Alveolar macrophages contribute to alveolar barrier dysfunction in ventilator-induced lung injury

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Mechanical ventilation with excessive end-inspiratory volume contributes to mortality in patients with acute lung injury and the acute respiratory distress syndrome (ARDS). The pathogenesis of ventilator-associated lung injury is incompletely understood; however, both clinical and experimental ventilation attributable lung injury are characterized by activation of the inflammatory response. For example, a variety of pro- and anti-inflammatory mediators have been correlated with the mechanical ventilation strategy in both experimental and clinical studies, including IL-1β, IL-6, IL-8, and IL-10 (5, 28, 33, 34). Previous reports have indicated that in vitro mechanical strain induces IL-8 release from alveolar epithelial-like (A549) cells and alveolar macrophages (32, 38). Because neutrophils contribute to ventilator-induced lung injury (VILI) (3), the release of neutrophil chemokines such as IL-8 likely constitutes an early step in the pathogenesis of VILI. Although animal studies using immunohistochemistry and in situ hybridization techniques have shown that a variety of lung cells produce inflammatory mediators in VILI (7), the relative contributions of resident lung cell types to the pathogenesis of VILI has not been fully explored. We hypothesized that alveolar macrophages have a central role in the initiation of VILI. The primary objective of the present study was to determine if alveolar macrophages contribute to the increase in lung vascular and alveolar epithelial permeability characteristic of experimental VILI using a previously described macrophage depletion technique (19). A second objective was to determine if injurious mechanical strain activates alveolar macrophages by inducing the release of soluble mediators from alveolar epithelial cells, or by a direct effect on alveolar macrophages.

METHODS

This protocol conforms to National Institutes of Health animal care and use guidelines and was approved by the University of California, San Francisco Institutional Animal Care and Use Committee (IACUC).

Clodronate liposome preparation and delivery. Liposomes were prepared as previously described (19). Briefly, liposomes were composed of phosphatidylserine, phosphatidylcholine, and cholesterol at a molar ratio of 1:6:4 in chloroform. The lipid solution was dried under low vacuum and dissolved in diethyl ether. A clodronate (Sigma, St. Louis, MO) stock solution or PBS was added, and the mixture was placed under nitrogen and sonicated for 3 min. The ether was then removed by rotary evaporation under reduced pressure at 30°C. Any gel phase that formed was disrupted by vortexing the sample to facilitate the removal of ether. The lipid suspension was then repeatedly extruded through 200-nm filters. The lipid solution was then delivered to anesthetized (ketamine 90 mg/kg ip) animals by aerosol (19). Fluorescent liposomes were prepared separately by adding the dye DiO to the lipid mixture (35). Compared with direct intratracheal instillation, aerosol delivery is associated with more macrophage depletion and fewer air space neutrophils (19).

VILI model. A previously described VILI model (17) with slight modification was used. Briefly, rats were anesthetized with 4% isoflurane and 50 mg/kg ip pentobarbital. A tracheostomy tube (15-gauge luer adapter) was placed, and mechanical ventilation was started with a tidal volume of 6 ml/kg, a positive end-expiratory pressure (PEEP) of 5 cmH2O, a respiratory rate of 60 breaths/min, and 21% oxygen. A

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right common carotid artery catheter (PE-50 tubing, BD) was placed for blood pressure monitoring and arterial blood gas measurement. Respiratory rate was adjusted to maintain normal arterial pH. Following a 10-min stable baseline period, tidal volume was increased to 30 ml/kg without PEEP. Tidal volume was decreased if necessary to limit airway pressure to 30 cmH2O throughout the protocol. These settings were used to approximate ventilation at total lung capacity. Respiratory rate was decreased to 30 breaths/min, and additional dead space was added to the ventilator circuit as needed to maintain normal arterial pH. Anesthesia was maintained with isoflurane (0.5–2%), and muscle relaxation was maintained with pancuronium (2 mg·kg\(^{-1}\)·h\(^{-1}\)). Ventilation was continued from 10 min to 4 h. Normal saline (1.5 mg·kg\(^{-1}\)·h\(^{-1}\)) was administered throughout the protocol. Airway pressures, blood pressure, and heart rate were monitored continuously using a computer-integrated data collection system. Arterial blood gases were measured at 1, 2, and 4 h, and plasma samples were collected at the start and end of the protocol.

