Mechanical ventilation causes airway distension with proinflammatory sequelae in mice

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Mechanical ventilation causes airway distension with proinflammatory sequelae in mice. Am J Physiol Lung Cell Mol Physiol 307: L27–L37, 2014. First published May 9, 2014; doi:10.1152/ajplung.00288.2013.—The pathogenesis of ventilator-induced lung injury has predominantly been attributed to overdistension or mechanical opening and collapse of alveoli, whereas mechanical strain on the airways is rarely taken into consideration. Here, we hypothesized that mechanical ventilation may cause significant airway distension, which may contribute to the pathological features of ventilator-induced lung injury. C57BL/6J mice were anesthetized and mechanically ventilated at tidal volumes of 6, 10, or 15 ml/kg body wt. Mice were imaged by flat-panel volume computer tomography, and central airways were segmented and rendered in 3D for quantitative assessment of airway distension. Alveolar distension was imaged by intravital microscopy. Functional dead space was analyzed in vivo, and proinflammatory cytokine release was analyzed in isolated, ventilated tracheae. CT scans revealed a reversible, up to 2.5-fold increase in upper airway volume during mechanical ventilation compared with spontaneous breathing. Airway distension was most pronounced in main bronchi, which showed the largest volumes at tidal volumes of 10 ml/kg body wt. Conversely, airway distension in segmental bronchi and functional dead space increased almost linearly, and alveolar distension increased even disproportionately with higher tidal volumes. In isolated tracheae, mechanical ventilation stimulated the release of the early-response cytokines TNF-α and IL-1β. Mechanical ventilation causes a rapid, pronounced, and reversible distension of upper airways in mice that is associated with an increase in functional dead space. Upper airway distension is most pronounced at moderate tidal volumes, whereas higher tidal volumes redistribute preferentially to the alveolar compartment. Airway distension triggers proinflammatory responses and may thus contribute relevantly to ventilator-induced pathologies.

mechanical ventilation; airways; strain; inflammation; ventilator-induced lung injury

Although mechanical ventilation is frequently a mandatory requirement to maintain adequate oxygenation and removal of carbon dioxide in critically ill patients, extensive experimental and clinical research over the past decades has revealed that mechanical ventilation itself may have detrimental effects and promote or aggravate existing lung injury. In the landmark study of the Airway Respiratory Distress Syndrome Network (1), the reduction of tidal volume (VT) from conventional 12 ml/kg body wt (bw) to 6 ml/kg bw resulted in a significant reduction of hospital mortality (31% vs. 39.8%). However, the implementation of lung-protective ventilation strategies is often hampered by clinical requirements to sustain sufficient gas exchange and by the heterogeneous distribution of airflow in injured lungs, resulting in overventilation of healthy, aerated lung regions (baby lung concept) (4). In line with this view, recent clinical trials indicate that further reductions of VT to values as low as 3 ml/kg when combined with extracorporeal CO2 elimination may have additional benefits over protective ventilation with 6 ml/kg (3, 31), stressing the need for further optimization and refinement of ventilation strategies to avoid or minimize ventilator-induced lung injury (VILI).

Conventionally, VILI has been attributed to pressure- or volume-related overdistension and the resulting mechanical injury of the alveolo-capillary barrier (baro- and volutrauma, respectively) (6). Subsequent studies identified that minor forms of alveolar overdistension also may promote lung injury through mecano-induced release of proinflammatory cytokines (biotrauma) (25, 30) and trigger pulmonary vascular dysfunction (18). In addition, cyclic opening and collapse of distal airways and alveoli has been proposed to generate high levels of mechanical stress on airway and alveolar epithelial cells with subsequent cell and organ injury (atelectrauma) (28).

In comparison, potential mechanical stretch effects in the airways and their contribution to VILI have been scarcely considered as of yet. In humans, trachea and main bronchi are mechanically stabilized through cartilage rings. Smaller airways, however, only show cartilage fragments or entirely lack a stabilizing scaffold. Although distal human airways may thus be subject to considerable stretch effects, analyses of distal airway dimensions during mechanical ventilation have so far evaded direct assessment by noninvasive imaging techniques. The lack of a cartilage scaffold in distal human airways is similar to the situation in the upper airways of the rodent lung.
where cartilage rings are limited to the trachea and cartilage fragments to the main bronchi (13). Hence, upper airway mechanics in rodents may serve as a surrogate for distal airway mechanics in larger mammals but, in contrast to the latter, have recently become accessible by state-of-the-art imaging techniques.

