Enhancement of the endotoxin recognition pathway by ventilation with a large tidal volume in rabbits


MATERIALS AND METHODS

Reagents

The ELISAs were specific for rabbit TNF-α and IL-8 (10). LPS from *Escherichia coli* O55:B5 was obtained from Sigma (St. Louis, MO).

Experimental Protocol

In the first series of experiments (series 1), which examined the effects of a large vs. a small Vt in the absence of LPS, intratracheal
(IT) LPS was not instilled, and mechanical ventilation was started immediately after the tracheotomy. In the second series of experiments (series 2), which compared the effects of a large vs. a small \( V_t \) in LPS-instilled lungs, mechanical ventilation was started after IT instillation of LPS.

**Animal Preparation**

The experimental protocol was reviewed and approved by the Keio University Council on Animal Care in accordance with the guidelines of the National Institutes of Health. Experiments were performed in Japanese white, male rabbits (SEASCO, Saitama, Japan) weighing 2.0–2.5 kg. The animals were free of respiratory tract infections and housed under standard conditions in an animal care facility. Sterile techniques were used for all interventions. The animals were premedicated with an injection of 50 mg/kg im ketamine. A 22-gauge catheter was inserted into the marginal ear vein for the continuous infusion of 20 mg·kg\(^{-1}\)·h\(^{-1}\) pentobarbital sodium. Additional 2-mg/kg iv boluses of pentobarbital sodium were administered during the procedure as needed. A midline incision was made in the right jugular area, and a 3.5-Fr feeding catheter was inserted into the artery for continuous monitoring of blood pressure and for blood sampling. Catheter patency was maintained by flushing with 4-U/ml doses of heparinized saline. A tracheotomy was performed, and a 4-mm internal diameter uncuffed endotracheal tube was inserted to a depth of 2 cm.

**Series 1: Effects of \( V_t \) on Expression of LPS Receptors**

**Study groups.** The 12 rabbits randomly assigned to 3 groups of 4 animals each included: 1) a spontaneously breathing group (control group), 2) a group ventilated with a 5-ml/kg \( V_t \) for 240 min after tracheotomy (small \( V_t \) group), and 3) a group ventilated with a 20-ml/kg \( V_t \) for 240 min after tracheotomy (large \( V_t \) group).

**Mechanical ventilation.** After tracheotomy, 0.2 mg/kg iv of pancuronium bromide was administered to facilitate ventilation with a Sechrist IV-100B, pressure-controlled ventilator (Sechrist Industries, Anaheim, CA). The inspired \( O_2 \) concentration was set at 60\% throughout the ventilation period. \( V_t \) was measured with a pneumotachograph (Krone, Tokyo, Japan) and set at 5 ml/kg in the small \( V_t \) group and at 20 ml/kg in the large \( V_t \) group. Positive end-expiratory pressure was not applied, since the goal of this study was to examine the effects of \( V_t \) on the lung injury. To avoid \( CO_2 \) rebreathing, \( V_t \) was adjusted to maintain \( PaCO_2 \) between 35 and 45 mmHg.

**Measurements Performed in Series 1**

mRNA expression of CD14 in whole lung homogenate. The lung tissues were homogenized and used for the estimation of mRNA level of CD14. To standardize the measurements, the ratio of each mRNA signal to \( \beta \)-actin was used. The expression of mRNA for CD14 in lung samples was determined by RT-PCR. Total RNA was extracted from frozen lung samples by the acid guanidinium phenol chloroform method (3) and treated with DNase I (Ambion, Austin, TX) according to the manufacturer’s instructions. Randomly primed, first-strand cDNA was prepared from 7 \( \mu \)g of total RNA in 20 \( \mu \)l of reaction volume containing 10 \( \mu \)M Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl\(_2\), 1 mM deoxyxynucleotide triphosphates, 1.6 \( \mu \)g of oligo(dT) primer, 40 units of RNase inhibitor, and 40 units of Moloney murine leukemia virus reverse transcriptase (Roche Molecular Biochemicals, Mannheim, Germany). PCR primers for CD14 and \( \beta \)-actin were designed based on GenBank sequence nos. D16545 and M10277, respectively. The sequences of these PCR primers were as follows:

- CD14 forward, 5′-TTGCTGCTCTGCTGGCACCAC-3′ (product size 538 bp);
- CD14 backward, 5′-GTTAAACCCAGGCTCCTTGAAAC-3′ (product size 538 bp);
- \( \beta \)-actin forward, 5′-CCATGTACTGGTCTATCAGG-3′;
- \( \beta \)-actin backward, 5′-ATCTCTTGCTCAGTCCAGGG-3′ (product size 289 bp).

