High tidal volume upregulates intrapulmonary cytokines in an in vivo mouse model of ventilator-induced lung injury

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Although ventilatory support is an indispensable tool in the treatment of critically ill patients, it is well documented that mechanical ventilation produces or worsens lung injury (10, 24, 31). Ventilator-induced lung injury (VILI) with the associated multiorgan dysfunction may lead to significant morbidity and mortality and thus remains one of the most important problems in the management of patients in the intensive care unit (32). The use of “protective” ventilator strategies designed to limit lung stretch has been shown to be useful in reducing VILI in patients with acute respiratory distress syndrome (ARDS), as demonstrated by the recent National Institutes of Health multicenter trial (1). However, it is not always possible, in some patients with ARDS who exhibit highly heterogeneous lung injury, to avoid overstretching the alveoli and the collapse and reopening of small airways in certain regions of their lungs (14). Alternative therapeutic strategies based on precise understanding of the pathophysiology are necessary to totally eliminate the iatrogenic consequences of VILI.

Studies in the literature have indicated a significant correlation between a pulmonary inflammatory response and the development of VILI. In the presence of underlying lung injury, most animal and clinical studies agree that mechanical ventilation exacerbates pulmonary inflammation and injury (6, 16–18, 26, 33–35). Such inflammation in the lungs appears to be mediated by proinflammatory cytokines, particularly tumor necrosis factor-α (TNF-α). In vivo saline-lavaged rabbit models, injurious ventilation has been found to increase intrapulmonary expression of TNF-α at both mRNA and protein levels (18, 35), and intratracheal administration of anti-TNF-α antibody attenuated the involvement of proinflammatory cytokines, particularly tumor necrosis factor-α (TNF-α). In vivo saline-lavaged rabbit models, injurious ventilation has been found to increase intrapulmonary expression of TNF-α at both mRNA and protein levels (18, 35), and intratracheal administration of anti-TNF-α antibody attenuated the physiological and pathological changes of VILI (16). In the absence of any preceding injury, however, it is unclear whether injurious ventilation per se can initiate cytokine-mediated pulmonary inflammation (9). Both in vitro and in vivo studies have given inconclusive results with respect to TNF-α involvement in VILI produced purely by mechanical ventilation (13, 19, 27, 28, 38–41). In contrast to TNF-α, macrophage inflammatory protein-2 (MIP-2), a neutrophil chemoattractant and the murine functional homolog of IL-8, has
been more consistently found in studies of purely mechanically induced VILI (3, 25, 28, 38). Because the expression of MIP-2 is mediated at least in part by TNF-α in a number of models of inflammatory lung injury (2, 7, 43), it is unclear whether VILI can induce MIP-2 expression without initially stimulating TNF-α release, but if not then the discrepancy between detection of TNF-α and MIP-2 adds to the confusion surrounding this area.

To address these conflicts in the literature, we have adapted an in vivo rat model of lung injury induced by high tidal volume ventilation (8, 13, 19, 25, 28, 41) for use in intact mice. Previous studies have tended to investigate lungs at a single stage of injury, and little consideration has been given to the possibility that cytokine expression may be transient during the course of VILI development. Experiments were therefore carried out to determine cytokine levels in lung lavage fluid at both an early and more advanced stage of VILI. The results demonstrated that high tidal volume-induced lung injury is sufficient to cause release of biologically active TNF-α and MIP-2 into the alveolar spaces of the lung. Furthermore, the data suggested that TNF-α protein expression is transient, peaking early in the course of injury, which may explain the previous literature controversy.

