The effect of lung-protective ventilation on severe *Pseudomonas aeruginosa* pneumonia and sepsis in rats.

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Running head: Severe pneumonia, sepsis, and protective ventilation

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ABSTRACT

Pneumonia caused by *Pseudomonas aeruginosa* carries a high rate of morbidity and mortality. A lung-protective strategy using low tidal volume (V\_T) ventilation for acute lung injury improves patient outcomes. The goal of the present study was to determine whether low V\_T ventilation has similar utility in the setting of severe *Pseudomonas aeruginosa* infection. A cytotoxic *Pseudomonas aeruginosa* strain, PA103, was instilled into the left lung of rats anesthetized with pentobarbital. The lung-protective effect of low V\_T (6 ml/kg) with or without high PEEP (10 or 3 cm H\_2O) was then compared with high V\_T with low PEEP ventilation (V\_T 12 ml/kg, PEEP 3 cm H\_2O). Severe lung injury and septic shock was induced. While ventilatory mode had little effect on the involved lung or septic physiology, injury to non-involved regions was attenuated by low V\_T ventilation as indicated by the wet to dry weight ratio (W/D) (6.13±0.78 vs. 3.78±0.26, respectively) and confirmed by histopathological examinations. High PEEP did not yield significant protective effect (W/D: 4.03±0.32), but rather caused overdistension of non-involved lungs. Broncho-alveolar lavage revealed higher concentrations of TNF-α in the fluid of non-involved lung undergoing high V\_T ventilation when compared to those animals receiving low V\_T. We conclude that low V\_T ventilation is protective in non-involved regions and that the application of high PEEP attenuated the beneficial effects of low V\_T ventilation, at least in the short-term.
Further, low $V_t$ ventilation cannot protect the involved lung, and high PEEP did not significantly alter lung injury over a short time-course.

Key words:

acute lung injury; ventilator-induced lung injury; lung protective strategy; cytokines; bacterial toxins
INTRODUCTION

Despite ongoing improvement in antimicrobial therapies, pneumonia still represents a life-threatening condition with high mortality and morbidity, especially for critically ill patients (10, 26). In fact, severe pneumonia and associated sepsis are the most common cause of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). While oxygen supplementation and mechanical ventilation is a common mode of support in these patients, ventilation itself has the potential to cause systemic spread of infection (20, 4) or lung injury (29). Randomized controlled trials involving ALI patients have shown that a lung-protective strategy with low tidal volume ($V_T$) ventilation ($V_T$ of 6 ml/kg) (1) and application of positive end-expiratory pressure (PEEP) (2) reduced patient mortality when compared with conventional mechanical ventilation ($V_T$ of 12 ml/kg).

Pneumonia is a common cause of ALI and usually presents inhomogenously, yielding discrete areas of infection and leaving other regions of the lung relatively spared. Therefore, we designed a study to investigate whether $V_T$ and PEEP maintained its beneficial effects on various areas of the lung in the context of severe pneumonia. We modified an animal model of pneumonia that we described previously (16, 18, 30-32) to mimic the course of unilateral pneumonia. *P. aeruginosa* was utilized to induce infection, as it is a common causative pathogen of nosocomial pneumonia (6-7, 27, 35). In addition, *P. aeruginosa* pneumonia, particularly when induced with the PA103 strain, frequently leads to bacteremia and sepsis (9, 11-12, 16, 18, 24, 30).
Thus, the first objective of this study was to test the hypothesis that low $V_T$ ventilation would reduce lung injury of the affected lungs and non-affected lungs following instillation of cytotoxic bacterial pathogen. Further we investigate whether this reduced lung injury would diminish the severity of sepsis and to test the hypothesis that application of high PEEP would further protect the lungs from ventilator induced lung injury.
METHODS

Pathogen and animals. Bacterial suspensions of *P. aeruginosa* strain PA103, which is a cytotoxic strain that produces the type III secreted toxins, ExoU and ExoT (11, 14), were prepared as previously described (31). Briefly, PA103 was subcultured on Vogel-Bonner minimal medium plates and inoculated into trypticase soy broth (Becton Dickinson, Sparks, MD) containing 10 mM nitrilotriacetic acid (Dojin, Kumamoto, Japan) and incubated overnight. The bacterial pellet was washed twice with lactated Ringer’s solution (L/R) (Otsuka, Tokyo, Japan) and diluted into the appropriate number of CFU per milliliter in L/R as determined by spectrophotometry. The number of bacteria in the solution was confirmed by serial dilution followed by culture on agar plates.

All protocols for animal experiments were approved by the Animal Research Committee of Yokohama City University. Specific pathogen-free male Sprague-Dawley rats, weighing 310 g to 400 g (9 to 11 weeks) (Japan SLC, Shizuoka, Japan), were used for all animal experiments.

