Introduction

One-lung ventilation (OLV) is one of the major causes of acute lung injury following thoracic surgery. Previous studies have suggested that OLV can induce lung injury as a result of mechanical ventilation, pulmonary capillary stress failure, oxidative stress, or ischemia-reperfusion (I/R) injury. Neutrophils play a key role in the development of acute lung injury. Cytoskeletal rearrangements in circulating neutrophils are induced by various stimuli including inflammatory cytokines and cause a decrease in the deformability of these cells. In much of the systemic circulation, neutrophil migration into inflamed tissue occurs after rolling of the cells in the postcapillary venules through the activity of the selectins. In the pulmonary circulation, however, much of the neutrophil migration occurs through the pulmonary capillaries which are too narrow for rolling to occur. The diameter of the spherical neutrophils measures 6–8 µm, whereas that of the pulmonary capillary segments measures 2–15 µm. Hence, neutrophils need to alter their shape to pass through these capillaries.
Neutrophil Cytoskeletal Rearrangements in Rat Lung

through the lung capillaries where they will be mechanically trapped if their deformability is decreased. This mechanical trapping of the neutrophils in the capillaries is referred to as sequestration. The cytoskeletal rearrangements that occur in circulating neutrophils are thus thought to represent the initiation of lung recruitment of these cells. However, the morphological and functional characteristics of these cells, that are associated with OLV, have not been fully elucidated to date.

Taken together, the cumulative evidence to date provides a basis for the hypothesis tested in our current study i.e., that the cytoskeletal changes which mediate the decreased deformability of neutrophils and recruitment of these cells into the lung are induced by OLV and subsequent re-expansion (RE). To examine this possibility, we utilized a rat model of OLV.

Materials and Methods

Animal preparation and a rat model of one-lung ventilation (OLV)

Male Wistar rats weighting 280g–330g were used in compliance with The Principles of Laboratory Animal Care, formulated by the National Society for Medical Research, and The Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996). The rats were anesthetized with pentobarbital sodium, which was first administered intraperitoneally at a dose of 50 mg/kg and then at 15 mg/kg at subsequent hourly intervals. An arterial line for blood sampling was placed in the left carotid artery. Following a tracheotomy, the rats were intubated with a 16-gauge catheter, and mechanical ventilation was maintained with a tidal volume of 2.5 ml in room air at a respiratory rate of 100 cycles/min. A positive end-expiratory pressure of 1.5 cm H2O was applied during the mechanical ventilation. After a right lateral thoracotomy in the fifth intercostal space, a tracheal catheter was inserted deep into the left main bronchus, and the tidal volume was decreased to 2.0 ml. After a specified period of OLV, the tracheal catheter was withdrawn from the left main bronchus to the trachea, and 8 ml of air was injected into the bilateral lungs through the tracheal catheter using a 10 ml syringe for the purpose of RE of the right lung. Subsequently, the tidal volume was increased to 2.5 ml and two-lung ventilation (TLV) was performed. This study was approved by the Animal Ethics Committee of Shinshu University School of Medicine.

Animal study design

In preliminary studies, OLV was found to induce a time-dependent increase in neutrophil sequestration...
In addition, we speculated that OLV in excess of three hours would not accurately replicate the conditions usually experienced during thoracic surgery and thus set this as the outer timepoint in the animal experiments. The protocol for our animal study is shown in Fig. 1. The experimental animal groups were TLV, OLV, and RE (n = 6 in each of these three groups). The TLV and OLV groups received their respective ventilations for 3.5 hours and the RE group was subjected to 3 hours of OLV followed by RE and 30 minutes of TLV. Whole blood samples were harvested on three occasions: (i) just before the start of ventilation (baseline, point 1); (ii) at 3 hours after the start of ventilation, i.e., just prior to lung RE in the RE group (point 2); and (iii) at 3.5 hours after the start of ventilation, i.e., at 30 minutes after lung RE in the RE group (point 3). The volumes of the whole blood samples were 0.6 ml at points 1 and 2, and 4.6 ml at point 3. At point 3, plasma was obtained from 4 ml out of 4.6 ml of the whole blood samples via ethylene diamine tetra-acetic acid (EDTA) treatment and centrifugation. Just after the point 3 blood sampling, rats were euthanized by blood loss from the descending aorta. Lung tissues were then harvested and frozen after perfusion of optimum cutting temperature (OCT) compound (Tissue-Tek™; Sakura Finetechnical Co., Ltd., Tokyo, Japan) into the airways.

