5-Lipoxygenase Deficiency Prevents Respiratory Failure during Ventilator-induced Lung Injury

Pietro Caironi, Fumito Ichinose, Rong Liu, Rosemary C. Jones, Kenneth D. Bloch, and Warren M. Zapol

Department of Anesthesia and Critical Care, and the Cardiovascular Research Center of the Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts

Rationale: **Mechanical ventilation with high V_T (HV_T) progressively leads to lung injury and decreased efficiency of gas exchange. Hypoxic pulmonary vasoconstriction (HPV) directs blood flow to well-ventilated lung regions, preserving systemic oxygenation during pulmonary injury. Recent experimental studies have revealed an important role for leukotriene (LT) biosynthesis by 5-lipoxygenase (5LO) in the impairment of HPV by endotoxin.** *Objectives:* **To investigate whether or not impairment of HPV contributes to the hypoxemia associated with HV_T and to evaluate the role of LTs in ventilator-induced lung injury.** *Methods:* **We studied wild-type and 5LO-deficient mice ventilated for up to 10 hours with low V_T (LV_T) or HV_T.** *Results:* **In wild-type mice, HV_T, but not LV_T, increased pulmonary vascular permeability and edema formation, impaired systemic oxygenation, and reduced survival. HPV, as reflected by the increase in left pulmonary vascular resistance induced by left mainstem bronchus occlusion, was markedly impaired in animals ventilated with HV_T. HV_T ventilation increased bronchoalveolar lavage levels of LTs and neutrophils. In 5LO-deficient mice, the HV_T-induced increase of pulmonary vascular permeability and worsening of respiratory mechanics were markedly attenuated, systemic oxygenation was preserved, and survival increased. Moreover, in 5LO-deficient mice, HV_T ventilation did not impair the ability of left mainstem bronchus occlusion to increase left pulmonary vascular resistance. Administration of MK886, a 5LO-activity inhibitor, or MK571, a selective cysteinyl-LT_1 receptor antagonist, largely prevented ventilator-induced lung injury.** *Conclusions:* **These results indicate that LTs play a central role in the lung injury and impaired oxygenation induced by HV_T ventilation.**

Keywords: hypoxic pulmonary vasoconstriction; leukotrienes; ventilator-induced lung injury

Excessive stretching of lung parenchyma, by ventilation with large V_T, progressively injures the structure and gas-exchanging efficiency of the lung (1–3). Common alterations of the respiratory system during ventilator-induced lung injury (VILI) include pulmonary edema, because of increased permeability of the alveolar–capillary membrane, and hypoxemia, as a consequence of intrapulmonary right-to-left shunting (especially in the terminal stages of VILI). During the development of acute lung injury, systemic oxygenation is maintained by hypoxic pulmonary vasoconstriction (HPV), a physiologic property of the pulmonary microvasculature that diverts blood flow from hypoxic/poorly ventilated lung regions toward more normally ventilated lung regions. Experimental and clinical studies have reported that HPV is impaired in lung injury induced by endotoxemia (4, 5) and in patients affected by acute respiratory distress syndrome (ARDS) (6). However, whether or not impairment of HPV contributes to the hypoxemia associated with VILI has not been reported.

The mechanisms contributing to the development of VILI have been extensively investigated (7). Excessive lung inflation can produce mechanical disruption of the alveolar–capillary membrane ("capillary stress failure") (8–10). In addition, the repetitive opening and closing of alveoli, especially in diseased lung regions, can contribute to the injury (11). Moreover, many studies have provided evidence that high V_T ventilation (HV_T) can induce lung inflammation via neutrophil infiltration and the production of inflammatory cytokines (12–17).

Leukotrienes (LTs) are potent lipid mediators of inflammation (18). The first step for LT biosynthesis requires the enzyme 5-lipoxygenase (5LO), which, in the presence of 5LO-activating protein (FLAP), converts arachidonic acid into LTA_4. An unstable intermediate, LTA_4 is metabolized by LTA_4 hydrolase into LTB_4 or, alternatively, is conjugated with glutathione by LTC_4 synthase to produce the cysteinyl-LTs (cysLTs), LTC_4, LTD_4, and LTE_4. LTB_4 acts as a powerful chemokinetic and chemotactic agent for leukocytes (19). CysLTs increase vascular permeability and modulate vascular smooth muscle tone (18) through the activation of the $cysLT_1$ and $cysLT_2$ receptors (20, 21).

LTs have been implicated as inflammatory mediators in experimental models of acute lung injury (22) and were found to be elevated in pulmonary edema fluid obtained from patients with ARDS (23–25). Moreover, cysLTs appear to play a critical role in mediating neutrophil-dependent inflammation (26). Recently, we reported that LTs contribute to the impairment of HPV associated with endotoxin-induced lung injury (27). This article describes a newly developed murine model of lung injury induced by long-term mechanical ventilation (up to 10 hours) and reports the impact of HV_T ventilation on the pulmonary vasoconstrictor response to alveolar hypoxia and the role of LTs in the pathogenesis of VILI. The results presented in this article have been previously reported, in part, in the form of abstracts (28, 29).

METHODS

Mouse Model of VILI

Mice were anesthetized by intraperitoneal injection of ketamine (120 mg/kg) and xylazine (8 mg/kg); then, a tracheostomy and arterial catheterization were performed as previously described (27). Mice received two differing types of mechanical ventilation. Low V_T (LV_T) ventilation provided a V_T equal to 12% of inspiratory capacity (IC), at a respiratory rate of 100 breaths/minute. HV_T ventilation provided a V_T equal to 43% of IC, at a respiratory rate of 90 breaths/minute. IC was obtained, for each animal, by constructing a pressure–volume (PV) curve of the respiratory system. Mechanical ventilation was ended either after 10 hours or when (after 7–10 hours of HV_T ventilation) a severe deterioration of the respiratory system compliance was detected by monitoring the PV curve, representing a preterminal state.

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Correspondence and requests for reprints should be addressed to Warren M. Zapol, M.D., Department of Anesthesia and Critical Care, Massachusetts General Hospital, Harvard Medical School, 55 Fruit Street, Boston, MA 02114. E-mail: wzapol@partners.org

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Gas Exchange Analysis

At the end of each experiment, arterial blood was obtained from the left carotid artery, and a blood gas analysis was performed.

Bronchoalveolar Lavage Fluid Analysis

Bronchoalveolar lavage was performed using 5 × 1 ml phosphate-buffered saline. CysLTs and LTB_4 concentrations in bronchoalveolar lavage fluid (BALF) supernatants were measured using an enzyme immunoassay (Neogen Corporation, Lexington, KY) after lipid extraction. The total number of cells obtained from BALF after centrifugation was counted with a hemocytometer, and a differential leukocyte count was measured after staining with Hema 3 (Fisher Scientific, Pittsburgh, PA).

Lung Edema and Microvascular Permeability Assay

To assess pulmonary edema, the amount of extravascular lung water (EVLW) in lung tissue was computed at the end of the experiment for the right lung as described previously (30). To estimate pulmonary vascular permeability, BALF supernatant was analyzed for total protein concentration using the Bradford method (Bio-Rad, Hercules, CA).

