One-hit Models of Ventilator-induced Lung Injury

Benign Inflammation versus *Inflammation as a By-product*

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ABSTRACT

Background: One important explanation for the detrimental effects of conventional mechanical ventilation is the biotrauma hypothesis that ventilation may trigger proinflammatory responses that subsequently cause lung injury. This hypothesis has frequently been studied in so-called one-hit models (overventilation of healthy lungs) that so far have failed to establish an unequivocal link between inflammation and hypoxemic lung failure. This study was designed to develop a one-hit biotrauma model.

Methods: Mice (six per group) were ventilated for up to 7 h (positive end-expiratory pressure 2 cm H_2O) and received 300 μl/h fluid support. Series_1: initial plateau pressures of 10, 24, 27, or 30 cm H_2O. Series_2: ventilation with pressure release at 34 cm H_2O and initial plateau pressure of 10, 24, 27, or 30 cm H_2O. To study the significance of inflammation, the latter groups were also pretreated with the steroid dexamethasone.

Results: Within 7 h, 20 of 24 mice ventilated with plateau pressure of 27 cm H_2O or more died of a catastrophic lung failure characterized by strongly increased proinflammatory markers and a precipitous decrease in pulmonary compliance, blood pressure, and oxygenation. Pretreatment with dexamethasone reduced inflammation, but prolonged median survival time by 30 min.

Conclusions: Our findings demonstrate a sharp distinction between ventilation with 24 cm H_2O that was well tolerated and ventilation with 27 cm H_2O that was lethal for most animals due to catastrophic lung failure. In the former case, inflammation was benign and in the latter, a by-product that only accelerated lung failure. The authors suggest that biotrauma—when defined as a ventilation-induced and inflammation-dependent hypoxemia—is difficult to study in murine one-hit models of ventilation, at least not within 7 h. **(Anesthesiology 2017; 126:909-22)**

In acute respiratory distress syndrome (ARDS), ventilation strategies affect outcome.[1,2] These findings have raised the question of how mechanical ventilation injures the lungs, major possibilities being physical tissue injury and the biotrauma hypothesis. As a result, in recent years, ventilator-induced lung injury (VILI) has been the most frequently used model in experimental ARDS studies.[3]

How quickly mechanical ventilation can injure the lungs was already demonstrated 40 yr ago when Webb and Tierney[4] showed that ventilation of rats without a positive end-expiratory pressure (PEEP) and with 45 cm H_2O inflation pressure destroys lungs within 20 min. Clinically, physical injury can be diagnosed as extraalveolar air, termed barotrauma.[5] Retrospective studies in patients suggested a safety threshold of about 35 cm H_2O plateau pressure (p_{plat}), below which barotrauma is rarely observed.[6]

In the National Institutes of Health, National Heart, Lung, and Blood Institute (NIH-NHLBI) ARDS Network study, where barotrauma was similar in both groups, the best correlation with survival was observed for proinflammatory mediators.[2,7,8] This has lent credibility to the biotrauma hypothesis that may be posited in a weak and in a strong form: in its weak form, it states that ventilator-induced inflammation contributes to the mortality of ARDS patients in conjunction with other factors (hits); in its strong form, it states that the inflammation induced by mechanical ventilation may suffice to injure the lungs much stronger than explained by the mechanical forces alone. The strong form of the biotrauma hypothesis has stimulated the so-called

What We Already Know about This Topic

- Laboratory models reveal key mechanisms of ventilator-induced lung injury; while multiple pharmacologics have demonstrated benefit in murine models of adult respiratory distress syndrome, none have proved beneficial in patients.

What This Article Tells Us That Is New

- Murine ventilator-induced lung injury (single "hit") is characterized by either mild inflammation with normal lung functions or—above a threshold plateau pressure—by fulminant lung failure. In this latter condition, pretreatment with corticosteroid suggests that ventilator-induced inflammation may have a bystander rather than a pathogenic impact on lung injury.

Corresponding article on page 766. Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org).

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one-hit models—with ventilation as the only hit—to examine its mechanisms.[9] It appears that such studies may be separated into two groups: (1) One-hit models that lead to a catastrophic breakdown of the lung that is sudden, strong, and final[4,10–12] and that is best explained by self-enforcing mechanical failure. In such studies, PEEP is typically absent (or at least very low) and p_{plat} is above 25 cm H_2O at the start and above 40 cm H_2O at the end of the experiment. (2) One-hit models with p_{plat} usually well below 25 cm H_2O where inflammation is present, but where severe lung injury and hypoxemia are absent.[13–15]

Because clinically, ARDS is graded by the P/F ratio,[16] in our opinion, a *biotrauma* model should show hypoxemia (Pao_2/Fio_2 ratio less than or equal to 300 mmHg) that is caused by ventilation-induced inflammation. Therefore, in the current study, it was our aim to develop a one-hit model of ARDS in mice that is (1) characterized by ARDS-like hypoxemia without a catastrophic breakdown and (2) dependent on inflammation.

To this end, we ventilated mice for up to 7 h with a PEEP of 2 cm H_2O and p_{plat} of 10, 24, 27, or 30 cm H_2O. When we found that all pressures above 24 cm H_2O caused a catastrophic lung failure with ventilation pressures rapidly precipitating after 3 to 6 h, we started a second series where we utilized a pressure valve to limit airway pressure to 34 cm H_2O—*i.e.*, below the safety threshold described by Boussarsar *et al.*[6] In order to address a possible contribution of inflammation in the progression of the disease, some animals were pretreated with the antiinflammatory steroid dexamethasone. Neither the pressure release strategy nor the steroid application prevented the catastrophic lung failure of animals ventilated with p_{plat} above 24 cm H_2O.

Materials and Methods

A complete description is available in Supplemental Digital Content 1 (http://links.lww.com/ALN/B392).

Animals

Female C57BL/6 N mice (aged 8 to 12 weeks, weighing 20 to 25 g) were kept under standard conditions. All experimental procedures were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (Recklinghausen, Germany; permission number: AZ 84-02.04.2013.A131).

Experimental Setup

Surgery was performed as described previously.[14] In short, mice were initially anaesthetized by intraperitoneal bolus injection of pentobarbital sodium (75 mg/kg) plus fentanyl (40 μg/kg). Anesthesia was maintained with pentobarbital sodium (20 mg/kg) *via* an intraperitoneal catheter every 30 to 60 min and fentanyl (20 μg/kg) every 3 h. A catheter was inserted into the carotid artery to permanently monitor blood pressure (AP_{mean}) and to supply saline (300 μl/h) to prevent hypovolemia and thrombus formation. Heart rate (HR) was calculated online from a three-lead electrocardiogram, and pulse oximetry was performed with a thigh clip.

Mice were ventilated for 7 h *via* a tracheal catheter with a mechanical ventilator (MidiVent Type 849; Hugo Sachs Elektronik - Harvard Apparatus GmbH, Germany) equipped with a flow-pressure sensor (Type 382 Mouse; Hugo Sachs Elektronik - Harvard Apparatus GmbH). Dynamic pulmonary compliance (C_{dyn}) and resistance were calculated online by the Pulmodyn software v 1.1 (Hugo Sachs Elektronik - Harvard Apparatus GmbH). ΔC_{dyn} (C_{dyn} end of ventilation – C_{dyn} start of ventilation) was calculated at the end of the experiments. Control animals were ventilated with a plateau airway pressure (p_{plat}) of 10 cm H_2O at 180 breaths/min with recruitment maneuvers every 20 min to prevent atelectasis.[14] Groups with p_{plat} of 24 cm H_2O or more were ventilated at a frequency of 90 breaths/min. End-tidal carbon dioxide ($ETco_2$) waveform was monitored with a microcapnograph (Type 340; Hugo Sachs Elektronik - Harvard Apparatus GmbH). A PEEP of 2 cm H_2O was applied, the fraction of inspired oxygen (Fio_2) was set to 0.3 and the inspiration/expiration ratio was 1:1 with a sinus-shaped waveform in all experiments.

At the end of experiment, mice were euthanized by exsanguinations *via* the carotid artery. Blood samples were analyzed for arterial oxygen saturation, arterial oxygen tension (Pao_2), $Paco_2$, and pH; the Horovitz ratio was calculated as Pao_2/Fio_2.

One-hit Model of VILI. We performed and analyzed two different series of experiments separately. In the first series (series_1), the following p_{plat}s were used in the ventilation protocol: (10 cm H_2O [p10], 24 cm H_2O [p24], 27 cm H_2O [p27], and 30 cm H_2O [p30]).