**Pulmonary edema, permeability, and bronchoalveolar lavage.** Pulmonary edema was measured as the blood-free, excess lung water determined by gravimetric methods (16). Lung endothelial and alveolar epithelial permeability to albumin were measured by determining the extravasation of intravascular 125I-labeled albumin into the lung as previously described (16). Permeability is expressed as the extravascular plasma equivalents in microliters. BAL fluid was performed with three aliquots of 7 ml of warmed normal saline instilled into the lungs and gently withdrawn. A differential cell count was determined on an aliquot of the total BAL fluid using a hemocytometer, and cytological centrifuge preparation was stained with Wright’s stain and eosin. For BAL fluid used in the nitrite assay, a single aliquot of 7 ml of warmed RPMI 1640 was instilled three times, centrifuged, and stored for later use.

**Experimental groups for the macrophage depletion studies.** A total of 44 rats were used for these studies. There were four groups: 1) empty liposomes, unventilated (n = 8); 2) clodronate liposomes, unventilated (n = 8); 3) empty liposomes, ventilated (n = 14); and 4) clodronate liposomes, ventilated (n = 14). BAL was done at the end of the experimental protocol on 4 subjects from each group. These four animals were not used for the pulmonary edema or permeability measurements.

**Measurement of plasma CXC ligand 1.** Plasma samples were collected from rats at the beginning of the protocol and at the end of the high tidal volume ventilation period. Levels of the chemokine CXC ligand 1 (CXCL1) were measured using a rat-specific ELISA with a detection threshold of 40 pg/ml (R&D Systems, Minneapolis, MN). Like human IL-8, CXCL1 is a ligand for CXC receptors 1 and 2. CXCL1 is also known as GRO, KC, MIP-2, and CINC-1 in rodents. All samples were tested in duplicate. CXCL1 levels were measured on four matched pairs of empty liposome-treated and clodronate liposome-treated rats (i.e., 4 subjects in each group of 14 subjects).

**Macrophage nitrite production assay.** Nitrite production was measured as a surrogate of macrophage nitric oxide production, a recognized functional marker of macrophage activation (25). Primary alveolar macrophages from 10 rats were plated at 75,000 cells/well in 96-well plates. Nitrite production was then measured 18 h after adding either BAL fluid from rats or after adding supernatants from cultured alveolar epithelial cells and macrophages exposed to mechanical strain in vitro. Nitrite concentration was determined with a colorimetric nitric oxide assay kit (Caymen, San Diego, CA) as previously described (17). For the BAL fluid studies, 200 μl of cell-free BAL fluid from unventilated rats (n = 4) or from rats exposed to mechanical ventilation for 10 (n = 3) or 20 min (n = 4) were added to the primary macrophages in 96-well plates, and cells were incubated for 18 h at 37°C in 5% CO2. There was a minimum of six wells of macrophages per experimental condition, and the experiment was repeated five times. To normalize nitrite production to cell number, cell density was determined as follows. Cells were washed with PBS and fixed in 70% ethanol for 10 min. Cells were then stained with 1% crystal violet (15 min) and thoroughly rinsed with tap water.

The stain was extracted from cells by adding 50 μl of 0.2% Triton X-100. Relative quantification was done by reading absorbance at 570 nm on plate reader. Total nitrite is reported as the increase in nitrite per 5 × 10^4 cells over the initial level in the BAL fluid (30). For the in vitro mechanical strain studies, alveolar epithelial type II cells were isolated from rat lungs (n = 10) as previously described (13). Epithelial cells were plated at 3 × 10^6 on 35-mm flexible membranes coated with fibronectin (Bioflex, Hillsborough, NC) and recoated with fibronectin (100 μg/ml, Calbiochem no. 341631; San Diego, CA). On day 5, epithelial monolayer confluence was confirmed, and media was changed to serum-free DME-H21, and primary rat alveolar macrophages (3 × 10^5) were plated on fibronectin-coated membranes and allowed to adhere for 1 h. Cells were exposed to mechanical strain (30% membrane surface area change at 0.5 Hz) for 30 min (Flexcell 4000T; Hillsborough, NC). Media was collected, centrifuged, and then added to naive primary alveolar macrophages on 96-well plates for measurement of nitrite production as described above. There was a minimum of three wells of cells per experimental condition, and the experiment was repeated four times.