Experimental groups: Sixty-three rats were used to evaluate lung injury and systemic responses of acutely infected animals. After initial anesthesia was given, rats were randomized to receive one of three ventilation settings: high $V_T$ + low PEEP ($V_T$ 12 ml/kg, PEEP 3 cm H$_2$O),
low $V_t$ + low PEEP ($V_t$ 6 ml/kg, PEEP 3 cm H$_2$O), or low $V_t$ + high PEEP ($V_t$ 6 ml/kg, PEEP 10 cm H$_2$O). Thirty rats (control group) received an airspace instillate that did not contain bacteria and followed the same procedure.

**Animal preparation:** Anesthesia was induced by injection of 25 mg of sodium pentobarbital into the peritoneal cavity. A tracheotomy was performed, and a 14-gauge plastic cannula (SR-OT1451C, Terumo, Tokyo, Japan) was inserted into the trachea to serve as an endotracheal tube. Mechanical ventilation was maintained by a constant volume pump (SN-480-7, Shinano, Tokyo, Japan) with an inspired oxygen fraction of 1.0 at a $V_t$ of either 12 or 6 ml/kg. The $V_t$ delivered by the ventilator was calibrated volumetrically by collecting expired gas. In brief, ventilator circuit was connected to a test lung that would generate intra-circuital pressure of approximately 20 cm H$_2$O while expiratory gas collected from the expiration port. Tidal volume was adjusted accordingly. A positive end-expiratory pressure of either 3 cm H$_2$O or 10 cm H$_2$O was applied. Ventilation frequency was adjusted to maintain the arterial carbon dioxide tension between 35 and 50 torr. A curved polyethylene tube, 0.58-mm inner diameter (PE50, Becton Dickinson, Sparks, MD), was carefully inserted through the endotracheal tube into the left lung for subsequent instillation of bacterial suspension. The right carotid artery was catheterized with a 24 gauge plastic cannula (SR-OT 2419C, Terumo) for measurement of blood pressure and
sampling of arterial blood. After completion of these preparations, rats were placed in the left lateral decubitus position on a warming device and were observed for at least 30 minutes until stabilization of respiration and circulation.

Next, instillation was performed over a 10-15 minute period (time 0). Arterial and airway pressures were monitored continuously using a hemodynamic monitor (Life Scope 12, Nihon Kohden, Tokyo, Japan). A rectal probe (ME-PDK061, Terumo) was used for continuous temperature monitoring (CTM-303, Terumo), and body temperature was maintained between 36 and 38 °C via a warming device. Blood (700 µl) was sampled every hour for blood gas analysis, measurement of the efflux of the airspace protein tracer into the circulation, and measurement of cytokines. Blood gas and acid-base analyses were performed using a critical care analyzer (OPTI3, AVL scientific, Roswell, GA). The volume of withdrawn blood was replaced by equi-volume intra-arterial administration of L/R, just after the blood sampling. In addition, 1 ml of L/R was given intra-arterially at an interval of 10, 5, or 2 minutes when systolic blood pressure decreased to 85, 75, or 65 mmHg, respectively. Six hours after infection, rats were deeply anesthetized and euthanized.

**Instillation of P. aeruginosa and alveolar protein tracers:** The instillate (3 ml/kg) contained PA103 (4 x 10^7 CFU/ml), 0.5 µCi of ^131^I-labeled human albumin (Daiichi Radioisotope, Tokyo,
Japan) as the airspace protein tracer, and 5% bovine serum albumin (Wako, Osaka, Japan) in L/R to evaluate transpulmonary protein leakage. The instillate also contained 10 μg of Evans blue (Sigma) to visualize the instillation on autopsy. Animals in which the instillate was not limited to one lung were excluded from study. The instillates were administered using a published method, and calculations were performed as described (37).

**Broncho-alveolar lavage (BAL).** A set of rats in each group underwent collection of BAL fluid 6 hours after the instillation. Five BALs were performed in each lung via instillation of 1.5 ml of PBS containing 0.1% EDTA per BAL. Approximately 100 μl of BAL fluid was set aside for bacterial cultures. The remaining volume was centrifuged at 3,400 g at 4°C for 20 minutes to obtain the supernatant, which was stored at –80°C until use.