In the second series of experiments (series_2), a respiratory pressure monitor and limiter (I; IPML type 870/01 for mouse; Hugo Sachs Elektronik - Harvard Apparatus GmbH) with pressure release above 34 cm H_2O (valve open time: 150 ms) was used to limit the maximum pressure in all animals. This value was chosen because clinical data suggest that pressures above 35 cm H_2O favor pneumothorax formation.[6] The following p_{plat}s were used during ventilation with the pressure release valve: 24 cm H_2O (p24 I), 27 cm H_2O (p27 I), and 30 cm H_2O (p30 I).

Treatments. The steroid dexamethasone was used to assess the inflammatory part in our model. Depending on group assignment, animals in the second series randomly received dexamethasone (1 mg/kg iv) directly after the start of mechanical ventilation. The following additional groups were examined: 24 cm H_2O (p24 I D), 27 cm H_2O (p27 I D), and 30 cm H_2O (p30 I D).

Sample Preparation

The right superior lobe was fixed in 4% formalin for histopathology, the right middle and inferior lobes were snap frozen in liquid nitrogen and stored at −80°C for real-time

quantitative polymerase chain reaction (qPCR) measurements. The wet/dry ratio was obtained from the right postcaval lobe after weight constancy at 42°C. Bronchoalveolar lavage of the left lung was performed by instilling twice 200 µl ice cold saline *via* a tracheal catheter. From each lung, about 350 µl bronchoalveolar lavage fluid (BALF) was recovered, centrifuged, and the supernatant was stored at −80°C until quantification of cytokines. All samples were analyzed in a blinded fashion. Because of limited amounts of material in a few cases or as a consequence of predefined internal quality controls, we could not perform all analyses on every lung.

Cytokine Detection

BALF cytokine levels of chemokine (C-X-C motif) ligand 1 (CXCL1, KC), chemokine (C-X-C motif) ligand 2 (CXCL2, MIP-2), chemokine (C-X-C motif) ligand 10 (CXCL10, IP-10), interleukin-6 (IL-6), and tumor necrosis factor (TNF) were quantified with enzyme-linked immunosorbent assays (R&D Systems GmbH, Germany) according to the manufacturer's protocols.

Real-time qPCR Analysis

Frozen lung tissues were grinded in liquid nitrogen. Total RNA was isolated from 20 mg lung powder with RNeasy® Mini Kit (QIAGEN GmbH, Germany) automated on a QIAcube robot (QIAGEN GmbH) with additional DNase digestion. RNA was quantified in buffered 10 mM TRIS-HCl (pH 7.5) using a spectrophotometer (NanoDrop 1000; Thermo Fisher Scientific Inc., USA). The details of PCR reaction are described in the Supplemental Digital Content 1 (http://links.lww.com/ALN/B392). Primer pairs used for the detection of chemokine (C-X-C motif) ligand 1 (*Cxcl1*), (C-X-C motif) ligand 10 (*Cxcl10*), dipeptidylpeptidase 4 (*Dpp4*), *Il6*, myeloperoxidase (*Mpo*), neuropeptide Y (*Npy*), TATA box binding protein (*Tbp*), and *Tnf* mRNA levels are listed in the Supplemental Digital Content 2 (http://links.lww.com/ALN/B393).

Quantification of real-time qPCR was performed with crossing point values, acquired *via* the second-derivative maximum method. Advanced relative quantification was performed with the LightCycler 480 software v 1.5 (Roche-Diagnostics GmbH, Germany) and efficiency-corrected by inrun standard curves from all samples.[17] Interrun calibrators from unventilated control animals were used to avoid interrun variations. Data were normalized to *Tbp*, which was validated as a very stably expressed reference gene out of 10 candidate genes, as determined by geNorm (average $M \leq 0.2$).[18] Gene expression is depicted as fold induction relative to unventilated controls. Real-time qPCR quality control was performed by inrun controls, melting curve profiles using the LightCycler 480 software (Roche-Diagnostics GmbH), and product separation in agarose gels.

Lung Histopathology

The right superior lobe was fixed with 4% formalin and embedded in paraffin for sectioning. Hematoxylin and eosin staining was performed with 3-mm-thick sections. Histopathology was evaluated in a blinded manner as reported previously.[14] In short, a scoring system based on four criteria was used: neutrophils in the alveolar or interstitial space, alveolar septal thickening, alveolar congestion, and formation of hyaline membranes. Each criterion scored one point, if present, resulting in a range of 0 to 4 points.

Statistical Analysis

Based on the data from Protti *et al.*[10] and on own preliminary data, the experiments were planned with a statistical power of 80% and an alpha error of 0.05 (corrected for multiple comparisons) in order to detect differences in the Pao_2/Fio_2 ratio of greater than 80 with an SD of 10 (JMP 10; SAS Institute Inc., USA). The data were analyzed using SAS® software v 9.4 (SAS Institute Inc.) using two-sided tests. Survival analysis was performed with log-rank tests (Proc Lifetest; SAS® software v 9.4), and *P* values were corrected by the Bonferroni–Holm procedure. Univariate tests were carried out using general linear mixed model analysis (Proc Glimmix; SAS® software v 9.4) assuming a log-normal distribution for all cytokine data and a normal distribution for all other parameters; residual plots were used as diagnostics. In case of heteroscedasticity (according to the covtest statement), the *df* were adjusted by the Kenward–Rogers method. *P* values were always adjusted by the simulated-Shaffer procedure.[19] $P < 0.05$ was considered significant. Data were plotted with GraphPad Prism® software v 6 (GraphPad Software Inc., USA).

Results

In the first series, mice were ventilated with 10, 24, 27, or 30 cm H_2O. All animals were ventilated for 7 h unless they died before. Death was defined as a drop in AP_{mean} < 80% of the initial value and is indicated (†). Since in the group p10 p_{plat} never exceeded 34 cm H_2O, these data are shown in the graphs of both experimental series (see below).

Series_1: Ventilation without Pressure Limitation

In series_1, animals ventilated with 10 cm H_2O or with 24 cm H_2O for 7 h showed stable p_{plat} (fig. 1A), HR, AP_{mean}, and $petCO_2$ (figs. S1–S3 in Supplemental Digital Content 3, http://links.lww.com/ALN/B394, Supplemental Digital Content 4, http://links.lww.com/ALN/B395, and Supplemental Digital Content 5, http://links.lww.com/ALN/B396). In contrast, most animals ventilated with 27 cm H_2O and all animals ventilated with 30 cm H_2O did not survive the 7-h period. Animals ventilated with 27 cm H_2O showed a mean survival time of 389 min and 80% mortality; during ventilation with 30 cm H_2O, the mean survival time