**Statistics.** Comparisons between two groups were made using an unpaired, two-tailed t-test for normally distributed data and the Mann-Whitney test for nonparametric data. Comparisons among three or more groups were made using one-way analysis of variance and Tukey post hoc test for multiple comparisons. P values less than 0.05 were considered significant. Data are expressed as means ± SD unless otherwise noted.

**RESULTS**

*High tidal volume ventilation rapidly decreases BAL macrophage counts.* BAL macrophage counts from rats ventilated with high tidal volume and without PEEP decreased with increasing duration of mechanical ventilation (Fig. 1). Macro-
phage recovery in BAL fluid was significantly decreased by 20 min and continued to decrease at 180 min. At baseline the total BAL cell count was $313 \times 10^3$ cells/ml, of which $291 \times 10^3$ were macrophages, $7 \times 9 \times 10^3$ were neutrophils, and $15 \times 4 \times 10^3$ were lymphocytes. After 30 min of ventilation, the total BAL cell count decreased to $190 \times 32 \times 10^3$ cells/ml with $169 \times 25 \times 10^3$ macrophages, $9 \times 5 \times 10^3$ neutrophils, and $12 \times 4 \times 10^3$ lymphocytes. Only the change in macrophages was statistically significant at the 30-min time point ($P < 0.05$).

**Macrophage depletion.** Rats were given increasing doses (0–20 mg/ml) of clodronate liposomes by aerosol. Maximal macrophage depletion was observed with a clodronate concentration of 20 mg/ml (Fig. 2A). The nadir of BAL macrophage counts was 3–4 days after administration (Fig. 2B). Macrophage uptake of liposomes occurred early and was confirmed by fluorescently labeled liposomes. Two hours after liposomes were given, ~70% of alveolar macrophages recovered in lavage fluid contained liposomes (not shown).

*Macrophage depletion preserves respiratory system elastance and oxygenation.* During baseline ventilation with low tidal volume and a PEEP level of 5 cmH$_2$O, elastance was comparable between animals treated with empty liposomes and clodronate containing liposomes (Fig. 3A). Upon increasing tidal volume and PEEP during ventilation, alveolar macrophage-depleted rats had significantly lower respiratory system elastance (Fig. 3B). Arterial oxygenation (mmHg) was significantly higher in alveolar macrophage-depleted rats compared with empty liposome-treated rats after 4 h of HV$_1$ ventilation ($*P < 0.05$).

Fig. 2. Clodronate liposomes depleted alveolar macrophages. A: dose response of alveolar macrophage depletion 4 days after clodronate was given. B: time course of macrophage depletion following clodronate treatment. An alveolar macrophage nadir representing 82% depletion was found at 4 days with a 10-ml aerosolized dose of 20 mg/ml clodronate liposome solution.

Fig. 3. Effect of macrophage depletion on respiratory system elastance and arterial oxygenation in ventilator-induced lung injury. A: compared with empty liposomes (vehicle), clodronate liposome administration resulted in significantly lower respiratory system elastance (cmH$_2$O/ml; $*P < 0.05$ compared with vehicle-treated group). B: arterial oxygenation (mmHg) was significantly higher in alveolar macrophage-depleted rats compared with empty liposome-treated rats after 4 h of HV$_1$ ventilation ($*P < 0.05$).
tidal volume and decreasing PEEP, elastance decreased to a similar level in both groups. One hour later, elastance was significantly lower in the clodronate-treated group (\(P < 0.05\)). This effect persisted for at least 4 h. Arterial oxygenation was comparable between the two groups for the first 2 h of the protocol, but by 4 h, PaO\(_2\) was significantly higher in the clodronate-treated group (Fig. 3B). There was no difference in arterial PCO\(_2\), tidal volume, mean arterial blood pressure, or heart rate at any time in the protocol.

Effect of macrophage depletion on pulmonary edema and protein permeability. Macrophage depletion with liposomal clodronate significantly decreased pulmonary edema in this model of VILI. Excess lung water in the vehicle-treated animals was significantly higher than in clodronate-treated animals (Fig. 4A). Although excess lung water in the ventilated group that received clodronate was significantly higher than in unventilated controls, the increase was less than 50% of that in the empty liposome-treated, ventilated group. There was no difference in excess lung water in unventilated rats given either empty liposomes or clodronate liposomes (not shown).