**Total and filamentous actin (F-actin) rimmed neutrophil counts**

The number of total leukocytes was determined using a hemocytometer and a 500 μl aliquot of blood. Blood smears were stained using a modified Wright’s method (Diff-Quik™; American Scientific Product, McGraw Park, Illinois, USA). Differential leukocyte counts were performed on 200 cells in each smear. Neutrophil cytoskeletal rearrangements were assessed by observing any changes in the localization of F-actin. After the fixation of whole blood samples at each time point using 0.5% paraformaldehyde, F-actin was labeled, and nuclei were stained by incubation with L-lysophosphatidylcholine, fluorescein isothiocyanate (FITC)-phalloidin, and ethidium bromide. Three hundred neutrophils were randomly identified by nuclear lobulation using fluorescence microscopy and the neutrophils were categorized as showing an F-actin rim beneath the plasma membrane or not, as previously described. F-actin rimmed neutrophil counts were obtained by multiplying the total neutrophil count by the percentage of neutrophils containing an F-actin rim.

**Capillary neutrophil counts**

Frozen lung tissues were cut into 2 μm sections using a cryostat. Immunohistochemistry was then performed using the following sequence: acetone fixation, blocking endogenous peroxidase activity using 1% bovine serum albumin in phosphate buffered saline, incubation with a rat granulocyte antibody (mouse IgM, Clone: HIS48; PharMingen, Franklin Lake, New Jersey, USA), incubation with a anti-mouse IgM antibody, incubation with streptavidin-horseradish peroxidase conjugate, and incubation with diaminobenzidine to detect antibody staining. A continuous group of 500 alveoli was counted with a light microscope at a 100X magnification. The neutrophils in the lung capillaries adjoining the walls of these 500 alveoli were also counted. The data were then expressed as the number of neutrophils per 100 alveoli.

**Cytokine-induced neutrophil chemoattractant 1 (CINC-1) levels**

The Cytokine-induced neutrophil chemoattractant 1 (CINC-1) levels in the plasma from the OLV rats were measured using a commercial ELISA kit (Rat CINC-1 Immunoassay; Quantikine R&D Systems Inc., Minneapolis, Minnesota, USA).

**Statistical analysis**

All of the data in this study are presented as the mean ± standard deviation (SD). The time course of the F-actin rimmed neutrophil counts was evaluated using a two-way repeated-measures analysis of variance (ANOVA) with Tukey’s post-hoc analysis. The capillary neutrophil counts and the cytokine levels were evaluated by one-way ANOVA with Tukey’s post-hoc analysis. A paired t test was used to compare the values between the collapsed lung and the ventilated lung. Statistical significance was set at \( p < 0.05 \).

**Results**

**Total and F-actin rimmed neutrophil counts**

The total neutrophil counts and F-actin rimmed neutrophil counts are listed in Table 1. The total neutrophil counts at point 2 tended to increase in comparison with the baseline neutrophil counts (point 1) in all of the animal groups. In the RE group however, the number of total neutrophils continued to increase for 30 minutes after RE, and the total neutrophil counts at point 3 were significantly higher than those at point 1 (\( p = 0.0067 \)). In the TLV and OLV groups in contrast, the total neutrophil
counts at point 3 did not increase in comparison with the neutrophil counts at point 2. There were no differences among the total neutrophil counts for each time course.