PCR amplifications were performed in a 25-\( \mu \)l reaction volume containing 1 \( \mu \)l of cDNA, 0.6 \( \mu \)M primers, and 12.5 \( \mu \)l of PCR Master Mix (Promega, Madison, WI), which contained Taq DNA polymerase, deoxynucleotide triphosphates, MgCl\(_2\), and reaction buffers at optimal concentrations for efficient amplification of cDNA templates by PCR. An initial denaturation at 95°C for 2 min was followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for CD14 or 55°C for \( \beta \)-actin for 30 s, and extension at 72°C for 1 min using a GeneAmp PCR System 9700 (Perkin-Elmer, Foster City, CA). Control samples, which were not subjected to reverse transcriptase, were used to detect the presence of contaminating DNA. The PCR products were separated by electrophoresis in 1.5% agarose gels (Promega) containing ethidium bromide in Tris-acetate/EDTA. PCR bands were scanned and imported to a personal computer, and the intensities of each band were analyzed using NIH Image software.

**Immunohistochemistry for CD14.** Immunohistochemistry was performed in 12 additional rabbits using a method previously described (14). Briefly, the left lung was inflated with formalin via the left main stem bronchus at a transpulmonary pressure of 25 cmH\(_2\)O. The left main stem bronchus was then ligated, and the lung was fixed for 18 h at 4°C. Sagittal sections, 2–5 mm thick, were embedded in paraffin and sliced sagittally into 3-\( \mu \)m-thick sections. The slides were deparaffinized and incubated with 0.3% \( H_2O_2 \) in methanol for 5 min to block endogenous peroxidases. They were then rinsed three times with PBS for 3 min and treated with a protein-blocking agent (IMMUNON; Shandon, Pittsburgh, PA) for 5 min at room temperature. The samples were then labeled with goat anti-rabbit CD14 polyclonal antibody overnight in a moist chamber at 4°C. The next day, tissue sections were rinsed three times with PBS for 3 min and then incubated with a biotinylated anti-goat secondary antibody (Shandon) for 10 min. The slides were rinsed three times with PBS for 3 min, followed by a 10-min incubation with horseradish peroxidase-streptavidin (LSAB2 kit; Dako, Carpinteria, CA). Tissue sections were then rinsed three times with PBS, and the chromogen was developed with diaminobenzidine for 10 min. Immunostaining controls included substitution of the primary anti-CD14 antibody with normal goat serum (Dako). Tissue sections were counterstained with Mayer’s hematoxylin. All slides were stained at the same time to control for the intensity of staining. Representative lung tissue sections were photographed using a microscope with a high-resolution color digital camera (FCD-725; Olympus, Tokyo, Japan). Digital images were prepared for illustration without altering color or image detail.

To determine the percentage of alveolar macrophages that had positive immunostaining for CD14, morphometric analysis was performed using semiquantitative techniques. Positive immunostaining was defined as cytoplasmic staining of the alveolar macrophages. For each slide, macrophages were counted in 100 randomly selected high-power fields. The morphometric analysis was performed by investigators blinded to the treatment group.