Alveolar epithelial and lung endothelial permeability to labeled albumin measured as the \(^{125}\)I-albumin activity in the extravascular space of the lung was significantly increased in both groups by high tidal volume ventilation. However, permeability was significantly lower in rats given clodronate liposomes compared with controls (\(P < 0.05\)) (Fig. 4B). There was no difference in permeability in unventilated rats given either empty liposomes or clodronate liposomes (not shown).

Macrophage depletion decreases chemokine levels and air space neutrophils. Plasma levels of the neutrophil chemokine CXCL1 were significantly higher in the vehicle-treated rats than in the clodronate-treated rats after high tidal volume ventilation (Fig. 5A). BAL neutrophil counts after 4 h of ventilation were significantly higher in rats receiving empty liposomes (Fig. 5B).

![Fig. 4. Effect of alveolar macrophages on alveolar barrier dysfunction in ventilator-induced lung injury (VILI). A: pulmonary edema measured as excess lung water (\(\mu\)l) increased with HVT ventilation (\(*P < 0.05\) compared with no ventilation). Macrophage depletion with clodronate resulted in significantly less pulmonary edema compared with rats treated with empty liposomes (vehicle; \(*P < 0.05\) compared with the ventilated, clodronate group). B: lung permeability to albumin measured as the extravasation of intravascular \(^{125}\)I-albumin into the extravascular space [extravascular plasma equivalents (EVPE) in microliters] was significantly decreased by macrophage depletion (\(*P < 0.05\) compared with no ventilation; \(*P < 0.05\) compared with the ventilated, clodronate group). Excess lung water and permeability were not different in unventilated rats given either empty liposomes or clodronate liposomes. Data from these two groups are combined here (No Ventilation).](http://ajplung.physiology.org/)

![Fig. 5. Effect of macrophage depletion on plasma chemokine levels and air space neutrophils in VILI. A: macrophage depletion with clodronate resulted in significantly lower plasma CXC ligand 1 (CXCL1) levels compared with empty liposome (vehicle)-treated rats following 4 h of high volume mechanical ventilation (\(*P < 0.05\) by Mann Whitney test, data are medians ± 25% and 75% confidence intervals). B: BAL fluid neutrophil counts (cells/ml) with clodronate treatment and after 4 h of HVT ventilation. There was no significant difference in BAL neutrophil counts in unventilated rats treated with either clodronate liposomes or empty liposomes. Following 4 h of high volume ventilation, BAL neutrophil counts increased to a greater extent in empty liposome-treated (vehicle) rats than clodronate-treated rats (\(*P < 0.05\) compared with all other groups).](http://ajplung.physiology.org/)
Macrophage nitrite production in vitro. Nitrite production in macrophages incubated with BAL fluid from ventilated rats was significantly higher than in macrophages incubated with BAL from unventilated animals (Fig. 6A). Nitrite levels from macrophages incubated with BAL from unventilated rats were not significantly different from levels in macrophages incubated with media alone (not shown). Cell-free media from cultured alveolar epithelial cells exposed to cyclic mechanical strain for 30 min did not significantly increase nitrite production in naïve alveolar macrophages compared with media from unstrained alveolar epithelial cells (Fig. 6B). Similarly, media from cultured primary alveolar macrophages exposed to mechanical strain did not increase nitrite production in naïve macrophages. However, media from alveolar epithelial cell and macrophage cocultures exposed to mechanical strain for 30 min significantly increased nitrite production in naïve macrophages compared with media from unstrained cocultures (Fig. 6B).