Histopathologic Analysis

Mouse lungs were perfusion-fixed via both the airway and pulmonary artery and embedded in JB-4 resin. Sections 2-μm thick were stained with 0.05% toluidine blue and microscopically examined by an investigator blinded as to the mouse genotype and ventilation protocol.

Measurement of HPV

After 6 hours of either LVT or HVT ventilation, mice were ventilated in a standard fashion with a VT of 7 ml/kg body weight and a respiratory rate of 100 breaths/minute. After a thoracotomy was performed, systemic arterial pressure (SAP), pulmonary arterial pressure, and left pulmonary arterial blood flow were continuously recorded as previously described (27). To assess HPV, the percentage increase in left pulmonary vascular resistance (LPVR) induced by left lung alveolar hypoxia (caused by left mainstem bronchus occlusion) was computed for each animal.

Pulmonary Vascular Response to Angiotensin II

To assess pulmonary vasomotor activity after mechanical ventilation, pulmonary vascular resistance was measured before and after intravenous infusion of angiotensin II (5 μg/kg/minute) as previously described (31). To examine the effect of angiotensin II on HPV, LPVR changes induced by left lung alveolar hypoxia were measured before and after angiotensin II infusion.

Statistical Analysis

Statistical analysis was performed using Sigma Stat 2.03 (SPPS, Inc., Chicago, IL). Statistical significance was defined as a p value of less than 0.05. All data are expressed as mean ± SEM.

Additional details on the methods used in these studies and a detailed description of experimental groups studied are provided in the online supplement.

RESULTS

Effects of HVT Ventilation in Wild-Type Mice

We developed a murine model of VILI, in which mice were subjected to long-term mechanical ventilation with either LVT or HVT (Table 1). Mechanical ventilation was ended either after 10 hours or when (after 7–10 hours of HVT ventilation) a severe deterioration of respiratory system compliance was detected by monitoring the PV curve (representing a preterminal state).

Hemodynamic parameters did not change during mechanical ventilation and were similar in mice ventilated at a LVT or HVT (*see* Table E1 on the online supplement). Similarly, the acid-base status did not differ between the two groups of ventilated mice and remained unchanged throughout the experiment (*see* Table E2). Mice ventilated at LVT routinely survived for the 10-hour study period. In contrast, mice ventilated at HVT consistently survived only until the 7th hour. Thereafter, they progressively developed severe acute lung injury and, by the 10th hour of ventilation, only 20% of the mice remained alive (Figure 1).

PV curves of the respiratory system were generated before and every hour during LVT or HVT ventilation. During HVT ventilation, lung compliance decreased, as reflected by a downward and rightward shift of the PV curve (Figure 2B), whereas during the entire period of LVT ventilation, the PV curve remained unchanged (Figure 2A). At the end of the experiment, EVLW was markedly greater in mice ventilated at HVT (6.1 ± 0.5 g H_2O/g dry lung) than in mice ventilated at LVT or mice studied at baseline (3.0 ± 0.2 and 3.2 ± 0.2, respectively; $p < 0.01$ for both; Figure 3A). After 6 hours of mechanical ventilation, the permeability of the alveolar–capillary membrane, as reflected by the total protein concentration in BALF, was similar between the HVT and LVT group (Figure 3B). In contrast, after longer periods of mechanical ventilation (up to 10 hours), BALF total protein concentration from mice ventilated at HVT was greater than that obtained from mice ventilated at LVT (Figure 3B), demonstrating that prolonged HVT ventilation markedly increases the permeability of the murine alveolar–capillary membrane.

The systemic Pa_{O_2} and the alveolar–arterial oxygen tension difference (A-aD_{O_2}) did not change during LVT ventilation (*see* Table E2 and Figure 3A, respectively), whereas HVT ventilation markedly decreased the Pa_{O_2} ($p < 0.001$; *see* Table E2 in the online supplement) and doubled the A-aD_{O_2} ($p < 0.01$; Figure 3A) at the end of the experiment. The increase in EVLW induced

TABLE 1. VENTILATORY SETTINGS AND RESPIRATORY MECHANICS

Ventilation Protocol	Strain of Mice	N	Body weight (*g*)	IC (*ml*)	VT/IC (%)	VT (*ml*)	VT plet/bw (*ml/kg*)	$PEEP_{t,dyn}$ (*cm H_2O*)	Peak Pressure (*cm H_2O*)
LVT	WT	25	23.0 ± 0.4	1.54 ± 0.02*	12	0.18 ± 0.00	11 ± 0	2.1 ± 0.1*	9.2 ± 0.1
HVT	WT	28	23.4 ± 0.3	1.56 ± 0.03*	43	0.67 ± 0.01*†	25 ± 0*†	1.8 ± 0.1*†	21.9 ± 0.4†
LVT	$5LO^{-/-}$	17	22.4 ± 0.2	1.73 ± 0.03	12	0.21 ± 0.00	12 ± 0	2.5 ± 0.1	8.9 ± 0.1
HVT	$5LO^{-/-}$	16	22.3 ± 0.2	1.71 ± 0.03	43	0.74 ± 0.01†	30 ± 0†	2.3 ± 0.1	22.7 ± 0.3†
LVT	MK886	14	22.8 ± 0.4	1.60 ± 0.04*	12	0.19 ± 0.00	11 ± 0	2.2 ± 0.1	9.8 ± 0.1
HVT	MK886	17	22.9 ± 0.3	1.55 ± 0.02*	43	0.67 ± 0.01*†	25 ± 0*†	1.6 ± 0.1*†	21.8 ± 0.5†
HVT	MK571	11	23.4 ± 0.5	1.59 ± 0.03	43	0.69 ± 0.02†	25 ± 1*†	1.5 ± 0.1*	23.4 ± 0.5†

Definition of abbreviations: bw = body weight; IC = inspiratory capacity; 5LO = 5-lipoxygenase; MK571 = wild-type mice treated with MK571; MK886 = wild-type mice pretreated with MK886; $PEEP_{t,dyn}$ = total dynamic positive end-expiratory pressure; VT plet = actual delivered VT estimated by plethysmographic measurements; WT = wild-type.

Respiratory mechanics at baseline in mice that were subsequently ventilated at LVT or HVT. All values were compared between different ventilation strategies and genotype/treatment groups by analysis of variance with a *post hoc* comparison.

* $p < 0.05$ versus 5LO-deficient mice with the same ventilation strategy.

† $p < 0.01$ versus LVT ventilation in the same genotype/treatment group and LVT ventilation in WT mice.