Innovative work by Sinclair and colleagues (21) using radiographic real-time imaging of rats following tantalum dust inhalation revealed the high distensibility of the upper airways in rodents by showing that the upper bronchi distend and retract in a cyclic manner with each single breath in spontaneously breathing rats. Prominent airway distension may directly contribute to hypoxemia subsequent to overventilation, as persistent increases in dead space will reduce alveolar ventilation after return to protective ventilation modes or spontaneous breathing, respectively. In addition, upper airway stretch may have proinflammatory effects and promote leukocyte recruitment into the airways, as suggested by the intravital microscopic observation that distension of the trachea promotes leukocyte-endothelial interaction in microvessels of the upper airway walls of ventilated mice (32). The notion of a potential role of the airways in the early inflammatory response to mechanical ventilation is further supported by genomic analyses by Copland and colleagues (5), who detected an upregulation of mitochondrial RNA levels of proinflammatory cytokines in bronchial epithelial cells of overventilated rats that markedly preceded genomic effects at the alveolar level.

On the basis of these findings, we hypothesized that overventilation will cause significant upper airway distension in small rodents, which may be relevant to the pathogenesis and characteristic pathological features of VILI. To test this concept, we analyzed the effects of mechanical ventilation on airway distension and subsequent proinflammatory effects in mice.

MATERIALS AND METHODS

Animals. Male adult C57BL/6J mice (25 ± 6 g bw) were purchased from Charles River (Sulzfeld, Germany). All animals received care in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996). The study was approved by the Animal Care and Use Committee of the local government authorities.

Surgical preparation. Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg bw) (Ketavet VET; Pfizer, New York, NY) and xylazine (20 mg/kg bw) (Rompun; Bayer HealthCare, Leverkusen, Germany) and placed in supine position on a thermostatic blanket (Homeothermic Blanket; Harvard Apparatus, Holliston, MA) that was feedback controlled via a rectal probe to maintain body temperature at 38°C (8). Animals were tracheotomized and intubated with a metal tracheal tube (1-mm OD, 16-mm length; Harvard Apparatus) for mechanical ventilation according to the individual protocols outlined below. Airway pressure was continuously monitored via a differential pressure transducer (MPX Type 399/2; Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany) and recorded by the software package DAISYlab (Datolog, Moenchengladbach, Germany). Anesthesia was maintained by intraperitoneal injections of 50% of the initial bolus combination every 30 min.

Volume computer tomography. Mice were positioned supine inside the tube (diameter: 93 cm) of a flat-panel volume CT scanner (Siemens Medical Solutions, Forchheim, Germany). This prototype combines the advantages of a CT scanner (high temporospatial resolution suitable for post hoc 3D reconstruction) with digital flat-panel detectors of conventional X-ray imaging, resulting in ultra-high-spatial resolution (voxel size: 150 μm³) and rapid image acquisition of 19 s/scan (7). This combination provides unique opportunities for repetitive imaging of murine upper airway dimensions under different ventilation conditions. From the acquired images, individual airways were segmented by an in-house-generated algorithm using the open source toolkit MITK (Version 0.99.3; German Cancer Research Center, Division of Medical and Biological Informatics, Heidelberg, Germany) (34) and 3D reconstructed as described before (33), allowing for the quantitative analysis of bronchial cross-sectional areas and total upper airway volumes (UAV). Cross-sectional areas were measured at the following locations based on defined anatomical landmarks and the branching pattern of the upper airways: the trachea just above the bifurcation (first branching) and the right and left main bronchi just above the branching off of the right upper lobar bronchus, whereas segmental bronchi were defined as the most distal branches that were detectable both under spontaneous breathing and mechanical ventilation. Density of lung parenchyma in the acquired CT scans was quantified using the software package CHILI PACS (Version 4.4.0; CHILI, Dossenheim/Heidelberg, Germany).

Intravital microscopy. Alveolar distension was visualized by intravital darkfield microscopy in the anesthetized and ventilated mouse as previously described (19). Images of the two-dimensional projection of subpleural alveoli were acquired during the end-inspiratory plateau phase, and alveolar boundaries were traced offline by an in-house software for calculation of alveolar dimensions.

Capnography. For capnographic assessment of functional dead space, a separate set of animals was anesthetized, tracheotomized, and ventilated as described above. The left carotid artery was cannulated with a polyethylene tube (0.28-mm ID, 0.61-mm OD; Smiths Medical International, Kent, UK), allowing for the determination of arterial partial pressure of CO₂ by blood gas analysis (RapidLab 348; Siemens HealthCare Diagnostic, Eschborn, Germany). The fraction of CO₂ in exhaled air was measured by the nondispersive infrared gas sensing method (O₂/CO₂ measuring Unit; TSE Systems, Bad Homburg, Germany), and functional dead space was calculated according to the Bohr equation (27).

