as shown in Figure 5A. Oxygen saturation index was recorded throughout the positive-pressure ventilation period, with significant differences between all groups, as shown in Figure 5C. Pa_{O_2}/Fi_{O_2} ratios were significantly lower in both the injurious and noninjurious *Duoxa*$^{-/-}$ groups compared with the *Duoxa*$^{+/+}$ groups, as depicted in Figure 5B. Oxygenation index was significantly increased in the *Duoxa*$^{-/-}$ injurious ventilation group compared with the *Duoxa*$^{+/+}$ group, as shown in Figure 5D, yet no differences were noted in the noninjurious ventilation groups.

Discussion

To determine whether neutrophils are important mediators of VILI, we hypothesized that *Duoxa*$^{-/-}$ mice would have reduced lung neutrophilia compared with *Duoxa*$^{+/+}$ mice after a VILI challenge, resulting in less lung injury. Duox has been shown to be essential for some, but not all, neutrophilic stimuli (9, 16, 17), and we show here for the first time that Duox is required for neutrophil recruitment in response to VILI. However, there was no evidence of worsening histologic lung injury score or alveolar–capillary leak between the *Duoxa*$^{-/-}$ and *Duoxa*$^{+/+}$ mice after injurious amounts of ventilation. This suggests little role for neutrophils early in the development of ARDS. Despite similar amounts of lung injury, the injurious ventilatory strategy induced severe hypoxemia in *Duoxa*$^{-/-}$ mice compared with the *Duoxa*$^{+/+}$ mice. This suggests an unexpected function for Duox in regulating oxygen exchange in the lung.

As predicted, *Duoxa*$^{-/-}$ mice had significantly reduced lung neutrophils compared with wild-type animals in response to injurious ventilatory strategies (Figures 3A and 3B). Differential neutrophilic influx into the lung does not appear to be due to lack of IL-8 production, similar to our previous findings with allergen challenge (8). Both *Duoxa*$^{-/-}$ and *Duoxa*$^{+/+}$ groups had significant increases in IL-8 homologs KC, MIP-2, and LIX compared with nonventilated control animals (Figure 3D). Similar neutrophil concentrations were present in the vascular space of both *Duoxa*$^{-/-}$ and *Duoxa*$^{+/+}$ mice, yet translocation across the capillary endothelium appears to be impaired in the *Duoxa*$^{-/-}$ mice (Figure 1C). These data suggest that neutrophil recruitment in response to VILI occurs through a noncanonical IL-8–independent mechanism, or that Duox-mediated signals are required for LIX-, MIP-2–, or KC-mediated recruitment of neutrophils from the vascular space into the lung. In fact, several chemokines and cytokines were significantly elevated in *Duoxa*$^{-/-}$ mice compared with wild-type mice after injurious ventilation, including IL-1α, IL-1β, IL-10, IL-12(p70), M-CSF, MIP-1α, MIP-2, and PAF (Figure 3C). The higher concentrations of these cytokines in conjunction with less neutrophil recruitment suggests disrupted Duox-dependent signaling pathways downstream of these cytokines.

Table 2. BAL Leukocytes and Differential Cell Counts

	Leukocytes	Neutrophils [% *Absolute*]		Macrophages [% *Absolute]*		Lymphocytes [% *Absolute*]	
NV$^{-/-}$	28	1	0	99	28	0	0
NV$^{+/+}$	19	0	0	100	19	0	0
LTV$^{-/-}$	34	9	3	90	31	1	0
LTV$^{+/+}$	34	14	5	86	29	1	0
HTV$^{-/-}$	34	7	3	92	31	1	0
HTV$^{+/+}$	34	18	6	80	27	2	1

Definition of abbreviations: HTV = high tidal volume; LTV = low tidal volume; NV=nonventilated.
Median values are recorded and presented as cells/μl.

