Mechanisms of early pulmonary neutrophil sequestration in ventilator-induced lung injury in mice

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Choudhury, Sharmila, Michael R. Wilson, Michael E. Goddard, Kieran P. O’Dea, and Masao Takata. Mechanisms of early pulmonary neutrophil sequestration in ventilator-induced lung injury in mice. Am J Physiol Lung Cell Mol Physiol 287: L902–L910, 2004.—Polymorphonuclear leukocytes (PMN) play an important role in ventilator-induced lung injury (VILI), but the mechanisms of pulmonary PMN recruitment, particularly early intravascular PMN sequestration during VILI, have not been elucidated. We investigated the physiological and molecular mechanisms of pulmonary PMN sequestration in an in vivo mouse model of VILI. Anesthetized C57/BL6 mice were ventilated for 1 h with high tidal volume (injurious ventilation), low tidal volume and high positive end-expiratory pressure (protective ventilation), or normal tidal volume (control ventilation). Pulmonary PMN sequestration analyzed by flow cytometry of lung cell suspensions was substantially enhanced in injurious ventilation compared with protective and control ventilation, preceding development of physiological signs of lung injury. Anesthetized, spontaneously breathing mice with continuous positive airway pressure demonstrated that raised alveolar pressure alone does not induce PMN entrapment. In vitro leukocyte deformability assay indicated stiffening of circulating leukocytes in injurious ventilation compared with control ventilation. PMN sequestration in injurious ventilation was markedly inhibited by administration of anti-L-selectin antibody, but not by anti-CD18 antibody. These results suggest that mechanical ventilatory stress initiates pulmonary PMN sequestration early in the course of VILI, and this phenomenon is associated with stretch-induced inflammatory events leading to PMN stiffening and mediated by L-selectin-dependent but CD18-independent mechanisms.

Mechanical ventilation produces of worsens lung injury, an event termed ventilator-induced lung injury (VILI) (8, 12, 32, 38). The initial stage of VILI has been shown to involve release of cytokines and other inflammatory mediators (4, 18, 19, 42, 50, 52, 54, 57, 59), whereas the advanced stage is characterized by infiltration of polymorphonuclear leukocytes (PMN) into the alveoli with development of physiological signs of lung injury (1, 12, 20, 22, 39, 49, 54, 55). Although PMN are considered to play a central role in the pathophysiology of VILI (1, 22, 39), the exact time profile of pulmonary PMN recruitment during VILI and underlying physiological and molecular mechanisms remain poorly understood.

PMN recruitment in the lung, particularly the initiating intravascular events of sequestration and adhesion, substantially differs from recruitment in other organs (5, 58). The lung harbors a large marginated PMN pool, because PMN must deform to pass through narrow pulmonary capillary segments, resulting in a longer transit time (7, 16, 17, 29). PMN sequestration occurs primarily within the pulmonary capillaries, not at the postcapillary venules (5, 58). Initial rapid PMN sequestration by the lung in response to inflammatory stimuli does not require selectins, in contrast to the systemic circulation (5, 10, 24), but instead involves changes in PMN biomechanical properties (loss of deformability), leading to enhanced physical PMN entrapment (5, 34, 60). L-selectin and β2-integrin (CD11b/CD18) are, however, required at a later stage for prolonged PMN sequestration within the pulmonary capillaries (6, 10, 24–26). PMN adhesion and migration into the alveolar space then occur, which can be CD11b/CD18 dependent or independent depending on the stimulus, again differing from the systemic circulation where migration is predominantly CD11b/CD18 dependent (5).

To our knowledge there have been no studies investigating the mechanisms of pulmonary PMN recruitment at the early intravascular stage in response to mechanical ventilatory stress. Injurious ventilation (IV) has recently been shown by our group (50, 59) and others (4) to upregulate cytokines and initiate inflammation in the very early phase of VILI before the development of obvious physiological signs of lung injury. Therefore, PMN sequestration may also be initiated earlier in VILI than previously considered, mediated by similar mechanisms of PMN sequestration in response to inflammatory stress as described above. Alternatively, different sequestration mechanisms may be involved in response to mechanical stress, considering that diverse sequestration/adhesion pathways are elicited in the lung in a stimulus-dependent fashion (5). Moreover, it has been proposed that raised alveolar pressure during application of positive end-expiratory pressure (PEEP) might contribute to PMN sequestration due to compression of alveolar capillaries (30, 31). Thus complex interactions of inflammatory and mechanical factors may be implicated in PMN sequestration during VILI.

The present study investigated the mechanisms of pulmonary PMN sequestration at an early stage of VILI in an in vivo mouse model. We developed a flow cytometry-based method.
to evaluate PMN sequestration and activation at the intravascular stage before intra-alveolar migration and determined the effects of different modes of mechanical ventilation as well as raised alveolar pressure. Finally, we examined the importance of PMN deformability and adhesion molecules in mediating pulmonary PMN sequestration during VILI.