Histology. Following 50 min of ventilation with either of the protocols outlined below, animals were euthanized by exsanguination, and lungs were harvested and fixed in 4% formalin. Paraffin-embedded and hematoxylin-eosin-stained slices were microscopically imaged, and areas of perivascular hemorrhage (n = 135 from ≥ 3 animals per group) were quantified using the software Neurolucida (MBF Bioscience, Williston, VT) and expressed relative to the lumen area of the corresponding vessel.

Experimental groups and protocols. For volume computer tomography (vCT) imaging, animals were randomly assigned to four different groups (n = 4–5 each). To test for effects of continuous mechanical ventilation on airway dimensions as a function of V₉, three different groups were continuously ventilated (Midivent; Hugo Sachs Elektronik, March-Hugstetten, Germany) over 50 min with V₉ of either 6, 10, or 15 ml/kg bw and a positive end-expiratory pressure (PEEP) of 2 cmH₂O. As low VT ventilation with room air did not reliably sustain adequate oxygenation in pilot experiments, the inspiratory fraction of O₂ (F(IO₂)) was set to 1.0 for all tested ventilation settings. Respiratory rate was set to 170, 150, or 100 breaths per minute (bpm), respectively, to maintain arterial partial pressure of CO₂ (Paco₂) between 35 and 45 mmHg during the different VT modes. For each group, vCT scans were taken during spontaneous breathing and at 5, 10, 15, 20, 30, 40, and 50 min after onset of mechanical ventilation with the respective V₉.

To test for the reversibility of V₉-dependent changes in airway dimensions, a fourth group was ventilated intermittently with V₉ of 6, 10, and 15 ml/kg bw in increasing order for 10 min each, yet animals were disconnected from the ventilator between different V₉ for a 10-min period of spontaneous breathing. vCT scans were taken at baseline during spontaneous breathing and subsequently at the end of
each 10-min interval of mechanical ventilation or spontaneous breathing, respectively.

For capnographic measurements of functional dead space, a separate set of mice (n = 5) was ventilated in supine position with \( V_T \) of 6, 10, and 15 ml/kg bw for 10 min each. At the end of each interval, \( \text{CO}_2 \) fraction in the exhalate was recorded, and an arterial blood sample obtained for measurement of \( \text{PaCO}_2 \) via an arterial line in the right carotid artery.

For imaging of alveolar distension, intravital microscopy was performed in a separate set of mice ventilated in random order with \( V_T \) of 6, 10, and 15 ml/kg bw (\( F_{\text{H}_2O} 0.21, \text{PEEP} 2 \text{cmH}_2\text{O} \)) for 10 min each.

**Isolated organ bath.** To probe for potential proinflammatory effects of airway stretch in mechanical ventilation, murine tracheae were harvested from euthanized mice, cannulated on both ends, and positioned in an isolated organ bath (isolated airway module FV-IAM-TB20, FlexiVent; Scireq, Montreal, Ontario, Canada). Both trachea and bath were filled with PBS, and the caudal cannula was closed to mimic the reciprocating ventilation pattern of the intact lung. Isolated tracheae were then ventilated via the cranial cannula for 4 h with a

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**Fig. 1.** Upper airway distension during mechanical ventilation in anesthetized mice. Representative volume computer tomography (vCT) scans from individual mice taken either during spontaneous breathing (top) or after 50 min of mechanical ventilation with 6, 10, or 15 ml/kg body wt (bw) tidal volume \( (V_T) \) (top middle, bottom middle, and bottom) show airway dimensions at the level of the main bronchi (arrows, left) and the lobar (arrows) and segmental (arrow heads) bronchi (right). Comparable image planes in the different mice were selected based on anatomical landmarks including the position of the scapula, heart shadow, and xiphoid process as well as the branching pattern of the upper airways. Considerable airway distension compared with spontaneous breathing is evident at all 3 mechanical ventilation modes and in all imaged bronchial segments.
A positive inspiratory pressure of 17 cmH₂O, a PEEP of 2 cmH₂O, and a respiratory rate of 100 bpm (Compact Animal Respirator, TSE Systems), mimicking the respiratory settings during ventilation with a VT of 15 ml/kg bw in vivo. Ventilator-induced cyclic distension of the isolated trachea was visualized by direct microscopic imaging (Axioptech vario 100HD; Zeiss, Jena, Germany) and quantified offline as difference in tracheal diameter between end-expiration and end-inspiration. Nonventilated, isolated tracheae served as controls (n = 10 each). After 4 h, the intratracheal fluid (volume: ~50 µl) was retrieved and stored at −80°C. Concentrations of the proinflammatory
cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and macrophage inflammatory protein-2 (MIP-2) in the intratracheal fluid were quantified by commercially available ELISA (QuantiKine; R&D Systems, Minneapolis, MN).