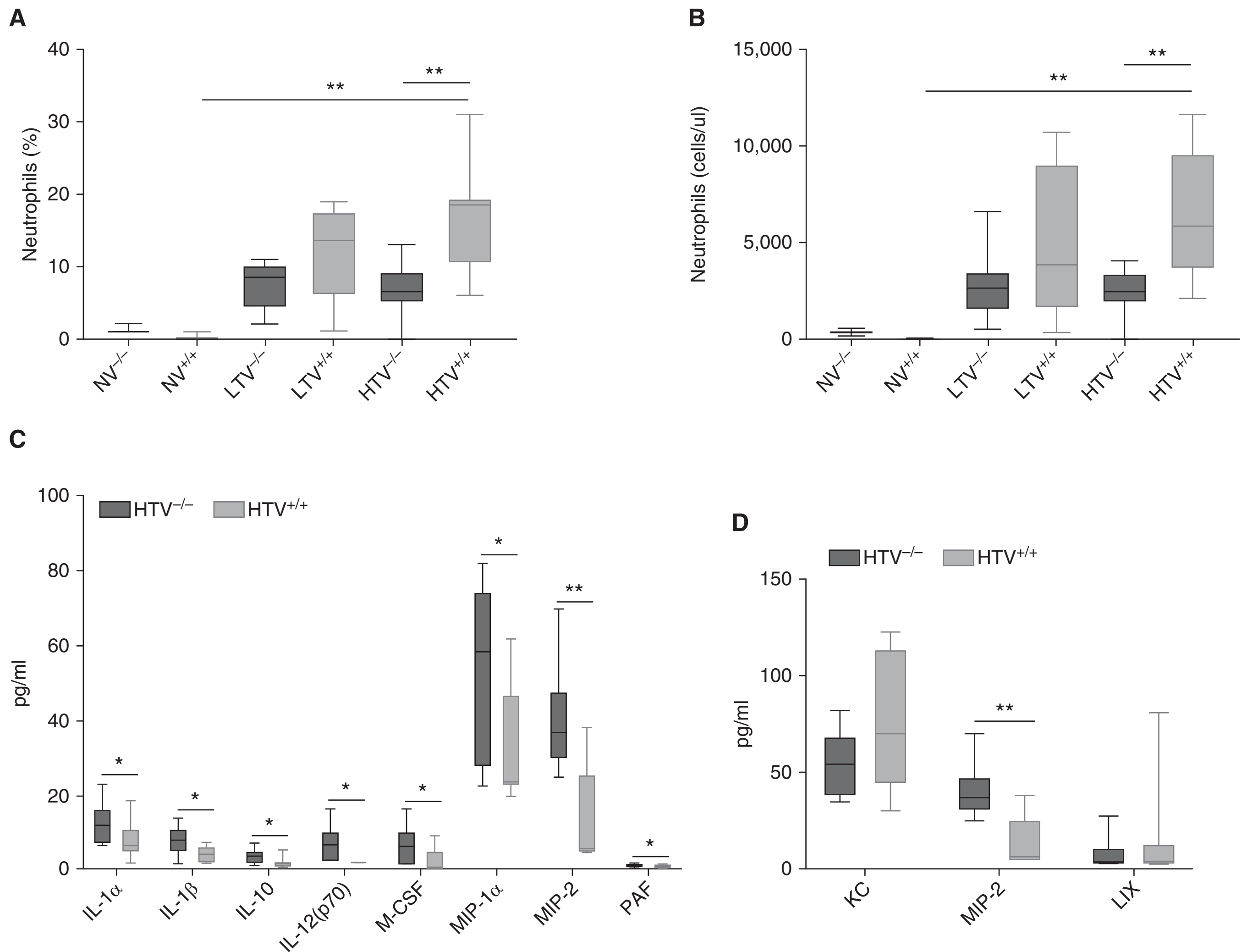

Figure 3. Airway cytokine concentrations do not correlate with airway neutrophil recruitment. (*A*) Percentage total neutrophil counts and (*B*) absolute airway neutrophils from BAL fluid recovered from NV, LTV, and HTV groups of *Duoxa*$^{-/-}$ and *Duoxa*$^{+/+}$mice. (*C*) Select cytokines and chemokines and (*D*) CXCL8 murine homologs in HTV groups of *Duoxa*$^{-/-}$ and *Duoxa*$^{+/+}$mice measured by Multiplex assay. Data are shown as median values with 95% confidence intervals. $n = 10$ per group. $^{*}P \leqslant 0.05$ and $^{**}P \leqslant 0.01$.