METHODS

Animal preparation. Experiments were carried out under the guidelines of the Animals (Scientific Procedures) Act 1986, United Kingdom. Male C57BL/6 mice (Harlan, Bicester, UK) aged 9–13 wk (25–29 g) were anesthetized by intraperitoneal injection of Hypnorm 2.5 ml/kg (fentanyl 0.8 mg/kg, fluanisone 25 mg/kg) and midazolam 2.5 ml/kg (12.5 mg/kg). An endotracheal tube (0.76 mm ID, 1.22 mm OD) was inserted via tracheotomy, and mice were ventilated by use of a custom-made mouse jet ventilator system, as described by Ewart et al. (12). In this system, inspiratory flow was controlled at a constant rate by a metering valve, and the timing of inspiration and expiration was regulated by electronically controlled solenoid valves. Airway pressure was monitored by a pressure transducer (MLT8380, ADInstruments, Chalgrove, UK), and airway flow was determined by a differential pressure transducer (PX137, Omega Engineering, Manchester, UK) connected to a miniature pneumotachograph in the ventilator circuit. Tidal volume (VT) was calculated by integrating airway flow during inspiration. During surgery and stabilization, the animals were ventilated with a peak inspiratory pressure (PIP) of 10–12 cmH2O, positive end-expiratory pressure (PEEP) of 2.5 cmH2O, and respiratory rate (RR) of 120 breaths/min, with the use of 100% O2. VT attained with these settings was 8–9 ml/kg, and inspiratory-expiratory ratio was kept constant at 1:2 throughout the experiment. A polyvinyl chloride catheter (0.28-mm ID, 0.61-mm OD) was introduced into the left carotid artery for monitoring of arterial blood pressure (BP) by use of a pressure transducer (MLT844, ADInstruments), blood-gas analysis, and infusion of fluids (0.9% NaCl containing 10 U/ml heparin at 0.4 ml/h). Another polyvinyl chloride catheter was placed into the intraperitoneal cavity for maintenance of anesthesia. Hypnorm 1.2 ml/kg and midazolam 1.2 ml/kg were administered approximately every 20 min or as assessed by mean BP fluctuations in response to gentle tail stimuli. Throughout the experiment, rectal temperature was monitored and main-
Tidal volume protocols were compared at three different lengths of ventilation (2 vs. 3 h), so only the differences in any of the measured variables between trials were analyzed by one-way ANOVA. Changes in parameters across the course of the experiments were expressed as units of TNF-α/ml; one unit was defined as the activity that results in 50% of WEHI cell death. Analysis of the standard curve using recombinant murine TNF-α (410-MT, R&D Systems) showed that the detection limit of this assay was 0.5 pg/ml.

Histopathological examination of the lungs. After lung lavage, the heart and lungs were removed en bloc via midline sternotomy. Lungs were fixed in 10% formalin, with the fixing solution introduced via a cannula passed down the endotracheal tube while the lungs were vertically suspended from the endotracheal tube. The cannula did not form a tight seal within the tube, limiting the lung inflation pressure to ~5 cmH2O, so that any artificial tissue disruption due to overinflation was minimized. Sections were then viewed by use of a hematoxylin and eosin stain by an observer blinded to the experimental group.

Data analysis. Data are expressed as means ± SD. Changes in parameters across the course of the experiments were analyzed by ANOVA for repeated measures, and differences between groups were analyzed by one-way ANOVA. Pairwise comparisons were made by Scheffé’s tests. A P value of <0.05 was considered significant.

RESULTS

Low-Vt series 3 animals exhibited no statistical differences in any of the measured variables between different lengths of ventilation (2 vs. 3 h), so only the combined data are presented.

Physiological measurements. Figure 1 shows changes in PIP during the ventilatory protocol. High-Vt ventilation led to an increase in PIP in all animals, starting at 110–120 min. PIP was virtually identical in both groups of high-Vt animals until 120 min when series 2 animals were terminated, and continued ventilation in series 1 animals led to further increases in PIP. Low-Vt ventilation had no significant effect on PIP.

Table 1 summarizes changes in mean BP and blood gas parameters during the course of the experiments. Baseline values for all the parameters, taken immediately before the start of ventilatory protocols, were not different among the three groups of animals. Mean BP was well maintained in all animals throughout the protocol, except for the final 10–20 min of the series 1 high-Vt experiments. PaO2 showed a decrease in series 1 (high Vt, late stage) mice but not in the other groups. With the use of supplemental inspiratory CO2, excessive hypocapnia was avoided and PaCO2 was maintained within a physiological range in high-Vt animals. Base excess showed small decreases in all groups, and as a result of the changes in PaCO2 and base excess, blood pH decreased in high-Vt animals but was still maintained more than 7.3 throughout the experiment.