**DISCUSSION**

High tidal volume ventilation induces alveolar epithelial and lung endothelial injury, increases barrier permeability, and decreases alveolar epithelial fluid clearance from the air spaces (14, 17, 18, 23, 39). Previous animal studies have shown that tidal volume reduction preserves epithelial and endothelial permeability and decreases pulmonary edema (14). In patients with acute lung injury and ARDS, tidal volume reduction reduces mortality (2). The precise mechanisms by which mechanical forces are translated into more severe lung injury are not fully understood. In many experimental and clinical studies, low tidal volume ventilation with PEEP results in significantly lower plasma and air space levels of inflammatory mediators (9, 10, 15, 28, 33, 34). Accordingly, it has been postulated that pathological mechanical forces are converted into proinflammatory signals early in the development of VILI (12). Although many resident lung cells can produce inflammatory mediators, alveolar macrophages have a large capacity for cytokine and chemokine production, as well as nitric oxide and reactive nitrogen species elaboration. Alveolar macrophages have been shown previously to be important in the pathogenesis of alveolar barrier dysfunction in *Pseudomonas* pneumonia (19, 22, 37), endotoxin (6, 8), and ischemia-reperfusion lung injury (11, 26, 27) models. The role of alveolar macrophages in the initial pathogenesis of VILI has not been fully explored; however, previous reports have shown that injurious mechanical ventilation results in decreased alveolar macrophage counts in bronchoalveolar lavage (BAL) fluid (40). We reasoned that alveolar macrophage activation in response to high tidal volume ventilation was an early event in the initiation of ventilator-attributable lung injury.

Initial studies confirmed that BAL macrophage counts precipitously dropped with the initiation of high tidal volume, zero PEEP ventilation. Ventilation for 20 min decreased macrophage counts 45% (Fig. 1). To determine the contribution of alveolar macrophages to the increase in alveolar epithelial and lung endothelial permeability characteristic of VILI, alveolar macrophages were depleted using a previously reported liposomal clodronate technique (19). Although macrophage depletion was not complete (82%; Fig. 2), depletion was sufficient to significantly decrease lung injury severity in this model. Macrophage depletion resulted in significantly lower respiratory system elastance (Fig. 3A) and preserved arterial oxygenation (Fig. 3B). It is notable that when ventilator settings were changed from a tidal volume of 6 ml/kg and a PEEP level of 5...
cmH₂O to a tidal volume of 30 ml/kg without PEEP, elastance decreased to a similar level in both groups. However, in the clodronate-treated rats, elastance continued to decrease for up to 1 h while elastance remained higher in the vehicle-treated controls. The initial decrease in elastance in both groups was likely the result of the change in ventilator settings. The subsequent decrease in elastance in the clodronate-treated group may have been due to changes in lung recruitment and surfactant secretion that were counterbalanced in the vehicle-treated rats by increased pulmonary edema. It is likely that the final differences in elastance and oxygenation are partly explained by differences in pulmonary edema severity between the groups (Fig. 4A). Furthermore, alveolar barrier permeability was significantly lower in macrophage-depleted rats following high tidal volume ventilation. These data support the hypothesis that alveolar barrier dysfunction occurs rapidly in VILI, and alveolar macrophages are important in the initial pathogenesis of VILI. The data also show that alveolar macrophages are not solely responsible for the increase in permeability in the model as macrophage depletion decreased, but did not completely prevent lung injury. For example, others have demonstrated that high volume ventilation results in endothelial cell activation, P-selectin expression, and lung neutrophil recruitment (41).

Previous studies have shown that VILI is in part dependent on the recruitment of neutrophils into the lung (3). One study found that blocking CXCL1 and CXCL2/3 signaling significantly decreased air space neutrophil counts and lung injury severity in experimental VILI (3). Therefore, one mechanism by which macrophages could initiate VILI is via the release of neutrophil chemokines and subsequent neutrophil recruitment into the lung. In the present study, circulating levels of the neutrophil chemokine CXCL1 and BAL neutrophil counts were significantly lower in macrophage-depleted rats (Fig. 5, A and B). Part of the protective effect of macrophage depletion could be attributable to decreased lung neutrophil recruitment.