Statistical analyses. All data are presented as means ± SE. Data were tested for intragroup differences at two time points by paired *t*-test and Wilcoxon signed-rank test according to normal distribution and Friedman repeated-measures ANOVA on ranks for multiple consecutive time points. For intergroup differences of two or more groups, respectively, data were analyzed by Mann-Whitney rank sum test or Kruskal-Wallis one-way ANOVA on ranks and appropriate post hoc test. Spearman’s rank correlation coefficient (r_s) was calculated and linear regression analysis performed (SigmaPlot 11.0; Systat Software, Chicago, IL). Statistical significance was assumed at P < 0.05.

RESULTS

Mechanical ventilation causes reversible, nonlinear distension of upper airways. Flat-panel vCT images of the thorax were recorded in anesthetized mice during either spontaneous breathing or continuous mechanical ventilation with VT of 6, 10, or 15 ml/kg bw. Representative vCT images show considerable widening of the upper airways at the level of the main, lobar, and segmental bronchi during all three mechanical ventilation modes compared with spontaneous breathing (Fig. 1). Segmentation and three-dimensional reconstruction of the airways from the obtained vCT images revealed a profound and homogeneous distension of the upper airways during mechanical ventilation, as exemplified in Fig. 2A for an individual animal intermittently ventilated with increasing VT. From 3D reconstructions, the total volume of the upper airways down to the level of the segmental bronchi, which represented the smallest size of airways clearly distinguishable in vCT images, was calculated. Only airways that were precisely identified under spontaneous breathing and during all subsequent mechanical ventilation modes were included into the analysis. Upon onset of mechanical ventilation, calculated UAV roughly doubled within <5 min (Fig. 2B). Thereafter, upper airway dimensions remained relatively constant over a subsequent period of 50 min during ventilation at 6 ml/kg bw VT. At a VT of 10 ml/kg bw, airway dimensions showed a tendency to increase further with time and significantly exceeded UAV of mice ventilated with 6 ml/kg bw after 30 min. Surprisingly, ventilation with a VT of 15 ml/kg bw did not cause further airway distension but in contrast resulted in airway dimensions that were only slightly larger than those at 6 ml/kg bw VT. Intermittent mechanical ventilation with increasing VT and alternating periods of spontaneous breathing in individual mice showed a similar pattern of airway distension as seen under continuous mechanical ventilation, in that all three VT modes caused marked upper airway distension, with 10 ml/kg bw showing again a higher UAV compared with VT of 6 and 15 ml/kg bw, respectively. Return to spontaneous breathing restored baseline UAVs within <10 min, demonstrating rapid reversibility of ventilator-induced upper airway distension (Fig. 2C). In contrast to the inverted U curve of the UAV vs. VT relationship, airway pressure (AWP), measured in a separate set of animals due to the restrictions of the vCT scanner, showed a progressive increase with higher VT (Fig. 2D). Upper airway compliance for each given VT was estimated as the ratio (ΔUAV/ΔAWP) of the changes in UAV and AWP, respectively. As UAV was discontinuously recorded, changes in UAV and AWP were systematically calculated relative to their individual baseline values during spontaneous breathing. Resulting values for ΔUAV/ΔAWP indicate a decrease in upper airway compliance when VT was increased from 10 to 15 ml/kg bw (Fig. 2D).

To gain further insights into the anatomic localization and homogeneity of respiratory tract distension, we individually analyzed ventilation-induced changes in cross-sectional area for the murine trachea, the two main bronchi, and the segmental bronchi at baseline (spontaneous breathing) and after 20 min of continuous ventilation with the specified VT. In the murine trachea, mechanical ventilation induced a slight dilation (P = 0.047), which, however, failed to reach statistical significance for each individual VT. All airways distal thereof showed a marked distension in response to all applied VT. Airway distension was most pronounced in the left and right main bronchus and was most prominent at a VT of 10 ml/kg bw (Fig. 3A). In line with the anatomical characteristics of the murine bronchial tree (13), the right main bronchus was found significantly larger under spontaneous breathing and during mechanical ventilation compared with the left main bronchus. In contrast to main bronchi, segmental bronchi showed a progressive distension with higher VT (r_s = 0.67, P < 0.05) and were consequently most expanded at a VT of 15 ml/kg bw (Fig. 3B).

The notion that more distal airways, which account for the bulk of total airway volume and thus anatomical dead space, distend progressively with higher VT is further substantiated by exhaled breath CO₂ analyses. Functional dead space showed a linear increase with higher VT analogous to the changes in segmental bronchi (Fig. 3B) and in contrast to the nonlinear pressure-volume relation of the upper airways. Parallel radiodensitometric analyses of the lung parenchyma showed reduced tissue density with higher VT, indicating again increased aeration of distal airways and/or alveoli (Fig. 3C).