Figure 2 illustrates changes in respiratory mechanics caused by the different ventilatory protocols. High-Vt ventilation produced decreases in Crs and increases in Rrs, with changes in series 1 > series 2.

Lung pathology. Figure 3 shows typical pathological findings obtained from series 1 (high Vt, late stage) and series 3 (low Vt) animals. All lungs from series 1 mice showed a moderate degree of injury, as characterized by the presence of hyaline membranes within alveoli and focal epithelial cell damage within the terminal and respiratory bronchioles. Lungs from low-Vt series 3 mice showed no such pathological signs of injury. Lungs from series 2 mice (not shown) displayed intermediate levels of injury.

Protein and cytokine measurements in lavage fluid. In preliminary studies, we compared MIP-2 levels in all three supernatants from the lavage procedure and found that the first wash contained the majority of cytokine recovered (1st wash 64.7 ± 5.4%, 2nd wash 26.1 ± 1.4%, 3rd wash 9.2 ± 6.7%, n = 4). Therefore, only the supernatants of the first wash were used for analyses of cytokines and protein concentrations, to avoid the possibility of diluting cytokines to levels below detectability. Fluid volumes recovered from the
concentrations in lung lavage fluid of experiments were as follows: series 1, 763 ± 41 μl; series 2, 606 ± 47 μl; series 3, 617 ± 56 μl.

Figure 4 shows protein concentration in the lung lavage fluid at the end of the ventilatory protocol. High-Vt ventilation led to increases in the protein concentration, with series 1 animals displaying the highest levels. Figure 5 illustrates TNF-α and MIP-2 concentrations in lung lavage fluid determined by ELISA. TNF-α concentrations were above the detection limit in 7 of 12 series 1 (high Vt, late stage) mice, all series 2 (high Vt, early stage) mice, and only 1 of 9 series 3 (low Vt, control) mice (Fig. 5A). To take into account the different lavage fluid volumes recovered in each group of animals, the amount of TNF-α protein recovery with the first wash (concentration of TNF-α in pg/ml, multiplied by lavage fluid volume recovered in ml) was also calculated: series 1, 15.3 ± 19.0 pg; series 2, 54.3 ± 36.1 pg; series 3, 3.1 ± 2.8 pg. Whether expressed as concentration or amount recovered, TNF-α in series 1 animals was not different from low-Vt series 3, whereas series 2 animals displayed significantly higher levels of TNF-α than both other groups (P < 0.01). MIP-2 concentrations were above the detection limit in all mice ventilated with high Vt and in 4 of 9 series 3 mice (Fig. 5B). The amount of MIP-2 protein recovery was also calculated: series 1, 57.7 ± 21.7 pg; series 2, 82.6 ± 76.6 pg; series 3, 2.6 ± 2.4 pg. Whether expressed as concentration or amount recovered, high-Vt series 1 and 2 animals exhibited similar MIP-2 values, which were significantly higher than low-Vt series 3 mice (P < 0.05).

In this study, we established a physiologically stable in vivo mouse model of VILI and using this model demonstrated that high-Vt ventilation alone, without any preceding lung injury, upregulates intrapulmonary cytokines TNF-α and MIP-2. Furthermore, we found that TNF-α expression in lung lavage fluid was positive in the early stage of lung injury (series 2) but then declined in the late stage (series 1), whereas MIP-2 expression was positive throughout the course. WEHI bioassay indicated the presence of bioactive TNF-α in lavage fluid from high-Vt series 1 animals, even when TNF-α protein levels were below the detection limit of the ELISA, but not in low-Vt animals. Thus TNF-α protein induced by high-Vt ventilation is likely to play a biological role in the alveolar space. The temporal profiles of TNF-α and MIP-2 protein upregulation, consistent with their general kinetic characteristics, may offer an explanation to previous controversy.