**Measurement of lung injury:** Lung injury was estimated in 4 different ways: a) the efflux of intra-alveolar 131I-labeled albumin into the circulation, b) the wet to dry weight ratio (W/D) of the lungs, c) the concentration of lactate dehydrogenase (LDH) in BAL fluid, and d) the lung histology.

a) The efflux of 131I-labeled albumin into the circulation was calculated by multiplying the counts measured in the blood sample by the systemic blood volume (body weight × 0.07), as previously reported (17, 38). Efflux was used as an indication of alveolar epithelia barrier destruction.
b) A set of rats in each group underwent measurement of W/D. Each lung was harvested 6 hours after the infection and individually homogenized, placed in a pre-weighed aluminum pan, and dried to constant weight in an oven at 80°C for 3 days. The W/D of the lungs was calculated in the established manner (34, 38) and taken as a reflection of lung edema.

c) The concentration of released LDH in the BAL fluid was measured according to the manufacturer’s protocol (CytoTox 96, Promega, Madison, WI). The values were compared with the serial dilution of LDH positive control (lysed L929 fibroblast cells) as standards. One unit of LDH in this study was the same level of the enzyme found in 1,000 lysed L929 fibroblast cells.

d) Lungs of the last set of rats were used for histological and immunohistochemical examination. The lungs were fixed with 4% formalin in PBS overnight. They were then infused with 10% formalin and sectioned and processed for paraffin-embedded sections. Four-micrometer-thick lung tissue sections were stained with hematoxylin and eosin (H-E) for routine histological examination.

**Bacterial Cultures.** To assess bacteremia, arterial blood (100 µl) was cultured on tryptic soy agar plates. For the quantification of bacteria in the lungs, BAL fluid was diluted with sterile PBS and streaked onto agar plates. Bacterial colonies were counted after incubation at 37°C for 12 hours, and the quantity of bacteria was determined by multiplying counted colonies by the dilution ratio.
**Leukocyte count:** The leukocyte count in the systemic circulation was quantified microscopically. Arterial blood (25 µl) obtained 6 h after the infection was added to 475 µl of 2% acetic acid containing 0.01% Gentian Violet, and cell counts were multiplied by the dilution factor to obtain the number of leukocyte in the sample.

**Assay for cytokines in BAL fluid and blood:** A biological TNF-α assay was performed using mouse sarcoma cells, WEHI-13VAR (CRL2148, American Type Cell Culture, Manassas, VA) as previously reported (8, 18). The TNF-α activity of each sample was calculated by comparing absorbance to that of a standard curve made from dilutions of rat TNF-α (PharMingen, San Diego, CA) between 1.2 pg/ml and 1250 pg/ml. The lower limit of detection for this assay was 1.2 pg/ml. One of the most potent CXC chemokines, growth related oncogene (GRO)/ cytokine-induced neutrophil chemoattractants (CINC)-1, was measured by enzyme-linked immunosorbent assay (ELISA) with a commercial ELISA kit following the manufacturer’s protocols (RPN2730, Amersham Pharmacia, Little Chalfont, Buckinghamshire, England). The lower limit of detection was 4.7 pg/ml.

**Immunohistochemistry of lungs:** For immunohistochemistry, sections were immersed in 0.3% hydrogen peroxide to inactivate intrinsic peroxidase activity. After antigen retrieval by microwave treatment in a citrate buffer and blocking with 10% normal rabbit serum/PBS,
sections were treated with the primary antibody against rat TNF-α (R&D systems, Minneapolis, MN; diluted 1:200) overnight at 4°C. After the labeled antigens were visualized with a catalyzed signal amplification system kit (Dako, Carpinteria, CA) and diaminobenthidine, the nuclei were counterstained with hematoxylin. Each sample was examined at 5 randomly selected fields, and the alveolar epithelial cells were scored 3 to 0 in a blinded fashion according to their intensity of staining: score 3 indicated intense staining, score 2 was moderate staining, score 1 was mild staining, and score 0 was faint or no staining. The medians of these scores were used to determine TNF-α positivity.

**Permeability of lungs to systemic TNF-α:** A separate group of rats (n=12) was used to determine the permeability of lungs to systemic TNF-α. The protocol for this experiment was the same as the general protocol except that 131I-albumin was not administered in this experiment. Two hours after instillation of bacteria, 125I-labeled TNF-α (IM206, Amersham Pharmacia) was administered intravenously. Arterial blood samples were collected 10 and 20 min after the injection, and radioactivity was measured as counts per minute (CPM)/g of plasma. Total CPM injected was calculated using a modification of an established method (16, 38) by multiplying CPM/g of plasma by estimated plasma volume (body weight (g) × 0.07 × (1−hematocrit/100)). Rats were killed at 6 hours, and BAL samples of both lungs were collected separately by the protocol.
outlined above. A portion of recovered BAL was weighed and measured for CPM/g, and total CPM in the airspace was calculated by multiplying the CPM/g with the total volume of the liquid instilled to obtain BAL (7.5 ml). The percentage of TNF-α in the airspace was calculated from total CPM in the airspace divided by total CPM injected. This series of experiments also included a set of spontaneously breathing control rats without instillation that were anesthetized and placed in the left lateral decubitus position for 6 hours without mechanical ventilation. Muscle relaxants were not administered to these spontaneously breathing control rats, and anesthesia was maintained with intraperitoneal pentobarbital.