The percentage of F-actin rimmed neutrophils and the F-actin neutrophil counts also tended to increase at point 2 in comparison with the baseline neutrophil counts (point 1) in each of the animal groups. This suggested that the surgical procedure alone increased the neutrophil activation levels. In the RE group however, the number of F-actin rimmed neutrophils continued to increase for 30 minutes after RE. In the TLV and OLV groups in contrast, the F-actin rimmed neutrophil counts at point 3 did not increase in comparison with the neutrophil counts at point 2. This suggests that additional neutrophil activation is induced by lung RE following OLV. In a comparison of each of the time courses of the F-actin neutrophil counts, there were significant differences found both between the RE and TLV groups, and between the RE and OLV groups (RE vs. TLV, \( p = 0.0479 \); RE vs. OLV, \( p = 0.0279 \), respectively). There was no significant difference observed between the time courses of the F-actin neutrophil counts in the TLV and OLV groups.

**Capillary neutrophil counts**

In ventilated lungs, the capillary neutrophil counts in the TLV, OLV, and RE groups were 42 ± 11, 72 ± 8, and 126 ± 18 per 100 alveoli, respectively. The number of sequestered neutrophils in the pulmonary capillaries in the OLV group was significantly higher than that in the TLV group. Furthermore, in the RE group, the number of neutrophils was significantly higher than that in either the TLV or OLV rats. This suggested that neutrophil sequestration in the pulmonary capillaries in the ventilated lung was induced not only by OLV itself, but also by the subsequent RE (Fig. 2). Histological analyses of the ventilated lungs also revealed numerous neutrophils in the lung capillaries in the RE group but indicated that few of these cells had been sequestrated in the TLV animals. In the OLV group, an intermediate number of capillary neutrophils between those in the TLV and RE groups was evident (Fig. 3). In the collapsed lung, the capillary neutrophil counts in the TLV, OLV, and RE groups were 39 ± 19, 99 ± 31, and 78 ± 5 per 100 alveoli, respectively. The counts in both the OLV and RE groups were significantly

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**Table 1**  
Time course data for total and F-actin-rimmed neutrophil counts

<table>
<thead>
<tr>
<th></th>
<th>Point 1</th>
<th>Point 2</th>
<th>Point 3</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total neutrophil counts (/μl)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLV</td>
<td>963 ± 451</td>
<td>2831 ± 1265</td>
<td>2741 ± 1263</td>
<td>0.2603</td>
</tr>
<tr>
<td>OLV</td>
<td>596 ± 285</td>
<td>2125 ± 1580</td>
<td>1941 ± 1411</td>
<td></td>
</tr>
<tr>
<td>RE</td>
<td>1044 ± 147</td>
<td>2780 ± 1349</td>
<td>4011 ± 1341*</td>
<td></td>
</tr>
<tr>
<td><strong>F-actin rimmed neutrophil counts (/μl)</strong> (percentage of F-actin rimmed neutrophils)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLV</td>
<td>166 ± 96 (22.9)</td>
<td>736 ± 521 (29.5)</td>
<td>621 ± 243 (26.2)</td>
<td></td>
</tr>
<tr>
<td>OLV</td>
<td>137 ± 123 (20.8)</td>
<td>548 ± 555 (29.2)</td>
<td>671 ± 497 (35.2)</td>
<td>0.0208‡</td>
</tr>
<tr>
<td>RE</td>
<td>208 ± 105 (19.6)</td>
<td>1036 ± 316 (40.6)</td>
<td>1750 ± 445 (46.8)†</td>
<td></td>
</tr>
</tbody>
</table>

\( P \) value means \( P \) value in a comparison of the time courses. *Significant difference compared with the baseline (\( P = 0.0067 \)); †Significant difference compared with the baseline (\( P = 0.0067 \)); ‡Significant differences compared with the other time courses of F-actin rimmed neutrophil counts (RE vs. TLV, \( P = 0.0479 \); RE vs. OLV, \( P = 0.0279 \)).