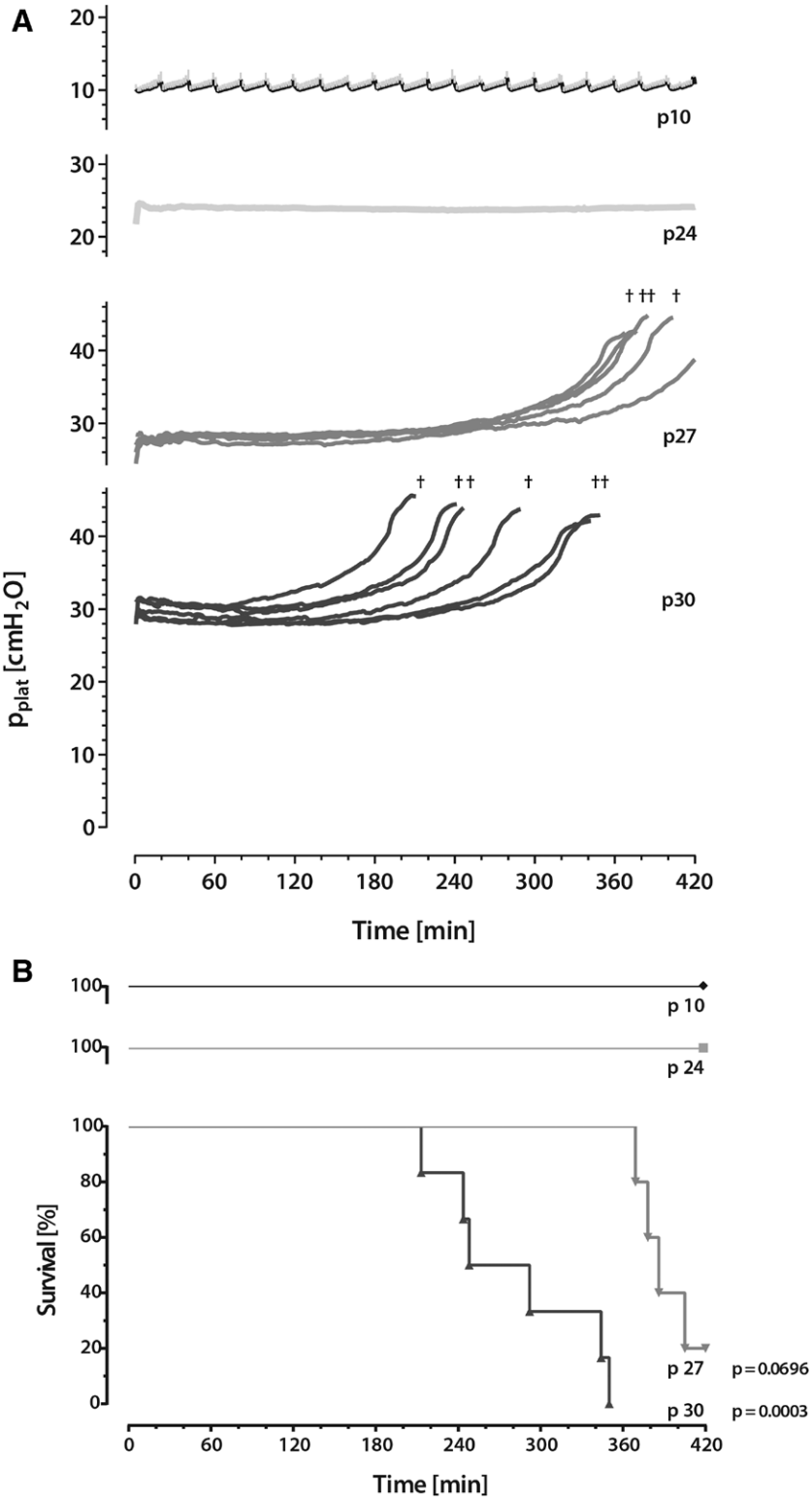

Fig. 1. High peak inspiratory pressures impair survival of ventilated mice (series_1). Mice were ventilated with 10 cm H_2O (p10), 24 cm H_2O (p24), 27 cm H_2O (p27), and 30 cm H_2O (p30) for 7 h. (*A*) Plateau airway pressure (p_{plat}) of mice during pressure unlimited mechanical ventilation. Data are shown as mean plus SD from groups 10 cm H_2O (p10) and 24 cm H_2O (p24); n = 6 each. Groups 27 cm H_2O (p27) and 30 cm H_2O (p30) are shown as single curve for each replicate, and time of death is indicated (†). (*B*) Kaplan–Meier plot of survival proportion of mice from (*A*). Statistical analysis was performed in comparison to p_{plat} 24 cm H_2O group in a log-rank test (censor = 420 min).

was 282 min and the mortality was 100% (fig. 1B; table S2 in Supplemental Digital Content 6, http://links.lww.com/ALN/B397, showing survival proportions).

Remarkably, before animals died, they showed a precipitating increase in p_{plat} (fig. 1A) and $petCO_2$ (fig. S3A, Supplemental Digital Content 5, http://links.lww.com/ALN/B396), as well as a matching decrease in C_{dyn} (fig. 2, A and B). This behavior is best illustrated if the p_{plat} data are normalized to the time point of the maximum gain in p_{plat} because then all animals follow a common kinetic (fig. S4, A and B in Supplemental Digital Content 7, http://links.lww.com/ALN/B398) such that once p_{plat} had increased by more than 18%, death occurred within about 30 min. The slope of that curve (first derivative, fig. S4, C, D in Supplemental Digital Content 7, http://links.lww.com/ALN/B398) shows that airway pressure increased continuously, but moderately, until shortly before death, when a dramatic increase occurred. This observation matches the dynamic pulmonary compliance data: the C_{dyn} curve showed a decrease by more than 20% about 30 min before death (fig. S5, A and B in Supplemental Digital Content 8, http://links.lww.com/ALN/B399). The slope of that curve shows that the pulmonary compliance was decreasing slowly, until suddenly a strong decrease started, which peaked shortly before death (fig. S5, C and D in Supplemental Digital Content 8, http://links.lww.com/ALN/B399).

In the two groups ventilated with $p_{plat} \geq 27$ cm H_2O, the wet/dry ratio was increased (above 7), the pulmonary compliance was decreased ($\Delta C_{dyn} < -9.7$), and the Horovitz ratio was less than 200 mmHg. In contrast to these observations, all parameters remained normal in the two groups ventilated with $p_{plat} \leq 24$ cm H_2O (fig. 2).

Animals ventilated with $p_{plat} \geq 27$ cm H_2O showed increased expression of proinflammatory mediators. In comparison to the 24 cm H_2O group, increased transcript levels of *Il6* (19-fold), *Cxcl1* (17-fold), *Tnf* (5-fold), and *Cxcl10* (4-fold) were detected for the p27 group. Expression ratios of those genes after ventilation with 30 cm H_2O were slightly less strongly increased, in comparison to those with 24 cm H_2O: *Il6* (12-fold), *Cxcl1* (11-fold), *Tnf* (4-fold), and *Cxcl10* (3-fold) (fig.

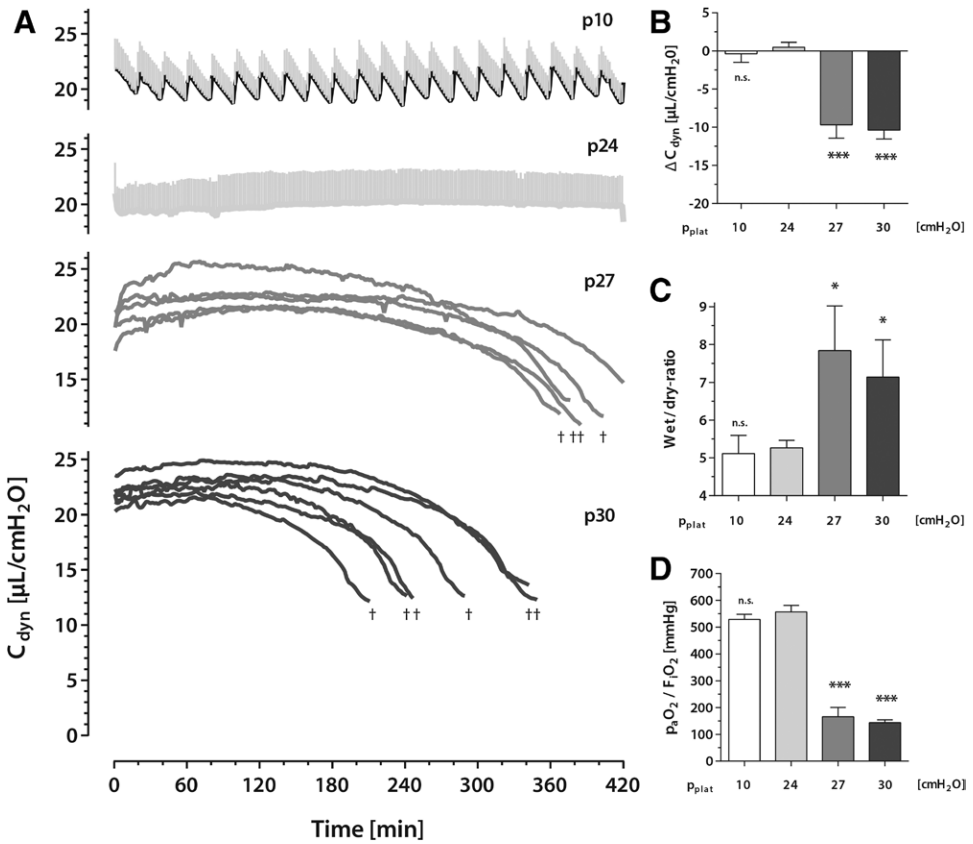

Fig. 2. Effect of inspiratory pressures impair lung functions in ventilated mice (series_1). Mice were ventilated with 10 cm H_2O (p10), 24 cm H_2O (p24), 27 cm H_2O (p27), and 30 cm H_2O (p30) for 7 h. (*A*) Dynamic pulmonary compliance (C_{dyn}) during mechanical ventilation. Data are shown as mean plus SD from groups 10 cm H_2O (p10) and 24 cm H_2O (p24); n = 6. Groups 27 cm H_2O (p27) and 30 cm H_2O (p30) are shown as single curve for each replicate, and time of death is indicated (†). (*B*) Δ dynamic pulmonary compliance was calculated by C_{dyn} end of ventilation – C_{dyn} start of ventilation; p10: n = 6, p24: n = 6, p27: n = 5, and p30: n = 6. (*C*) The lung wet/dry ratio was obtained from the right postcaval lobe; p10: n = 6, p24: n = 6, p27: n = 5, p30: n = 5. (*D*) The Horovitz ratio was calculated as Pao_2/fraction of inspired oxygen (F_{IO_2}); p10: n = 6, p24: n = 6, p27: n = 5, p30: n = 5. Data are shown as mean ± SD. $^*P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ in comparison to the 24 cm H_2O group. n.s. = not significant.