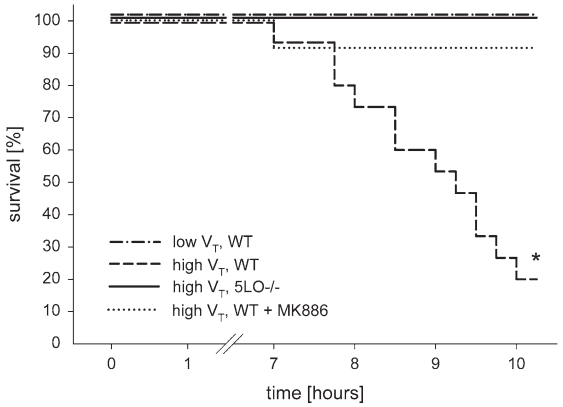

Figure 1. Survival during long-term mechanical ventilation in wild-type (WT) mice, during low VT (LVT; n = 12) or high VT (HVT; n = 15) ventilation, and in 5-lipoxygenase (5LO)–deficient mice (n = 8) and in MK886-pretreated WT mice (n = 12) during HVT ventilation (*$p < 0.01$ vs. other groups).

by HVT ventilation was highly correlated with the decrease in Pa_{O_2} and the increase in A-aD_{O_2} ($r^2 = 0.91$ and $r^2 = 0.86$, respectively; $p < 0.05$ for both).

To assess the histopathologic consequences of mechanical ventilation in our murine model of VILI, lung sections were prepared from mice ventilated at LVT or HVT and examined microscopically. In comparison to mice ventilated at LVT (Figure 4A) and mice studied at baseline (data not shown), the alveolar–capillary membrane in the central lung regions from mice subjected to HVT was thin and lacked a patent capillary network, associated with epithelial surface disruption and a moderate degree of inflammatory cell infiltration (Figure 4B). Lung subpleural regions were less affected as judged by the presence of open capillaries and less alveolar surface injury (data not shown).

HVT Ventilation Impairs HPV

To assess the impact of HVT ventilation on HPV, changes in LPVR during left lung alveolar hypoxia were examined in mice after 6 hours of either LVT or HVT ventilation. To induce left lung alveolar hypoxia, the left mainstem bronchus was reversibly occluded with a microvascular clip. Before left mainstem bronchus occlusion (LMBO), hemodynamic parameters were similar between the two groups of ventilated mice (*see* Table E3). In mice ventilated at LVT, LMBO consistently decreased left pulmonary arterial blood flow, resulting in a 99 ± 10% increase in LPVR ($p < 0.01$; Figure 5). The same magnitude of increase in LPVR during LMBO was measured in mice studied at baseline (100 ± 10%), demonstrating that long-term ventilation with LVT does not affect HPV. In contrast, LMBO did not increase LPVR in mice ventilated at HVT (17 ± 8%, p = not significant vs. HVT ventilation before LMBO and $p < 0.01$ vs. LVT ventilation after LMBO; Figure 5). These results show that HVT ventilation impairs HPV.

To examine the possibility that impaired HPV after HVT ventilation was attributable to a generalized mechanical disruption of pulmonary vasomotor responsiveness, we studied the pulmonary vasomotor response to the intravenous infusion of angiotensin II (5 μg/kg/minute) in mice ventilated for 6 hours. After LVT ventilation, angiotensin II increased pulmonary vascular resistance from 57 ± 15 mm Hg/ml/minute/g to 106 ± 7 mm Hg/ml/minute/g ($p < 0.05$). Similarly, in mice ventilated at HVT, pulmonary vascular resistance increased from 64 ± 8 to 125 ± 8 mm Hg/ml/minute/g after angiotensin II infusion ($p < 0.01$). To investigate the hypothesis that impairment of HPV after HVT ventilation is caused by an alteration in the balance

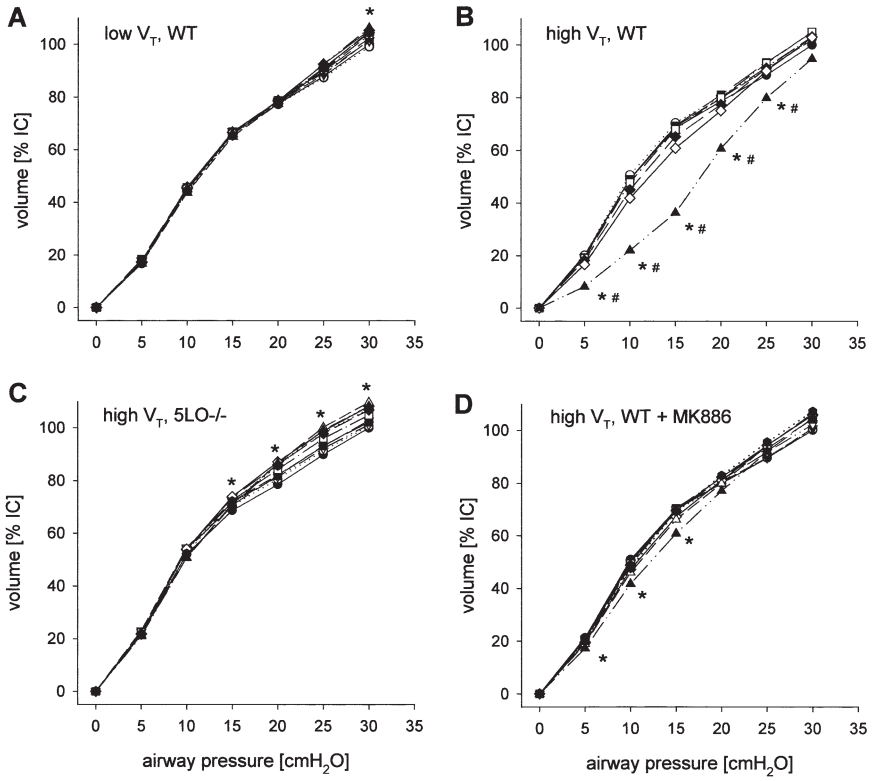

Figure 2. The pressure–volume (PV) curve of the respiratory system in WT mice ventilated either (*A*) at LVT (n = 12) or (*B*) at HVT (n = 15), and in (*C*) 5LO-deficient mice ($5LO^{-/-}$; n = 8) and (*D*) MK886-pretreated WT mice (MK886; n = 12), during mechanical ventilation with HVT. *Solid circles* represent PV curve performed at baseline, *open circles* represent PV curve performed after 1 hour of ventilation, *inverted solid triangles* after 2 hours, *inverted open triangles* after 3 hours, *solid squares* after 4 hours, *open squares* after 5 hours, *solid diamonds* after 6 hours, *open diamonds* after 7 hours, *solid hexagons* after 8 hours, *open triangles* after 9 hours, and *solid triangles* represent PV curve performed at the end of the experiment. For clarity, only mean values are reported. PV curve values obtained at the end of the experiment represent mean values of the last PV curve performed in each animal (either after 10 hours of LVT ventilation or after a period between 7 and 10 hours of HVT mechanical ventilation). *$p < 0.05$ versus baseline; #$p < 0.05$ versus WT mice ventilated at LVT and 5LO-deficient and MK886-pretreated WT mice ventilated at HVT at the end of the experiment. IC = inspiratory capacity of the respiratory system, defined as the inflation volume needed to achieve 30 cm H_2O airway pressure.