Direct visualization of subpleural alveoli by intravital microscopy (Fig. 3D) revealed that alveolar dimensions did not increase linearly but progressively with VT. In other

Fig. 2. Upper airways distend reversibly and as a function of VT. A: representative images of segmented vCT scans from an individual animal show 3-dimensional projections of the upper airways and parts of the lungs. Mechanical ventilation induced distinct airway distension, which extends homogeneously across the visualized bronchial tree. B: quantitative analyses of upper airway volumes from segmented vCT scans show changes in airway volume after switching from spontaneous breathing to mechanical ventilation and over 50 subsequent minutes of ventilation with VT of either 6, 10, or 15 ml/kg bw. Data are means ± SE from n = 4–5 animals each; *P < 0.05 vs. spontaneous breathing. #P < 0.05 vs. 6 ml/kg bw VT. C: group data showing the effects of sequential switching between spontaneous breathing and mechanical ventilation at 6, 10, and 15 ml/kg bw VT on upper airway volume demonstrate rapid reversibility of ventilation-induced airway distension. Different VT applied over 10-min intervals each are depicted as bars. Lines connecting the individual data points do not necessarily reflect the exact time course of airway dimension changes. Data are means ± SE from n = 4 mice; *P < 0.05 vs. subsequent spontaneous breathing interval, #P < 0.05 vs. 10 ml/kg bw VT. D: group data show upper airway volume, mean airway pressure, and airway compliance as a function of VT and relative to spontaneous breathing in anesthetized mice. Data are means ± SE from n = 4–5 mice each.
Fig. 3. Segmental analysis of respiratory tract distension. A: group data show expansion of the bronchial tree after 20 min of mechanical ventilation at 6, 10, and 15 mL/kg bw VT relative to the preceding spontaneous breathing interval. Cross-sectional area analyses were performed separately for the distal trachea, the left and right main bronchi, and the segmental bronchi as schematically illustrated (left) and repeated in triplicate at the identical locations during mechanical ventilation and spontaneous breathing. Whereas tracheae only show modest distension, main bronchi and segmental bronchi expanded prominently in nonlinear or linear fashion, respectively, during mechanical ventilation with increasing VT. Data are means ± SE from 3–5 mice each; *P < 0.05 vs. corresponding spontaneous breathing interval, #P < 0.05 vs. 10 mL/kg bw VT, †P < 0.05 vs. left main bronchus. B: group data show cross-sectional area of segmental bronchi and functional dead space as a function of VT. Data are means ± SE from 3–5 mice each; *P < 0.05 vs. 6 mL/kg bw VT. C: group data show radiodensity of lung tissue as a function of VT. Data are means ± SE from 3–5 mice each; *P < 0.05 vs. 6 mL/kg bw VT. D: lung intravital microscopic images acquired in the end-inspiratory phase of mechanical ventilation with 6, 10, and 15 mL/kg bw VT show expansion of representative alveoli (encircled) with higher VT. Scale bars = 100 μm. E: quantitative analysis of intravital microscopic recordings show disproportional increase in alveolar area as a function of VT. Data are means ± SE from 49 alveoli each in 3 mice; *P < 0.05 between the increments in alveolar expansion per 1 mL/kg bw VT.
words, the increase in alveolar size per ml $\Delta V_T$ was significantly larger when $V_T$ increased from 10 to 15 ml/kg bw, compared with alveolar expansion in response to the increase in $V_T$ from 6 to 10 ml/kg bw (Fig. 3E). Taken together, the above findings indicate that upper airway distension decreased as lung inflation increased, whereas inversely a higher fraction of $V_T$ became located to the alveolar compartment.

High $V_T$ ventilation causes vascular hemorrhage in peribronchial blood vessels and inflammatory cytokine release in isolated tracheae. Next, we analyzed histological lung sections and isolated airways to gain insight into potential structural and functional sequelae of upper airway distension. Representative images of hematoxylin and eosin-stained sections (Fig. 4A) from lungs obtained after 50 min of mechanical ventilation show worsening signs of structural damage with increasing $V_T$ in the form of overinflated lung regions with overdistended and partially ruptured alveoli. In addition, 6 ml/kg bw $V_T$ caused mild-to-moderate thickening of the alveolar septal walls, whereas 10 ml/kg bw $V_T$ consistently resulted in marked perivascular hemorrhage of larger blood vessels adjacent to nonrespiratory bronchioles. Vascular barrier failure was even more pronounced when $V_T$ was increased further to 15 ml/kg bw, resulting in the occasional occurrence of red blood cells in