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**Fig. 2**  
Capillary neutrophil counts in the ventilated rat lungs. The capillary neutrophil count in the OLV group was significantly higher than that in the TLV group. Furthermore, the number of neutrophils was significantly higher in the RE group than in either the TLV or OLV groups. Each of the error bars indicates the standard deviation. OLV: one lung ventilation; TLV: two lung ventilation; RE: re-expansion. * significant differences were found between the two groups (\( p < 0.05 \)).
higher than those of the TLV rats. RE of the collapsed lung had no effect on the numbers of neutrophils within capillaries beyond that induced by OLV. In the RE group, the capillary neutrophil counts in the ventilated lung were higher than that in the collapsed lung ($p < 0.05$). In the OLV and TLV groups, however, there were no significant differences in the capillary neutrophil counts between the collapsed and ventilated lungs.

**Plasma CINC-1 levels**

The plasma CINC-1 levels in the TLV, OLV, and RE groups were measured at 274 ± 9, 451 ± 129, and 1177 ± 136 pg/ml, respectively. The cytokine levels in the RE group were significantly higher than those in the TLV and OLV groups. There were no significant differences however, between the plasma CINC-1 levels in the TLV and OLV groups (Fig. 4).

**Discussion**

The results of our present study in a rat OLV model indicate that after lung RE, the number of circulating F-actin rimmed neutrophils increases. Furthermore, the number of neutrophils that became sequestered in the lung capillaries also increases after RE, particularly in the ventilated lung. The plasma CINC-1 level was further found to be elevated after RE.

We focus in our present analyses on neutrophil cytoskeletal rearrangement as one of the key mechanisms underlying neutrophil recruitment. Various stimuli, including

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![Fig. 3](image)

**Fig. 3** Immunohistochemical detection of neutrophils in the OLV rat model. Immunohistochemical localization of sequestered neutrophils in ventilated lungs from the TLV, OLV, and RE groups, respectively. A few neutrophils are detectable in the lung capillaries in the TLV group. In contrast, numerous sequestrated neutrophils are evident in the RE group. In the OLV group, capillary neutrophils intermediate between those in the TLV and RE groups could be observed. Small areas of interstitial edema were also observed in the RE rats, but no major alveolar damage such as alveolar edema, hemorrhage, or hyaline membrane formations could be detected in any groups. The high magnification image shows the sequestered neutrophils in the lung capillaries. The diameters of these neutrophils are about 8 to 10 μm. OLV: one lung ventilation; TLV: two lung ventilation; RE: re-expansion.
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OLV can induce lung injury in the ventilated lung as a result of mechanical ventilation, pulmonary capillary stress failure, or oxidative stress. However, OLV alone cannot explain our present findings that the number of capillary neutrophils in the ventilated lung in the RE group was higher than that in the OLV group. RE should be involved in the neutrophil recruitment into the ventilated lung. RE pulmonary edema is a rare complication of lung collapse treatments that is secondary only to pneumothorax, pleural effusion, or atelectasis. Although, this disorder has been historically believed to occur after chronic lung collapse, its incidence after relatively short period lung collapse has also been reported. However, RE pulmonary edema usually develops unilaterally in a collapsed lung and also does not fully explain our current findings.

On the other hand, I/R lung injury, which is a major clinical issue in the early phase of lung transplantation and a significant cause of early morbidity and mortality after these procedures, has also been thought to occur after a thoracotomy because blood flow to the collapsed lung is reduced and reperfusion injury can occur after RE. Several previous studies have shown that I/R of the lung induces a rapid release of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), interleukin (IL)-1β, IL-6, and IL-8. De Perrot, et al. have reported that although most cytokine levels decrease after reperfusion, those of IL-8 significantly increase, and are a predictor of the success of early graft function after human lung transplantation. Previous in vitro studies demonstrate that hypoxia-reoxygenation induces IL-8 production in monocytes, epithelial cells, endothelial cells, pulmonary fibroblasts and vascular smooth muscle cells. Some studies have also suggested that microvascular endothelial cells can store IL-8 in Weibel-Palade bodies and secrete IL-8 into blood vessels very rapidly.

