

# Increased Susceptibility to Ventilator-associated Lung Injury Persists after Clinical Recovery from Experimental Endotoxemia

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**Background:** Endotoxin, when delivered shortly before or during mechanical ventilation, increases susceptibility to ventilation-associated lung injury. However, it is unclear whether increased susceptibility to ventilator-associated lung injury is still present after clinical recovery from a transient endotoxin challenge.

**Methods:** Anesthetized rats were submitted to a 4-h period of mechanical ventilation with low (8 ml/kg) or high (24, 27, or 30 ml/kg) tidal volumes ( $V_T$ s) 24 h after transient illness had been provoked by a single nonlethal intravenous injection of *Escherichia coli* endotoxin. Control animals were injected with phosphate-buffered saline and underwent the same protocol.

**Results:** At 24 h, endotoxin-treated nonventilated animals showed no symptoms of clinical illness, and oxygenation was comparable with that of controls, but lung neutrophil counts were increased. Compared with controls, mechanical ventilation with high  $V_T$  induced a stronger pulmonary inflammatory response and more severe lung injury in endotoxin-treated animals, as indicated by impaired oxygenation, increased lung wet-to-dry weight ratio, and increased levels of protein, neutrophils, and cytokines in lung lavage fluid. In addition, the highest  $V_T$  resulted in increased mortality in endotoxin-treated animals. Low  $V_T$  after endotoxin treatment did not cause functional pulmonary impairment but induced an inflammatory response.

**Conclusions:** In this animal model, a 24-h delay after a single systemic injection of endotoxin resulted in clinical recovery and preserved pulmonary function but did not prevent increased susceptibility to ventilator-associated lung injury provoked by high  $V_T$ . Residual pulmonary inflammation and neutrophilic infiltration at initiation of mechanical ventilation probably contribute to these findings.

THE systemic inflammatory response induced by endotoxin is characterized by cytokine release, leukocyte activation and emigration, and lung injury.<sup>1,2</sup> Endotoxin

release has been demonstrated in operative medicine, e.g., during major abdominal, vascular, and cardiac surgery<sup>3–5</sup> and may contribute to the postoperative inflammatory response, which is usually transient and self-limiting.

Mechanical ventilation (MV) with high tidal volumes ( $V_T$ s) may also lead to lung injury and systemic dissemination of inflammation.<sup>6–8</sup> *In vivo* and *ex vivo* experiments exposing animals to both systemic endotoxin and MV show that when applied simultaneously or in a timely, closely related fashion, these two stimuli synergistically increase lung injury and systemic inflammation.<sup>9,10</sup> Because after a single systemic injection the response to endotoxin evolves rapidly within a few hours,<sup>11</sup> presumably, in these studies, MV is performed at a time when the inflammatory process is at its maximum, with high levels of systemic cytokines and cellular activation. This experimental condition may reflect the clinical situation of intraoperative endotoxemia or of MV during the acute phase of sepsis.

In clinical practice, MV may for various reasons be required (e.g., for surgical repair of trauma or for prolonged respiratory support after major surgery) when the clinical response to an inflammatory stimulus has resolved and systemic cytokine levels are low. However, the effects of MV on lung injury and pulmonary inflammation in this late phase after transient systemic inflammation are not well known.

Increased pulmonary infiltration with inflammatory cells may still be present after constitutional and functional recovery from endotoxemia.<sup>11,12</sup> We hypothesize that these subclinical findings predispose the lungs to injurious effects of MV even after clinical recovery from systemic inflammation.

To study this hypothesis, we subjected rats to MV with low  $V_T$  and positive end-expiratory pressure as well as to increasing levels of  $V_T$  24 h after we applied a nonlethal dose of endotoxin. In pilot studies, this dose of endotoxin caused transient clinical illness and rapid clinical recovery, did not impair oxygenation at 24 h, and led to 100% survival at 48 h in nonventilated animals.

## Materials and Methods

This study was approved by the institutional and local Committee on the Care and Use of Animals (Thüringer Landesamt für Lebensmittelsicherheit und Verbraucher-

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schutz, Weimar, Germany). Male Wistar rats weighing 350–380 g were used for this study.

#### *Treatment with Endotoxin or PBS*

Animals were anesthetized with 1.5% isoflurane in 100% oxygen *via* a face-shoulder mask. Tail artery blood (200  $\mu$ l) was collected to obtain baseline blood gas analysis (ABL 50; Radiometer, Copenhagen, Denmark), blood leukocyte counts, and interleukin 6 (IL-6) concentration. Subsequently, 1 mg endotoxin (lipopolysaccharide, serotype *Escherichia coli* O55:B5) dissolved in 1 ml phosphate-buffered saline (PBS) or 1 ml PBS (control group) was injected intravenously in a tail vein. This dose of endotoxin had resulted in transient clinical illness and rapid recovery but no mortality at 48 h in preliminary experiments by our group. Anesthesia was discontinued, and the animals were transferred to their cages with free access to water and food. After 24 h, control and endotoxin-treated animals were randomly assigned to undergo no MV or MV with  $V_T$  8, 24, 27, or 30 ml/kg body weight ( $n = 10$ /group). A range of high  $V_T$  was chosen to be able to identify gradual pulmonary deterioration (dose-response relation) and to maximize lung injury without causing mortality. Selection of the highest  $V_T$  was based on preliminary experiments demonstrating that severe lung injury and hypoxemia occurred in healthy (PBS-injected) rats at  $V_T$  30 ml/kg.

#### *Experiments and Measurements in Nonventilated Animals*

Inhalation anesthesia was repeated at 6 and 24 h after treatment to obtain blood samples for arterial blood gas analysis, leukocyte counts, and IL-6 levels. After drawing the 24-h blood sample, ketamine (70 mg) and midazolam (4 mg) were injected intraperitoneally, resulting in deep anesthesia. The abdomen was opened, and animals were exsanguinated by rapid collection of 8–10 ml blood from the inferior vena cava. Sternotomy was performed, and the lungs were removed *en bloc*. Further measurements and sample collection were performed identically as in ventilated animals.