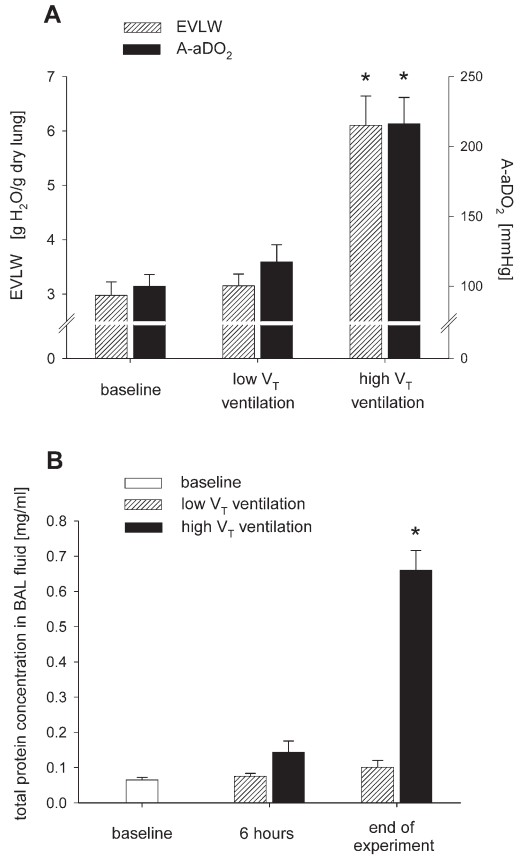

Figure 3. Extravascular lung water (EVLW) and alveolar–arterial oxygen tension difference (A-aDO$_2$; *A*) and total protein concentration in bronchoalveolar lavage fluid (BALF; *B*) in WT mice subjected to mechanical ventilation. After long-term mechanical ventilation, EVLW (n = 5) and A-aDO$_2$ (n = 15) were increased in mice ventilated with HVT compared with mice ventilated with LVT (n = 3 for EVLW, n = 12 for A-aDO$_2$) and mice studied at baseline (n = 3 for EVLW, n = 10 for A-aDO$_2$; *$p < 0.01$). Similarly, the total protein concentration in BALF was higher in mice after long-term HVT ventilation (n = 6) than in mice studied at baseline (n = 4), in mice after long-term LVT ventilation (n = 6), and in mice after ventilation with HVT (n = 5) for only 6 hours (*$p < 0.001$, all groups).

of vasoconstrictors and vasodilators, changes in LPVR induced by LMBO before and after beginning an angiotensin II infusion were measured in a subset of mice after mechanical ventilation. In animals ventilated at HVT, angiotensin II infusion restored HPV, enhancing the LMBO-induced increase in LPVR to 92 ± 15% ($p < 0.05$ vs. wild-type mice ventilated at HVT before angiotensin II infusion), but angiotensin II did not alter HPV in mice ventilated at LVT (data not shown). Taken together, these results demonstrate that the impairment of HPV associated with HVT ventilation is not attributable to a generalized mechanical dysfunction of the vasomotor contractile apparatus, and that administration of a vasoconstrictor to mice ventilated for 6 hours at HVT can restore the ability of the pulmonary vasculature to constrict in response to hypoxia.

HVT Ventilation Induces Pulmonary Neutrophil Recruitment and LT Production

To examine the mechanisms by which HVT ventilation induces pulmonary injury and impairs HPV, we measured the levels of neutrophils in BALF obtained from mice after LVT or HVT ventilation. In mice ventilated at HVT, neutrophil levels in BALF were significantly higher than in mice ventilated with LVT, both after 6 hours (31 ± 6 vs. $7 \pm 2 \times 10^3$, $p < 0.01$) and after the entire duration of the experiment (27 ± 5 vs. $14 \pm 1 \times 10^3$, $p < 0.01$; *see* Table E4).

To investigate whether mechanical ventilation induces LT production, BALF concentrations of cysLTs and LTB_4 were measured. In comparison to mice studied at baseline, long-term LVT ventilation did not alter the BALF levels of either cysLTs or LTB_4 (Figure 6). In contrast, BALF cysLT levels in mice receiving HVT ventilation were greater than in mice ventilated at LVT, both after 6 hours (0.22 ± 0.04 vs. 0.12 ± 0.01 ng/ml, $p < 0.01$) and after the entire duration of mechanical ventilation ($p < 0.01$; Figure 6A). BALF LTB_4 levels did not differ between LVT and HVT group after 6 hours of mechanical ventilation (1.8 ± 0.3 vs. 2.3 ± 0.4 ng/ml). In contrast, after long-term mechanical ventilation, BALF LTB_4 concentrations in mice ventilated at HVT were twofold greater than in mice ventilated at LVT ($p < 0.05$; Figure 6B).

Congenital Disruption of 5LO Decreases Mortality and Attenuates VILI

To learn whether or not LTs are involved in the pathogenesis of VILI, we studied the effects of LVT and HVT ventilation in mice deficient for 5LO (Table 1). The two genotypes did not differ in terms of hemodynamic measurements (*see* Table E1) and acid-base parameters (data not shown). Mice deficient for 5LO routinely survived during LVT ventilation for the 10-hour study period. Survival during HVT ventilation was longer in 5LO-deficient than in wild-type mice ($p < 0.01$; Figure 1): 5LO-deficient mice ventilated at HVT consistently survived for 10 hours. The PV curve of the respiratory system of 5LO-deficient mice ventilated at HVT for 10 hours was shifted upwards in comparison with the baseline, as well as in comparison with wild-type mice ventilated at HVT at the end of the experiment (Figure 2C). Pulmonary microvascular permeability, as reflected by the BALF total protein concentration, increased in 5LO-deficient mice during HVT ventilation in comparison to LVT ventilation, but was less than that in wild-type mice ventilated at HVT (Figure 7A). HVT ventilation did not increase the A-aDO$_2$ of 5LO-deficient mice (90 ± 11 vs. 99 ± 10 mm Hg at baseline), and this value was markedly less than that of wild-type mice receiving HVT ventilation (216 ± 19 mm Hg, $p < 0.001$). Similarly, the Pa_{O_2} of 5LO-deficient mice ventilated at HVT did not differ from the values recorded after LVT ventilation (data not shown) and was markedly higher than that of wild-type mice receiving HVT ventilation ($p < 0.001$; Figure 7B). Neutrophil levels in BALF during HVT ventilation were lower in 5LO-deficient mice than in wild-type mice, both after 6 hours (Figure 8) and at the end of the experiment ($18 \pm 2 \times 10^3$ vs. $27 \pm 5 \times 10^3$, $p < 0.05$; *see* Table E4). After 10 hours of HVT ventilation, histologic examination of lung sections from 5LO-deficient mice showed essentially normal morphology similar to that observed in lungs of wild-type or 5LO-deficient mice ventilated at LVT and markedly different from that found in wild-type mice ventilated at HVT (Figure 4).