Table 1. Physiological parameters during the course of experiments

<table>
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<th>Baseline</th>
<th>Start of Protocol</th>
<th>End of Protocol</th>
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<tbody>
<tr>
<td><strong>Series 1 (high Vt, late stage)</strong></td>
<td></td>
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<td></td>
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<tr>
<td>Mean BP, mmHg</td>
<td>61 ± 9</td>
<td>67 ± 7</td>
<td>44 ± 2†</td>
</tr>
<tr>
<td>PaO2, Torr</td>
<td>396 ± 58</td>
<td>425 ± 47</td>
<td>343 ± 52†</td>
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<td>PaCO2, Torr</td>
<td>26 ± 3</td>
<td>37 ± 2</td>
<td>43 ± 2†</td>
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<td>pH</td>
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<td>7.43 ± 0.03</td>
<td>7.32 ± 0.02†</td>
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<td>Base excess</td>
<td>1.6 ± 2.1</td>
<td>0.2 ± 2.0</td>
<td>-4.5 ± 1.4†</td>
</tr>
<tr>
<td><strong>Series 2 (high Vt, early stage)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean BP, mmHg</td>
<td>64 ± 11</td>
<td>62 ± 11</td>
<td>55 ± 9</td>
</tr>
<tr>
<td>PaO2, Torr</td>
<td>416 ± 41</td>
<td>427 ± 52</td>
<td>411 ± 79</td>
</tr>
<tr>
<td>PaCO2, Torr</td>
<td>28 ± 2</td>
<td>41 ± 6</td>
<td>43 ± 6†</td>
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<tr>
<td>pH</td>
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<td>7.41 ± 0.05</td>
<td>7.35 ± 0.05†</td>
</tr>
<tr>
<td>Base excess</td>
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<td>-2.6 ± 1.8†</td>
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<tr>
<td><strong>Series 3 (low Vt, control)</strong></td>
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<td>Mean BP, mmHg</td>
<td>61 ± 6</td>
<td>61 ± 6</td>
<td>65 ± 7</td>
</tr>
<tr>
<td>PaO2, Torr</td>
<td>404 ± 31</td>
<td>421 ± 23</td>
<td>439 ± 31</td>
</tr>
<tr>
<td>PaCO2, Torr</td>
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<td>29 ± 3</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>pH</td>
<td>7.56 ± 0.03</td>
<td>7.51 ± 0.05</td>
<td>7.47 ± 0.03</td>
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<tr>
<td>Base excess</td>
<td>1.9 ± 3.1</td>
<td>0.9 ± 3.2</td>
<td>-2.2 ± 2.7†</td>
</tr>
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Values are means ± SD; n = 8–12 for all observations. Note that inspiratory gas containing 4–5% CO2 was used in high-tidal volume (Vt) animals. Series 1 (high Vt, late stage) animals were euthanized as soon as blood pressure (BP) fell below 45 mmHg. Start of protocol measurements were made either immediately on changing ventilatory protocol (BP) or after 30 min of ventilatory protocol (blood-gas parameters). PaO2 and PaCO2, arterial PO2 and PCO2, respectively. *P < 0.05 vs. start of protocol. †P < 0.01 vs. start of protocol.

Figure 6 shows the results of WEHI cytotoxicity assay performed on lavage samples from series 1 (high Vt, late stage) and series 3 (low Vt, control) mice. All lavage samples from series 1 mice demonstrated positive TNF-α bioactivity, whereas those from series 3 animals were virtually negative.

DISCUSSION

In this study, we established a physiologically stable in vivo mouse model of VILI and using this model demonstrated that high-Vt ventilation alone, without any preceding lung injury, upregulates intrapulmonary cytokines TNF-α and MIP-2. Furthermore, we found that TNF-α expression in lung lavage fluid was positive in the early stage of lung injury (series 2) but then declined in the late stage (series 1), whereas MIP-2 expression was positive throughout the course. WEHI bioassay indicated the presence of bioactive TNF-α in lavage fluid from high-Vt series 1 animals, even when TNF-α protein levels were below the detection limit of the ELISA, but not in low-Vt animals. Thus TNF-α protein induced by high-Vt ventilation is likely to play a biological role in the alveolar space. The temporal profiles of TNF-α and MIP-2 protein upregulation, consistent with their general kinetic characteristics, may offer an explanation to previous controversy.
in the literature regarding the involvement of cytokines in the pathogenesis of VILI.