Alternative mechanisms for neutrophil recruitment include Duox-dependent EGFR (epidermal growth factor receptor) or purinergic signaling. Several publications have shown Duox1 to be essential for EGFR-mediated signaling, IL-8 production in cell culture, and neutrophil recruitment *in vivo* (17, 18). Similarly, the release of mitochondrial DNA, formyl-Met-Leu-Phe peptide, and ATP during alveolar stretch has been shown to be important for neutrophil chemotaxis in VILI (19). DUOX is localized in type II alveolar epithelial cells (20), and DUOX-dependent purinergic signaling from airway epithelium has been shown to be an important mechanism for multiple pathways of gene regulation and cellular response (20–23).

To standardize the definition of acute lung injury in animals across research studies, Matute-Bello and colleagues devised a validated scoring system in which neutrophilic inflammation is one component of the total acute lung injury score (14). Based on this scoring system, no significant differences were noted between the *Duoxa*$^{-/-}$ and *Duoxa*$^{+/+}$ injurious ventilation groups (Figure 1A). Because the *Duoxa*$^{-/-}$ mice had minimal lung neutrophilia, the degree of lung injury in the *Duoxa*$^{-/-}$ mice may be underrepresented based on lung histology alone. The degree of severe hypoxemia observed in the *Duoxa*$^{-/-}$ group suggests that lung histology alone inadequately reflects the degree of physiologic impairment in these mice. In our study, several indicators of oxygenation were significantly decreased in the *Duoxa*$^{-/-}$ injurious ventilation group compared with all other groups (Figure 5). This profound hypoxemia is a critical feature of clinically important acute lung injury and, in our model, occurs independently of neutrophil migration into the lung. We are unable to fully address the mechanism for severe hypoxemia observed in the *Duoxa*$^{-/-}$ mice, but several plausible mechanisms are suggested by our data and published literature.