METHODS

Animals

Animal experiments were carried out on male C57/BL6 mice (Harlan, Bicester, UK) aged 9–12 wk (20–29 g) under the guidelines of the Animals (Scientific Procedures) Act 1986, UK.

Mechanical Ventilation in Mice

To investigate the effects of injurious mechanical ventilation on pulmonary PMN sequestration, we used a modification of our previous mouse model of high tidal volume (V T)-induced VILI (59). Mice were anesthetized by intraperitoneal injection of Hypnorm (0.8 mg/kg Fentanyl, 25 mg/kg Fluanisone) and Midazolam (12.5 mg/kg). Anesthesia was maintained by further administration of these agents (1/3 of the initial dose every 20 min) via an intraperitoneal catheter. An endotracheal tube was inserted via tracheotomy, and mice were ventilated with air via a custom-made mouse ventilator (13), with a peak inspiratory pressure (PIP) of 9–11 cmH 2 O, PEEP of 2.5–2.8 cmH 2 O, inspiratory-expiratory ratio of 1:2, and respiratory rate (RR) of 120 breaths/min. The left carotid artery was cannulated for blood pressure (BP) monitoring, blood gas analysis, and fluid infusion. Airway pressure, airway flow (using a miniature pneumotachograph), BP, and rectal temperature were monitored throughout. V T was calculated by integrating inspiratory flow and respiratory system compliance (Cr) and resistance (Rrs) were measured by the end-inflation occlusion technique (13, 59). After instrumentation, sustained inflation of 35 cmH 2 O for 5 s was given two times to standardize volume history of the lungs, and animals received one of the following ventilatory protocols.

Injurious ventilation. Mice were ventilated with an initial PIP of 43–48 cmH 2 O, zero PEEP, RR of 90/min, using air supplemented with 4% CO 2 . V T attained was 44.1 ± 3.2 (SD) ml/kg with a mean airway pressure (MAP) of 8.9 ± 0.2 cmH 2 O, and this V T was maintained throughout. We have shown similar ventilator settings to produce substantial VILI in mice if maintained for longer (>2 h) (59), but in this study the experiment was intentionally terminated when PIP started to show 5–10% increase from the initial value. The average duration of the experiments was 58.5 ± 10 min.

Protective ventilation. Mice were ventilated with a PIP of 12–15 cmH 2 O, PEEP of 6–7 cmH 2 O, RR of 240/min, using air for 1 h. This protective ventilation (PV) protocol was designed to produce the same MAP as IV, but with a “protective” mode of ventilation using a low V T and high PEEP. V T attained was 6.1 ± 0.4 ml/kg and MAP was 8.9 ± 0.1 cmH 2 O.

Control ventilation. Mice were ventilated with the settings used during the instrumentation period, i.e., PIP of 9–11 cmH 2 O, PEEP of 2–2.5 cmH 2 O, RR of 120/min, using air for 1 h. This control ventilation (CV) protocol was to ventilate the animal with a normal V T expected for spontaneously breathing C57/BL6 mice (51). V T attained was 8.6 ± 0.3 ml/kg, and MAP was 4.3 ± 0.2 cmH 2 O.

Except for IV animals, sustained inflation of 35 cmH 2 O for 5 s was performed every 30 min to minimize atelectasis. In a separate group of IV animals, the right external jugular vein was cannulated, and one of the following antibodies, i.e., monoclonal rat anti-mouse L-selectin antibody (30 μg, MEL-14; BD Pharmingen, Oxford, UK), monoclonal rat anti-mouse CD18 antibody (30 μg, GAME-46; BD Pharmingen), or control rat IgG (30 μg, BD Pharmingen), was administered by intravenous infusion over 15 min just before starting protocol.

Spontaneous Respiration in Mice

To investigate the effects of raised alveolar pressure on pulmonary PMN sequestration, we used anesthetized mice breathing spontaneously with or without continuous positive airway pressure (CPAP).

Mice were anesthetized with isoflurane (1.5–3%) in air, and an endotracheal tube was inserted via tracheotomy and then connected to a breathing circuit incorporating a pneumotachograph and pressure transducer. The left carotid artery was cannulated, and airway pressure, airway flow, BP, and rectal temperature were monitored. Animals were allowed to breathe spontaneously with or without CPAP hemodynamically tolerated by the anesthetized C57/BL6 mice in our experiments, or 0–1 cmH 2 O (low CPAP) for 1 h. MAP attained was 6.7 ± 0.4 (high CPAP) or 0.7 ± 0.1 cmH 2 O (low CPAP). Isoflurane concentration was adjusted to maintain RR between 60 and 90/min, and adequate gas exchange was confirmed by blood gas analysis.