#### *Experiments and Measurements in Mechanically Ventilated Animals*

**Instrumentation.** Twenty-four hours after treatment, animals were anesthetized using an intraperitoneal injection of ketamine (70 mg) and midazolam (4 mg). Cefuroxime, 12.5 mg, was injected subcutaneously for antibiotic prophylaxis (Cefuroxim-ratiopharm® 250 mg; Ratiopharm GmbH, Ulm, Germany). The right external jugular vein and the right carotid artery were surgically exposed and polyethylene catheters (ID 0.5 mm) were placed in each vessel and secured with ligatures. A venous blood sample was drawn for leukocyte counts. Pressure transducers were connected to each catheter, and central venous and arterial blood pressures were

displayed on a monitor continuously (SMU 611; PPG Hellige GmbH, Freiburg, Germany). Infusion of balanced electrolyte solutions at a rate of 10 ml/h (Thomaejonin; Delta Pharma GmbH, Pfullingen Germany) and intravenous anesthetics (18 mg/h ketamine and 0.18 mg/h midazolam) at a rate of 0.6 ml/h was started and maintained throughout the ventilation period. The trachea was exposed and incised, and a blunt cannula (OD 2 mm) was placed in the tracheal lumen. Skin incisions were closed with metal clips.

**Ventilation Period.** Mechanical ventilation was started (respiratory rate, 40 breaths/min; inspiratory-to-expiratory ratio, 1:1; fraction of inspired oxygen, 0.4; positive end-expiratory pressure, 4 cm H<sub>2</sub>O; Animal Ventilator CIV 101; Columbus Instruments, Columbus, OH), and  $V_T$  was adjusted according to group allocation. Carbon dioxide was added to the inspiratory gas if high  $V_T$  was applied, to attain arterial normocapnia at the beginning of the ventilation period. No further corrections of carbon dioxide were made throughout the ventilation period. A pressure transducer was connected to the tracheal cannula, and airway pressures were displayed on a monitor (SMU 611). Pancuronium bromide, 0.2 mg, was injected intravenously for muscle relaxation. Body temperature was maintained at 38°C using a feedback warming system (Theraterm de Luxe, 250W; Osram, Munich, Germany; with HSE temperature regulator; Hugo Sachs Elektronik, March, Germany). Heart rate, arterial and central venous blood pressures, peak airway pressure, and arterial blood gases were recorded every 30 min throughout the ventilation period. After 4 h of MV, animals were killed, and lungs were harvested as described above (see Experiments and Measurements in Nonventilated Animals).

#### *Measurements and Sample Collection*

**Lung Lavage.** Using blunt clamps, a small part of the lower lobe of the right (in nonventilated animals, also of the left) lung was excluded from the lavage procedure. Lung lavage was performed with 10 ml PBS using 2.5-ml aliquots. The pooled effluent (7.5–8.5 ml) was centrifuged at 3,000 rpm for 10 min. The cell pellet was resuspended in 100  $\mu$ l PBS, and absolute numbers of neutrophils and macrophages were counted using a hemocytometer and corrected for the total amount of lavage fluid obtained. The supernatant of the lavage fluid was stored at –70°C until cytokine levels and protein concentration were determined.

**Lung Lavage Protein Content.** Protein content in the supernatant of the lung lavage fluid was determined using turbidimetry (Roche Diagnostics, Mannheim, Germany; analyzer: Hitachi 717) with a detection threshold of 60 mg/l.

**Blood Samples.** Total leukocyte counts were determined using a hemocytometer. The percentage of blood neutrophils was obtained from a cell smear (May-Gruen-

wald stain; 100 leukocytes were counted at a magnification of 500 $\times$ ), and absolute neutrophil numbers (cells/ $\mu$ l) were calculated using the corresponding total leukocyte counts. The supernatant from centrifuged (4,000 rpm for 10 min) blood samples was stored at  $-70^{\circ}\text{C}$  until IL-6 assays were performed.

**Cytokines.** Levels of IL-6 in plasma and of IL-6, interleukin-10 (IL-10), and macrophage inflammatory protein 2 (MIP-2) in the supernatant of lung were assessed by enzyme-linked immunosorbent assay using commercially available kits specific for rats (IL-6: KRC0062; MIP-2: KRC1022; IL-10: KRC0101; BioSource, Solingen, Germany). Measurements were made according to manufacturer guidelines. The detection thresholds were 8 pg/ml for IL-6, 1 pg/ml for MIP-2, and 5 pg/ml for IL-10.

**Lung W/D Ratio.** The part of the right lung that had been excluded from the lavage procedure was weighed, desiccated ( $48^{\circ}\text{C}$  for 48 h), and weighed again. The wet-to-dry weight (W/D) ratio was calculated and normalized to the ratio in nonventilated control animals.

**Lung Histology.** The nonlabeled part of the left lung that had been obtained from nonventilated animals was fixed in 10% buffered formalin, and hematoxylin and eosin-stained slides were prepared for light microscopy. The average number of neutrophils from 10 randomly selected (excluding conducting airways and blood vessels) high-power fields (magnification: 500 $\times$ ) in each slide was recorded.

Animals were excluded from data analysis if they died before the end of the period of MV. Because mortality was high in animals ventilated with  $V_T$  30 ml/kg, this group was stopped after 11 animals (6 endotoxin, 5 controls) had been enrolled.

### Statistical Analysis

Data are presented as mean  $\pm$  SEM. Two-way analysis of variance was used to assess significant effects of treatment (endotoxin *vs.* control) and  $V_T$  on variables. A repeated-measures term was used where appropriate and Bonferroni *post hoc* correction was made in case of multiple comparisons. A *t* test was used to assess differences between two groups.  $P < 0.05$  was considered statistically significant. The statistics software SPSS Version 11 (Cary, NC) was used for data analysis.

## Results

### Effects of Endotoxin Injection in Nonventilated Animals

Endotoxin-treated animals exhibited symptoms of illness (tachypnea, reduced locomotion, piloerection) for 4–6 h but appeared unaffected at 12 and 24 h after the injection.