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**Fig. 4.** High $V_T$ ventilation causes vascular hemorrhage in peribronchial blood vessels in the murine lung. **A:** representative images show hematoxylin and eosin (H&E)-stained lung sections obtained from spontaneously breathing control mice or after 50 min of mechanical ventilation with $V_T$ of either 6, 10, or 15 ml/kg bw at low (top) and high (bottom) magnification. All mechanical ventilation modes caused mild to moderate thickening of the alveolar septal walls. In addition, $V_T \geq 10$ ml/kg bw resulted in vascular hemorrhage of peribronchial blood vessels (arrows) and occasional intra-alveolar bleeding at $V_T$ of 15 ml/kg bw (arrowheads). Scale bars = 100 $\mu$m. **B:** group data show area of perivascular hemorrhage as determined by planimetric analyses of H&E-stained lung sections relative to the area of the corresponding blood vessel for spontaneously breathing mice (where perivascular hemorrhage was entirely absent) and for mice after 50 min of mechanical ventilation with $V_T$ of 6, 10, or 15 ml/kg bw. Data are means $\pm$ SE from a total of $n = 135$ measurements in 3–5 mice per group; *$P < 0.05$ vs. 6 ml/kg bw $V_T$. 

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the alveolar space in addition to the described perivascular hemorrhages. Planimetric analysis of hemorrhage area quantitatively documents the onset and progression of perivascular bleeding with higher VT (Fig. 4B). To test whether upper airway distension suffices to trigger the release of inflammatory mediators characteristic for VILI, we ventilated isolated murine tracheae with pressures corresponding to those measured during ventilation with 15 ml/kg bw VT in vivo. Microscopic imaging showed a moderate yet significant ventilation-dependent distension of the isolated tracheal segments by 6.8 ± 0.8% (Fig. 5, A and B), which is in reasonable agreement with the 8.5 ± 5.7% tracheal diameter distension upon ventilation with VT of 15 ml/kg bw detected by vCT imaging in vivo. Analysis of cytokine release into the tracheal lumen revealed a significant release of TNF-α and IL-1β in response to 4 h of mechanical ventilation compared with nonventilated tracheae (Fig. 5, C and D). Conversely, MIP-2, which is predominantly expressed by monocytes and macrophages (2), remained largely unchanged (Fig. 5E).

Fig. 5. Mechanical ventilation causes proinflammatory cytokine release from isolated murine airways. A: representative images of an isolated, cannulated, and sealed trachea ventilated in an organ bath with pressures similar to those measured at ventilation with 15 ml/kg bw VT in vivo, i.e., a positive inspiratory pressure of 17 cmH2O and a positive end-expiratory pressure of 2 cmH2O show distinct dilation between cartilage rings in end-inspiration compared with expiration. Scale bars = 500 μm. B: diameter measurements in isolated tracheae show ventilation-dependent airway distension. Data are means ± SE from n = 14 intercartilage rings in 3 tracheae; *P < 0.05 vs. end-expiration. C–E: group data show release of the early response cytokines tumor necrosis factor (TNF)-α (C), interleukin (IL)-1β (D), and the macrophage/monocyte-derived chemokine macrophage inflammatory protein (MIP)-2 (E) from isolated tracheae with or without mechanical ventilation. Cytokine concentrations in the intratracheal fluid were measured after 4 h by ELISA. Data are means ± SE from 10–13 tracheae per group; *P < 0.05 vs. nonventilated control.
DISCUSSION

Using flat-panel vCT, we demonstrate that mechanical ventilation in mice causes a rapid, pronounced, and reversible distension of upper airways distal to the trachea. Distension of segmental bronchi, functional dead space as quantified by capnography, and lung parenchymal aeration as an inverse of CT tissue density increased progressively with higher V\textsubscript{T}. In contrast, distension of main bronchi reached its maximum at a V\textsubscript{T} of 10 ml/kg bw but decreased at higher V\textsubscript{T} due to a decline in airway compliance that was associated with the progressive formation of perivascular hemorrhages adjacent to the large airways. The lack of upper airway distension at V\textsubscript{T} >10 ml/kg bw was paralleled by a disproportional expansion of the alveolar compartment, signifying a redistribution of V\textsubscript{T} fractions from upper to lower airspaces with increasing V\textsubscript{T} that is expected to be of both physiological and clinical relevance. In isolated tracheae, mechanical ventilation caused airway distension comparable to that observed in vivo and triggered the release of early-response cytokines. Hence, upper airways act as a volume reservoir over the physiological range of V\textsubscript{T} that may protect the alveolar membrane from excessive expansion. Upper airway distension, however, concomitantly recapitulates proinflammatory cytokine release as a key aspect of overventilated lungs and may thus play an important and thus far unrecognized role in the pathology of VILI.