We have successfully adapted an in vivo model of lung injury induced by high-pressure, high-Vt mechanical ventilation as used by others (8, 13, 19, 25, 28, 41) for use in intact mice. The Vt employed in the present study (34 ml/kg) is four to five times more than that of spontaneously breathing C57BL6 mice (6–9 ml/kg) (36). Although much higher than would be used within the clinical setting, it has been suggested that such very high Vt may produce a similar degree of alveolar overdistention to that observed regionally in ARDS patients receiving much lower Vt (38). The heterogeneous nature of the lung injury in ARDS causes certain lung regions to preferentially receive the bulk of the delivered gas, leading to regional overdistention (14). Moreover, the Vt employed is similar to those used in previous in vivo and ex vivo animal studies in the literature (8, 19, 28, 38, 39), which have substantially contributed to our understanding of the mechanisms of VILI. Furthermore, this level of Vt was able to reproducibly induce acute lung injury in mice without preceding lung insult, thereby enabling us to investigate the effects of pure mechanical stretch. The processes of VILI development in our model were essentially consistent with those observed in other species. Compared with control low-Vt mice, all high-Vt mice exhibited progressive decreases in Crs, increases in Rrs, and increases in protein levels in lavage fluid. Series 1 mice, ventilated with a high Vt until the lung injury progressed to such a point that substantial alveolar edema developed and BP could not be maintained, displayed a greater level of injury than series 2 mice, ventilated in an identical manner but for only 2 h. Histological examination was also carried out to validate the pathological evolution of VILI. Lungs from series 1 animals showed epithelial cell damage and hyaline membrane formation, which were not present in control series 3 animals. Alveolar proteinaceous edema and hyaline membranes are two characteristic features of high pressure- or high volume-induced VILI observed in other species (8, 10).

In the present model, arterial BP was monitored continuously in all animals and despite the large Vt used was well maintained within a reasonable range expected for C57BL6 mice anesthetized with Hypnorm and midazolam (44). Only in series 1 (high Vt, late stage) animals did deterioration of BP occur, but this was only in the last 10–20 min, and the experiments were terminated as soon as mean BP fell below 45 mmHg. The absence of severe metabolic acidosis and hypoxemia, even at the point just before euthanasia in series 1 animals, suggests that adequate blood perfusion and tissue oxygenation were likely achieved in our experiments. The use of inspired gas containing supplementary CO2 contributed to maintenance of normocapnia during high-Vt ventilation, but small decreases in base excess resulted in a slightly lower pH in high-Vt mice than low-Vt mice. However, this difference in pH would not have altered the validity of this model, because acidosis is likely to have attenuated, rather than exacerbated, the injury induced by high Vt.
Thus we believe that our attempts to monitor these homeostatic parameters and maintain them within reasonable ranges should help to minimize any confounding effects produced by impaired tissue perfusion and metabolism.

Previous studies in the literature have produced at times confusing results regarding the involvement of proinflammatory cytokines, particularly TNF-α, in the progression of VILI (9). Most studies using animals with “preinjured” lungs suggest that injurious ventilation stimulates cytokine release within the lungs, as detected by ELISA in bronchoalveolar lavage (BAL) fluid (6, 16, 18, 35), but not all studies have found an increase in TNF-α protein levels (41). Moreover, there is considerable debate as to whether pure mechanical ventilatory stress per se, without preceding lung injury, can initiate cytokine-mediated pulmonary inflammatory responses (27, 28). Using an ex vivo isolated nonperfused lung preparation, Slutsky and colleagues found that injurious high-VT ventilation in the absence of underlying lung injury increased lavage fluid levels of many cytokines, including TNF-α and MIP-2 in rats (38) and mice (40). Increased release of TNF-α into vascular perfusate has also been shown with such injurious ventilation in isolated perfused mouse lungs (42). Consistent with these, Foda et al. (13) reported a significant increase in TNF-α in BAL fluid in an in vivo rat model of VILI produced by high-Vt ventilation without underlying injury. In contrast, using a similar in vivo rat model of VILI, Ricard et al. (28) showed no effect of injurious ventilation on BAL fluid TNF-α protein levels. The authors also failed to reproduce the findings of Slutsky’s group in isolated nonperfused rat lungs, postulating that such ex vivo VILI models may be inherently unstable owing to lack of perfusion and/or contamination. Similarly, Verbrugge et al. (41) found no increase in TNF-α in BAL fluid in healthy rats subjected to high-Vt ventilation. At the mRNA level, Tremblay et al. (39) found in isolated rat lungs that injurious ventilation produced widespread pulmonary epithelial expression of TNF-α mRNA, whereas Imanaka and colleagues (19) reported no significant increase in TNF-α mRNA in rats ventilated with high Vt. These apparent discrepancies, at least in part, may come from the differences in experimental designs and conditions in each model employed.