**Respiratory Variables.** Respiratory variables are shown in table 1. Oxygenation at baseline and at 6 and

**Table 1. Effects of Intravenous Injection of Endotoxin (LPS) or PBS (Control) on Respiratory and Inflammatory Variables in Blood Samples of Nonventilated Animals**

	0 h	6 h	24 h
$\text{PaO}_2$ , mmHg			
LPS	374 $\pm$ 8	367 $\pm$ 10	363 $\pm$ 16
Control	369 $\pm$ 10	370 $\pm$ 9	353 $\pm$ 8
$\text{Paco}_2$ , mmHg			
LPS	44.7 $\pm$ 2.6	38.8 $\pm$ 1.2*	35.2 $\pm$ 0.9*
Control	40.8 $\pm$ 1.4	44.4 $\pm$ 1.7	45.3 $\pm$ 1.3
pH			
LPS	7.38 $\pm$ 0.05	7.39 $\pm$ 0.03	7.38 $\pm$ 0.04
Control	7.36 $\pm$ 0.06	7.42 $\pm$ 0.05	7.4 $\pm$ 0.04
TLC, cells/ $\mu$ l			
LPS	8,633 $\pm$ 648	2,750 $\pm$ 380†	11,950 $\pm$ 1,302†
Control	7,816 $\pm$ 837	9,472 $\pm$ 1,070	8,727 $\pm$ 623
Neutrophils, cells/ $\mu$ l			
LPS	745 $\pm$ 101	1,371 $\pm$ 147†	6,744 $\pm$ 499†
Control	842 $\pm$ 162	3,978 $\pm$ 478†	1,570 $\pm$ 384

Values are given as mean  $\pm$  SEM.

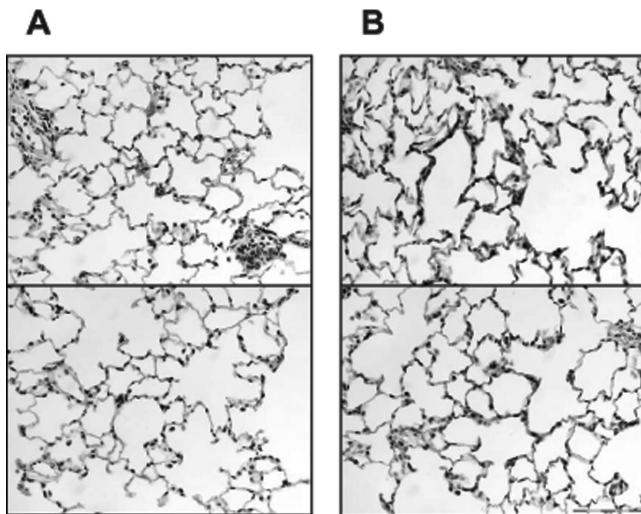
\*  $P < 0.05$  vs. control group. †  $P < 0.05$  vs. 0 h within group.

0 h = before injection of LPS or PBS; 6 h = 6 h after injection of LPS or PBS; 24 h = 24 h after injection of LPS or PBS; LPS = lipopolysaccharide;  $\text{Paco}_2$  = arterial carbon dioxide tension;  $\text{PaO}_2$  = arterial oxygen tension; PBS = phosphate-buffered saline; TLC = total leukocyte count.

24 h after treatment was comparable in both groups. In endotoxin-treated animals, arterial carbon dioxide tension was lower at 6 and 24 h after treatment as compared with baseline and as compared with controls. There were no differences in arterial pH between groups at any time point.

**Systemic Inflammation.** Markers of systemic inflammation are shown in table 1. Total leukocyte counts were significantly lower at 6 h and significantly higher at 24 h after endotoxin injection compared with baseline. No significant changes in total leukocyte counts were seen in control animals. Blood neutrophil counts in endotoxin-treated animals increased at 6 and 24 h after treatment. In control animals, blood neutrophil counts were significantly higher at 6 but not at 24 h compared with baseline. IL-6 levels in blood were below the detection threshold before endotoxin treatment in all but one animal, measurable in all animals (mean  $\pm$  SEM: 638.6  $\pm$  221 pg/ml) at 6 h, and below the detection threshold again in all but one animal at 24 h after endotoxin injection (the same animal that had detectable IL-6 before the injection). IL-6 was not detectable in most of the control animals at any time. Low levels were found in three control animals before PBS injection and did not change at 6 or 24 h after injection.

**Pulmonary Injury and Inflammation.** There were no gross differences in lung histology between groups (fig. 1), but neutrophil numbers in lung tissue ( $12.9 \pm 1$  *vs.*  $5.0 \pm 1$  cells/high-power field;  $P < 0.001$ ) were significantly higher in endotoxin-treated than in control animals. Lung W/D ratio (fig. 2) and macrophage count and protein content in lung lavage fluid (fig. 3) were not different between endotoxin-treated and control ani-



**Fig. 1.** Representative hematoxylin and eosin photomicrographs of lung tissue sections obtained from nonventilated animals 24 h after intravenous injection of endotoxin (A) or phosphate-buffered saline (control; B). Magnification is 500 $\times$ .

mals, but neutrophil counts in lung lavage fluid were significantly higher in endotoxin-treated animals (fig. 3). MIP-2 was detectable in lung lavage fluid in all control and endotoxin-treated animals, and levels were not different between groups (fig. 4). IL-6 levels in lung lavage were below the detection threshold in six of nine endotoxin-treated animals and in eight of nine control animals and low (< 55 pg/ml) in the remaining four animals. IL-10 in lung lavage fluid was below the detection threshold in seven of nine endotoxin-treated animals and eight of nine control animals, and less than 160 pg/ml in the remaining three animals.