LPS-induced Lung Injury in Mice

For studies to validate flow cytometric measurement of PMN sequestration, mice received an intravenous injection of 0.2–200 μg of LPS (Escherichia coli O111:B4; Sigma-Aldrich, Poole, UK) via tail vein, and after 0.5–2 h, lungs were analyzed by both flow cytometry and myeloperoxidase (MPO) assay. For studies to validate the efficacy of blocking antibodies for CD18, mice were anesthetized with isoflurane (1.5–3%) in air, with an endotracheal tube inserted via tracheotomy, and received intratracheal instillation of LPS (20 μg in 25 μl of saline). Anti-CD18 antibody (30 μg) was administered by infusion over 15 min via the external jugular vein before LPS instillation. Mice were allowed to breathe spontaneously under anesthesia for a further 3 h, and their lungs were analyzed for intra-alveolar PMN infiltration by lung lavage.

Sample Harvesting

Mice were intravascularly heparinized and killed by anesthetic overdose. After blood was obtained by cardiac puncture, the mice were subjected to pulmonary arterial perfusion for preparation of lung cell suspension, and both lung and blood samples were analyzed by flow cytometry. In a separate group of animals, blood samples were obtained for peripheral blood PMN counts and leukocyte deformability assay, and the mice were subjected to lung lavage using three separate washes of 750 μl of sterile saline, as described previously (59), to evaluate intra-alveolar PMN migration. Lavage fluids recovered were centrifuged, and cell pellets were resuspended and combined. Cell counts were determined by hemocytometer, with differential cytology performed by cytoospin and Diff-Quik staining.

Preparation of Lung Cell Suspension

Pulmonary arterial perfusion was performed before dissection of lung to remove residual blood within the pulmonary vasculature and reduce nonadherent leukocytes from subsequent flow cytometric analysis. The chest was opened, plastic cannulas were placed into the main pulmonary artery (via right ventricle) and left atrium (via left ventricle), and the left lung was perfused with PBS for 3 min via a syringe pump at a flow rate of 0.6 ml/min (i.e., 20–30 ml·kg -1 ·min -1 for a mouse) and perfusion pressure <10 mmHg. Perfusion of the right lung was prevented by a ligature placed around the right hilum. The draining fluid from the left atrial cannula was confirmed to be macroscopically transparent at the end of perfusion. The left lung was then removed, and connective tissues at the hilum were dissected out. The remaining lung parenchyma was rinsed in PBS and subjected to pulmonary arterial perfusion for preparation of lung cell suspension, and both lung and blood samples were analyzed by flow cytometry. In a separate group of animals, blood samples were obtained for peripheral blood PMN counts and leukocyte deformability assay, and the mice were subjected to lung lavage using three separate washes of 750 μl of sterile saline, as described previously (59), to evaluate intra-alveolar PMN migration. Lavage fluids recovered were centrifuged, and cell pellets were resuspended and combined. Cell counts were determined by hemocytometer, with differential cytology performed by cytoospin and Diff-Quik staining.
Flow Cytometric Analysis

Flow cytometry was used for the identification and quantification of PMN, as well as evaluation of PMN adhesion molecule expression in lung and blood samples. Samples (lung cell suspension or blood) were incubated with the different antibodies for 30 min at 4°C in the dark, erythrocytes were lysed, and cells were fixed with FACS lyses solution (BD Pharmingen). Cells were washed twice and then analyzed with a FACSCalibur flow cytometer (BD) and CellQuest Pro software. A minimum of 50,000 cells in each sample was analyzed. PMN were identified by FITC-conjugated rat anti-mouse Ly-6G/C (Gr-1) antibody (BD Pharmingen), and the percentage of PMN to total cells in the sample was determined. Gr-1 is strongly expressed on PMN and to a much lesser extent expressed on monocytes (27), and monocytes were further differentiated from PMN by their lower side scatter properties. The absolute cell counts in each sample were measured using Perfect-Count microspheres (Caltag Medsystems, Towcester, UK) according to manufacturer’s instructions, and the total PMN recovered per left lung or per milliliter of blood were calculated. In some experiments, surface expression of L-selectin or Mac-1 (CD11b) on PMN in lung and blood samples were measured by simultaneously staining with phycoerythrin-conjugated rat anti-mouse L-selectin (MEL-14, BD Pharmingen) or rat anti-mouse Mac-1 (M1/70, BD Pharmingen).