**Statistical analysis.** The mean values of measurements made only once during the protocol were compared using unpaired *t* tests. Measurements made more than once per animal were compared using repeated measures ANOVA. Pairwise comparisons were made by one-factor ANOVA followed by Scheffe’s post-hoc analysis. Scattered data were analyzed with Mann-Whitney’s U test. Significance was set at *p* <0.05. Values are reported as mean ± SEM or as median with 25 and 75 percentiles.
**Results**

*Exclusion of animals*

Two animals in the high $V_T + \text{low PEEP}$ group and one animal in the low $V_T + \text{high PEEP}$ group were excluded from the study, because they died before completion of the experiment. These deaths were attributed to intra-abdominal hemorrhage secondary to intraperitoneal pentobarbital injection and from exsanguination from a neck incision. One animal in the high $V_T + \text{low PEEP}$ group, two animals in the low $V_T + \text{low PEEP}$ group, and one animal in the low $V_T + \text{high PEEP}$ group were excluded from the study because of instillation of the right lung or both lungs. This was confirmed by multiple methods after completion of the study, including bacterial counts in BAL fluid, staining of lungs with dye in the instillate, and examination of the placement of an instillation catheter.

*Respiration and airway status*

Ventilation frequency required to maintain $P_aCO_2$ in the desired range was highest in the low $V_T + \text{high PEEP}$ group followed by the low $V_T + \text{low PEEP}$ group (Fig. 1A). Peak airway pressure increased after the instillation except the low $V_T + \text{high PEEP}$ group and the value was significantly lower in the low $V_T + \text{low PEEP}$ group at the end of the experiment (Fig. 1B).
**Injury in the bacterial-instilled lungs**

After instillation of bacteria, the presence of alveolar protein tracer in the circulation increased in a time-dependent manner, indicating injury to the alveolar epithelial cells (Fig. 2-A). The tracer levels were significantly less in the low V\(_T\) + low PEEP group when compared with that in the high V\(_T\) + low PEEP group and the low V\(_T\) + high PEEP group. In control animals, the alveolar protein tracer did not leak into the circulation with any of the ventilation settings, indicating no alveolar epithelial injury (Fig. 2-B). Two other indices for the injury of the instilled lung, LDH concentration in the BAL fluid and W/D of the lung, were high in all groups (Fig. 3-A, B), and there were no differences when comparing the groups.

Concentrations of TNF-\(\alpha\) and GRO/CINC-1 in the BAL fluid of the instilled lungs were elevated in all 3 groups, and there were no differences in TNF-\(\alpha\) and GRO/CINC-1 concentrations when comparing the groups (Fig. 3-D, E).

Histopathological study of the instilled lungs revealed severe lung injury, i.e. intra-alveolar hemorrhage, protein precipitation and leukocyte infiltration into the alveoli, and edematous thickening of perivascular space in the instilled lungs (Fig. 4-A). There was no difference in histopathology when comparing the three groups.
**Bacteremia and systemic responses**

All rats were bacteremic at the end of the experiment, and there was no difference in the quantity of bacterial colonies when comparing the groups (Table I).

Mean arterial pressure decreased in a time-dependent fashion and was significantly lower than the baseline values in all groups (Fig. 5-A). There was no difference in the mean arterial pressure when comparing the three groups. The volume of extra fluid given to the animals to maintain systolic blood pressure averaged 11-13 ml, with no difference in the volume of administered fluid when comparing the groups (Table I). Blood base excess decreased continuously after infection was induced, and the animals developed severe acidosis 6 hours after the instillation (Fig. 5-B). There was no significant difference in the degree of base excess when comparing the groups.

Oxygenation, calculated as alveolar-arterial oxygen difference (A-aDo$_2$), deteriorated after bacterial instillation. A-aDo$_2$ increased in a time-dependent manner and was significantly higher at 6 hours when compared with baseline values in the high $V_T$ + low PEEP group and in the low $V_T$ + low PEEP group (Fig. 5-C). In contrast, oxygenation was well maintained in the low $V_T$ + high PEEP group, even after the instillation of bacteria. Accordingly, A-aDo$_2$ was significantly lower in the low $V_T$ + high PEEP group when compared to the other two groups.

The leukocyte count in the circulation at 6 hours after the instillation of bacteria was low
There was no significant difference in the leukocyte count in the circulation when comparing the groups.