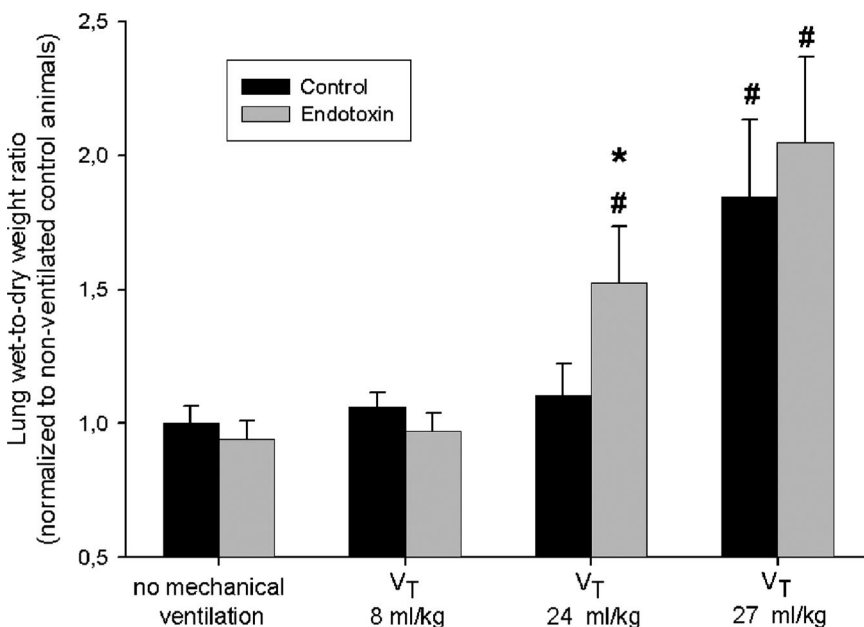
#### Effects of Mechanical Ventilation

**Mortality.** No animal died in the groups with  $V_T$  8 ml/kg. One control and two endotoxin-treated animals

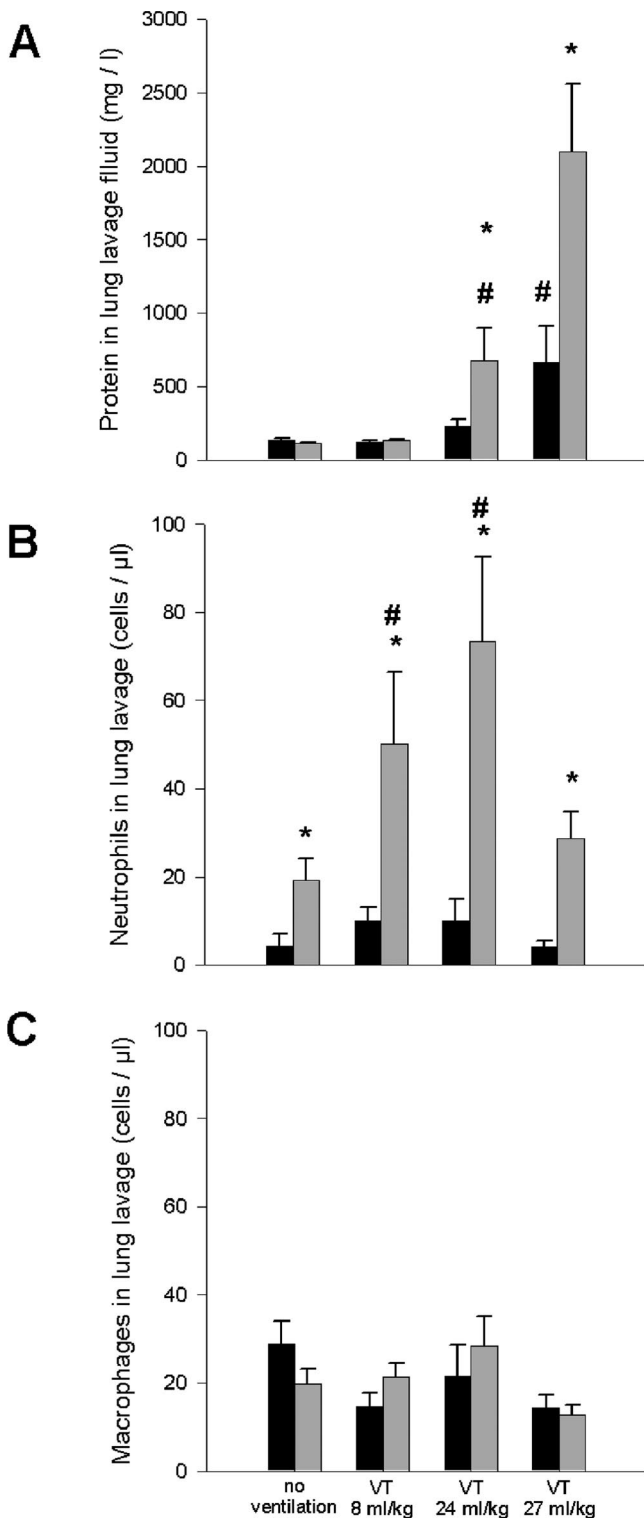
in the  $V_T$  24 ml/kg groups and two endotoxin-treated and two control animals in the  $V_T$  27 ml/kg groups became progressively hypotensive and died. In the groups ventilated with  $V_T$  30 ml/kg, five of six endotoxin-treated and one of five control animals died. All animals ventilated with  $V_T$  30 ml/kg had development of severe pulmonary edema and became progressively hypotensive. Because of the low number of surviving animals ventilated with  $V_T$  30 ml/kg, no further parameters and no statistics are reported from these groups.

**Hemodynamic and Respiratory Effects.** In endotoxin-treated but not in control animals ventilated with  $V_T$  27 ml/kg, arterial oxygen tension decreased significantly at the end of the MV period. Arterial oxygen tension remained unchanged over time in all other groups and did not differ between endotoxin-treated and control animals (fig. 5). Further hemodynamic and respiratory variables at the beginning and at the end of MV are shown in table 2. Heart rate and arterial pressure decreased significantly over time in all groups regardless of  $V_T$  and pretreatment. Peak airway pressure at the beginning and at the end of the ventilation period was comparable in both groups ventilated with  $V_T$  8 ml/kg but was significantly higher in endotoxin-treated animals ventilated with 24 or 27 ml/kg as compared with the respective control groups. Arterial carbon dioxide tension was higher and pH was lower in both groups ventilated with  $V_T$  8 ml/kg as compared with animals ventilated with 24 or 27 ml/kg, but there were no differences regarding these variables between endotoxin-treated and control groups at the same  $V_T$ .