**Cell culture experiments.** To evaluate the effect of increased CD14 expression, we compared the LPS-induced TNF-\( \alpha \) release by alveolar macrophages obtained from bronchoalveolar lavage (BAL) fluid, cultured as follows: BAL fluid was filtered through a sterile gauge and centrifuged at 250 g for 5 min. The cell fractions were then washed with RPMI 1640 supplemented with 100 mg/ml streptomycin, 100 U/ml penicillin, and 10% heat-inactivated FBS (GIBCO-BRL, Gaithersburg, MD) and recentrifuged three times. The final pellet was resuspended in RPMI 1640. Cell viability was assessed by trypan blue exclusion, and cells were counted. A differential cell count was made with Diff-Quick (Harleco, Philadelphia, PA). The cells suspended in the culture medium were seeded at a concentration of 2.5 × 105
cells/ml (5 × 105 cells/well) on six-well culture plates (Corning Laboratory Science, Corning, NY). After 2 h of incubation (37°C at 5% CO₂), nonadherent cells were removed, and 2 ml of fresh RPMI 1640 were added to the adhered alveolar macrophages. In the positive control groups, the cells were stimulated with 1,000 ng/ml of LPS. Cell-free culture supernatants were collected after 6 h and kept at −70°C until assay of the cytokines.

**Measurement of cytokines.** Concentrations of rabbit IL-8 and TNF-α were measured with rabbit-specific immunoassays (10). The lower limit of detection for both cytokines was 10 pg/ml.

**Series 2: Effects of V_t in Lungs Instilled with LPS**

**Study groups.** Immediately after tracheotomy, 24 rabbits were randomly assigned to 1 of 3 groups: 1) small V_t ventilation with IT LPS instillation (small V_t + IT LPS group), 2) large V_t ventilation with IT saline instillation (large V_t + IT saline group), and 3) large V_t ventilation with IT LPS instillation (large V_t + IT LPS group).

**IT instillation.** Throughout the instillation procedure, spontaneous breathing was allowed to avoid overinflation of the lung, and 100% O₂ was delivered to avoid hypoxia. The rabbits were placed in the right lateral decubitus position, on a heating pad, under a radiant heat lamp. The endotracheal tube was advanced into the right main bronchus at a depth of 7–8 cm. A balloon-tipped catheter was advanced through the endotracheal tube into the right lower lobe, and the balloon was inflated with 0.2 ml of air. IT instillation consisted of either 1 ml of 0.9% sterile saline or 50 mg/kg of LPS dissolved in 1 ml of sterile saline containing 3 mg of anhydrous Evans blue to mark the instilled lung, followed by a flush with 2 ml of air (27). After 10 min, the balloon catheter was removed, the endotracheal tube was repositioned to a depth of 2 cm, and an alveolar recruitment maneuver was performed by inflating the lungs at a pressure of 30–40 cmH₂O for 15 s.

**BAL of the instilled lung.** After 240 min of mechanical ventilation, the rabbits were killed by injection of 250 mg of pentobarbital sodium. BAL was performed with 8 ml of saline instilled via an 18-gauge catheter placed in the right lower lobe. The mean fluid recovery ratio was 57.6 ± 2.2% and was similar in all groups. The BAL fluid was centrifuged at 400 g and 4°C for 10 min, and the supernatant was stored at −70°C until analysis.

**Measurements Performed in Series 2**

**Pulmonary albumin permeability.** Albumin concentrations in BAL fluid and in plasma were measured by colorimetric assay kits (Beckman, Fullerton, CA). The ratio of albumin in BAL fluid to that in plasma was calculated and used as an index of pulmonary albumin permeability.

**Measurement of cytokines.** IL-8 and TNF-α protein concentrations in BAL fluid and plasma were measured as described earlier.

**Statistical analyses.** Results are expressed as means ± SD. Data were analyzed by the Statview 5.0 computer program (Abacus Concepts, Berkeley, CA). Analyses were performed by one-way factorial analysis of variance, followed by Fisher’s least significant differences test. Statistical significance was set at P < 0.05.

**RESULTS**

**Series 1**

**Effect of V_t on mRNA expression of CD14.** mRNA expression of CD14, expressed as the ratio to that of β-actin, was 0.37 ± 0.06, 1.12 ± 0.21, and 2.60 ± 0.76 in the control, small V_t, and large V_t groups, respectively. mRNA expression of CD14 was significantly increased only in the large V_t group compared with the control and the small V_t groups (P < 0.05). This observation indicates that 240 min of mechanical ventilation with a large V_t increased the mRNA expression of CD14 in the lung.