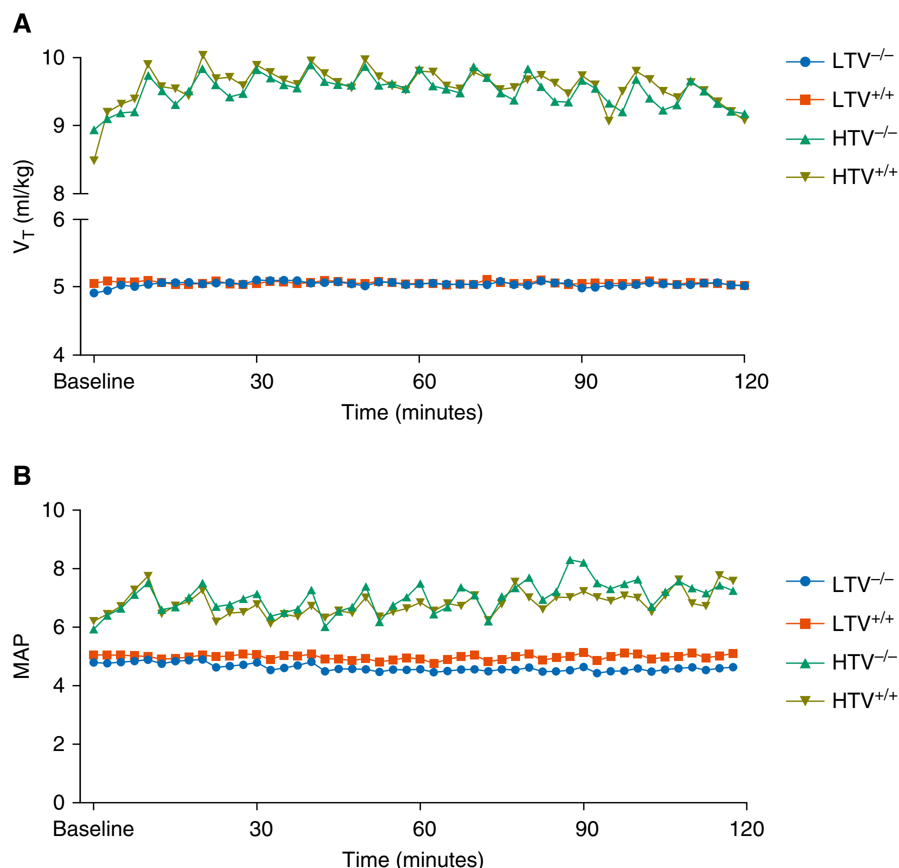

Figure 4. Ventilator settings were similar between *Duoxa*$^{-/-}$ and *Duoxa*$^{+/+}$ mice. (*A*) Measurement of V_T and (*B*) MAP over time in LTV and HTV groups of *Duoxa*$^{-/-}$ and *Duoxa*$^{+/+}$mice. Data are shown as means. $n = 10$ per group. MAP = mean airway pressure; V_T = tidal volume.

A simple explanation for worsening hypoxemia is decreased alveolar epithelial barrier function in the *Duoxa*$^{-/-}$ mice. DUOX is recognized to be important component of epithelial regeneration and wound repair in response to injury in isolated tracheobronchial epithelial cells (16, 24). However, we did not observe differences in barrier function between the wild-type and knockout animals (Figure 2). Albumin was significantly elevated in both groups during high-V_T ventilation compared with the nonventilated animals, confirming VILI induced alveolar edema, but no differences were observed between the *Duoxa*$^{-/-}$ and *Duoxa*$^{+/+}$ mice (Figure 2). Similarly, evaluation of baseline airway barrier function and channel activity was assessed by Ussing chamber measurements in isolated mouse tracheoepithelial cell cultures. No significant differences were observed in the *Duoxa*$^{-/-}$ mice compared with the *Duoxa*$^{+/+}$ mice (data not shown). This argues against barrier dysfunction being a predominant driver of hypoxemia in the knockout animals.

Altered cross-talk signaling from type II alveolar epithelial cells to the pulmonary vascular endothelium is a highly plausible explanation for the disproportionate hypoxemia seen in the *Duoxa*$^{-/-}$ mice. The most compelling of multiple possible mechanisms is DUOX-dependent paracrine signaling from type II alveolar epithelial cells inducing nitric oxide (NO) production in pulmonary vascular endothelium. There is clear evidence that alveolar stretch induces endothelial NO release (25), which is necessary for appropriate pulmonary capillary vasodilation and avoidance of ventilation–perfusion mismatch. This stretch-induced NO is dependent on the calcium-dependent release of ATP and the activation of P2Y2 receptors in the alveolar epithelium. This immediately suggests a plausible mechanism of stretch-induced, DUOX-dependent paracrine signaling from type II alveolar cells to regulate NO release from the pulmonary epithelium. In the absence of DUOX, there is potential loss of this paracrine signaling pathway that results in ventilation–perfusion mismatch and the hypoxemia we observed in the knockout mice.

Alternatively, increased platelet activation or coagulation in pulmonary vessels may be a mechanism for worsening hypoxemia in the *Duoxa*$^{-/-}$ mice. In support of this possibility, cytokine expression of PAF was significantly increased in the knockout mice during LTV and HTV ventilation compared with unventilated animals, and PAF concentrations were higher in the *Duoxa*$^{-/-}$ mice compared with the *Duoxa*$^{-/-}$ mice after both the protective and injurious ventilatory strategies (Table 1).

It is highly unlikely that pulmonary vascular endothelium alone is responsible for the derangements seen in the knockout mice, as DUOX has not been identified in this cell type, nor has it been identified in related tissues, including vascular smooth muscle, fibroblasts, or perivascular adipocytes (25). However, the interdependence between the alveolar epithelium and pulmonary capillary bed during early development may have resulted in deranged alveolar–capillary architecture and function that is elicited by our injurious ventilation protocol.