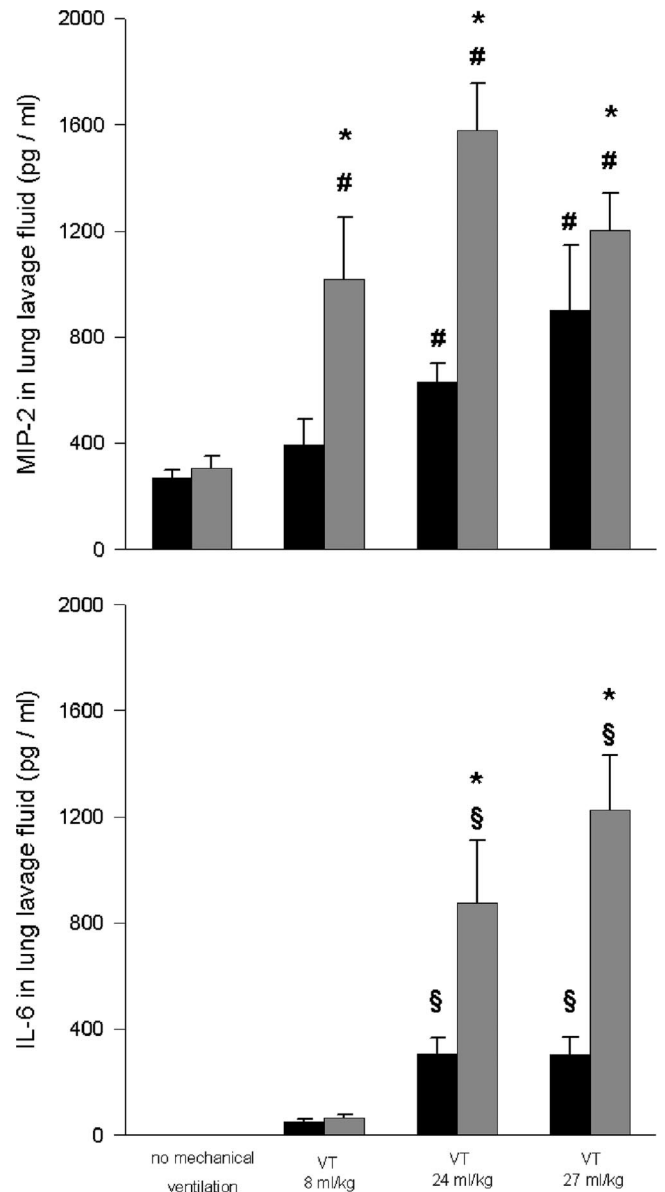
Regardless of pretreatment, most animals ventilated with  $V_T$  27 ml/kg had development of pulmonary edema at the end of the ventilation period, with frothy fluid filling the airways and tracheal cannula. During MV,



**Fig. 2.** Lung wet-to-dry weight ratio (mean  $\pm$  SEM) in endotoxin-treated (gray bars) and control animals (black bars). Values in nonventilated animals were obtained 24 h after treatment. The other animals underwent a 4-h period of mechanical ventilation 24 h after treatment. Wet-to-dry weight ratios were normalized to values obtained in nonventilated control animals. \*  $P < 0.05$  versus control group ventilated with same tidal volume ( $V_T$ ). #  $P < 0.05$  versus nonventilated animals.



**Fig. 3.** Protein content (A), neutrophil counts (B), and macrophage counts (C) in lung lavage fluid obtained from endotoxin-treated (gray bars) and control animals (black bars). Values (given as mean  $\pm$  SEM) were obtained in nonventilated animals 24 h after treatment. The other animals underwent a 4-h period of mechanical ventilation 24 h after treatment. \*  $P < 0.05$  versus control group ventilated with same tidal volume ( $V_T$ ). #  $P < 0.05$  versus nonventilated animals.



**Fig. 4.** Levels (mean  $\pm$  SEM) of macrophage inflammatory protein 2 (MIP-2) and interleukin 6 (IL-6) in lung lavage fluid obtained from endotoxin-treated (gray bars) and control animals (black bars). Values in nonventilated animals were obtained 24 h after treatment. The other animals underwent a 4-h period of mechanical ventilation 24 h after treatment. Irrespective of treatment, IL-6 was undetectable in most nonventilated animals (see text for details). \*  $P < 0.05$  versus control. #  $P < 0.05$  versus nonventilated animals. §  $P < 0.05$  versus tidal volume ( $V_T$ ) of 8 ml/kg.

edema fluid was removed if it extended beyond the tracheal cannula into the ventilator tubes (eight animals). Quantification of neutrophils and macrophages in the removed fluid was attempted but was successful only in four endotoxin-treated animals. Cell counts were fourfold to eightfold higher in edema than in lavage fluid.

**Lung W/D Ratio.** Lung W/D ratio in both groups of animals ventilated with low  $V_T$  was comparable to that of nonventilated animals (fig. 2). As compared with nonventilated animals, ventilation with  $V_T$  24 and 27 ml/kg