MPO Assay

To establish the validity of the flow cytometric quantification of pulmonary PMN sequestration, the MPO assay was performed on the same lung from mice challenged by intravenous LPS, and the data were compared with those obtained by flow cytometry. MPO activity quantification was carried out as previously described (45). In brief, lung tissue was homogenized, sonicated two times, freeze-thawed three times, and centrifuged. The supernatant was mixed with 50 mM potassium phosphate (pH 6) containing O-dianisidine (Sigma-Aldrich) and hydrogen peroxide and analyzed in triplicate on a spectrophotometric plate reader (MRX II; Dynex, Worthing, UK) at 460 nm. Change in absorbance was measured over 3 min, and results are expressed as optical density per minute per gram lung wet weight.

In Vitro Leukocyte Deformability Assay

Blood samples (500–800 µl) were subjected to gentle hypotonic erythrocyte lysis (RBC Lyse, Sigma-Aldrich), centrifuged, and washed. Leukocytes were resuspended in HBSS containing 0.5% BSA (3 × 10⁵ cells/ml, total volume 5 ml). The filtration assay to measure leukocyte deformability was performed according to the method described by Selby and colleagues (44). In brief, samples were filtered through a 5-µm-pore polycarbonate membrane (Poretics) housed in a filter holder (Swin-Lok; Whatman, Kent, UK) at a constant flow rate of 1 ml/min maintained with a syringe infusion pump. Filtration pressure, defined as the rate of increase in pressure upstream to the filter holder over a period of time (cmH₂O/min), was monitored during filtration. HBSS/BSA was filtered alone for 15 min to minimize nonspecific cell binding and to establish the baseline pressure generated by filtration of medium for each membrane. Leukocyte suspensions were then filtered over 5 min, and results are expressed as filtration pressure of leukocyte suspension with baseline pressure subtracted.

Data Analysis

Data were expressed as means ± SD and analyzed either by t-tests or by ANOVA with Scheffe’s tests for multicomparsion. A P value of <0.05 was considered significant.

RESULTS

Flow Cytometric Analysis of Pulmonary PMN Sequestration

In this study, we have developed a flow cytometry-based method for the quantification and analysis of pulmonary PMN. PMN were identified and counted by their positive Gr-1 staining and side scatter properties (Fig. 1A). Quantification of lung-sequestered PMN by this method was found to be sensitive and reproducible (interassay variability <15% for 0.5 × 10⁵ PMN/lung). The method was validated by comparison with MPO assay, a widely used indirect method for the quantification of PMN sequestration in tissues. LPS-challenged mice showed a more than tenfold increase in MPO activity compared with control untreated mice, and the PMN numbers obtained by flow cytometry exhibited a good positive correlation with the MPO data (Fig. 2). Furthermore, expression of surface markers was easily quantified on pulmonary PMN populations, e.g., L-selectin shedding and Mac-1 upregulation could be detected in lungs from LPS-challenged mice (Fig. 1B).

IV Produces Pulmonary PMN Sequestration in the Early Stage of VILI

To investigate the early events of PMN sequestration during VILI, we studied the effects of various modes of mechanical ventilation in mice at an ~1-h time point, before the development of significant physiological signs of lung injury. Throughout the ventilation protocol, cardiorespiratory parameters (BP, blood gas, and respiratory mechanics) were maintained within a physiological range in all animals studied (Table 1). At the end of the protocol, the IV animals exhibited only a small decrease in Crs and increase in Rrs without large changes in arterial P0₂, a picture consistent with a very early phase of VILI in this model, as previously described (59). However, flow cytometric analysis demonstrated that IV had already produced a substantial increase in the number of PMN in the lung compared with CV (P < 0.01) or PV (~2-fold increase, P < 0.05, Fig. 3A). PV did not produce greater PMN sequestration than CV, although the MAP (8.9 ± 0.1 cmH₂O) was similar to IV (8.9 ± 0.2 cmH₂O) and much higher than CV (4.3 ± 0.2 cmH₂O). The observed increase in pulmonary PMN number with IV could not be explained by blood neutrophilia because the IV animals tended to show lower, rather than higher, blood PMN counts than CV or PV animals, and the peripheral blood PMN counts did not show any significant differences between the three groups (Fig. 3B). Moreover, we found no overall correlation between lung and blood PMN numbers in all three ventilated groups (n = 15, R = 0.333, P = 0.226). The total cell counts in lavage fluid were lower in the IV than in the CV group (P < 0.05), in agreement with previous observations (20), but differential cytology showed negligible PMN in all groups (Table 2). This indicates that the PMN were still at the sequestration/adhesion stage and largely confined to the intravascular compartments, with extravasation and transepithelial migration into the alveoli not yet occurred.

Raised Alveolar Pressure Does Not Induce Pulmonary PMN Sequestration

To investigate the effects of raised alveolar pressure on pulmonary PMN sequestration independent of any compound physiological influence of positive pressure ventilation, we studied the effects of CPAP in spontaneously breathing mice. All cardiorespiratory parameters were maintained within a physiological range throughout the 1-h protocol (Table 3). The
flow cytometry data exhibited no difference in pulmonary PMN numbers between low CPAP and high CPAP mice (Fig. 4).