The concentration of TNF-α in the circulation measured at 6 hours after the instillation was elevated in all groups, but there was no statistical difference when comparing the groups (Table I).

**Effect of ventilation on the contralateral lung**

A negligible amount of LDH was found in the BAL fluid of non-instilled lungs (Fig. 3-A). The W/D of the non-instilled lungs in the high $V_T$ + low PEEP group was elevated (Fig. 3-B). The W/D of the non-instilled lungs in the low $V_T$ + low PEEP group and in the low $V_T$ + high PEEP group was significantly lower than that in the high $V_T$ + low PEEP group, which were comparable to the values of control, non-infected animals (Fig. 3-C). Histopathological study of the non-instilled lungs showed an enlargement of the peri-vascular space in the high $V_T$ + low PEEP group (Fig. 6-A). Overdistension of alveoli was also observed in the high $V_T$ + low PEEP group and in the low $V_T$ + high PEEP group (Fig. 6-A, C). These abnormal findings were not seen in the low $V_T$ + low PEEP group (Fig. 6-B).

The concentration of TNF-α in the BAL fluid of non-instilled lung in the high $V_T$ + low PEEP group was significantly higher than that in the other 2 groups (Fig. 3–D). The concentration of
GRO/CINC-1 in BAL fluid of non-instilled lungs was elevated above baseline, but was still lower than concentrations seen in the BAL of instilled lungs (Fig. 3–E). Immunohistochemical analysis showed positive labeling for TNF-α in the alveolar epithelial cells of the non-instilled lungs in the high $V_T$ + low PEEP group (Fig. 6-D). In contrast, the signal intensity of these cells in the low $V_T$ + low PEEP group and in the low $V_T$ + high PEEP group was low (Fig. 6-E, F).

The positivity score of TNF-α in the high $V_T$ + low PEEP group (median was 3, and 25th and 75th percentiles were 1 and 3) was significantly higher when compared with that in the low $V_T$ + low PEEP group (median was 1, and 25th and 75th percentiles were 1 and 2) or in the low $V_T$ + high PEEP group (median was 2, and 25th and 75th percentiles were 1 and 2). The fraction of TNF-α in the BAL fluid from non-instilled lungs that was attributed to entry from the systemic circulation in any infected groups was comparable to that in non-infected, unventilated control rats (Fig. 7).
Discussion

The present study demonstrated that the lung-protective ventilatory strategy could not ameliorate damage in the affected lung during the acute phase of severe pneumonia and concomitant sepsis induced by a cytotoxic strain of \textit{P. aeruginosa}. While lung edema and inflammation also occurred in the non-involved lungs when using relatively higher $V_T$ of 12 ml/kg, which is still clinically acceptable, the use of low $V_T$ ventilation protected non-involved lungs from ventilator-induced lung injury. Physiological changes in the non-involved lung were confirmed morphologically (Fig. 6-A-C), demonstrating that the use of low $V_T$ ventilation prevented development of perivascular fluid accumulation that was observed in the high $V_T +$ low PEEP group.

To investigate mechanisms of lung edema in the non-involved lung, TNF-$\alpha$ concentration in the BAL fluid was assessed. The increase in TNF-$\alpha$ seen with conventional ventilation was attenuated by the use of low $V_T$ ventilation with or without PEEP, which was also consistent with the degree of pulmonary edema seen in each group. Hamacher and colleagues (15) investigated the BAL fluid of early-phase ARDS patients and found high concentrations of TNF-$\alpha$ in their BAL fluid. Further, incubation of human lung microvascular endothelial cells with the BAL fluid from early-phase ARDS was cytotoxic to the endothelial cells, and neutralization of TNF-$\alpha$
inhibited the cytotoxic activity of the BAL fluid. Therefore, TNF-α detected in the BAL fluid of the non-infected lungs may be responsible for at least a portion of the lung endothelial cell damage and associated pulmonary edema in the non-infected lungs of the high V_{T} + low PEEP group.

The influx of TNF-α in the non-instilled lungs from the circulation was negligible in all experimental groups, and immunohistochemical findings confirmed production of TNF-α in alveolar macrophages and in bronchial- and alveolar-epithelial cells with conventional ventilation. Furthermore, the trend in the positivity score of TNF-α of the cells in the non-instilled lungs correlated well with the concentration of TNF-α in the BAL fluid of non-instilled lungs (Fig. 3-D). These observations suggest that pulmonary epithelial cells and alveolar macrophages of non-infected lungs release TNF-α in the alveolar space when lungs are ventilated with high-stretch, high V_{T} ventilation in the presence of sepsis physiology and/or with infection of other part of lungs. This idea is supported by a report from Pugin and coworkers (22), which demonstrated marked elevation of TNF-α in the supernatant of the culture medium when alveolar macrophages were stretched in the presence of the gram-negative bacterial toxin, lipopolysaccharide (LPS). In an ex vivo study performed by Ricard and colleagues (25), isolated non-perfused rat lungs were ventilated for 2 hours with a V_{T} of 42 ml/kg. When LPS was given intravenously 50 minutes
before lungs were removed, TNF-α concentration in BAL fluid was elevated in the ventilated lungs.