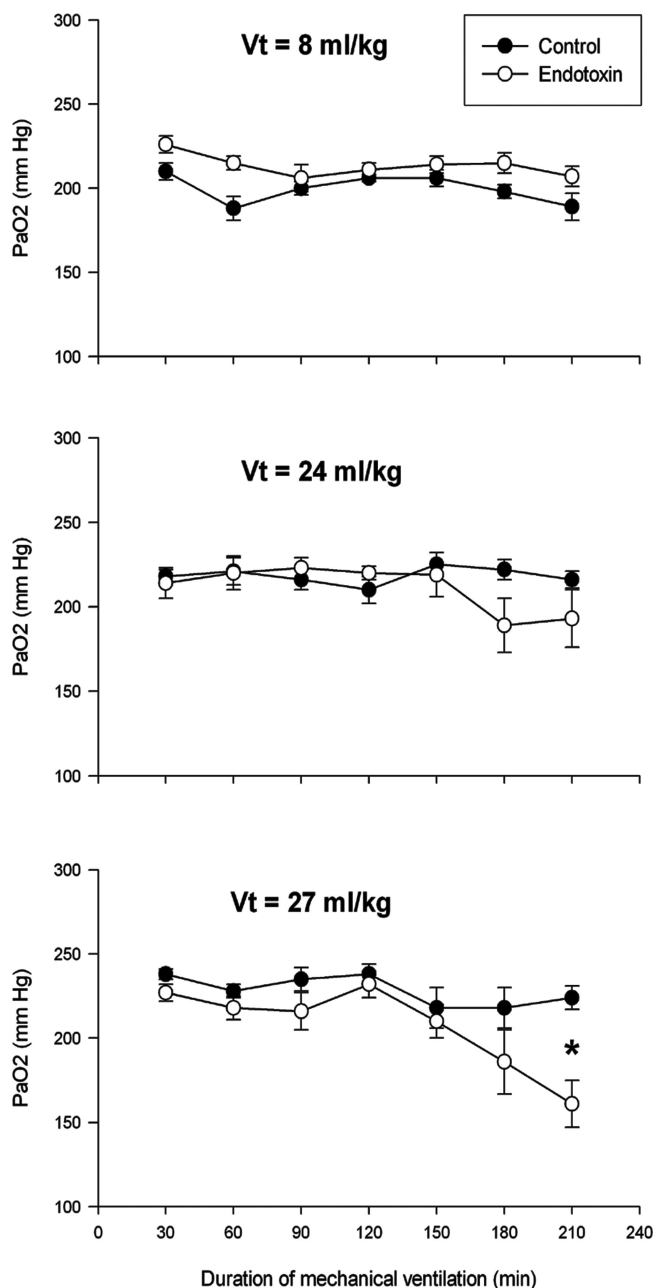


Fig. 5. Arterial oxygen tension ( $P_{aO_2}$ ; mean  $\pm$  SEM) in animals undergoing mechanical ventilation with different tidal volumes ( $V_T$ s) 24 h after intravenous injection of endotoxin (lipopolysaccharide) or phosphate-buffered saline (control). \*  $P < 0.05$  versus  $P_{aO_2}$  at 30 min within group and versus  $P_{aO_2}$  in the control group at the same time.

significantly increased lung W/D ratio in endotoxin-treated animals (fig. 2). In control animals, lung W/D ratio increased significantly with  $V_T$  27 ml/kg only (fig. 2).

#### Lung Lavage Fluid.

**Protein Content.** In endotoxin-treated animals, lung lavage protein content was comparable with that in nonventilated animals after MV with  $V_T$  8 ml/kg but increased significantly after MV with 24 and 27 ml/kg (fig. 3A). In control animals, lung lavage protein increased significantly only in animals ventilated with  $V_T$  27 ml/kg (fig. 3A).

**Cell Counts.** Neutrophils in lung lavage fluid at any  $V_T$  were significantly higher in endotoxin-treated animals than in control animals (fig. 3B). In endotoxin-treated animals ventilated with 8 or 24 but not with 27 ml/kg, lung lavage neutrophil counts were significantly higher than in nonventilated animals (fig. 3B). No significant differences in lung lavage neutrophil counts were seen in control animals (fig. 3B). Irrespective of pretreatment or  $V_T$ , there was no significant change in macrophage counts in lung lavage fluid (fig. 3C).

**Cytokines.** Interleukin 6 and MIP-2 levels are shown in figure 4.

**Interleukin 6.** Interleukin-6 levels in lung lavage fluid at low  $V_T$  were comparable between endotoxin-treated and control animals. High  $V_T$  resulted in increased IL-6 levels in both groups, but more so in endotoxin-treated animals.

**Macrophage inflammatory protein 2.** Macrophage inflammatory protein-2 levels in lung lavage fluid were significantly higher in endotoxin-treated than in control animals at corresponding  $V_T$ . In endotoxin-treated animals, MIP-2 at each  $V_T$  was higher than in nonventilated animals. In control animals, only MIP-2 levels in animals ventilated with  $V_T$  24 or 27 ml/kg were higher than in nonventilated animals.

**Interleukin 10.** In control animals, IL-10 levels were detectable only in one or two animals at each  $V_T$ . In endotoxin-treated animals, IL-10 was detectable in one animal in the 3-ml and in three animals in the 24-ml group. All values were less than 200 pg/ml. No statistical analysis was performed in these groups. IL-10 was detected in all but one animal in the 27-ml/kg group (mean  $\pm$  SEM: 95.4  $\pm$  29 pg/ml).

**Blood Neutrophil Counts.** Blood neutrophil counts before MV were significantly higher in endotoxin-treated animals than in control animals (fig. 6). There was a significant increase in blood neutrophil counts at the end of MV in all  $V_T$  groups in control animals, whereas a significant decrease was seen in endotoxin-treated animals ventilated with  $V_T$  27 and 30 ml/kg (fig. 6).

## Discussion

We studied the effects of MV with increasing  $V_T$ s on pulmonary inflammation and lung injury in animals after recovery from an endotoxin challenge. By establishing a 24-h delay between the endotoxin injection and the ventilation period, our study represents a two-hit model of lung injury. Our study thus differs substantially from previous studies,<sup>9,10,13</sup> which exposed animals to artificial ventilation and endotoxin simultaneously, thereby creating a situation where the injurious effects of MV occur during the maximal acute systemic inflammatory response to endotoxin. We established a range of  $V_T$ s, which enabled us to identify not only gradual increases in the severity of lung injury but also subtle differences

**Table 2. Hemodynamic and Respiratory Variables in Animals Pretreated with Endotoxin (LPS) or PBS (Control) at 30 min after Initiation and 30 min before the End of MV**

	LPS			Control		
	V <sub>T</sub> 8 ml/kg	V <sub>T</sub> 24 ml/kg	V <sub>T</sub> 27 ml/kg	V <sub>T</sub> 8 ml/kg	V <sub>T</sub> 24 ml/kg	V <sub>T</sub> 27 ml/kg
HR, beats/min						
Begin	426 ± 16	450 ± 22	452 ± 15	405 ± 12	463 ± 24	414 ± 27
End	362 ± 20*	332 ± 33*	289 ± 20*	330 ± 14*	347 ± 22*	260 ± 21*
AP, mmHg						
Begin	108 ± 22	129 ± 8	132 ± 9	106 ± 13	122 ± 14	118 ± 12
End	91 ± 5*	87 ± 6*	71 ± 8*	78 ± 6*	77 ± 10*	75 ± 7*
Ppeak, mmHg						
Begin	10.5 ± 0.4	22.7 ± 0.8‡	25.5 ± 0.8‡	10.5 ± 0.4	18.3 ± 0.4	23.7 ± 0.7
End	11.2 ± 0.2	20.3 ± 0.9‡	24.8 ± 1.0‡	11.1 ± 0.3	16.8 ± 0.3	23.3 ± 1.0
Paco <sub>2</sub> , mmHg						
Begin	51 ± 1.4	33 ± 1.6†	34 ± 0.8†	53 ± 1.6	35 ± 2†	35 ± 2.1†
End	42 ± 1	34 ± 1.1†	32 ± 1.4†	41 ± 1.3	36 ± 0.8†	33 ± 1.2†
pH						
Begin	7.27 ± 0.02	7.40 ± 0.02	7.38 ± 0.02	7.30 ± 0.01	7.45 ± 0.02	7.43 ± 0.02
End	7.38 ± 0.01	7.41 ± 0.04	7.37 ± 0.02	7.43 ± 0.01	7.45 ± 0.02	7.42 ± 0.05

Values are given as mean ± SEM.