**IV Decreases the Deformability of Circulating PMN**

To investigate the potential contribution of PMN stiffening in mediating sequestration during VILI, we compared the average deformability of circulating leukocytes between CV and IV animals by in vitro cell filtration assay. The filtration pressure required to pass IV leukocytes through the 5-μm polycarbonate membrane was greater than CV leukocytes (0.67 ± 0.25 for IV vs. 0.23 ± 0.02 cmH₂O/min for CV, *P < 0.05*, Fig. 5). The pressure generated by untreated leukocytes from nonventilated animals was similar (0.1–0.3 cmH₂O/min) to CV leukocytes. As a positive control, leukocytes were exposed ex vivo to a priming dose of N-formyl-methionyl-leucyl-phenylalanine (FMLP) (0.5 nM, 5 min; filtration pressure 0.8–1 cmH₂O/min). These results suggest that a loss of PMN deformability occurred and persisted during the early phase of VILI.

**IV Does Not Change Adhesion Molecule Expression on Pulmonary PMN**

To investigate the potential role of adhesion molecules in mediating PMN sequestration during VILI, we evaluated whether in situ expression of L-selectin and Mac-1 (CD11b) on pulmonary PMN differed between CV and IV mice. In both groups, flow cytometric analysis showed lower L-selectin and higher Mac-1 expression on lung PMN compared with peripheral blood PMN (*P < 0.05*, Fig. 6). This phenomenon was also
observed in nonventilated control mice (data not shown), suggesting that L-selectin shedding and CD11b/CD18 upregulation might occur to a certain degree as normal processes of pulmonary PMN sequestration/migration. However, no differences in L-selectin and Mac-1 levels were detected between IV and CV animals on lung or blood PMN.

**IV Induces PMN Sequestration via L-Selectin-Dependent and CD18-Independent Mechanisms**

To further investigate whether constitutively expressed L-selectin and CD11b/CD18 are required for early PMN sequestration during VILI, we studied the effects of blocking antibodies for L-selectin and CD18 in mice ventilated by IV. The IV mice given control IgG showed similar levels of PMN numbers (1.86 ± 0.70 × 10^5/lung) to IV mice not given antibody. The IV mice given anti-L-selectin antibody exhibited substantially lower pulmonary PMN numbers (0.81 ± 0.13 × 10^5/lung) than those given control IgG (P < 0.05, Fig. 7). Compared with the CV animals (0.65 ± 0.20 × 10^5/lung, see Fig. 3A), this implies that >80% of IV-induced PMN sequestration was inhibited by anti-L-selectin antibody. In contrast, no difference was observed in PMN numbers between the IV animals given anti-CD18 antibody (1.75 ± 0.48 × 10^5/lung) and control IgG. In separate experiments using mice challenged with intratracheal LPS, a well-established model of CD18-dependent transepithelial PMN migration (43), we confirmed that this dose of anti-CD18 antibody attenuated PMN percentages in lung lavage cells by ~70% (8.3 ± 4.5% for anti-CD18 vs. 26 ± 3.6% for controls) (P < 0.01, n = 3 per group).

**DISCUSSION**

In this study, we demonstrated that injurious mechanical ventilation in mice produced significant PMN sequestration by the lung at the very early stage of VILI before the development of substantial physiological signs of lung injury. We found that the mechanisms of this early PMN sequestration during VILI could be attributed to a combination of physical stiffening of circulating PMN, presumably in response to inflammatory mediators released during the course of VILI, and L-selectin-mediated leukocyte-endothelial cell interactions. Furthermore, the sequestration was independent of mechanical effects of raised alveolar pressure compressing pulmonary capillaries, and in contrast to PMN sequestration in the lung induced by intravenous inflammatory agents, it was also independent of CD18-mediated leukocyte-endothelial cell interactions.

Pulmonary PMN recruitment has been shown to play a crucial role in the progression of VILI. Kawano et al. (22)