Histological study revealed only the sparse presence of inflammatory cells in non-instilled lungs. While the chemoattractant molecule, GRO/CINC-1, was present in the BAL fluid of non-instilled lungs, the concentration was relatively low in all three experimental groups. In a model of intraperitoneal inflammation, Call and associates (3) found that the ratio of local to systemic chemokine concentration was a significant factor for local neutrophil recruitment to the peritoneal cavity. Thus, the relatively low concentration of GRO/CINC-1 in the unaffected lung compared to that of affected lung may account for the absence of infiltrates in the non-instilled lungs. Further, Wilson and associates (39) determined that the BAL fluid of lungs ventilated with high $V_T$ was associated with increased TNF-α at the early stage of lung injury but not in the latter stages. Thus, the high concentration of TNF-α, low concentration of chemokine, and absence of inflammatory cells in the non-involved lungs in the present study may represent an early stage of ventilator induced lung injury, and TNF-α-mediated inflammation may be evident at later stages of high $V_T$ + low PEEP ventilation.

In the present study, all animals were bacteremic by the end of the experiment, and there was no difference in the degree of bacteremia among groups. The PA103 strain used in the present
study produces a type III secreted toxin, ExoU (11), which is cytotoxic to lung epithelial cells and alveolar macrophages in vitro (11, 18). Instillation of PA103 into the airspaces of animals produced severe lung injury (30-31, 33) and septic shock (18), which is consistent with the results of the present study. This injury likely limits the utility of lung-protective ventilation in the affected lung in this P. aeruginosa pneumonia model.

Careful attention was given to the determination of $V_T$ in this experiment. Using the P. aeruginosa pneumonia model, Savel and colleagues (29) demonstrated that low $V_T$ ventilation correlated with decreased alveolar permeability; however, they failed to show the difference in alveolar protein permeability indicated by % of instilled $^{125}$I-albumin in blood. Further they demonstrated that large $V_T$, 15 ml/kg, in the absence of pneumonia resulted in lung injury (e.g., increased epithelial protein permeability and alveolar infiltrates); indicating that ventilation with large $V_T$ per se caused lung injury. In another study by Frank and coworkers (13), mechanical ventilation of rats with a $V_T$ of 12 ml/kg did not result in lung injury. In the present study, a $V_T$ of 12 ml/kg did not result in lung injury in rats that were not infected as demonstrated by alveolar protein tracer analysis (Fig. 2-B), lung W/D (Fig. 3-C), and light microscopic analysis of H-E-stained lung section (Fig. 4-B).

As ventilation frequency was adjusted to control $P_{a}CO_2$ within a certain range, high frequency,
e.g. greater than 120 /minute, was necessary in the low $V_T$ groups. This extreme of ventilation frequency may produce some error in the determination of tidal volumes actually delivered to the lung; when using short inspiratory time, actual $V_T$ delivered to the lung is highly dependent on the flow rates generated by the ventilator and the corresponding compressible losses in the ventilator tubing. To estimate actual lung delivered $V_T$, we measured compressible volume in the ventilator tubing. The compressible volume was 0.03 ml/cm H$_2$O; therefore, the compressible losses are relatively small when compared to $V_T$ and can therefore be regarded as negligible. It is still possible that a short inspiratory time without an end-inspiratory pause may result in some difference in an inspiratory volume distribution within the lungs; short expiratory time might have generated intrinsic PEEP, resulting is increased total PEEP above externally-applied PEEP. Although a concomitant decrease in $V_T$ was likely to limit the generation of intrinsic PEEP in the low $V_T$ groups, this could exaggerate the overdistending effect of PEEP in the non-affected lung.

High PEEP (10 cm H$_2$O) was utilized in the present study to protect lungs from ventilator induced lung injury, in accordance with previous reports (13, 36). However, the present study showed that an increased PEEP had no benefit in terms of W/D ratio of lungs, LDH concentration in BAL fluid, or cytokine concentrations in BAL fluid. While high PEEP resulted in better oxygenation, likely due to recruitment of dependent lungs, it also resulted in a translocation of
alveolar protein tracer from the infected lungs into the circulation, possibly secondary to a higher peak airway pressure or larger lung volume. Further, high PEEP resulted in overdistension of alveoli in the non-affected lung, which is consistent with previous studies (23, 28). Rouby et al. (28) warned that lung recruitment and overinflation occurred simultaneously after an increase in intrathoracic pressure and that selecting the optimal PEEP level should focus on limiting lung overinflation. Accordingly, when lung injury is inhomogeneous, as in the present experiment, application of high PEEP may not be justified. However, the long-term effects of PEEP were not assessed in the present study. Further, these data do not exclude a beneficial effect of high PEEP in more homogenous lung disease, i.e. early stage of RDS in the infant or early stage ALI/ARDS in secondary ARDS.