There are potential confounders in this study. The use of *Duoxa*$^{+/+}$ mice purchased from an exogenous vendor as control animals versus the in-house *Duoxa*$^{-/-}$ mice may have resulted in important differences in the gut or lung microbiome that influenced our results. Similarly, most of the mice were females, and we were unable to equally match all groups based on sex. We do not anticipate a profound skew on the major findings of this study based on these confounders; we expect cytokine expression levels to be at the highest risk for biased results. *Duoxa*$^{-/-}$ mice are functionally hypothyroid and are routinely supplemented with thyroxine in their drinking water to maintain a euthyroid state. We periodically measure thyroid function in our knockout colonies, but we did not measure thyroid function in the mice used for this study. Because inadequate supplementation leads to underdeveloped, underweight mice, the equal weights between all mice in our study,

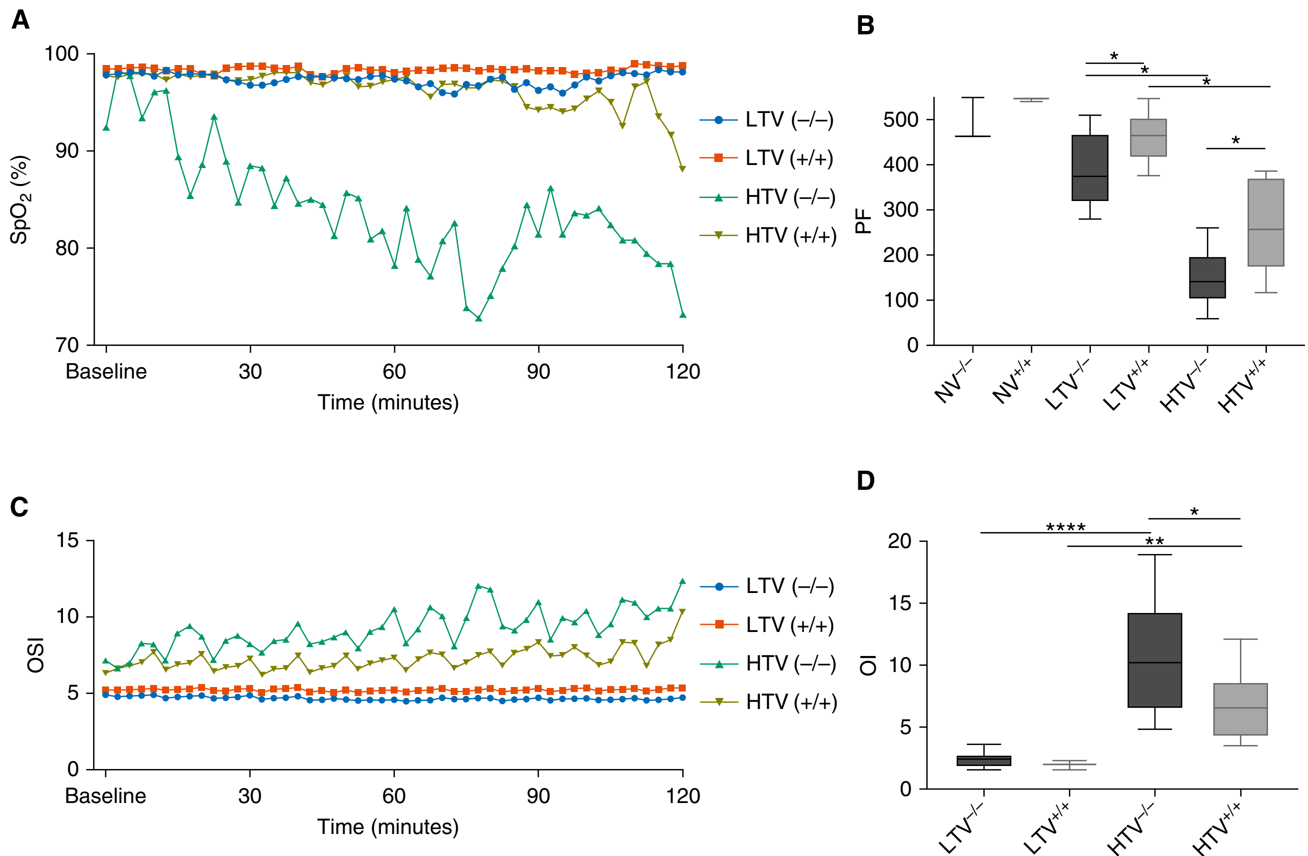

Figure 5. *Duoxa*$^{-/-}$ mice have severe hypoxemia in response to HTV ventilation. Measurement of (*A*) Sp_{O_2} over time, (*B*) average PF ratio, (*C*) OSI over time, and (*D*) average OI in NV, LTV, and HTV groups of *Duoxa*$^{-/-}$ and *Duoxa*$^{+/+}$mice. Data are shown as means for time courses and as median values with 95% confidence intervals for averaged data. $n = 10$ per group. *$P \leqslant 0.05$, **$P \leqslant 0.01$, and ****$P \leqslant 0.0001$. OI = Oxygenation Index; OSI = Oxygen Saturation Index; PF = arterial oxygen tension/fraction of inspired oxygen; Sp_{O_2} = oxygen saturation.

combined with our periodic direct measurements of thyroid function, reassure us that the knockout mice were adequately supplemented.