In this study, we evaluated cytoskeletal rearrangements in the circulating rat neutrophils by counting the number of F-actin rimmed neutrophils. These cells were increased in number during OLV and found to be further elevated after RE of the lung. This increase in F-actin rimmed neutrophils after RE was also observed to be well synchronized with the elevated number of neutrophils in the pulmonary capillaries in the ventilated lung.

interleukin-8 (IL-8), the activated fragment of the fifth component of complement (C5a), and N-formylmethionylleucyl-phenylalanine (fMLP), can induce rapid increases in actin polymerization in neutrophils, leading to a dramatic redistribution of the generated actin microfilaments, i.e., F-actin, to the subcortical region of the neutrophils. In the systemic circulation, these neutrophils are more rigid due to their cytoskeletal rearrangements and cannot make contact with the activated endothelial surface, so that no rolling within the postcapillary venules or migration into the inflammatory tissues would occur. In the pulmonary circulation, however, the diameter of the pulmonary capillary segments is marginally smaller than that of the spherical neutrophils and most neutrophils must become more elongated to pass through the segments. Hence, neutrophils which are stimulated and have a decreased deformability cannot transit through the lung capillaries and will therefore sequester and migrate to the alveoli.

In this study, we evaluated cytoskeletal rearrangements in the circulating rat neutrophils by counting the number of F-actin rimmed neutrophils. These cells were increased in number during OLV and found to be further elevated after RE of the lung. This increase in F-actin rimmed neutrophils after RE was also observed to be well synchronized with the elevated number of neutrophils in the pulmonary capillaries in the ventilated lung.

that in the TLV group, in which hypoxia did not occur, and because the cytokine level in the OLV group was significantly lower than that in the RE group, in which the hypoxic duration was shorter than that in the OLV group. In another preliminary study, we measured the CINC-1 levels in the broncho-alveolar lavage fluid (BALF) and the corresponding transcript levels in the lung tissue, but no significant increases were found in either case (data not shown). We speculate that the elevation of CINC-1 in our rat OLV model is not caused by its increased synthesis in the lung, but through its rapid release from a stored pool in the endothelial cells of the pulmonary capillary, induced by RE. In considering our present findings, i.e., the CINC-1 elevation in the plasma, the cytoskeletal rearrangement in the circulating neutrophils, and the neutrophil recruitment in the ventilated lung, we also speculate that circulating neutrophils alter their cytoskeleton after RE due to the release of various stimuli from the re-expanded lung tissue, e.g. CINC-1, and that these neutrophils with decreased deformability will sequester in the ventilated lung which has already been damaged during OLV. Further studies will be needed however to fully clarify these possibilities.

There are several noteworthy anomalies in our current findings. First, OLV is believed to induce lung injury, but we found no evidence of this in our OLV rats other than neutrophil recruitment. We examined the wet/dry ratios of the lungs and the protein levels in the BALF in our preliminary study, but no significant changes were detected during OLV or after RE (data not shown). A longer duration of OLV or TLV after RE might therefore be needed to induce lung injury. Further studies will be needed to clarify these possibilities. It is also worth noting that CINC-1 was not identified as a cause of neutrophil recruitment after RE in our present experiments because we did not perform cytokine blocking analyses.

In conclusion, our present findings indicate that lung RE following OLV induces cytoskeletal rearrangements in circulating neutrophils and thereby promotes their pulmonary capillary sequestration, particularly in the case of ventilated lungs. Rapid CINC-1 elevation in the plasma after RE is likely to be involved in neutrophil recruitment.

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Disclosure Statement

None to declare.

References