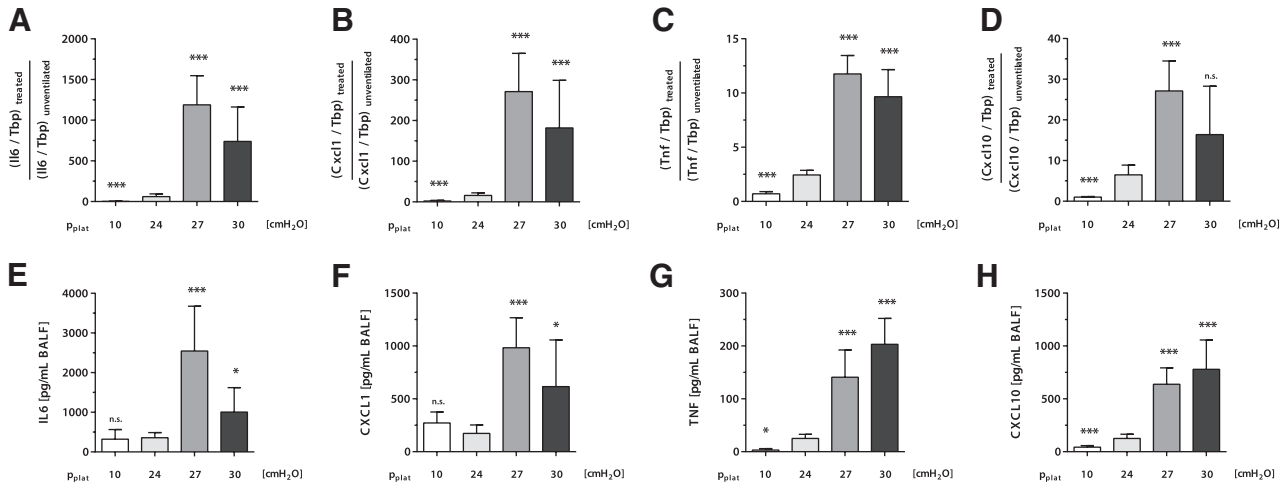

Fig. 3. Expression of proinflammatory mediators after ventilation of mice with various inspiratory pressures (series_1). Mice were ventilated with 10 cm H_2O (p10), 24 cm H_2O (p24), 27 cm H_2O (p27), and 30 cm H_2O (p30) for 7 h. (*A–D*) Increased expression of proinflammatory response genes in mice. Relative mRNA levels were determined by qPCR in lung tissue. Transcript levels of interleukin 6 (*Il6*; p10: n = 6, p24: n = 5, p27: n = 5, p30: n = 6), tumor necrosis factor (*Tnf*; p10: n = 6, p24: n = 5, p27: n = 5, p30: n = 6), chemokine (C-X-C motif) ligand 1 (*Cxcl1*; p10: n = 6, p24: n = 5, p27: n = 5, p30: n = 6), and C-X-C motif ligand 10 (*Cxcl10*; p10: n = 5, p24: n = 5, p27: n = 5, p30: n = 6) were normalized to TATA box binding protein (*Tbp*). Gene expression is depicted as fold induction relative to unventilated controls. (*E–H*) Increased release of cytokines in mice. Cytokine levels were determined by enzyme-linked immunosorbent assays in bronchoalveolar lavage fluid (BALF). Cytokine quantity of IL6 (p10: n = 5, p24: n = 5, p27: n = 4, p30: n = 6), TNF (p10: n = 4, p24: n = 5, p27: n = 4, p30: n = 5), chemokine (C-X-C motif) ligand 1 (CXCL1) (p10: n = 5, p24: n = 4, p27: n = 4, p30: n = 5), and C-X-C motif ligand 10 (CXCL10) (p10: n = 5, p24: n = 4, p27: n = 4, p30: n = 6) were analyzed. (*A–H*), Data are shown as mean plus SD. $^*P \le 0.05$, $^{**}P \le 0.01$, and $^{***}P \le 0.001$ in comparison to the 24 cm H_2O group. n.s. = not significant.

3A–D). The cytokine levels in the BALF matched the mRNA patterns. After ventilation with 27 cm H_2O or 30 cm H_2O, BALF protein levels were enhanced in comparison to the p24 group: IL-6 (3- to 7-fold), CXCL1 (4- to 6-fold), TNF (6- to 8-fold), and CXCL10 (5- to 6-fold) (fig. 3E–H).

Histopathologic scoring demonstrated significant alterations in all lungs ventilated with $p_{plat} \ge 27$ cm H_2O (fig. 4). In most lungs ventilated with high pressures, neutrophils in combination with alveolar septal thickening were observed, resulting in a score of 1.8 (p27 group) and 1.6 (p30 group). Ventilation with 10 cm H_2O or with 24 cm H_2O let to a largely normal histopathologic score (0.1 for both groups).

Series_2: Pressure-limited Ventilation

The steep increase in p_{plat} shortly before the death of the animals suggested a mechanical failure of the lung with a possible positive (catastrophic) feedback loop between p_{plat} and lung injury. To further examine this hypothesis, in series_2, lungs were ventilated with a pressure release valve (IPML, I) that did not permit $p_{plat} > 34$ cm H_2O (fig. 5A). In addition, to explore a possible pathogenic role of inflammation (*i.e.*, biotrauma), some animals were pretreated with dexamethasone.

Using pressure release ventilation, the mean survival time was 372 min in the p27 I group with a mortality of 100% (fig. 5B). In the p30 I group, mean survival time was 366 min (as opposed to 282 min in the p30 group without IPML) and the mortality was 50% (table S3, in Supplemental Digital Content 9, http://links.lww.com/ALN/B400). In these groups, HR and AP_{mean} (figs. S1–S2B in Supplemental Digital Content 3, http://links.lww.com/ALN/B394 and Supplemental Digital Content 4, http://links.lww.com/ALN/B395) remained stable until lung

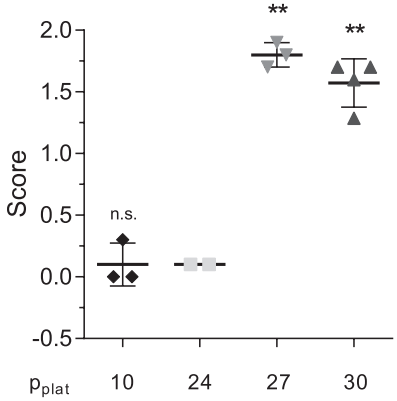

Fig.4. Lung histopathologic score (series_1). Mice were ventilated with 10 cm H_2O (p10), 24 cm H_2O (p24), 27 cm H_2O (p27), and 30 cm H_2O (p30) for 7 h. Histopathologic scoring parameters were neutrophils in the alveolar or interstitial space, alveolar septal thickening, alveolar congestion, and formation of hyaline membranes. Each criterion scored one point, resulting in a range of 0 to 4 points. Data are shown as scatter plot with mean ± SD. p10: n = 3, p24: n = 4, p27: n = 3, p30: n = 4. $^{**}P \le 0.01$ in comparison to the 24 cm H_2O group. n.s. = not significant; p_{plat} = plateau airway pressure.