### Table 1. Changes in physiological parameters in mechanically ventilated mice after 1 h

<table>
<thead>
<tr>
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<th>Baseline</th>
<th>End of Protocol</th>
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<tbody>
<tr>
<td>CV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean BP, mmHg</td>
<td>60±9</td>
<td>54±7</td>
</tr>
<tr>
<td>Pao2, mmHg</td>
<td>122±11</td>
<td>111±8</td>
</tr>
<tr>
<td>Paco2, mmHg</td>
<td>36±5</td>
<td>36±4</td>
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<td>pH</td>
<td>7.46±0.05</td>
<td>7.43±0.05</td>
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<tr>
<td>Base excess</td>
<td>1.6±2.7</td>
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</tr>
<tr>
<td>ΔCrs, %</td>
<td>−8.5±2.5</td>
<td>−5.5±5</td>
</tr>
<tr>
<td>ΔRrs, %</td>
<td>−5.8±6</td>
<td></td>
</tr>
<tr>
<td>PV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean BP, mmHg</td>
<td>67±10</td>
<td>54±6</td>
</tr>
<tr>
<td>Pao2, mmHg</td>
<td>124±10</td>
<td>94±13</td>
</tr>
<tr>
<td>Paco2, mmHg</td>
<td>38±4</td>
<td>50±6*</td>
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<tr>
<td>pH</td>
<td>7.44±0.05</td>
<td>7.32±0.04*</td>
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<tr>
<td>Base excess</td>
<td>1.0±3.4</td>
<td>−1.9±2.1</td>
</tr>
<tr>
<td>ΔCrs, %</td>
<td>6.4±6.7*</td>
<td>−5.8±6</td>
</tr>
<tr>
<td>ΔRrs, %</td>
<td>−5.8±6</td>
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<tr>
<td>IV</td>
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<tr>
<td>Mean BP, mmHg</td>
<td>62±9</td>
<td>51±8</td>
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<tr>
<td>Pao2, mmHg</td>
<td>125±16</td>
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<td>Paco2, mmHg</td>
<td>38±7</td>
<td>38±1</td>
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<tr>
<td>pH</td>
<td>7.44±0.04</td>
<td>7.41±0.03</td>
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<tr>
<td>Base excess</td>
<td>1.3±2.5</td>
<td>−0.6±1.6</td>
</tr>
<tr>
<td>ΔCrs, %</td>
<td>−12.5±3.8</td>
<td>−19±5.7*</td>
</tr>
<tr>
<td>ΔRrs, %</td>
<td>−5.8±6</td>
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</table>

Values are means ± SD, n = 9–12 for all groups. *P < 0.01 compared with both other groups. At baseline before the start of the ventilation protocols, no differences were observed among the groups in any of the parameters. Blood pressure (BP) was within a reasonable range expected for C57/BL6 mice anesthetized with Hypnorm and Midazolam (59). Use of supplemental CO2 with injurious ventilation (IV) ensured a normal Pa CO2, whereas the low tidal volume (VT) used in protective ventilation (PV) led to a slightly higher Pa CO2 and lower pH than IV or control ventilation (CV). Crs, respiratory system compliance; Rrs, respiratory system resistance.

### Table 2. Lung lavage cell counts in mechanically ventilated mice

<table>
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<tr>
<th></th>
<th>CV</th>
<th>PV</th>
<th>IV</th>
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<tr>
<td>Total cell count, ×10^3</td>
<td>33.8±20.5</td>
<td>30.8±13.5</td>
<td>8.4±2.4*</td>
</tr>
<tr>
<td>% PMN</td>
<td>0.7±0.4</td>
<td>0.6±0.3</td>
<td>1.1±0.7</td>
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</table>

Values are means ± SD, n = 4–6 for all groups. *P < 0.05 compared with CV. PMN, polymorphonuclear leukocyte.
found that depletion of circulating PMN attenuated VILI in surfactant-depleted rabbits, and Belperio et al. (1) and Quinn et al. (39) recently reported that in vivo inhibition of CXC chemokines reduces pulmonary PMN infiltration and lung injury in rodent models of VILI. Despite such evidence, the mechanisms of PMN recruitment during VILI, in particular the initiating events of PMN sequestration within the pulmonary circulation, have drawn little attention. Most studies on PMN involvement in VILI have investigated the end point or late stages, by which time clear physiological signs of lung injury such as abnormalities in gas exchange and lung mechanics have developed, and/or substantial PMN infiltration is evident in the “intra-alveolar” space, as detected by lung lavage or pathology examination of the lungs (1, 18, 19, 22, 39, 49, 50, 55). Hence, PMN involvement in VILI is generally considered to take place at relatively advanced stages in response to extensive tissue damage and release of mediators (1, 12, 18, 19, 39, 55). The present study was designed to specifically investigate early “intravascular” events of PMN sequestration/adhesion. On the basis of a flow cytometry-based technique that enables quantification of total pulmonary PMN, including intravascular, interstitial, and intra-alveolar cell populations, our results provide the first direct evidence that injurious ventilation can initiate pulmonary PMN sequestration in the early stage of VILI. Without any sign of PMN infiltration within the alveoli, the acutely increased PMN population during the short experimental protocol in this study should largely be confined to intravascular compartments. Moreover, this sequestration phenomenon occurs much earlier than previously considered, i.e., before the development of physiological signs of lung injury and before the process of extravasation.