Upper airway distension and proinflammatory signaling in response to mechanical ventilation. In this study, we made use of a novel prototype flat-panel volume CT scanner with an unprecedented combination of spatial and temporal resolution, which facilitated the visualization of the murine upper airways down to the level of the segmental bronchi during different mechanical ventilation modes. Although this approach allowed for repeated scans in short intervals within the same animal, the image acquisition speed does not suffice to allow for a temporal resolution of the single respiratory cycle. In pilot experiments, triggering of the acquisition sequences to the inspiratory or expiratory plateau, respectively, was tested but, because of the high respiratory rate in mice, yielded considerably worse resolution (data not shown) compared with averaged image acquisition over the full respiratory cycle while concomitantly extending the total data acquisition time disproportionately. In the present study, we therefore opted for a continuous rather than triggered acquisition protocol. As a result, the obtained images and data reflect temporal averages of 32 (for 15 ml/kg bw V\textsubscript{T}) to 54 (for 6 ml/kg bw V\textsubscript{T}) successive respiratory cycles.

Notably, resolution of upper airway dynamics over a single breath was recently realized in an elegant study in ventilated rats by microfocal X-ray imaging following inhalation of tantalum dust (21). Real-time tantalum bronchograms revealed airway distension in both spontaneous breathing and mechanically ventilated rats at end-inspiration compared with end-expiration and in addition showed an increase in circumferential airway strain with higher airway pressures. These findings are essentially in agreement with data from our present study, in that they demonstrate the profound yet reversible effect of ventilation on upper airway dimensions in small rodents. In addition, the present ability for three-dimensional rendering of the flat-panel CT data allowed for volumetric quantification, which revealed that, even with lung protective ventilation at 6 ml/kg bw, the anatomical dead space in the upper airways approximately doubled compared with spontaneous breathing and accounted by itself (i.e., without consideration of the distal airspaces) for almost 1/3 of the approximated total V\textsubscript{T} of 150 μl in a 25-g mouse. Further increase of V\textsubscript{T} from 6 to 10 ml/kg bw resulted in even more pronounced airway distension. However, as ventilation-induced upper airway distension was rapidly reversible, ventilator-induced increases in anatomical dead space are unlikely to contribute to persistent hypoxemia after return to spontaneous breathing.

Overdistension of the airways may, however, trigger or promote proinflammatory responses to mechanical ventilation and high V\textsubscript{T}, which have previously been attributed almost exclusively to alveolar overdistension. Ventilation of isolated airways with pressures similar to those applied during high V\textsubscript{T} ventilation in vivo resulted in tracheal distension and concomitant release of proinflammatory early-response cytokines, which is a hallmark of the biotrauma concept of VILI (25, 30). Notably, ventilation-induced cytokine release was evident in isolated tracheae, i.e., the segment of the upper airways that is the least susceptible to mechanical strain. Although more distal airways at present evade experimental testing in isolated organ baths due to their small size and short segment length, it is tempting to speculate that cytokine responses in the more distensible airway segments distal to the trachea may be even more pronounced. In contrast to TNF-α and IL-1β, no release of MIP-2 was detectable in overventilated tracheae, a finding that is consistent with the predominant expression of this chemokine in inflammatory cells (2) rather than the epithelial and parenchymal cells constituting the upper airways. Previously, whole rat lung gene arrays had revealed differential regulation of several genes including IL-1β within 90 min of overventilation with a V\textsubscript{T} of 25 ml/kg bw (5). Importantly, spatial analyses using in situ hybridization and laser capture microdissection revealed that upregulation of IL-1β as well as of three other target genes, Egr-1, c-Jun, and HSP70, was primarily localized to the bronchiolar airway epithelium but absent in the alveolar regions. These findings stress the potential relevance of upper airway distension in the initiation or propagation of the inflammatory response to mechanical ventilation. In addition, our present data indicate that these effects may not be confined to excessive overventilation but occur in the range of clinically relevant V\textsubscript{T}. This view is further substantiated by elegant studies from Wagner and colleagues (15, 32), who showed by intravital microscopy that airway distension by high PEEP stimulates leukocyte adhesion in postcapillary venules of the airways in both rats and mice. Notably, PEEP-induced leukocyte adhesion was blocked by the nonspecific selectin inhibitor fucoidin (15) and absent in mice deficient in either P-selectin or intercellular-adhesion molecule-1 (32), suggesting that airway distension may trigger endothelial expression of leukocyte adhesion molecules in peribronchial blood vessels. Injurious effects of mechanical ventilation on peribronchial vessels were also evident in the present study in the form of peribronchial vascular hemorrhages at V\textsubscript{T} of 10 ml/kg bw or more. Importantly, these findings indicate that the effects of upper airway distension are not confined to the airway epithelium but transgress into the adjacent vascular compartment.