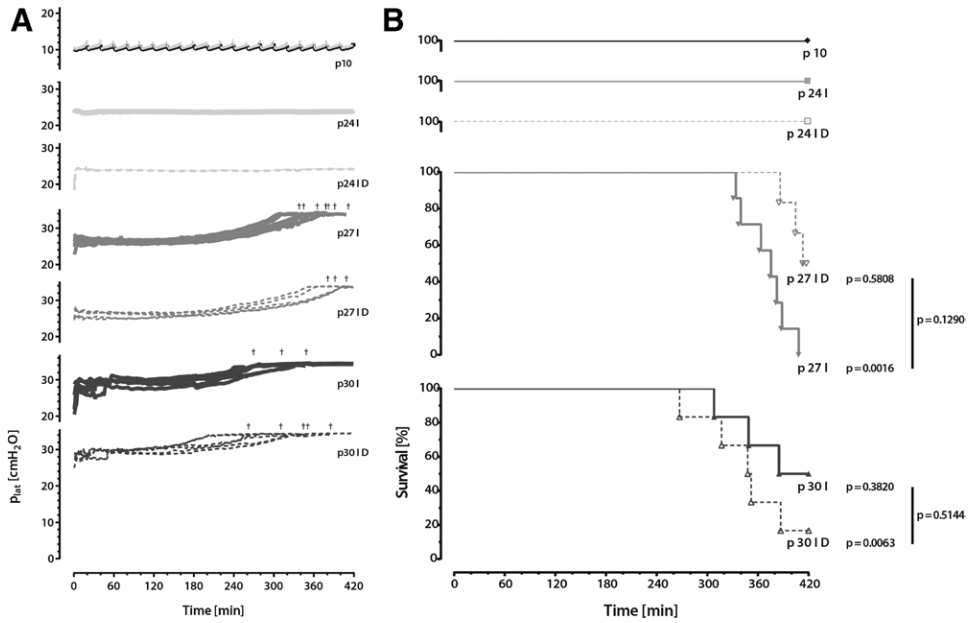

Fig. 5. (*A*) Plateau airway pressure (p_{plat}) of mice during 7 h of pressure-limited mechanical ventilation (series_2). Animals randomly received dexamethasone (D, 1 mg/kg iv) directly after start of mechanical ventilation including pressure release valve (I, P^{max}_{plat} 34 cm H_2O). Data are shown as mean plus SD from groups 10 cm H_2O (p10), 24 cm H_2O (p24 I), and 24 cm H_2O plus D (p24 I D); n = 6. Groups 27 cm H_2O (p27 I), 27 cm H_2O plus D (p27 I D), 30 cm H_2O (p30 I), and 30 cm H_2O plus D (p30 I D) are shown as single curve for each replicate, and time of death is indicated (†). (*B*) Kaplan–Meier plot of survival proportions of mice from (*A*). Statistical analysis was performed in comparison to 24 cm H_2O group and in comparisons of D *versus* corresponding pressure control groups in log-rank test (censor = 420 min).

failure occurred. Lung failure is reflected by an accelerating increase in airway pressure (fig. 5A) and $petCO_2$ (fig. S3B, Supplemental Digital Content 5, http://links.lww.com/ALN/B396) and by a precipitating decrease in compliance (fig. 6, A and B). In the p27 I and the p30 I groups, the pulmonary compliance was decreased ($\Delta C_{dyn} < -10.1$; fig. 6B), the wet/dry ratio was increased (fig. 6C), and the oxygenation index was less than 200 mmHg (fig. 6D).

All physiologic parameters remained normal in the two groups with $p_{plat} \leq 24$ cm H_2O: p_{plat} (fig. 5A), C_{dyn}, ΔC_{dyn}, wet/dry ratio, and Pa_{O_2}/F_{IO_2} (fig. 6A–D), as well as HR, AP_{mean}, $petCO_2$ (fig. S1–S3B, Supplemental Digital Content 3, http://links.lww.com/ALN/B394, Supplemental Digital Content 4, http://links.lww.com/ALN/B395, and Supplemental Digital Content 5, http://links.lww.com/ALN/B396).

In comparison to p24 I, pressure release ventilation with p_{plat} 27 or 30 cm H_2O led to strongly increased transcript levels of: *Il6* (16- to 18-fold), *Cxcl1* (13- to 19-fold), *Tnf* (6-fold), and *Cxcl10* (4- to 8-fold; fig. 7). Transcript levels in mice ventilated with 24 cm H_2O were only a little higher than in those ventilated with 10 cm H_2O. Compared to the p24 I group, BALF levels were enhanced for IL-6 (4- to 10-fold), CXCL1 (2- to 5-fold), TNF (5- to 7-fold), and CXCL10 (2- to 4-fold) in the p27 I and p30 I groups (fig. 8). IL-6 and CXCL1 levels in mice ventilated with 24 cm H_2O were similar to those ventilated with 10 cm H_2O; TNF and CXLC10 levels were somewhat higher (fig. 8). As in series_1, histopathologic scoring revealed significant alterations in all lungs ventilated with $p_{plat} \geq 27$ cm H_2O despite the pressure release valve (fig. 9). Neutrophils in combination with alveolar septal thickening were observed in most lungs, resulting in scores of 1.4 (p27 I group) and 1.5 (p30 I group). Ventilation with $p_{plat} \leq 24$ cm H_2O did not affect the histopathologic score.

Dexamethasone pretreatment diminished mRNA transcription of *Il6*, *Cxcl1, Tnf*, and *Cxcl10* (fig. 7) and decreased the BALF protein levels of IL-6 and CXCL1 (fig. 8) in all animals ventilated with $p_{plat} \geq 27$ cm H_2O. TNF BALF content was slightly reduced by dexamethasone in the p27 I D group, whereas the TNF and CXCL10 levels in the p30 I D group were not affected compared to those in the respective groups without dexamethasone.

Although dexamethasone pretreatment reduced inflammatory parameters after ventilation with $p_{plat} \geq 27$ cm H_2O (figs. 7 and 8), survival proportions were not affected (fig. 5B). In line with these data, dexamethasone pretreatment did improve neither lung wet/dry ratio (fig. 6B) nor ΔC_{dyn} (fig. 6C). Further, dexamethasone pretreatment did not improve gas exchange, in mice ventilated with neither 27 cm H_2O nor 30 cm H_2O (fig. 6D).

Histopathologic examination (fig. 9) revealed no effect of dexamethasone pretreatment on the ventilation-induced structural alterations. In most lungs, neutrophils were observed in addition to alveolar septal thickening.