We have demonstrated that, in our present in vivo mouse model, both TNF-α and MIP-2 levels in lung lavage fluid were elevated with high-Vt ventilation, without any preceding lung injury. Even when TNF-α protein was undetectable by ELISA, bioactive TNF-α was detected by WEHI assay in series 1 (high Vt, late stage) animals. This implies that the TNF-α induced by high-Vt ventilation could play an actual biological role of underlying lung injury increased lavage fluid levels of many cytokines, including TNF-α and MIP-2 in rats (38) and mice (40). Increased release of TNF-α into vascular perfusate has also been shown with such injurious ventilation in isolated perfused mouse lungs (42). Consistent with these, Foda et al. (13) reported a significant increase in TNF-α in BAL fluid in an in vivo rat model of VILI produced by high-Vt ventilation without underlying injury. In contrast, using a similar in vivo rat model of VILI, Ricard et al. (28) showed no effect of injurious ventilation on BAL fluid TNF-α protein levels. The authors also failed to reproduce the findings of Slutsky’s group in isolated nonperfused rat lungs, postulating that such ex vivo VILI models may be inherently unstable owing to lack of perfusion and/or contamination. Similarly, Verbrugge et al. (41) found no increase in TNF-α in BAL fluid in healthy rats subjected to high-Vt ventilation. At the mRNA level, Tremblay et al. (39) found in isolated rat lungs that injurious ventilation produced widespread pulmonary epithelial expression of TNF-α mRNA, whereas Imanaka and colleagues (19) reported no significant increase in TNF-α mRNA in rats ventilated with high Vt. These apparent discrepancies, at least in part, may come from the differences in experimental designs and conditions in each model employed.

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We have demonstrated that, in our present in vivo mouse model, both TNF-α and MIP-2 levels in lung lavage fluid were elevated with high-Vt ventilation, without any preceding lung injury. Even when TNF-α protein was undetectable by ELISA, bioactive TNF-α was detected by WEHI assay in series 1 (high Vt, late stage) animals. This implies that the TNF-α induced by high-Vt ventilation could play an actual biological role.
expected of this cytokine, e.g., initiating an inflammatory cascade in the alveolar space. Low-VT series 3 animals exhibited effectively negative TNF-α and MIP-2 by ELISA as well as the more sensitive WEHI assay, suggesting that the model is free from endotoxin contamination.

The present study was designed specifically to look at two distinct points within the course of the development of VILI. Series 2 mice, terminated at an early point of VILI when the physiological signs of injury just become apparent (i.e., when the PIP started to increase), expressed significantly higher levels of both TNF-α and MIP-2 than control series 3 animals. In comparison, series 1 mice with a more advanced lung injury also showed increased levels of MIP-2, but TNF-α protein was lower and not always detectable by ELISA (but bioactivity was detectable by a more sensitive WEHI bioassay) and therefore not significantly different from control series 3 animals. This indicates that TNF-α protein expression in the alveolar space may be transient, peaking for a relatively short period of time and then rapidly declining to levels that may be below the detection limit of the ELISA, whereas MIP-2 levels may be more sustained during the progression of VILI. It is unlikely that this apparent transience in TNF-α protein levels can be explained by dilution effects due to the increased edema fluid accumulation in high-VT series 1 animals (as represented by the increased Rrs and protein levels in lavage fluid, and more directly by the increased fluid volumes recovered by the lavage). Intrapulmonary cytokines recovered by the whole lung lavage procedure were inevitably diluted with a large and fixed amount of saline (750 μl, i.e., 25–30 ml/kg), which is 2/3 to 3/4 of total lung capacity of C57BL6 mice (37) and should be far in excess of any alveolar edema fluid expected in these mice. Moreover, TNF-α levels exhibited the same transience whether expressed as concentration or amount of protein recovered, to take into account the different lavage fluid volumes recovered.