\*  $P < 0.05$  vs. begin. †  $P < 0.05$  vs. tidal volume 8 ml/kg. ‡  $P < 0.05$  vs. control at same tidal volume.

AP = systolic arterial pressure; HR = heart rate; LPS = lipopolysaccharide; MV = mechanical ventilation; Paco<sub>2</sub> = arterial carbon dioxide tension; PBS = phosphate-buffered saline, Ppeak = peak inspiratory pressure.

between lipopolysaccharide-treated and control animals. In addition, we included a group of animals ventilated with low V<sub>T</sub> and positive end-expiratory pressure, thereby simulating ventilator settings close to recommended clinical practice in preinjured lungs.<sup>14</sup> We found an increased susceptibility to ventilator-associated lung injury despite clinical recovery from a single injection of endotoxin. The detrimental effects of increasing V<sub>T</sub>s on the lungs of endotoxin-pretreated as compared with control animals were characterized by an increase in cytokine release and neutrophil infiltration. This inflammatory injury was accompanied by functional pulmonary impairment and by increased mortality in animals ventilated with high V<sub>T</sub>. However, even a low V<sub>T</sub> applied 24 h after endotoxin exposure induced a detectable pulmonary inflammatory response.

We aimed to expose the animals to an only moderate initial injury. Therefore, the endotoxin dose used in our study was adjusted to result in 100% survival at 48 h. Results from a comprehensive murine study demonstrated that survival at 24 h after a systemic endotoxin challenge always translated to long-term survival.<sup>12</sup> We saw only transient clinical deterioration in our study, and 24 h after endotoxemia (before MV), blood and lung cytokine levels and oxygenation were comparable between the endotoxin and control groups. In endotoxin-treated animals, arterial carbon dioxide levels at 6 and 24 h after treatment were lower as compared with baseline and as compared with control animals. This suggests that residual effects of endotoxin on pulmonary function were still present; however, this finding was not reflected by overt tachypnea or other clinical symptoms of respiratory impairment. In addition, comparable hemo-

dynamics in endotoxin-treated and control animals during MV support our assumption of constitutional recovery from the endotoxin exposure.

Twenty-four hours after the endotoxin challenge, blood and lung lavage neutrophil counts were significantly higher in the endotoxin group as compared with the control group. In previous studies, maximum neutrophil infiltration in the lung interstitium has been found at 3–6 h after a systemic endotoxin challenge, whereas neutrophil counts in lung lavage fluid peaked at 24 h.<sup>11,15</sup> Given this time course, our findings likely reflect residual pulmonary infiltration, rather than ongoing neutrophil recruitment to the lungs. In our study, the higher baseline level of activated neutrophils in the circulation and lungs may have predisposed these animals to an enhanced inflammatory response to MV.

During ventilation, blood neutrophil counts in endotoxin-pretreated animals decreased during ventilation (with increasing V<sub>T</sub>) while lung lavage neutrophils increased. In control animals, blood neutrophil levels increased during MV, but lung lavage neutrophil counts did not. This suggests that in the endotoxin group, previous neutrophilia and neutrophil activation led to its increased sequestration in the lung, an effect not seen in the control group.

During ventilation, lung lavage cytokine levels (MIP-2, IL-6) increased with increasing V<sub>T</sub> with or without endotoxin pretreatment. The occurrence and relevance of increased pulmonary cytokine levels in the setting of MV alone has been a matter of debate.<sup>16</sup> Our results regarding IL-6 and MIP-2 within the context of moderate increases in neutrophil counts in control animals support the view of a proinflammatory pulmonary reaction with

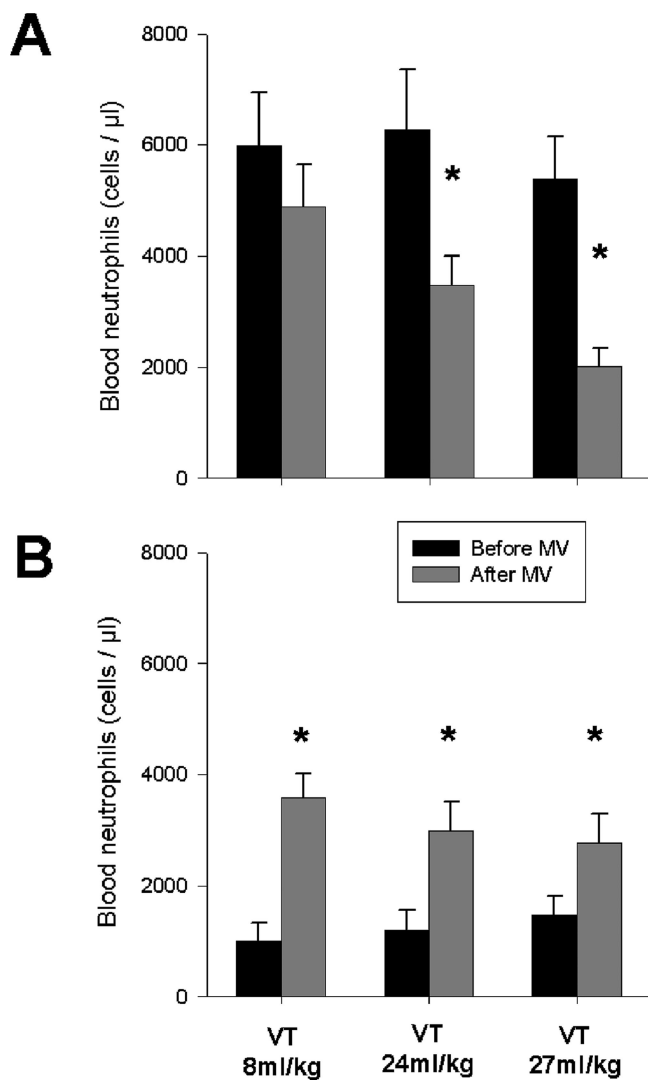


Fig. 6. Blood neutrophil counts (mean  $\pm$  SEM) in endotoxin-treated (A) and control animals (B) before (black bars) and after (gray bars) mechanical ventilation (MV) with increasing tidal volumes ( $V_T$ s). \*  $P < 0.05$  versus value before MV within the same group.

increasing  $V_T$ . Furthermore, measurable IL-10 levels in lung lavage fluid in endotoxin-treated animals ventilated with  $V_T$  27 ml/kg suggest a simultaneous antiinflammatory response in this group. The exaggerated increase in lung cytokine levels in the endotoxin group suggest higher production rates due to previous activation of neutrophils and, possibly, macrophages in this group.