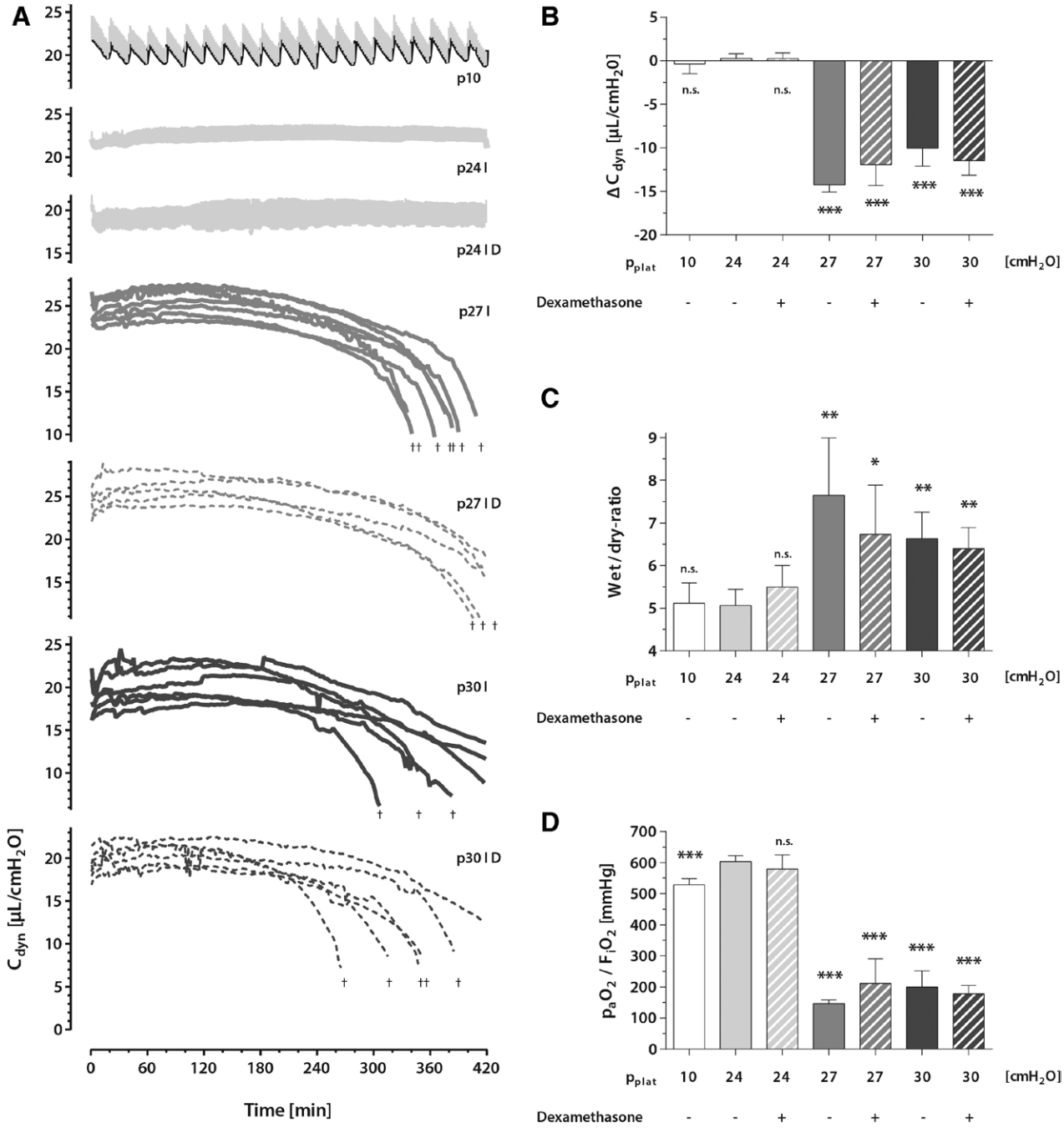

Fig. 6. Effect of dexamethasone (D) on lung functions in pressure-limited ventilation (series_2). Mice were ventilated with 10 cm H_2O (p10), 24 cm H_2O (p24 I), 27 cm H_2O (p27 I), or 30 cm H_2O (p30 I) including pressure release valve (I, P_{plat}^{max} 34 cm H_2O) for 7 h (or until death). Animals randomly received D (1 mg/kg iv) directly after start of mechanical ventilation. (*A*) Dynamic pulmonary compliance (C_{dyn}) during mechanical ventilation. Data are shown as mean plus SD from groups 10 cm H_2O (p10), 24 cm H_2O (p24 I), and 24 cm H_2O plus dexamethasone (p24 I D); n = 6 each. Groups 27 cm H_2O (p27 I), 27 cm H2O plus D (p27 I D), 30 cm H_2O (p30 I), and 30 cm H_2O plus D (p30 I D) are shown as single curve for each replicate, and time of death is indicated (†). (*B*) Δ dynamic pulmonary compliance was calculated by C_{dyn} end of ventilation – C_{dyn} start of ventilation. (*C*) Lung wet/dry ratio was obtained from the right postcaval lobe. (*D*) The Horovitz ratio was calculated as Pa_{O_2}/fraction of inspired oxygen (F_{IO_2}). (*B–D*) Data are shown as mean ± SD; p10: n = 6, p24 I: n = 6, p24 I D: n = 6, p27 I: n = 7, p27 I D: n = 6, p30 I: n = 6, p30 I D: n = 6. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ in comparison to the 24 cm H_2O group. n.s. = not significant.

Discussion

We observed that—even with a PEEP as low as 2 cm H_2O—p_{plat} of 24 cm H_2O were well tolerated for at least 7 h. However, slightly above this pressure (greater than or equal to 27 cm H_2O), we observed a catastrophic type of lung failure that rapidly developed once a critical threshold value

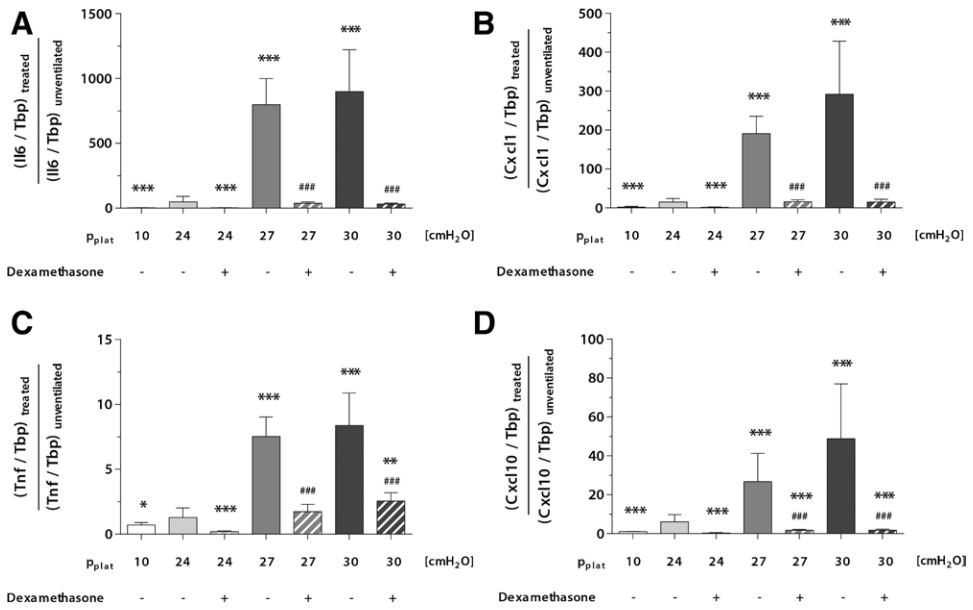

Fig. 7. Effect of dexamethasone (D) on the expression of proinflammatory genes in pressure-limited ventilation (series_2). Mice were ventilated with 10 cm H_2O (p10), 24 cm H_2O (p24 l), 27 cm H_2O (p27 l), or 30 cm H_2O (p30 l) including pressure release valve (l, P^{max}_{plat} 34 cm H2O) for 7 h (or until death). Animals randomly received D (1 mg/kg iv) directly after start of mechanical ventilation. Relative mRNA levels were determined by qPCR in lung tissue. Transcript levels of (*A*) interleukin 6 (*Il6*; p10: n = 6, p24 l: n = 6, p24 l D: n = 6, p27 l: n = 6, p27 l D: n = 6, p30 l: n = 6, p30 l D: n = 6), (*B*) chemokine (C-X-C motif) ligand 1 (*Cxcl1*; p10: n = 6, p24 l: n = 6, p24 l D: n = 6, p27 l: n = 6, p27 l D: n = 6, p30 l: n = 6, p30 l D: n = 6), (*C*) tumor necrosis factor (*Tnf*; p10: n = 6, p24 l: n = 6, p24 l D: n = 6, p27 l: n = 6, p27 l D: n = 6, p30 l: n = 6, p30 l D: n = 6), and (*D*) C-X-C motif ligand 10 (*Cxcl10*; p10: n = 5, p24 l: n = 6, p24 l D: n = 6, p27 l: n = 6, p27 l D: n = 6, p30 l: n = 6, p30 l D: n = 6) were normalized to TATA box binding protein (*Tbp*). Gene expression is depicted as fold induction relative to unventilated controls and shown as mean plus SD. **P* ≤ 0.05, ***P* ≤ 0.01, and ****P* ≤ 0.001 in comparison to 24 cm H_2O group and ###*P* ≤ 0.001 in comparisons of D *versus* corresponding pressure control groups.

was reached. We believe that this behavior is best explained in terms of material fatigue of stress bearing elements. Our findings further suggest that under these conditions, inflammation is the result rather than the cause of lung injury and as such it may accelerate an inevitable organ failure.

Ventilation with 24 cm H_2O Is Safe for At Least 7 h

We have recently described a setup for a mouse intensive care unit providing anesthesia, ventilation, volume support, and thermoregulation that yielded stable lung mechanics and hemodynamics for 6 h.[14] Here, we extend those findings and show that mice tolerate ventilation with p_{plat} = 24 cm H_2O for at least 7 h. Within this period, lung functions, oxygenation, and cardiovascular functions all remained completely stable despite the presence of inflammation. Similar observations have been made in human patients ventilated for hours or even a week.[20,21] Notably, the intensity of inflammation was similar to that at p_{plat} = 10 cm H_2O, being higher than inflammation in naive mice,[14] but considerably lower than that at $p_{plat} \geq 27$ cm H_2O. These findings do further support our notion that pulmonary inflammation occurs readily but that this does not necessarily compromise lung functions.[22] We therefore believe that the current definition of experimental ARDS,[3] which allows us to declare ARDS in perfectly functional lungs only because of inflammation, lacks discriminatory power.

Ventilation Greater Than or Equal to 27 cm H_2O Results in Catastrophic Lung Failure

Ventilation with $p_{plat} \geq 27$cmH_2O resulted in a type of lung failure that always followed the same characteristic pattern, even though—depending on the individual animal—the actual time of onset differed. These results are comparable to those described by Protti *et al.*[10] who observed in pigs that all animals ventilated with 22 ml/kg (initial mean p_{plat} = 16 cm H_2O) survived ventilation for 54 h, whereas most animals ventilated with 38 ml/kg (initial mean $p_{plat} \geq 26$ cm H_2O) died between 8 and 50 h. Unfortunately, Protti *et al.*[10] did not report the time course of lung injury development although they state that lung injury progressed quickly once it had started.