Table 3. Changes in physiological parameters in spontaneously breathing mice given low and high CPAP for 1 h

<table>
<thead>
<tr>
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<th>Baseline</th>
<th>End of Protocol</th>
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<tr>
<td><strong>Low CPAP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory rate</td>
<td>77±8</td>
<td>67±21</td>
</tr>
<tr>
<td>Mean BP, mmHg</td>
<td>87±12</td>
<td>87±8</td>
</tr>
<tr>
<td>(P_{O_2}), mmHg</td>
<td>116±7</td>
<td>97±14</td>
</tr>
<tr>
<td>(P_{O_2}), mmHg</td>
<td>35±4</td>
<td>36±5</td>
</tr>
<tr>
<td>pH</td>
<td>7.36±0.04</td>
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</tr>
<tr>
<td>Base excess</td>
<td>−5.2±2.5</td>
<td>−2.1±1.4</td>
</tr>
<tr>
<td><strong>High CPAP</strong></td>
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<tr>
<td>Respiratory rate</td>
<td>85±16</td>
<td>82±10</td>
</tr>
<tr>
<td>Mean BP, mmHg</td>
<td>88±7</td>
<td>87±6</td>
</tr>
<tr>
<td>(P_{O_2}), mmHg</td>
<td>118±7</td>
<td>109±8</td>
</tr>
<tr>
<td>(P_{O_2}), mmHg</td>
<td>32±4</td>
<td>36±6</td>
</tr>
<tr>
<td>pH</td>
<td>7.40±0.06</td>
<td>7.39±0.04</td>
</tr>
<tr>
<td>Base excess</td>
<td>−4.1±2.0</td>
<td>−2.8±0.7</td>
</tr>
</tbody>
</table>

Values are means ± SD, \(n = 4\) per group. No significant changes were observed in any of the parameters between low and high continuous positive airway pressure (CPAP) groups, and all parameters were maintained within a physiological range.

Fig. 4. Pulmonary PMN sequestration in spontaneously breathing mice given low (0–1 cmH2O) and high (6–7 cmH2O) continuous positive airway pressure (CPAP) for 1 h. Pulmonary PMN sequestration analyzed by flow cytometry expressed as \(10^5\) PMN per lung. High CPAP did not produce significant PMN sequestration compared with low CPAP (\(n = 4\) per group).

Fig. 5. Leukocyte deformability measured by in vitro cell filtration assay in CV and IV in mice. Filtration pressure was monitored over 5 min, and rate of increase is expressed in cmH2O/min. Leukocytes from IV animals produced a higher filtration pressure than leukocytes from CV animals (\(n = 5\) per group, *\(P < 0.05\)).

A

![A](http://ajplung.physiology.org/)

B

![B](http://ajplung.physiology.org/)

Fig. 6. L-selectin and Mac-1 expression on pulmonary and circulating peripheral blood PMN in CV and IV in mice. L-selectin and Mac-1 were measured on lung and blood PMN populations by flow cytometry and expressed as mean fluorescent intensity. A: L-selectin expression is lower on lung PMN than blood PMN, but there is no difference between CV and IV in both lung and blood PMN. B: Mac-1 expression is higher on lung PMN than blood PMN, but there is no difference between CV and IV in both lung and blood PMN (\(n = 5\) per group, *\(P < 0.05\)).
and transepithelial migration of PMN into the alveolar space, as recently suggested by Uhlig et al. (36).

The present study also investigated the mechanisms of how pure mechanical ventilatory stress leads to this early PMN sequestration in the lung. It has been reported that raised alveolar pressure (and hence raised transpulmonary pressure) may produce temporary PMN entrapment due to the compression of alveolar capillaries, resulting in a greater discrepancy between PMN and capillary diameters. Markus and colleagues (31) found a transient arterio-venous gradient in circulating PMN counts during brief application of PEEP in ventilated rabbits, whereas Loick et al. (30) reported similar findings with PEEP in ventilated patients after cardiopulmonary bypass. These authors agreed that PEEP induces a transient PMN entrapment in the lung, but it remained to be investigated whether a longer application of PEEP results in a prolonged or steady-state sequestration of PMN. To ascertain the contribution of sustained elevation in alveolar pressure toward PMN sequestration, we studied an additional ventilatory protocol, PV, which has a high MAP similar to IV, but used a protective ventilator mode with low $V_T$. We found that PV did not increase PMN numbers more than CV despite the increased MAP. To confirm this finding in an experimental setting that was independent of any compound influence of different modes of positive pressure ventilation, we directly studied the effects of different levels of MAP in isoflurane-anesthetized, spontaneously breathing mice. Although it may be difficult to compare these two experiments directly due to potential effects of different anesthetics used, the results in spontaneously breathing animals also indicate that PMN numbers were not enhanced by increasing CPAP and MAP. Thus the previously reported “mechanical” PMN entrapment by PEEP is indeed a transient phenomenon as speculated by these authors, and raised mean alveolar pressure during mechanical ventilation is by itself unlikely to produce prolonged PMN sequestration.