Although particular care was made to avoid bacterial instillation in the right lung, some contamination was unavoidable, and animals were excluded from study if right lung BAL bacterial counts were greater than $3 \times 10^3$ CFU/ml. Of the remaining rats, the highest bacterial count in the right lung BAL was 630 CFU/ml (Table II). Because previous studies demonstrated a threshold of $10^5$ CFU/ml of bacteria in 100 ml of BAL fluid of intubated patients to qualify for a diagnosis of ventilator-associated pneumonia (19), the right lungs in these animals were considered as non-involved lungs for the purposes of experimentation.
Plasma and BAL fluid TNF-α levels were measured using a bioassay, which has several advantages over an immunoassay (5, 21). First, the bioassay corrects for the presence of soluble receptors for TNF, which may buffer TNF-α activity. Second, the immunoassay cannot distinguish biologically-active TNF-α from biologically-inactive precursors of TNF-α or protein-bound TNF-α. While the bioassay may possess lower sensitivity when compared with the immunoassay, this problem can be overcome through the use of cells with increased sensitivity to TNF-α (8).

The present study possesses some limitations. First, there are likely pathophysiological differences between pneumonia in humans and rodents. Second, pneumonia involves a process of inoculation, incubation, and subsequent appearance of clinical symptoms of infection. In contrast, the experimental animals in the present study received abrupt inoculation of high dose bacteria in the airspaces. Third, animals were observed for only 6 h following infection. Thus, this study cannot be generalized to conditions of ventilation for longer durations. Lastly, the precise relationship between TNF-α and lung edema was not explored in the present study. Further experiments are warranted to clarify these issues.

We conclude that ventilatory mode cannot protect the involved lung in the context of severe pseudomonal pneumonia, but low $V_T$ ventilation is protective in non-involved regions. Further,
the application of 10 cmH₂O of PEEP attenuated the beneficial effects of low $V_T$ ventilation over a short-term period.

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**Figure Legends**

**Fig. 1**

Ventilation frequency and peak airway pressure. (A) Ventilation frequency was adjusted to maintain $P_aCO_2$ between 35 and 50 torr. (B) Peak airway pressure was continuously measured. Bacterial suspension instillation was performed at time 0. $V_T$, tidal volume. Mean±SEM. *$p<0.05$ vs. high $V_T$ + low PEEP group. †$p<0.05$ vs. low $V_T$ + low PEEP group. ¶$p<0.05$ vs. baseline value. Number of samples in parentheses.

**Fig. 2**

Airspace protein tracer in blood. The quantity of $^{131}$I-albumin that entered the circulation via the lungs was calculated and is shown as a percentage of the initial dose. Instillation was performed at time 0. Mean±SEM. ¶$p<0.05$ vs. baseline value. †$p<0.05$ vs. low $V_T$ + low PEEP group. Number of samples in parentheses. $V_T$, tidal volume.

**Fig. 3**

Assessment of lung injury. Unless otherwise mentioned, data are shown from rats that underwent instillation of bacterial suspension into the left lung, and lungs were harvested 6 hours after the
instillation. A, Concentration of lactate dehydrogenase (LDH) in broncho-alveolar lavage (BAL) fluid. Broncho-alveolar lavage of both lungs was performed separately, 6 hours after the instillation. B, Lung wet to dry weight ratio (W/D). C, W/D of rats instilled vehicle solution instead of the bacterial suspension (control). D, The concentration of tumor necrosis factor (TNF)-α in the BAL fluid. E, The concentration of growth related oncogene / cytokine-induced neutrophil chemoattractants-1 (GRO/CINC-1) in the BAL fluid. Mean±SEM. *p<0.05 vs. high \( V_T \) + low PEEP group. Number of samples in parentheses. \( V_T \), tidal volume.

Fig. 4

Representative micrographs of lung tissue stained with hematoxylin and eosin. A: Instilled lung in the high \( V_T \) + low PEEP group; B: Non-instilled lung of un-infected rats ventilated with high \( V_T \) + low PEEP for 6 hours: no injury of lung components or inflammatory cells were seen. \( V_T \), tidal volume.