To visualize the pattern of lung failure, we shifted the time scales of each experiment, so that the time points of the maximum gain (slope) in ventilation pressure were superimposed on each other (Supplemental Digital Content 7, http://links.lww.com/ALN/B398, and Supplemental Digital Content 8, http://links.lww.com/ALN/B399, showing the time-shifted data for p_{plat} and C_{dyn}). This analysis revealed that once p_{plat} had increased by more than 18% or

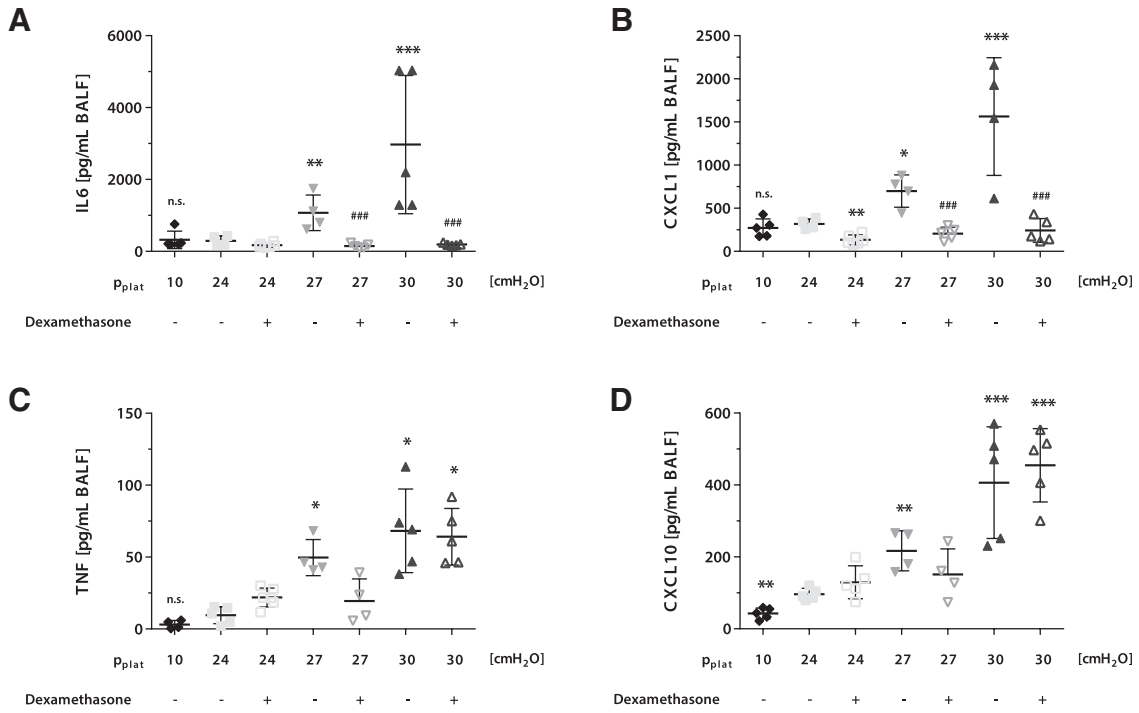

Fig. 8. Effect of dexamethasone (D) on cytokine release in pressure-limited ventilation (series_2). Mice were ventilated with 10 cm H_2O (p10), 24 cm H_2O (p24 l), 27 cm H_2O (p27 l), or 30 cm H_2O (p30 l) including pressure release valve (l, P_{plat}^{max} 34 cm H_2O) for 7 h (or until death). Animals randomly received D (1 mg/kg iv) directly after start of mechanical ventilation. Cytokine levels were determined by enzyme-linked immunosorbent assays in bronchoalveolar lavage fluid (BALF). Cytokine quantity of (*A*) interleukin 6 (IL6; p10: n = 5, p24 l: n = 4, p24 l D: n = 6, p27 l: n = 4, p27 l D: n = 5, p30 l: n = 5, p30 l D: n = 5), (*B*) chemokine (C-X-C motif) ligand 1 (CXCL1; p10: n = 5, p24 l: n = 4, p24 l D: n = 6, p27 l: n = 4, p27 l D: n = 5, p30 l: n = 4, p30 l D: n = 5), (*C*) tumor necrosis factor (TNF; p10: n = 4, p24 l: n = 4, p24 l D: n = 6, p27 l: n = 4, p27 l D: n = 4, p30 l: n = 5, p30 l D: n = 5), and (*D*) C-X-C motif ligand 10 (CXCL10; p10: n = 5, p24 l: n = 5, p24 l D: n = 5, p27 l: n = 4, p27 l D: n = 4, p30 l: n = 5, p30 l D: n = 5) are shown as scatter plot with mean ± SD. **P* ≤ 0.05, ***P* ≤ 0.01, and ****P* ≤ 0.001 in comparison to 24 cm H2O group and ##*P* ≤ 0.01 and ###*P* ≤ 0.001 in comparisons of D *versus* corresponding pressure control groups. n.s. = not significant.

compliance had decreased by more than 20%, death inevitably occurred within 30 min.

This type of lung failure was characterized by two properties: (1) it occurred at random times and (2) once it occurred, it developed in a rapid, self-enforcing way. Such a behavior is typical for fatigue failure, in the sense used in structural material mechanics, such as the failure of brittle polymers.[23,24] Here, fatigue describes the weakening of a material that is subjected to cyclic loading and unloading with stresses below the material's ultimate tensile stress limit. In many cases, material fatigue is a function of the number of cycles,[25] which may be relevant here, because not only controls were ventilated with twice the number of cycles (180 breaths/min) than the other groups (90 breaths/min), but also this may help to understand species differences (see paragraph after next). One brittle stress-bearing element in the lung is collagen, a material that can develop propagating cracks.[23,24] Unfortunately, the material fatigue of collagen fibers has not yet been explored, but it is well known that collagen fibers may rupture upon minimal strain (less than 5%).[23,26] Another potential stress-bearing element are the basement membranes, whose destruction would explain the severe hemorrhage in the current and several previous studies.[27] Clearly, the nature of the stress-bearing elements requires further study.

At $p_{plat} \geq 27$ cm H_2O, murine lungs approach their total lung capacity,[28] which means that the stress-bearing elements experience cyclic load under these conditions. This interpretation is also supported by the results seen in series_2, where the pressure release valve limited p_{plat} to 34 cm H_2O, which is much lower than in many previous studies where lung failure occurred in the presence of $p_{plat} > 40$ cm H_2O.[4,10] The final consequences have been well described: rupture of the alveolar plasma membrane and of the thin pulmonary capillary wall with consequential hemorrhage.[4,9,26,29–31]

Certainly, p_{plat} alone is not sufficient to explain the threshold of mechanical lung failure, and the underlying forces need to be described by a more comprehensive approach such as the energy/power concept developed by Protti *et al.*[32] and Gattinoni *et al.*[33] According to their analysis, the strain (total lung capacity to functional residual capacity [FRC] ratio) is critical for the time to generate lung injury[34] and the relevant total lung capacity to FRC ratios of 2 to 3 are comparable across mammalian species. Differences between species are explained by lung dimensions and by differences in specific lung elastance (the bulk modulus calculated as FRC/compliance), which is about