Irrespective of pretreatment and  $V_T$ , MV did not significantly affect alveolar macrophage counts. Interestingly, the lowest individual counts were seen in animals ventilated with the highest  $V_T$ . Consistent with our results, other groups reported decreased alveolar macrophage counts in rats submitted to MV with high  $V_T$  in both *ex vivo* and *in vivo* experiments.<sup>17,18</sup> Extraalveolar relocation of macrophages was discussed as a possible reason, but explanations remained speculative.<sup>17,19</sup> In our study, a technical aspect might partially explain the

macrophage numbers: Most of the animals in the  $V_T$  27 ml/kg groups developed severe pulmonary edema, and a variable amount of edema fluid (as much as 2 ml in some animals) was lost before lung lavage. Because cell counts were considerably higher in edema than in lavage fluid, this loss may have resulted in underestimation of inflammatory markers in lavage fluid. The fact that neutrophil counts and MIP-2 levels in lung lavage fluid, irrespective of pretreatment, did not increase further with the highest  $V_T$  supports this hypothesis.

In general, our animal model exemplifies the two-hit theory in the pathogenesis of organ injury. According to this theory, the first intervention (hit) primes the organism for an exaggerated response to a second intervention (hit). Endotoxin is frequently used as a first intervention in these models. In our study, endotoxin and ventilation are the two interventions that were applied in a clinically relevant time-related fashion.

There are a number of similarities between our study (delayed MV after endotoxemia) and previous studies in which endotoxin was mostly immediately followed by MV. In one study, endotoxin alone did not impair oxygenation but caused a significant decrease in blood neutrophil counts and an increase in lung lavage neutrophils in nonventilated animals at 4 h, indicating a pulmonary inflammatory response similar to that in our study.<sup>9</sup> Endotoxin with immediate ventilation with a moderate  $V_T$  of 10 ml/kg resulted in aggravated lung injury as compared with either MV or endotoxin alone. In contrast to our findings with a  $V_T$  of 8 ml/kg, the  $V_T$  of 10 ml/kg also caused functional pulmonary impairment, suggesting that pulmonary susceptibility to detrimental effects of MV may be higher in the early phase of the endotoxin response, when pulmonary permeability changes are most pronounced. In another study, rabbits received a low dose of endotoxin intravenously while being ventilated with  $V_T$  15 ml/kg without positive end-expiratory pressure.<sup>13</sup> In accord with our findings, combination of endotoxin and MV aggravated the pulmonary release of inflammatory mediators as compared with MV or endotoxin treatment alone.

Our results, in keeping with the two-hit theory of experimental organ injury, are clinically relevant. Transient endotoxemia occurs in various clinical scenarios, including major surgery,<sup>3-5</sup> trauma,<sup>20,21</sup> endoscopic procedures,<sup>22,23</sup> ischemia-reperfusion,<sup>24</sup> and after enteral refeeding.<sup>25</sup> Residual subclinical pulmonary alterations may persist after these procedures and may leave these patients at risk for ventilator-associated injury. While clearly recommended in patients with acute respiratory distress syndrome,<sup>14</sup> recent data indicate that the use of low  $V_T$  in patients with presumably healthy lungs is less well established.<sup>26</sup> Therefore, these patients are at increased risk of being exposed to a potentially harmful ventilation strategy.

When translating our findings to clinical scenarios, some

limitations of our study must be mentioned. First, to obtain measurable pulmonary effects within a short period of time, we applied  $V_T$ s far beyond settings used in clinical practice. One may hypothesize, however, that detrimental pulmonary effects may also be provoked by less excessive  $V_T$  and a longer duration of MV. Second, to provoke clinical effects in rodents, much higher endotoxin doses are required than in human volunteers.<sup>1</sup> Nevertheless, despite differences in sensitivity, responses to endotoxin exposure seem to be qualitatively similar in different species.<sup>27</sup> Third, although increased alveolar neutrophil counts have been shown in humans after bronchial endotoxin instillation,<sup>28</sup> we are missing from clinical studies clear evidence that neutrophil translocation into the lung interstitium and alveolar space occurs after systemic exposure to endotoxin. However, basic pulmonary reactions to systemic endotoxin in humans resemble effects observed in animals,<sup>29</sup> and increased adhesion and accumulation of neutrophils in the pulmonary vasculature, a process commonly occurring before extravasation, has been demonstrated in human endotoxemia.<sup>30</sup> Therefore, our findings likely maintain clinical relevance. Last, our method of determining lung W/D ratio does not take in account residual intravascular blood in the lung. Therefore, results may have been affected by different amounts of lung perfusion secondary to different inflammatory states of the lung. However, because animals were exsanguinated before lung harvest (thereby reducing the amount of residual blood in the pulmonary circulation) and because of the collaborative finding of frank edema fluid in the proximal airways of animals ventilated with high  $V_T$ , we assume that this limitation was of minor relevance and that our findings regarding lung W/D ratio truly reflect pulmonary edema.

In conclusion, in this experimental study, a transient period of endotoxemia with subsequent clinical recovery before initiation of MV resulted in increased susceptibility to the deleterious effects of increasing  $V_T$ . Our data provide indirect evidence that pulmonary neutrophil infiltration before MV predisposes to these pulmonary alterations. Our results further suggest that it may be advisable to avoid high  $V_T$  in subjects with presumably healthy lungs even after clinical recovery from a period of endotoxemia.

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