**Immunohistochemistry for CD14.** Although there was no evident alveolar flooding in the stained sections, a more prominent immunostaining for CD14 on alveolar macrophages was observed in the large V_t group (Fig. 1C) than in the control (Fig. 1A) and the small V_t groups (Fig. 1B). In contrast, the CD14 expression on polymorphonuclear neutrophils (PMNs) was much weaker. The percentage of CD14-positive alveolar macrophages by morphometric analysis was significantly higher in the large V_t group than in the control and the small V_t groups (Fig. 2, P < 0.001 for both comparisons).

**Effect of V_t on cytokine release by alveolar macrophages in vitro.** In the absence of LPS stimulation, TNF-α was undetectable in all groups (Fig. 3). When induced by LPS, the release of TNF-α was higher in the small V_t group than in the control group (Fig. 3, P < 0.05). Furthermore, the LPS-induced TNF-α release in the large V_t group was significantly higher than in the control and the small V_t groups (Fig. 3, P < 0.001 for both comparisons) and was 20-fold higher than in the small V_t group.

In contrast with the undetectable release of TNF-α in the absence of LPS stimulation, including in the large V_t group, the release of IL-8 was detectable in the absence of LPS stimulation in all groups. Release of IL-8 in the absence of LPS was 385 ± 44, 539 ± 54, and 1,573 ± 67 pg/ml in the control, small V_t, and large V_t groups, respectively. This release in the absence of LPS was significantly greater in the small V_t than in the control group (P < 0.001) and significantly greater in the large V_t group than in the other two groups (P < 0.001 for both comparisons).

**Series 2**

Because ventilation with a large V_t upregulated the expression of CD14 on alveolar macrophages and increased the LPS-induced TNF-α release in cell culture experiments, we hypothesized that ventilation with a large V_t would exacerbate the inflammatory response in LPS-instilled lungs in vivo. To test this hypothesis, lung injury and inflammatory response were examined after 240 min of mechanical ventilation after the IT instillation of LPS.

**Ventilatory conditions.** The ventilatory settings used in each group are summarized in Table 1. Peak airway pressure was significantly higher in the large V_t groups with or without IT LPS than that in the small V_t group (Table 1, P < 0.001 for both comparisons). The arterial pH was 7.38 ± 0.09, 7.38 ± 0.05, and 7.36 ± 0.13 in the small V_t + IT LPS, large V_t + IT saline, and large V_t + IT LPS groups, respectively. PaCO₂ was 42.4 ± 10.7, 39.6 ± 5.8, and 39.9 ± 7.7 mmHg in the small V_t + IT LPS, large V_t + IT saline, and large V_t + IT LPS groups, respectively. Despite adjustments by increasing the respiratory rate, the mean PaCO₂ tended to be higher in the small V_t than in the large V_t groups, although the difference did not reach statistical significance.

**PMN count in BAL fluid.** Although the mean count was higher in the large V_t + IT LPS group, the differences in PMN counts in BAL fluid were not significant because of great variability among the study groups (Fig. 4A).

**Pulmonary albumin permeability.** The pulmonary albumin permeability was significantly higher in the large V_t + IT LPS group than in the other groups (Fig. 4B, P < 0.05 for both comparisons), indicating that ventilation with a large V_t in-
creased the lung epithelial and endothelial permeability when applied to LPS-instilled lungs.

Cytokine release in plasma and BAL fluid. TNF-α release was undetectable in the large Vt + IT saline group (Fig. 5, A and B). Whereas the TNF-α release in BAL fluid was detectable and similar in the LPS-instilled groups (Fig. 5A), its release in plasma was significantly higher in the large Vt + IT LPS group than in the small Vt + IT LPS group (Fig. 5B, P < 0.05).