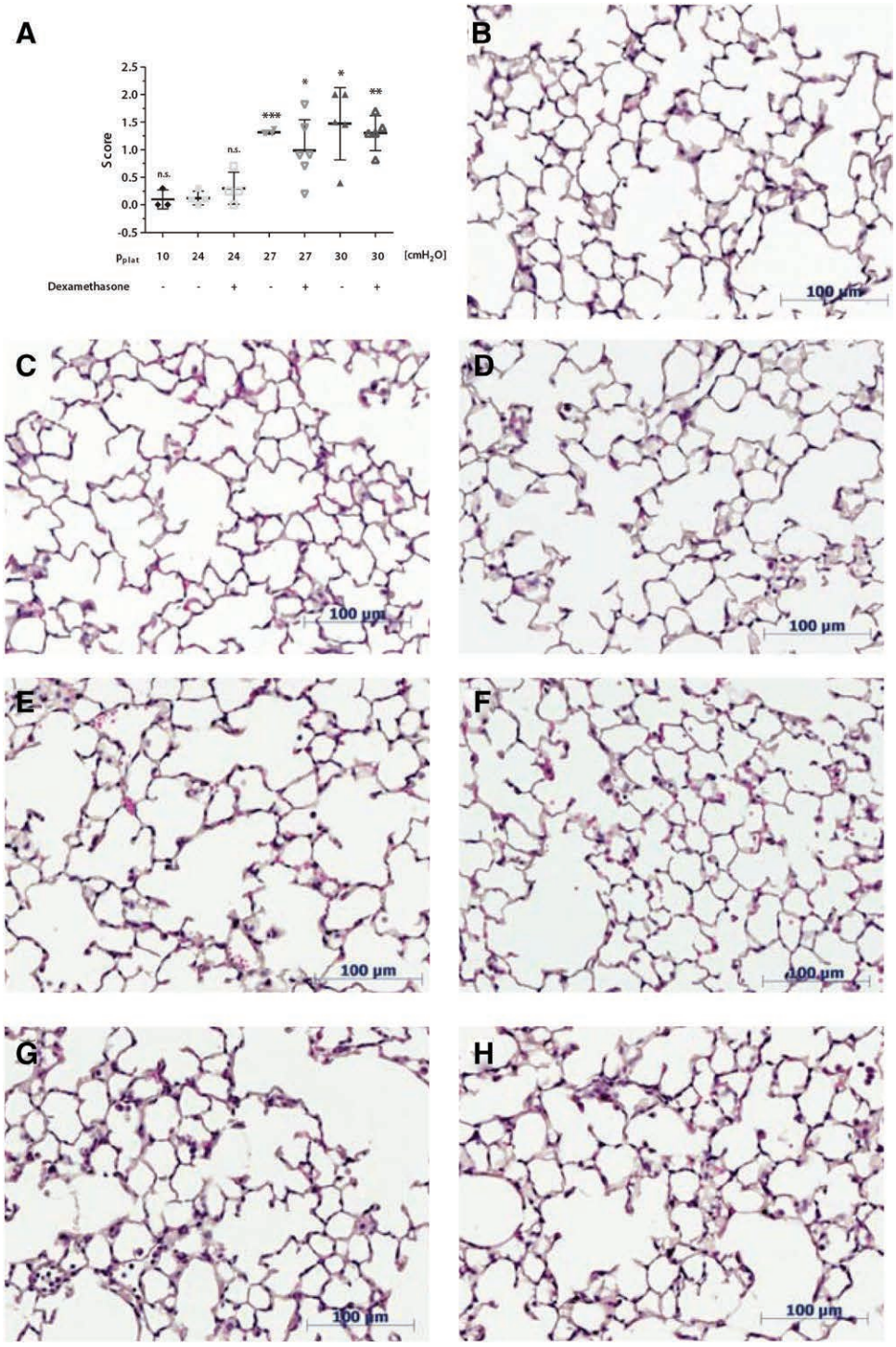

Fig. 9. Effect of dexamethasone on histopathologic scores (series_2). Mice were ventilated with 10 cm H_2O (p10), 24 cm H_2O (p24 I), 27 cm H_2O (p27 I), or 30 cm H_2O (p30 I) including pressure release valve (I, P_{plat}^{max} 34 cm H_2O) for 7 h (or until death). Animals randomly received D (1 mg/kg iv) directly after start of mechanical ventilation: 24 cm H_2O plus D (p24 I D), 27 cm H_2O plus D (p27 I D), and 30 cm H_2O plus D (p30 I D). (*A*) Histopathologic scoring parameters were neutrophils in the alveolar or interstitial space, alveolar septal thickening, alveolar congestion, and formation of hyaline membranes. Each criterion scored one point, resulting in a range of 0 to 4 points. (*B–H*) Representative images of lung sections stained with hematoxylin and eosin from (*B*) p10, (*C*) p24 I, (*D*) p24 I D, (*E*) p27 I, (*F*) p27 I D, (*G*) p30 I, and (*H*) p30 I D. Magnification ×200. (*A*) Data are shown as scatter plot with mean ± SD; p10: n = 3, p24 I: n = 4, p24 I D: n = 5, p27 I: n = 5, p27 I D: n = 6, p30 I: n = 5, p30 I D: n = 5. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ in comparison to 24 cm H_2O group. n.s. = not significant.

7 to 12 cm H_2O in mice,[28,35,36] 6 cm H_2O in pigs,[10] and 12 cm H_2O in humans.[37] These values suggest that human lungs are more resistant to injurious ventilation than those of mice or pig. This analysis, however, cannot explain the survival differences between pigs[10] and mice, which may be explained by the more fragile alveolar structures in mice or alternatively by the breathing frequency that was six times higher in mice: 90 *versus* 15 breaths/min; thus, after 7 h of ventilation, our mice had experienced the same number of cycles as the pigs had after 42 h.[10] Notably, respiratory rate exponentially increases the mechanical power delivered to the lungs during mechanical ventilation.[33]

In summary, we believe that material fatigue of stress-bearing elements in the lungs due to cyclic load is the most likely explanation for our findings. However, at present, we cannot rule out other explanations such as the repeated opening and closure of alveolar units that are also associated with high rates of stress.[38]

Lack of Protection by Dexamethasone

Another alternative explanation for the massive lung damage would be biotrauma, *i.e.*, the destruction of the lungs by inflammation. In fact, the current series of experiments was initiated in order to establish a model of biotrauma with ventilation as the only hit. The experiments with dexamethasone, a well-known antiinflammatory drug, were designed to examine this hypothesis. Despite being able to reduce most of the inflammatory responses, dexamethasone treatment had little effect on physiologic parameters (p_{plat}, C_{dyn}, $petCO_2$, wet/dry ratio, and Pao_2/Fio_2), histopathologic scoring, or on survival proportions. The fact that infiltrating neutrophil numbers were not affected by the pretreatment with dexamethasone can be explained by the presence of hemorrhage (fig. 9). The results of the dexamethasone experiments suggest that inflammation was a result and not the cause of the lung failure. However, application of dexamethasone is only one method to reduce inflammation, and we cannot exclude the possibility that other methods may be more effective although the random-type pattern of lung failure in the present study is rather different from the gradual and well-timed lung failure that is known to be induced by inflammation, *e.g.*, in acid-induced lung injury.[39] We would also like to acknowledge that the mouse lung is too small for gravitational effects to come into play, while in large animal one-hit models, severe regional inflammation can be generated after barrier breakdown.

Our findings also demonstrate the necessity to continuously monitor cardiovascular and pulmonary functions. Without monitoring blood pressure, we might have ventilated dead animals, and without monitoring lung functions, the interpretation of the lung failure would have been difficult. In addition, the random occurrence of lung failure mandates sufficiently long ventilation times. A thought experiment might illustrate the problems that can arise from curtailed observation times. If we had stopped the experiments in series_2 only 34 min earlier (after 386 min instead of 420 min), the percentage of animals surviving would have increased from 50% (table S3, Supplemental Digital Content 9, http://links.lww.com/ALN/B400) to 100% (table S4, Supplemental Digital Content 10, http://links.lww.com/ALN/B401) and dexamethasone would have had a significant effect on survival (P = 0.04; fig. S6B, Supplemental Digital Content 11, http://links.lww.com/ALN/B402, showing the Kaplan-Meier plots with different censor times) and lung compliance (P = 0.03; fig. S7B, Supplemental Digital Content 12, http://links.lww.com/ALN/B403, showing C_{dyn} with different censor times). These results again indicate that inflammation may accelerate an impending mechanical lung failure. Notably, in 38 murine VILI studies reviewed by us,[9] the mean duration was 3.6 ± 1.8 h and we wonder how many treatments had remained effective had the animals been ventilated longer.

Conclusions

Our findings demonstrate a relatively sharp distinction between ventilation with 24 cm H_2O that was well tolerated and ventilation with 27 cm H_2O—only 3 cm H_2O more—where nearly all animals died because of fatal lung failure. With the caveat that 7 h may have been too short to see lung injury in those animals ventilated with 24 cm H_2O, it seems possible that there may be a threshold for the occurrence of mechanical lung injury, a suggestion that was also made in pig studies that lasted for 3 days.[10] Such a threshold would be reached when brittle stress-bearing elements are exposed to high degrees of cyclic load. As a result, the onset of lung failure occurs at random time points. Our findings also suggest—at least in mice—that in one-hit models of VILI, inflammation accelerates the inevitable lung failure and that the relationship between ventilation and inflammation in ARDS patients may better be studied in multiple hit models.

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Competing Interests

The authors declare no competing interests.

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Dr. Adoniram J. Shurtleff: How "Uncle Ad" Became an "Ad" Against Self-Administration of Anesthesia

Affectionately called "Uncle Ad" by his nieces and nephews, Dr. Adoniram J. Shurtleff (1841 to 1885, *left*) had expanded his Massachusetts dental practice from the town of Natick to an office at Boston's Hotel Boylston (*right*). Shortly after 6 PM on February 26, 1885, a close friend had followed Dr. Shurtleff's instructions and had administered a proprietary brand of nitrous oxide to the 43-year-old dentist, who, ironically, was suffering from a severe toothache. At 10 PM the janitor discovered "the doctor lying upon the floor dead," and "the body was not cold." The dead man's left hand clutched a piece of tubing "with one end of it held tightly between the teeth [and] the other of course attached to the cylinder," which was "completely empty of [laughing] gas." A few months after Shurtleff's death, a leading dental authority, Dr. Albion Dudley, published a wise admonition: "No dentist should ever undertake to administer an anesthetic to himself under any circumstances." (Copyright © the American Society of Anesthesiologists' Wood Library-Museum of Anesthesiology.)

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