Preservation of HPV after HVT Ventilation in 5LO-deficient Mice

To investigate the effects of 5LO deficiency on the impairment of HPV induced by HVT ventilation, we analyzed the changes of LPVR induced by LMBO in 5LO-deficient mice ventilated with either LVT or HVT for 6 hours. Before LMBO, systemic arterial pressure and pulmonary arterial pressure were similar in both groups of 5LO-deficient mice and did not differ from

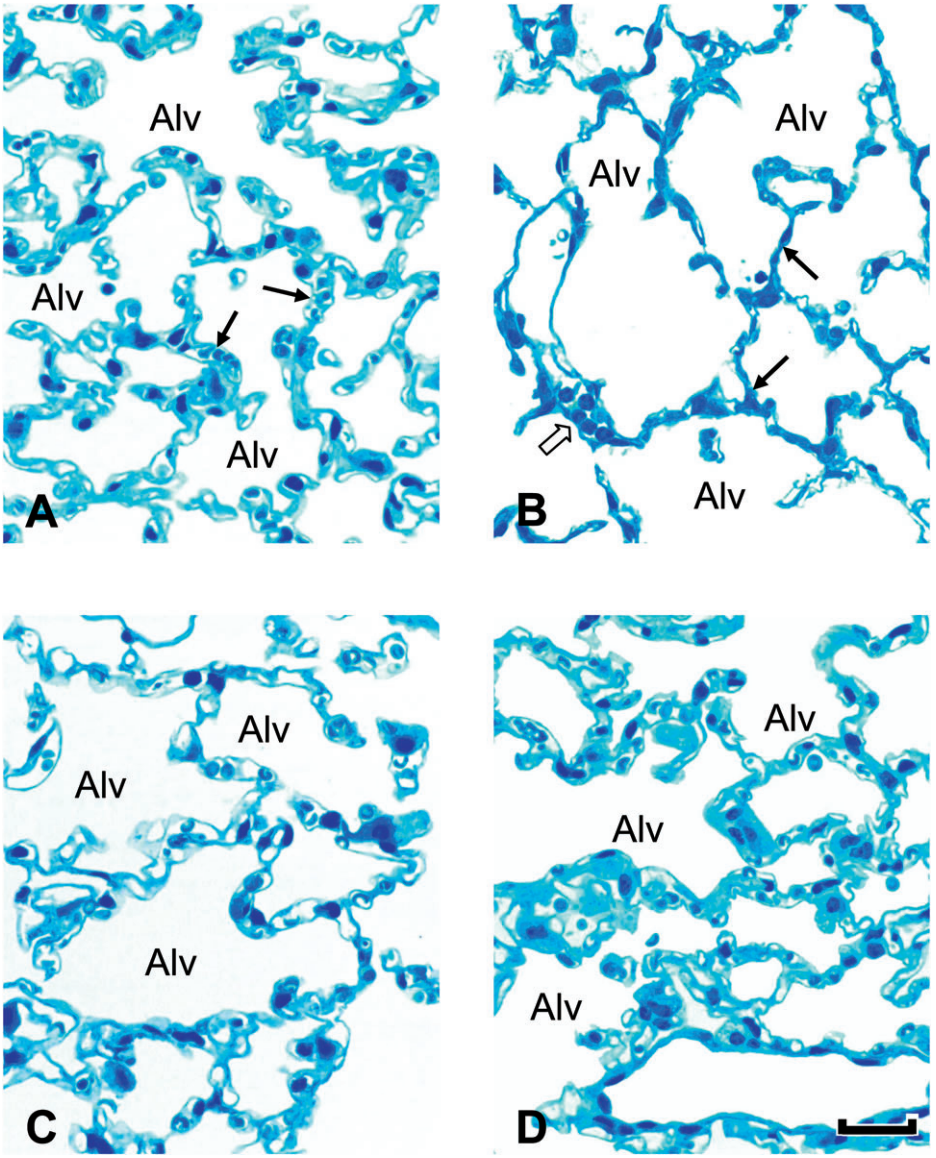

Figure 4. Lung sections 2-μm thick from WT mice ventilated at LVT (*A*) and HVT (*B*), and from 5LO-deficient mice (*C*) and MK886-pretreated WT mice (*D*) both ventilated at HVT after long-term mechanical ventilation. Comparable areas from central lung regions are shown. Note the lacey network of open capillaries after (*A*) LVT ventilation (*arrows*) and the loss of these features after (*B*) HVT ventilation (*arrows*). The narrow alveolar–capillary membrane after HVT ventilation reflects capillary collapse and epithelial surface disruption. Note also the presence of neutrophils within the intravascular space (*open arrow* in *B*). The absence of these structural changes in 5LO-deficient mice (*C*) and WT mice pretreated with MK886 (*D*) demonstrates protection against injury induced by HVT ventilation. *Bar* = 25 μm; all other panels are at the same magnification. Alv = alveolar space.

wild-type mice in corresponding experimental groups (*see* Table E3). In 5LO-deficient mice after LVT ventilation, LMBO decreased left pulmonary arterial blood flow, resulting in a 98 ± 15% increase of LPVR ($p < 0.01$; Figure 5). Similarly, after 5LO-deficient mice were ventilated at HVT, LMBO decreased left pulmonary arterial blood flow, leading to an increased LPVR, which was markedly greater than that measured in wild-type mice (94 ± 9 vs. 17 ± 8%, $p < 0.001$; Figure 5).

Inhibition of 5LO-activating Protein Decreases VILI

Because strain differences in the background of wild-type and 5LO-deficient mice might potentially lead to differing responses to HVT ventilation (32), we sought to confirm that 5LO deficiency attenuates VILI by studying wild-type mice pretreated with the FLAP inhibitor MK886 (Calbiochem, San Diego, CA). Survival during HVT ventilation of MK886-pretreated wild-type mice did not differ from 5LO-deficient mice and was longer than that in untreated animals ($p < 0.01$; Figure 1). HVT ventilation of MK886-pretreated wild-type mice shifted the PV curve slightly downward at low lung inflation volumes, but the shift was less marked than that caused by HVT ventilation of untreated wild-type mice (Figure 2D). Pretreatment of wild-type mice with MK886 prevented the increase in EVLW associated with HVT ventilation (4.1 ± 0.6 vs. 6.1 ± 0.5 g H_2O/g dry lung in untreated wild-type mice, $p < 0.05$). Moreover, HVT ventilation of MK886-pretreated wild-type mice increased BALF total protein concentration to a lesser extent than was observed in untreated wild-type mice (Figure 7A). HVT ventilation did not impair gas exchange in MK886-pretreated mice: the A-aD_{O_2} (data not shown) and Pa_{O_2} (Figure 7B) after 10 hours of HVT ventilation were similar to the values recorded in mice at baseline. Pretreatment with MK886 significantly reduced the pulmonary neutrophil recruitment associated with HVT ventilation, as reflected by the decreased neutrophil levels in BALF obtained from MK886-pretreated wild-type mice in comparison to untreated animals (Figure 8 and Table E4). Histopathologic evaluation of lung sections from MK886-pretreated mice ventilated at HVT showed protection against VILI to the same extent as that observed in 5LO-deficient mice (Figure 4). Pretreatment of wild-type mice with MK886 prevented the increase of cysLTs and LTB_4 concentrations in BALF after HVT ventilation (Figure 6). Of note, when wild-type mice were treated with the vehicle used to dissolve MK886, they mani-