Although the release of TNF-α was undetectable in the absence of IT LPS, IL-8 release was detectable in both the plasma and the BAL fluid, including the IT saline-instilled group. IL-8 release in BAL fluid was higher in the large Vt + IT LPS group than in the large Vt + IT saline group (Fig. 5C, P < 0.05). IL-8 release in plasma was higher in the large Vt groups than in the small Vt + IT LPS group (Fig. 5D, P < 0.05). These results indicate that ventilation with large Vt does not, by itself, induce the release of TNF-α, although it does

Fig. 1. Representative positive immunostaining for CD14 in lung tissues after mechanical ventilation. Positive staining for CD14 is brown. Hematoxylin was used as a counter stain. A: control group (spontaneous breathing). B: small tidal volume (Vt; 5 ml/kg) group. C: large Vt (20 ml/kg) group. D: negative control of the large Vt group by normal goat serum. Positive staining was observed on alveolar macrophages (C, arrows). However, polymorphonuclear neutrophils (PMNs) were not distinctly stained by the CD14 antibody.

Fig. 2. Effect of Vt on percentages of CD14-positive alveolar macrophages after immunostaining. Cytoplasmic pattern cells were considered as "positive cells" and counted in 100 high-power fields. **P < 0.001 vs. control group, ††P < 0.001 vs. small Vt group; n = 4 in each group.
induce the release of IL-8, even without subsequent LPS stimulation.

**DISCUSSION**

This study compared the effects of a large vs. a small Vt on the expression of CD14 in rabbit lungs. In series 1, we showed that ventilation with a large Vt alone, compared with a small Vt, upregulated the expression of CD14 on alveolar macrophages and induced a 20-fold increase in LPS-induced TNF-α release in cell culture experiments. In series 2, although mechanical ventilation with a large Vt did not, by itself, induce the release of TNF-α, ventilation with a large Vt after LPS instillation did increase the release of TNF-α in plasma as well as the lung epithelial and endothelial permeability. These results suggest that mechanical stress of a large Vt sensitizes the alveolar macrophages to LPS stimulation, a phenomenon that may occur via the upregulation of CD14.

In this study, we chose a Vt of 20 ml/kg as a large, however, “noninjurious” Vt. Although this Vt is obviously larger than that routinely used in humans, hemodynamic stability was maintained, and no pneumothorax occurred throughout the 4-h experiments. In addition, no evident alveolar flooding was observed morphologically, and lung epithelial and endothelial permeability, ascertained by the pulmonary albumin permeability, was not increased. An important finding of our study is that such a large, noninjurious Vt upregulated the expression of CD14 on alveolar macrophages, causing a 20-fold increase in the LPS-induced TNF-α release.

Although there is discordance among studies (4), several recent observations in animals have shown that the mechanical stress associated with large Vt ventilation does not, by itself, induce the release of TNF-α in the lung, even when it causes severe alveolar injury (1, 19, 22). Bregeon et al. (1) have reported that a Vt of 10 ml/kg upregulated the transcription of the TNF-α gene, although it did not induce the release of TNF-α in BAL fluid in rabbits. Ricard et al. (22) detected no in vivo TNF-α release in BAL fluid after ventilation with a 42-ml/kg Vt in rats and reported that both a small (7 ml/kg) and a large (42 ml/kg) Vt increased the LPS-induced TNF-α release compared with nonventilated lungs. Pugin et al. (19) showed in vitro that stretch to alveolar macrophages induced no TNF-α release by itself but increased the LPS-induced TNF-α release. In our experiments, alveolar macrophages harvested after in vivo stimulation by mechanical ventilation showed no TNF-α release without adding an inflammatory stimulus, although the TNF-α release was increased after LPS stimulation. Therefore, we hypothesize that this increase may be associated with an upregulated expression of CD14.