Unexpectedly, total upper airway distension did not increase progressively with higher V\textsubscript{T} but reached a maximum around
10 ml/kg bw with a subsequent decline when VT increased further to 15 ml/kg bw. This nonlinear response was primarily localized to the main bronchi, i.e., the airway segments with the most pronounced distension during mechanical ventilation. In contrast, airways distal thereof showed a progressive distension as a function of VT as reflected by respective nonlinear increases in segmental bronchi area and total functional dead space. The most distal airspaces, namely the alveoli, even showed a disproportionate expansion with increasing VT, as visualized by direct imaging of alveolar dynamics. Notably, the true magnitude of this effect is expected to be even larger due to the mathematical underestimation of three-dimensional expansion by a two-dimensional imaging technique. Taken together, these findings indicate marked differences in stress-strain relationships between the different sections of the respiratory tract with important functional consequences: at VT in the physiological range (i.e., ~6 ml/kg bw) a considerable fraction of VT remains within the distensible upper airways, resulting in an increase of functional dead space. At higher VT, however, upper airway compliance decreases, whereas alveolar expansion increases, resulting in a redistribution of VT fractions from proximal to distal airspaces. This decline in upper airway compliance can be expected to be largely attributable to the fact that upper airways reach their maximal diameter at inflation with VT around 10 ml/kg bw or corresponding airway pressures, respectively (12, 21). Notably, however, the finding that, not only upper airway compliance, but also absolute airway distension during mechanical ventilation with a VT of 6 ml/kg bw despite a concomitant increase in mean airway pressure indicates that, in addition to anatomical restrictions, structural changes may limit airway distension at higher VT. In the present study, potential histological correlates were evident in the form of perivascular hemorrhages adjacent to the upper airways at higher VT, which may directly impinge on airway diameter and hence distension (16). As the opposing behavior of upper airways and alveoli in response to increasing VT will compensate for each other, airway pressure as a reflection of the sum stress-strain relationship in the respiratory tract increased linearly over the range of applied VT. The proposed preferential distribution of moderate yet not high VT to the extra-alveolar airspaces is also reflected in the classic landmark paper by Tschermerlin and Margulies (26), in that total air volume increased in a hyperbolic curve with transpulmonary pressures, whereas alveolar epithelial basement membrane surface area increased in a linear fashion.

**Potential clinical relevance.** The present concept of VT redistribution as a function of lung inflation may be of important physiological and clinical implications. First, it suggests that the upper airways may act as a volume reservoir over the physiological range of VT that protects the alveolar membrane from excessive distension. Second, it postulates the existence of a rather distinct VT-threshold, which is reached when airway distension approaches its maximum. Beyond this threshold, further increases in VT become disproportionally harmful to the alveolar membrane. Third, it provides a mechanistic explanation for the previously reported finding that the pressure-volume curve of the lung is a poor predictor of alveolar expansion (20).

When considering the relevance of the present findings for the clinical scenario, it is important to keep in mind that the anatomy of the murine respiratory tract differs markedly from the situation in humans in that it lacks cartilage rings below the level of the trachea (14) and cartilage fragments downstream of the main bronchi (13). Hence, murine main and segmental bronchi are structurally more representative of distal human airways, which likewise lack cartilage fragments (13), rather than of the proximal bronchi. Even between murine and human airways of similar structure and size, stress-strain relationships will differ as a function of the tethering forces exerted by the parenchymal attachments to the outside of the airway wall (17). Although these important considerations are likely to impact quantitatively on the extent of airway distension and VT redistribution in different species, they do, however, not call into question the general qualitative concept proposed here.

In support of the notion that relevant airflow distension may likewise occur in humans, Hedenstierna (10) detected an ~50% increase in anatomical dead space with a corresponding increase in VT in patients with respiratory failure receiving prolonged ventilator treatment. Notably, Hedenstierna and Lundberg (11) also found airway compliance to be markedly higher during anesthesia and mechanical ventilation compared with spontaneously breathing subjects, suggesting that anesthetized and ventilated patients already at risk for VILI may be particularly prone to airway overdistension. It is further noteworthy that even mechanical ventilation with a VT as low as 6 ml/kg bw causes considerable airflow stretch. This finding may relate to the observation that even protective ventilation strategies with a VT in the range of those of spontaneously breathing mice (22, 23) or humans (24), respectively, still cause distinct proinflammatory responses in mice (9, 29) and men (35) that cannot be readily explained by alveolar overdistension. Notably, in the present study, airway distension during ventilation with a VT of 6 ml/kg bw was likely augmented by the concurrent application of a modest PEEP of 2 cmH2O; however, this does not diminish the clinical relevance of this finding, as similar or higher levels of PEEP would be expected in most clinical ventilator settings. Taken together, airflow distension may contribute importantly to the injurious effects of mechanical ventilation and should be taken into consideration in pathomechanistic studies of VILI, which tend to focus exclusively on stretch-induced alveolar epithelial damage.

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**DISCLOSURES**

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**AUTHOR CONTRIBUTIONS**


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