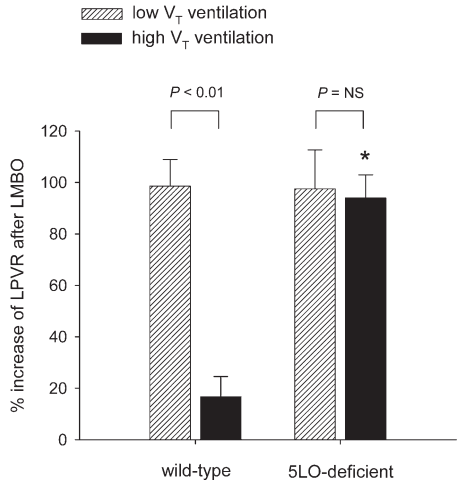

Figure 5. Effects of congenital deficiency of 5LO on the left mainstem bronchus occlusion (LMBO)–induced increase in left pulmonary vascular resistance (LPVR) in mice ventilated at LVT or HVT for 6 hours. In mice ventilated at LVT, LMBO markedly increased LPVR both in WT (n = 8) and 5LO-deficient mice (n = 4). In contrast, after HVT ventilation, LMBO did not increase LPVR in WT mice (n = 8), whereas it did increase LPVR in 5LO-deficient mice (n = 3; *p < 0.001 vs. WT mice). NS = not significant.

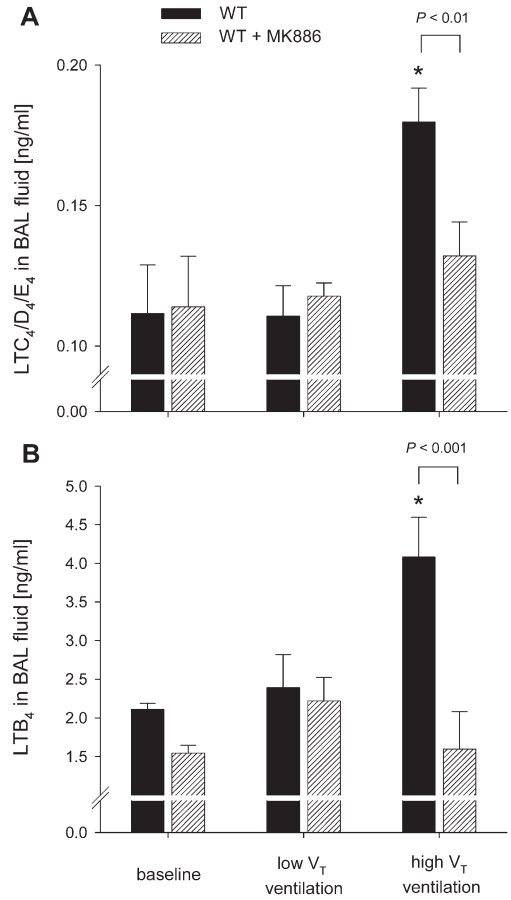

Figure 6. Leukotriene (LT) $C_4/D_4/E_4$ (*A*) and LTB_4 (*B*) levels in BALF obtained from untreated and MK886-pretreated WT mice after long-term mechanical ventilation with LVT (n = 6, both groups) or HVT (n = 6, both groups) and from mice studied at baseline (n = 3, both groups). Both $LTC_4/D_4/E_4$ and LTB_4 levels were significantly increased in untreated WT mice after HVT ventilation (*p < 0.05 vs. LVT ventilation and baseline). In contrast, pretreatment with MK886, a 5LO-activating protein (FLAP) inhibitor, blocked the increase of LT production associated with HVT ventilation.

fested a similar development of respiratory failure during HVT ventilation as did untreated wild-type mice (data not shown).

Effects of a CysLT Receptor 1 Antagonist on Lung Injury Caused by HVT Ventilation

To elucidate the role of cysLTs in the pathogenesis of VILI, wild-type mice were treated with MK571, a cysLT receptor 1 antagonist (Cayman Chemical, Ann Arbor, MI). In MK571-treated wild-type mice ventilated at HVT, survival did not differ from 5LO-deficient or MK886-pretreated wild-type mice and was longer than that observed in untreated wild-type mice (100% survival after 10 hours of HVT ventilation, $p < 0.01$). At the end of the experiment, total protein concentration in BALF from MK571-treated wild-type mice was lower than that obtained from untreated animals (Figure 7A) and was similar to that measured in BALF from 5LO-deficient mice and MK886-pretreated wild-type mice. Moreover, treatment of wild-type mice with MK571 prevented the increase in EVLW associated with HVT ventilation (3.7 ± 0.2 vs. 6.1 ± 0.5 g H_2O/g dry lung in untreated wild-type mice, $p < 0.05$). The Pa_{O_2} after HVT ventilation was greater in MK571-treated than in untreated wild-type mice ($p < 0.01$) and did not differ from that measured in 5LO-deficient and MK886-pretreated wild-type mice (Figure 7B). In contrast, pulmonary neutrophil recruitment into BALF induced by HVT ventilation was not attenuated by MK571 treatment (Figure 8 and Table E4).

DISCUSSION

Our study demonstrates that long-term mechanical ventilation of mice with HVT induces severe acute lung injury, characterized by pulmonary inflammation and edema accumulation and by severely altered gas exchange with evidence of impaired HPV and systemic arterial hypoxemia, ultimately leading to death. Congenital deficiency of 5LO markedly attenuates the development of acute respiratory failure during HVT ventilation, reducing lung inflammation and preserving the effectiveness of HPV and systemic oxygenation. Treatment with the FLAP inhibitor MK886 or the selective $cysLT_1$ receptor antagonist MK571 reduced pulmonary microvascular permeability and edema formation associated with HVT ventilation and maintained systemic oxygenation to the same extent as did congenital 5LO deficiency. These findings suggest an important role for LTs, particularly cysLTs, in the pathogenesis of VILI.

In contrast to previous studies, both in mice (14–16, 33) and other species (2, 7, 34), in which VILI was investigated after exposure to mechanical ventilation for shorter periods of time or without producing severe lung injury, our animal model enabled exposure of mice to mechanical ventilatory stress until fatal respiratory failure was produced. This design permitted us to investigate the impact on survival of different ventilation strategies and potential treatments and to characterize the sequence of events that contribute to the pathophysiology of murine VILI. Mice were ventilated at HVT for a maximal period of 10 hours. To have a reproducible severity and time course of the development of the lung injury, VT was set in each animal as a percentage of the IC of the respiratory system (35). From pilot studies, we learned that mechanical ventilation with a VT near 40% of IC