Due to the rapidity of the change in macrophage counts with high volume, zero PEEP ventilation, we suspected that increased macrophage activation and adhesion, rather than cell death or emigration, was most likely. Although macrophage adhesion was not directly measured, increased adhesion is indicative of activation. In addition, previous studies have reported that high volume ventilation induced increased intracellular expression of GADD45 (1) and membrane expression of CD14 (24) in alveolar macrophages (markers of activation) and that LPS-induced TNF-α production was significantly higher in macrophages isolated from rabbits ventilated with high volumes for 6 h (24). Macrophage activation during high tidal volume ventilation could be the result of direct mechanical sensing by alveolar macrophages. For example, Pugin and colleagues (32) have previously shown that alveolar macrophages respond to mechanical stress in vitro by increasing IL-8 and IL-6 production. Alternatively, macrophage activation could be initiated by mediators released from alveolar epithelial cells or other resident lung cells in response to mechanical stress; however, this hypothesis has not been thoroughly investigated. We postulated that BAL fluid from rats ventilated with high tidal volume would induce nitric oxide synthase activity and nitrite production in primary alveolar macrophages in vitro. When macrophages were incubated with BAL fluid from rats exposed to 20 min of high volume ventilation, nitrite production was significantly increased compared with BAL fluid from unventilated rats (Fig. 6A). These data indicated that soluble mediators in the BAL fluid accounted for at least part of the observed increase in macrophage activation. To determine if alveolar epithelial cells were a source of soluble mediators responsible for macrophage activation, we cultured alveolar epithelial cells and alveolar macrophages separately and in coculture and exposed the cells to mechanical strain. Media from stretched or unstretched alveolar epithelial cells or alveolar macrophages alone did not increase nitrite production in naïve primary alveolar macrophages (Fig. 6B). However, media from epithelial monolayers cultured with alveolar macrophages during a 30-min period of stretch increased macrophage nitrite production by 39% (P < 0.05) in the subsequent macrophage nitrite assay (Fig. 6B). Taken together, these data provide evidence that alveolar macrophage activation is up-regulated early in VILI and that soluble mediators contribute to additional macrophage activation in VILI. Although either epithelial cells or macrophages could be potential sources of these mediators, the data suggest that an interaction between the cell types is required for mediator release. These data are not inconsistent with the hypothesis that alveolar macrophages detect and respond to mechanical strain; however, in the culture conditions studied, mechanical strain alone was not sufficient to induce the release of mediators capable of inducing nitrite production in naïve macrophages. Of course, mechanical forces may affect alveolar epithelial cell membranes directly and influence cytoskeletal organization (4, 18, 39); however, data from the present study support an important role for macrophage-epithelial cell interaction in the loss of epithelial barrier function in this model.

Although clodronate treatment is a widely used tool for alveolar macrophage depletion, it is possible that clodronate had other effects on resident lung cells as well. These data must be interpreted in this context. For example, in one study, up to 30% of alveolar epithelial cells contained liposomes following intratracheal instillation (31). The precise effects of clodronate liposomes on epithelial function are not clear, but, consistent with previous reports (19, 22), we did not observe a significant difference in permeability in unventilated rats treated with clodronate liposomes or empty liposomes. It has also been reported that clodronate liposomes inhibit cytokine production in a macrophage-like cell line (29); therefore, decreased cytokine production by macrophages remaining in the lung is an additional potential mechanism for the observed protective effect in the present study. It is noteworthy that the absence of alveolar macrophage function has been shown to result in more severe lung injury in some models, as macrophage phagocytosis of neutrophils and other apoptotic cells is an important step in the regulation of the inflammatory response (20–22, 36). Therefore, it is uncertain if sustained macrophage inactivation would have a net beneficial effect in clinical ventilator-associated lung injury.

In summary, we found that depletion of alveolar macrophages in vivo decreased lung endothelial and alveolar epithelial permeability, resulting in lower plasma levels of CXCL1 and lower neutrophil counts in the BAL fluid. These data suggest that alveolar macrophages are a key contributor to the early proinflammatory milieu and increased permeability pulmonary edema characteristic of VILI. In addition, soluble mediators released into the air spaces during high volume
ventilation activated naïve macrophages, as did media from stretched alveolar epithelial cell and macrophage cocultures. These data support the hypothesis that soluable factors released into the air spaces as a result of the interaction between alveolar epithelial cells and alveolar macrophages trigger additional macrophage activation in VILI. However, the potential role of the alveolar epithelium or other resident lung cells in initiating macrophage activation has not been entirely elucidated. This study provides important insights into the pathogenesis of ventilator-attributable lung injury. First, VILI is initiated in part by alveolar macrophages. These data also suggest that one mechanism by which alveolar macrophages contribute to VILI is through recruitment of neutrophils into the air spaces. Second, the onset of ventilator-attributable lung injury is rapid and begins within minutes of starting mechanical ventilation. Accordingly, in the clinical setting, protective ventilation strategies may need to be initiated as early as possible in the course of mechanical ventilation.

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