We hypothesized that in a VILI model, *Duoxa*$^{-/-}$ mice would have reduced lung neutrophilia, resulting in less severe lung injury compared with *Duoxa*$^{+/+}$ mice. To the contrary, we did not observe a correlation between lung injury and lung neutrophilia, which suggests that neutrophil migration into the lung is minimally important for early features of acute lung injury. Importantly, the *Duoxa*$^{-/-}$ mice had significantly impaired oxygenation compared with the *Duoxa*$^{+/+}$ mice. These data suggest that dual oxidases are important in limiting profound hypoxemia and regulating hypoxic pulmonary vasoconstriction during VILI. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

References

1. Brower RG, Matthay MA, Morris A, Schoenfeld D, Thompson BT, Wheeler A; Acute Respiratory Distress Syndrome Network. Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. *N Engl J Med* 2000;342:1301–1308.
2. Slutsky AS, Ranieri VM. Mechanical ventilation: lessons from the ARDSNet trial. *Respir Res* 2000;1:73–77.
3. Grommes J, Soehnlein O. Contribution of neutrophils to acute lung injury. *Mol Med* 2011;17:293–307.
4. Matthay MA, Eschenbacher WL, Goetzl EJ. Elevated concentrations of leukotriene D4 in pulmonary edema fluid of patients with the adult respiratory distress syndrome. *J Clin Immunol* 1984;4: 479–483.
5. Parsons PE, Fowler AA, Hyers TM, Henson PM. Chemotactic activity in bronchoalveolar lavage fluid from patients with adult respiratory distress syndrome. *Am Rev Respir Dis* 1985;132: 490–493.
6. Steinberg KP, Milberg JA, Martin TR, Maunder RJ, Cockrill BA, Hudson LD. Evolution of bronchoalveolar cell populations in the adult respiratory distress syndrome. *Am J Respir Crit Care Med* 1994;150: 113–122.
7. Abraham E, Carmody A, Shenkar R, Arcaroli J. Neutrophils as early immunologic effectors in hemorrhage- or endotoxemia-induced acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2000;279: L1137–L1145.
8. Chang S, Linderholm A, Franzi L, Kenyon N, Grasberger H, Harper R. Dual oxidase regulates neutrophil recruitment in allergic airways. *Free Radic Biol Med* 2013;65:38–46.