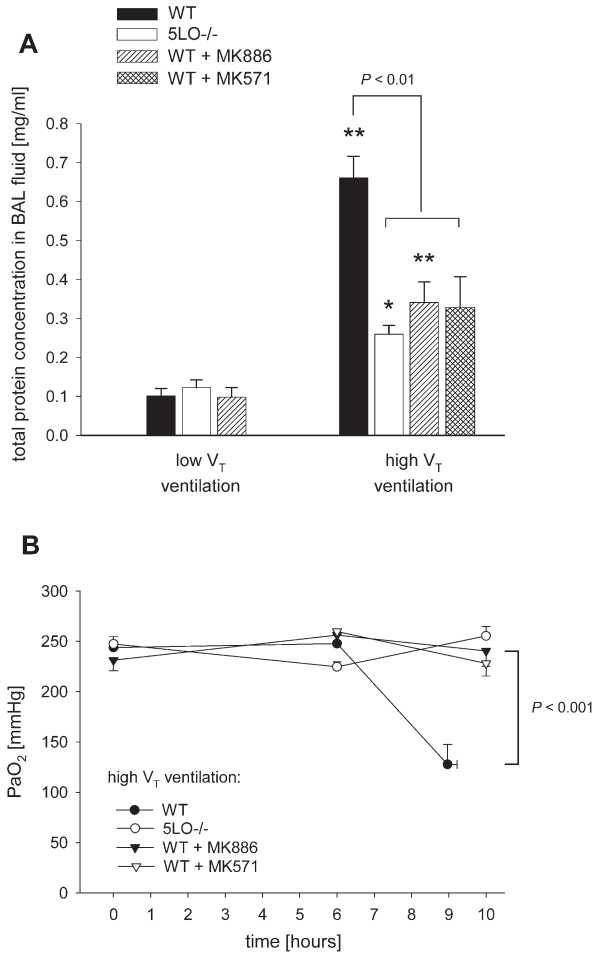

Figure 7. Total protein concentration in BALF (*A*) and Pa_{O_2} (*B*) in untreated WT mice, 5LO-deficient mice, and WT mice treated with MK886 or MK571 after prolonged ventilation. Total protein concentration in BALF at the end of the experiment (*A*) was markedly higher in untreated WT mice ventilated at HVT (n = 6) than in WT mice ventilated at LVT (n = 6). Congenital deficiency of 5LO (n = 5) and treatment with MK886 (n = 6) or MK571 (n = 3) significantly reduced the increase in BALF total protein concentration caused by HVT ventilation (*$p < 0.05$, **$p < 0.01$ vs. LVT ventilation). Pa_{O_2} (*B*) markedly decreased in untreated WT mice after HVT ventilation (n = 10 at baseline, n = 5 at 6 hours, and n = 15 at the end of the experiment). In contrast, Pa_{O_2} remained unchanged during HVT ventilation in 5LO-deficient mice (n = 7 at baseline, n = 5 at 6 hours, and n = 8 at the end of the experiment), in MK886-pretreated WT mice (n = 6 at baseline, n = 5 at 6 hours, and n = 12 at the end of the experiment), and in MK571-treated WT mice (n = 5 at 6 hours, n = 6 at the end of the experiment).

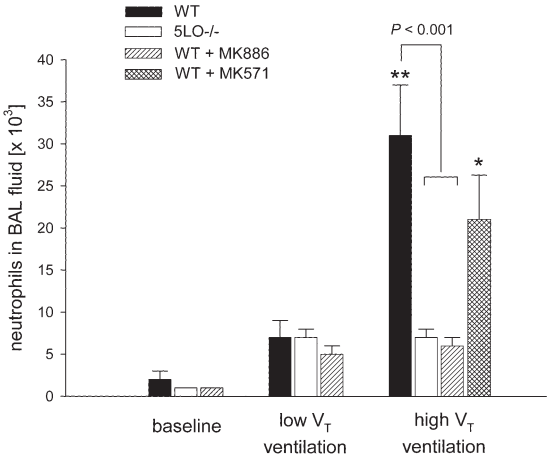

Figure 8. Neutrophil levels in BALF obtained from untreated WT mice, 5LO-deficient mice, and WT mice treated with MK886 studied at baseline (n = 4, each group) and after 6 hours of LVT or HVT ventilation (n = 5, each group), and from MK571-treated WT mice ventilated at HVT for 6 hours. HVT ventilation significantly increased neutrophil levels only in untreated WT and in MK571-treated WT mice (**$p < 0.001$ vs. baseline and LVT ventilation, *$p < 0.05$ vs. 5LO-deficient and MK886-pretreated WT mice ventilated at HVT). Congenital deficiency of 5LO and pretreatment with MK886 prevented the increased levels of neutrophils in BALF associated with HVT ventilation ($p < 0.001$ vs. untreated WT mice).

produced severe lung injury after 6 to 7 hours of ventilation. In contrast, VT set according to the body weight of the animals led to a greater variability of the severity and the time of onset of severe lung injury. For LVT ventilation, VT was set equal to 12% of IC. In an attempt to examine only the variation of VT, a similar respiratory rate was used for both ventilatory strategies (100 and 90 breaths/minute during LVT and HVT ventilation, respectively). Because of the marked difference of minute ventilation between the LVT and HVT group, different fractions of carbon dioxide were added to the inspiratory gas mixture to maintain Pa_{CO_2} within a physiologic range. Thus, we were able to avoid respiratory acidosis or alkalosis, which might influence the production of inflammatory mediators and the pathophysiology of acute lung injury (36–38).

Small animals have been reported to require a shorter period of time to develop VILI (as short as 1 hour) (2) than larger animals (24–48 hours) (3), suggesting that mechanical stress–driven injury predominates in small animals, whereas inflammation-induced injury has a more important role in larger animals (7). In the present study, the survival time during HVT ventilation was significantly longer than that previously reported in rodents (7, 33). Moreover, wild-type mice were ventilated with an HVT of approximately 25 ml/kg, generating a peak airway pressure of 22 cm H_2O (Table 1), a lung stretch of milder intensity than was produced in previous murine models of VILI (14, 16, 33). Of note, the deterioration of the respiratory system during HVT ventilation was preceded by pulmonary neutrophil and cysLT accumulation. These findings support the hypothesis that HVT ventilation induces murine lung injury, at least partially, mediated and amplified by inflammation, as recently suggested (14–16). It is conceivable that VILI develops as a result of a wide spectrum of mechanisms, in which two extremes may be recognized (39): short-term VILI, induced by higher stretch/VT with a predominance of mechanical stress–induced mechanisms, and long-term VILI, induced by a milder stimulus leading to inflammation-induced mechanisms of lung injury.

Because a deterioration of arterial oxygenation is frequently observed in association with severe VILI, and because HPV serves to prevent the systemic hypoxemia associated with alveolar edema and intrapulmonary shunting, we investigated whether HVT mechanical ventilation itself could alter the pulmonary vasoconstrictor response to alveolar hypoxia. We chose to examine the effect of alveolar hypoxia on HPV before the development of pulmonary edema, which typically commences after 6 hours of HVT ventilation. In wild-type mice ventilated at HVT, LMBO did not significantly increase the LPVR, consistent with a severe impairment of HPV. This impairment did not occur

with LVT ventilation. To our knowledge, these findings demonstrate for the first time that the decreased gas exchange efficiency associated with VILI is caused not only by the accumulation of lung edema but also by inhibiting the ability of the pulmonary circulation to divert blood flow from hypoxic/poorly ventilated to well-ventilated lung regions. Of note, at the time of HPV assessment, arterial oxygenation remained preserved, likely because alveolar edema was not yet present, as suggested by our analysis of BALF total protein concentration.