Although the CD14 expression on alveolar macrophages is rapidly upregulated in response to inflammatory stimulation, its constitutive expression is much weaker than on blood monocytes or peritoneal macrophages (12, 15). By flow cytometry, Maus et al. (15) showed that resident alveolar macrophages did not express CD14, whereas recruited alveolar monocytes strongly expressed CD14, and these CD14-positive cells showed a fourfold increase in LPS-induced TNF-α release. Because mechanical stress causes the release of not only α- but also of β-chemokines (8), it is plausible that, in our model, monocytes and lymphocytes were also recruited to the lung by mechanical stress. Because this upregulated CD14 expression was associated with increased TNF-α after subsequent LPS stimulation only, we thought that this noninjurious large Vt ventilation may “prime” rather than “fully activate” the alveolar macrophages. Although the underlying mechanisms remain unclear, mechanical stress during ventilation activates the mechanoreceptors of alveolar cells, which activates the immune system (8, 19). Alveolar macrophages are sensitive to cyclic pressure stretching (19), and mechanical stress increases the expression of NF-κB (8, 19). We have shown that mechanical stress upregulates the expression of CD14 and that CD14-expressing alveolar macrophages are prominently responsive to LPS challenge. As Chen et al. (2) reported that the release of TNF-α by LPS stimulation reflects a sepsis-induced suppression of the antibacterial host defense.

**Table 1. Ventilatory conditions in each experimental group**

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, kg</th>
<th>Vt, ml/kg</th>
<th>PAP, cmH2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small Vt</td>
<td>2.36±0.15</td>
<td>5.03±0.58</td>
<td>7.75±1.50</td>
</tr>
<tr>
<td>Large Vt</td>
<td>2.27±0.17</td>
<td>20.06±0.55*</td>
<td>21.67±2.58*</td>
</tr>
<tr>
<td>Large Vt</td>
<td>2.21±0.20</td>
<td>21.02±1.31*</td>
<td>20.20±1.79*</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 8 in each group. Vt, tidal volume; PAP, peak airway pressure; IT, intratracheal. *P < 0.001 compared with the small Vt + IT LPS group.
it is possible that the upregulated CD14 expression in our model reflected an activation of the immune system.

Another noteworthy observation in our in vivo experiments was an increase in cytokine release by a large Vt, not only in BAL fluid but also in plasma. Previous studies have shown that the alveolar barrier is important to compartmentalize the inflammatory response in the lung (26) and that ventilation with a large Vt caused the loss of this compartmentalization (7, 17).
Haitsma et al. (7) have reported that the loss of this compartmentalization caused leakage of inflammatory mediators from the lung into the systemic circulation, resulting in increased TNF-α concentrations in plasma and decreased concentrations in BAL fluid. Our results showed that, compared with a small Vt, ventilation with a large Vt after LPS instillation increased the release of TNF-α into the plasma. Because no TNF-α release in plasma was observed in the saline-instilled lung experiments, it is likely that large Vt ventilation caused the leakage of TNF-α, produced in the air space in response to LPS, into the blood stream along with IL-8. Therefore, we hypothesize that the increased release of TNF-α in plasma may, in part, reflect an increase in the release of TNF-α in the lung, although the source of plasma TNF-α measured in our experiments remains unclear. Further studies of the underlying mechanisms by which large Vt ventilation causes this loss of compartmentalization are warranted and were beyond the scope of our experiments.

Although the peak inspiratory pressure was greater than 20 cmH2O, the Vt we applied was relatively lower than that routinely used in humans. However, recent studies have shown that, in patients with ARDS, the lungs are nonuniformly inflated, and that relatively intact lungs can be severely overdistended with injured lungs (6). Because endotracheal intubation introduces bacterial products into the lungs (16), it is likely that LPS plays important roles during mechanical ventilation, especially in patients with acute lung injury. The amounts of LPS used in this study were distinctly higher than those expected during a clinical situation. However, the finding that large Vt ventilation enhanced the response to LPS is clinically relevant since it may promote lung inflammation, even in disease-free lungs.

In conclusion, ventilation with a large Vt alone upregulated the expression of CD14 on alveolar macrophages and induced a 20-fold increase in LPS-induced TNF-α release in cell culture experiments. In in vivo experiments, a large Vt applied to LPS-instilled lungs increased the lung alveolar permeability and the release of TNF-α in plasma. These results suggest that the mechanical stress of a large Vt sensitizes the alveolar macrophages to LPS stimulation, a phenomenon that may occur via upregulation of CD14.

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REFERENCES