To the best of our knowledge, transience of TNF-α protein levels in lavage fluid with injurious ventilation has not previously been reported, although it is consistent with the general kinetic characteristics of these cytokines, e.g., transient increases in TNF-α levels but sustained increases in MIP-2 levels are observed in plasma after endotoxin administration in animals (20). We postulate that much of the literature controversy regarding the involvement of TNF-α in purely mechanically induced VILI may be ascribed to this transient nature of TNF-α expression. The progression of lung injury would be determined by various factors, including the degree (ventilator settings) and length (length of ventilation) of the mechanical stress imposed and the susceptibility of the animals used. By examining lung injury that is already well advanced, TNF-α protein levels may have peaked and declined in many in vivo animal studies to levels undetectable by ELISA techniques, even though the length of injurious ventilation may be similar to that used in the present study (28). On the other hand, MIP-2 expression is relatively more sustained, which may explain why this cytokine has consistently been found to be increased in VILI models (3, 25, 28). Although Tremblay et al. (39) reported in isolated rat lungs ventilated with a high Vt that the proportion of epithelial cells expressing TNF-α mRNA peaked at 30 min and returned to baseline thereafter, they did not find such transience in lavage fluid TNF-α protein. An apparent lack of transience in TNF-α protein levels in ex vivo models of lung injury may be related to reduced removal of the cytokine via blood flow and lymphatic drainage (28) and possibly to reduced action of proteases and anti-inflammatory cytokines compared with intact animals. This may also explain at least in part why some ex vivo studies (38–40) display higher levels of cytokines than observed in our study and the previous in vivo study by Foda et al. (13), both with injurious and noninjurious ventilation.

In the present study, we used ELISA to measure TNF-α and MIP-2 levels, which is a well-established, highly specific, and reproducible method to evaluate cytokine kinetics at the protein expression level (15, 22). The WEHI bioassay was used first to assess whether TNF-α could be detected in amounts that were undetectable by ELISA with its better sensitivity (0.5 pg/ml of ELISA sensitivity) and second to confirm whether TNF-α in lavage fluid was bioactive. Because the WEHI assay was not performed in series 2 mice (high Vt, early stage), we are unable to draw a conclusion as to whether TNF-α bioactivity would have shown a similar decline during the progression of VILI. Although it is not unreasonable to expect that the bioactivity profile of a cytokine would in general follow its protein expression profile, very little is known about the kinetics of various intrinsic factors that may affect TNF-α bioactivity in the alveolar space, e.g., soluble TNF-α receptors, β-macroglobulin, and natural antibodies (15). Precise determination of TNF-α bioactivity profile and its biological role and consequences in the progression of VILI remains to be further investigated.

This study supports the growing body of data suggesting that ventilation strategy influences outcome, and this may be due in part to the release of proinflammatory cytokines. Further studies are necessary to determine whether pharmaceutical blockade of such cytokines could be used as treatments to attenuate either the development of VILI or the systemic progression to multiple organ failure that occurs with mechanical ventilation of ARDS patients. The mouse model of VILI developed in the present study may provide a useful investigative tool to clearly define the importance of various inflammatory pathways involved in VILI by use of genetically engineered mice. The present study also indicates that the timing of any treatment regimen may be critical, depending on the expression profile of the cytokines involved.

In conclusion, using an in vivo mouse model of VILI, we demonstrated that high-Vt ventilation per se, without any preceding lung injury, upregulates intrapulmonary cytokines TNF-α and MIP-2. The transient nature of intra-alveolar TNF-α protein expression...
found in this study may help to resolve the previous controversies in the literature as to the significance of cytokine involvement in the pathogenesis of VILI.

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REFERENCES