Fig. 5

Mean arterial pressure (MAP), base excess, and alveolar-arterial oxygen difference (A-a\(D_{O_2}\)) for 6 hours. Instillation was performed at time 0. A, calculated MAP as an indicator of
hemodynamics. B, base excess as an indicator of acid-base status. C, A-aD_{O2} as an indicator of oxygenation. Mean±SEM. \*p< 0.05 vs. baseline value. \#p< 0.05 vs. low V_{T} + high PEEP group. Number of samples in parentheses. V_{T}, tidal volume.

Fig. 6

Representative micrographs of lung tissue stained with hematoxylin and eosin (A-C) or immunohistochemistry of TNF-\(\alpha\) (D-F). A,D: non-instilled lung in the high V_{T} + low PEEP group: enlargement of the peri-vascular space (arrow) and overdistension of alveoli (*) were seen, and intense staining for TNF-\(\alpha\) was observed in alveolar epithelial-cells, bronchioepithelial-cells, and alveolar macrophages; B, E: non-instilled lung in the low V_{T} group; C, F: non-instilled lung in the low V_{T} + high PEEP group: overdistension of alveoli (*) were seen. V_{T}, tidal volume.

Fig 7

The fraction of \(^{125}\)I-labeled TNF-\(\alpha\) in the airspace over total TNF-\(\alpha\) injected into the circulation. \(^{125}\)I-TNF-\(\alpha\) was injected 2 hours after the instillation of \(P. aeruginosa\). Lungs were harvested 6 hours after the instillation. \(^{125}\)I-TNF-\(\alpha\) in the airspace of instilled lungs and non-instilled lungs were calculated as a fraction of the amount injected. As a reference, the value for the non-
infected non-ventilation control rats is presented. Number of samples in parentheses. $V_T$, tidal volume.
Table I. Leukocyte and bacterial counts, TNF-α concentration, and fluid supplementation in experimental groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Bacterial counts (CFU/ml)</th>
<th>Number of leukocytes (x1000 /mm$^3$)</th>
<th>Serum TNF-α (ng / ml)</th>
<th>Fluid replacement volume to maintain blood pressure (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>high $V_t$ + low PEEP</td>
<td>60 (12.5, 840)</td>
<td>2.1 ± 0.50</td>
<td>0.07 ± 0.06</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>low $V_t$ + low PEEP</td>
<td>110 (15, 285)</td>
<td>1.7 ± 0.44</td>
<td>0.14 ± 0.12</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>low $V_t$ + high PEEP</td>
<td>140 (57.5, 325)</td>
<td>2.2 ± 0.32</td>
<td>0.17 ± 0.10</td>
<td>13 ± 5</td>
</tr>
</tbody>
</table>

Bacterial counts are expressed as median, with 25th and 75th percentiles in parentheses. Number of leukocytes, serum TNF-α, and fluid replacement are expressed as mean and SEM. Note that fluid supplementation includes the volume of fluid administered to maintain blood pressure but does not include the volume of infusion used to substitute for the volume of blood sampling.
**Table II. Bacterial counts in the BAL fluid from instilled and non-instilled lungs.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Bacterial count (CFU/ml)</th>
<th></th>
<th>Non-instilled lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Instilled lungs (x10^4)</td>
<td></td>
</tr>
<tr>
<td>high V&lt;sub&gt;T&lt;/sub&gt; + low PEEP</td>
<td>71 (2, 88)</td>
<td>180 (120, 180)</td>
<td></td>
</tr>
<tr>
<td>low V&lt;sub&gt;T&lt;/sub&gt; + low PEEP</td>
<td>140 (50, 178)</td>
<td>85 (60, 91)</td>
<td></td>
</tr>
<tr>
<td>low V&lt;sub&gt;T&lt;/sub&gt; + high PEEP</td>
<td>150 (123, 165)</td>
<td>170 (163, 260)</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as median, with 25<sup>th</sup> and 75<sup>th</sup> percentiles in parentheses.
Figure 1

A. Ventilation frequency ( /min)

- high VT + low PEEP
- low VT + low PEEP
- low VT + high PEEP

B. Peak airway pressure (cm H₂O)

- high VT + low PEEP
- low VT + low PEEP
- low VT + high PEEP

Time after the instillation (hour)
Airspace protein tracer in blood (%)

A. infected animals

- high $V_T$ + low PEEP
- low $V_T$ + low PEEP
- low $V_T$ + high PEEP

B. un-infected animals

- high $V_T$ + low PEEP
- low $V_T$ + low PEEP
- low $V_T$ + high PEEP

Figure 2
Figure 3
Figure 5
Figure 6
Figure 7

Entry of TNF-α into airspace from the circulation (%)