The impairment of HPV was unlikely to be caused by mechanical disruption of the pulmonary vascular contractile apparatus induced by high-stretch/VT ventilation. The observation that angiotensin II induced a similar increase of pulmonary vascular resistance both in mice ventilated at LVT and HVT clearly demonstrates the preservation of vascular reactivity to an exogenous agonist. Intravenous infusion of angiotensin II also restored HPV after HVT ventilation. HPV is considered an intrinsic property of pulmonary vascular smooth muscle cells that is modulated by vasoactive mediators (40). The magnitude of HPV at any time results from the balance between vasodilating and vasoconstricting factors (31). Thus, HVT ventilation is likely to alter the levels of vasodilators and vasoconstrictors, favoring vasodilation and impairing HPV. The infusion of angiotensin II is likely to have readjusted the balance toward vasoconstriction, thereby restoring HPV.

Enhanced levels of both LTB_4 and cysLTs were detected in BALF obtained from mice after HVT mechanical ventilation, in comparison with mice after LVT ventilation and animals studied at baseline. LT concentrations have been reported to be elevated in other experimental models of acute lung injury (22, 27). Moreover, increased levels of LTs have been detected in pulmonary edema fluid obtained from patients with ARDS (23–25). LTs are primarily synthesized by activated cells of myeloid origin, such as neutrophils, eosinophils, mast cells, and monocytes/macrophages (26). It has been demonstrated, both in animal models (7, 14) and patients with ARDS (41), that high stretch/VT ventilation can induce activation and pulmonary recruitment of neutrophils and alveolar macrophages. We found that HVT ventilation induced pronounced neutrophil accumulation in BALF both after 6 hours of ventilation and at the end of the study. It is probable that HVT ventilation recruits/activates myeloid cells responsible for pulmonary LT biosynthesis. Increased concentration of LTs, particularly the chemoattractant LTB_4, may recruit additional leukocytes, thereby amplifying the inflammatory response to HVT. Taken together, these findings led us to hypothesize a role for LTs in the pathogenesis of VILI.

Congenital disruption of 5LO or pretreatment with the FLAP inhibitor MK886 during HVT ventilation significantly preserved respiratory mechanics and prevented the increase of alveolar–capillary membrane permeability. Systemic arterial oxygenation was maintained, and survival was increased. Because products of the 5LO pathway, especially cysLTs, are vasoactive mediators (18) and have been previously implicated in the impairment of HPV induced by endotoxemia (27), we investigated the role of 5LO in the impairment of HPV associated with VILI. We found that 5LO-deficient mice were protected from the deleterious effects of HVT ventilation on HPV. Thus, it is possible that the production of LTs associated with HVT ventilation alters the balance of vasodilators and vasoconstrictors regulating pulmonary vascular tone in wild-type mice, leading to an impaired HPV. Although other vasoactive mediators produced during HVT ventilation may contribute to the loss of HPV, such as nitric oxide (42) or prostacyclin (13), our results suggest that activation of 5LO is required to impair HPV during the development of VILI.

The reduction in lung neutrophil accumulation in 5LO-deficient and in MK886-pretreated wild-type mice ventilated at HVT suggests a possible role for the chemoattractant LTB_4 in neutrophil recruitment associated with VILI. Despite preventing the ventilator-induced pulmonary accumulation of leukocytes measured after 6 hours of ventilation, 5LO-deficient mice and MK886-pretreated wild-type mice showed increased BALF neutrophil levels after more prolonged HVT ventilation, albeit to a lesser extent than was observed in wild-type mice. It is conceivable that additional LT-independent mechanisms, such as the recently described activation of the chemokine receptor CXCR2 (14), contribute to the pulmonary accumulation of leukocytes during VILI.

Because increased concentrations of cysLTs were detected in BALF from mice ventilated at HVT before the development of lung injury, we studied the effects of treatment with MK571, a selective $cysLT_1$ receptor antagonist, during HVT ventilation. Treatment of wild-type mice with MK571 reduced BALF plasma protein concentration to the same extent as was measured in 5LO-deficient and MK886-pretreated wild-type mice. In addition, MK571-treated wild-type mice ventilated at HVT did not develop pulmonary edema, and survival was enhanced to a similar extent as that observed for 5LO-deficient and MK886-pretreated mice. MK571 also prevented the impaired gas exchange induced by HVT. We previously observed that pretreatment with MK571 prevented the impairment of gas exchange and loss of HPV induced by endotoxin challenge. Although not measured directly in this study, it is probable that MK571 preserves systemic oxygenation in mice subjected to HVT ventilation, at least in part, by maintaining the ability of the pulmonary vasculature to vasoconstrict in response to hypoxia. These observations suggest that activation of the $cysLT_1$ receptor contributes to the pathogenesis of VILI. Interestingly, as previously reported for endotoxin challenge (27), treatment with MK571 did not reduce pulmonary neutrophil accumulation in BALF associated with HVT ventilation, suggesting that pulmonary neutrophil infiltration alone is not sufficient to cause VILI. The $cysLT_1$ receptor has been detected in pulmonary smooth muscle cells as well as in circulating blood leukocytes (20), and it has been recently suggested that cysLTs may act as activating ligands for peripheral leukocytes (43). It is conceivable that MK571 may partially prevent the development of VILI by inhibiting the activation of leukocytes recruited to the lung by HVT ventilation. The incomplete reduction of pulmonary plasma protein extravasation after HVT ventilation in 5LO-deficient mice and in MK886- and MK571-treated wild-type mice suggests that additional LT-independent mechanisms also contribute to impaired alveolar–capillary membrane function during HVT ventilation (7, 9, 44).

VILI significantly contributes to the mortality associated with ARDS (45). In a recent randomized controlled clinical trial (46), the use of LVT ventilation in patients with ARDS led to a 22% reduction of mortality in comparison with a traditional HVT ventilatory strategy. Despite improvements in care, the mortality rate of patients affected by ARDS remains approximately 30 to 40% (45). Moreover, during acute lung injury, because of the inhomogeneity of the disease, even LVT ventilation may result in high levels of stretch applied to local lung regions, thereby further worsening lung injury (47). A common feature of ARDS is severe arterial hypoxemia, which is attributed in part to an attenuation of the vasoconstrictor response to hypoxia (HPV) (6). The current study demonstrated in mice that mechanical ventilation at HVT impairs HPV. Moreover, interruption of the 5LO pathway attenuated VILI and prevented the impairment of HPV associated with HVT ventilation, suggesting a critical role of LTs, especially cysLTs, in the pathogenesis of murine VILI. If observations in mice may be extrapolated to humans,

these findings suggest that pharmacologic inhibition of LT production or LT receptor blockade may prevent the injurious effects of mechanical ventilation on the efficiency of the respiratory system and HPV in patients with acute respiratory failure.

***Conflict of Interest Statement*:** P.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. F.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.C.J. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.D.B. has presented studies described in this manuscript to employees of Critical Therapeutics, Inc. (CTI), and received an honorarium for his presentation. CTI is interested in testing an inhibitor of 5LO in clinical acute lung injury. W.M.Z. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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