9. van der Vliet A. NADPH oxidases in lung biology and pathology: host defense enzymes, and more. *Free Radic Biol Med* 2008;44: 938–955.
10. Cagle LA, Franzi LM, Linderholm AL, Last JA, Adams JY, Harper RW, *et al*. Effects of positive end-expiratory pressure and recruitment maneuvers in a ventilator-induced injury mouse model. *PLoS One* 2017;12:e0187419.
11. Cagle L, Linderholm A, Franzi L, Harper RW, Kenyon NJ. Role of dual oxidase in airway neutrophilia in ventilator-induced lung injury. *Am J Respir Crit Care Med* 2018;197:A2967.
12. Grasberger H, De Deken X, Mayo OB, Raad H, Weiss M, Liao XH, *et al*. Mice deficient in dual oxidase maturation factors are severely hypothyroid. *Mol Endocrinol* 2012;26:481–492.
13. Rawat M, Chandrasekharan PK, Williams A, Gugino S, Koenigsknecht C, Swartz D, *et al*. Oxygen saturation index and severity of hypoxic respiratory failure. *Neonatology* 2015;107:161–166.
14. Matute-Bello G, Downey G, Moore BB, Groshong SD, Matthay MA, Slutsky AS, *et al*.; Acute Lung Injury in Animals Study Group. An official American Thoracic Society workshop report: features and measurements of experimental acute lung injury in animals. *Am J Respir Cell Mol Biol* 2011;44:725–738.
15. Aeffner F, Bolon B, Davis IC. Mouse models of acute respiratory distress syndrome: a review of analytical approaches, pathologic features, and common measurements. *Toxicol Pathol* 2015;43: 1074–1092.
16. Chang S, Linderholm A, Harper R. DUOX-mediated signaling is not required for LPS-induced neutrophilic response in the airways. *PLoS One* 2015;10:e0131810.
17. Habibovic A, Hristova M, Heppner DE, Danyal K, Ather JL, Janssen-Heininger YM, *et al*. DUOX1 mediates persistent epithelial EGFR activation, mucous cell metaplasia, and airway remodeling during allergic asthma. *JCI Insight* 2016;1:e88811.
18. Nakanaga T, Nadel JA, Ueki IF, Koff JL, Shao MX. Regulation of interleukin-8 via an airway epithelial signaling cascade. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L1289–L1296.
19. Grazioli S, Dunn-Siegrist I, Pauchard LA, Blot M, Charles PE, Pugin J. Mitochondrial alarmins are tissue mediators of ventilator-induced lung injury and ARDS. *PLoS One* 2019;14:e0225468.
20. van der Vliet A, Bove PF. Purinergic signaling in wound healing and airway remodeling. *Subcell Biochem* 2011;55:139–157.
21. Sham D, Wesley UV, Hristova M, van der Vliet A. ATP-mediated transactivation of the epidermal growth factor receptor in airway epithelial cells involves DUOX1-dependent oxidation of Src and ADAM17. *PLoS One* 2013;8:e54391.
22. Schiffers C, Hristova M, Habibovic A, Dustin CM, Danyal K, Reynaert NL, *et al*. The transient receptor potential channel vanilloid 1 is critical in innate airway epithelial responses to protease allergens. *Am J Respir Cell Mol Biol* 2020;63:198–208.
23. Massart C, Hoste C, Virion A, Ruf J, Dumont JE, Van Sande J. Cell biology of H_2O_2 generation in the thyroid: investigation of the control of dual oxidases (DUOX) activity in intact *ex vivo* thyroid tissue and cell lines. *Mol Cell Endocrinol* 2011; 343:32–44.
24. van der Vliet A, Janssen-Heininger YM. Hydrogen peroxide as a damage signal in tissue injury and inflammation: murderer, mediator, or messenger? *J Cell Biochem* 2014;115:427–435.
25. Mura M, Binnie M, Han B, Li C, Andrade CF, Shiozaki A, *et al*. Functions of type II pneumocyte-derived vascular endothelial growth factor in alveolar structure, acute inflammation, and vascular permeability. *Am J Pathol* 2010;176:1725–1734.