In vitro cell filtration assay demonstrated a small but significant steady-state decrease in the deformability of circulating leukocytes in IV compared with CV mice. In vitro exposure of PMN to inflammatory agents, e.g., complement and bacterial components or chemokines, induces a very rapid (within minutes) yet “transient” (reversible by 15–20 min) decrease in PMN deformability (5, 9, 21, 37, 60) due to the polymerization of G-actin to F-actin (21, 34, 60). Such PMN stiffening is considered to play a major role in vivo in triggering the initial rapid (<5 min) PMN sequestration by the lung in response to intravenous challenge of these inflammatory agents (5). Some clinical studies have found a “steady-state” decrease in PMN deformability in blood taken from patients with sepsis (23, 47) and acute exacerbation in chronic obstructive pulmonary disease (44), suggesting that stiffening can persist and potentially contribute to prolonged PMN sequestration and lung inflammation in these diseases. On the basis of these findings, our results suggest that loss of PMN deformability could contribute, at least in part, to the enhanced PMN sequestration during VILI. We speculate that the loss of PMN deformability is produced by systemic (in the central circulation) and/or local (in the pulmonary microcirculation as PMN pass through the capillary bed) exposure of PMN to inflammatory mediators, which are released and persisted throughout our 1-h ventilation protocol. Potential candidates for such mediators of PMN stiffening during VILI include CXC chemokines, e.g., macrophage inflammatory protein-2, for which an important role in the progression of VILI has been demonstrated (1, 39). Another CXC chemokine, cytokine-induced neutrophil chemoattractant in rats, a homolog of keratinocyte-derived chemokine in mice, has recently been shown to move from the lung into the vascular compartment where it may promote PMN migration into the lung (40). Other chemoattractants that may be involved include platelet-activating factor, prostaglandins, and leukotriene B4 (2, 19, 33, 58).

We further investigated whether PMN cell adhesion molecules, L-selectin and the β2-integrin complex CD11b/CD18, are involved in the early PMN sequestration in VILI. During the general migration process of PMN, L-selectin is shed from the cell surface and Mac-1 is upregulated upon activation (28, 58), and several studies reported such changes in expression during acute lung injury. In patients with acute respiratory distress syndrome (ARDS), increased Mac-1 and decreased L-selectin on blood and lavage PMN have been observed (3, 46). In a rat model of VILI, Imanaka et al. (20) and Ohta et al. (35) detected Mac-1 upregulation on PMN that had migrated into the alveolar space. Although their VILI model was similar to ours, using a short period (40 min) of injurious high $V_T$ ventilation, the model was also associated with changes in lung histology and substantial intra-alveolar PMN infiltration, thus representing a more advanced stage of VILI. In our mouse model of an early stage of VILI before the intra-alveolar migration of PMN, L-selectin and Mac-1 expression on both lung-sequestered and circulating PMN were similar between IV and CV. This suggests that the IV protocol used in our study was not sufficient to produce changes in adhesion molecule expression on PMN in mice. Alternatively, the time point of our measurement (1 h) may have been too early to detect any potential changes in these molecules’ expression in mice.
do not appear to be involved. This is in sharp contrast to PMN sequestration in the lung with intravenous challenge of inflammatory agents, whereby both L-selectin and CD11b/CD18 are essential for prolonged sequestration in the pulmonary capillaries in response to complement fragments (6, 10, 24), FMLP (36) and LPS (25). Our results provide strong evidence that different and unique pathways are involved in pulmonary PMN sequestration in response to mechanical ventilatory stress compared with sequestration in response to pure inflammatory stress.

Our mouse model provided a reproducible physiological picture consistent with an early phase of VILI. Throughout the 1-h ventilation period the mice displayed stable cardiopulmonary function with only small changes in lung mechanics, no deterioration in blood gas, and no evidence of intra-alveolar PMN infiltration. Our previous study confirmed that a longer period of similar IV (2–3 h) produces progressive pulmonary edema, deteriorated lung mechanics, cardiorespiratory collapse, and lung pathology representative of VILI with hyaline membrane formation and epithelial damage (59). Although the VT employed in this study was considerably higher than those used in clinical settings, it is similar to those used in many previous animal studies (11, 14, 20, 35, 41, 52, 53) that have contributed greatly to our understanding of the mechanisms of VILI. Moreover, it has been suggested that such high VT in animals may be experimentally comparable to the excessive overdistension expected in some part of the ARDS lungs, because the heterogeneous nature of lung injury in ARDS often leads to certain lung regions receiving the bulk of the delivered gas (8, 15, 38). Finally, Soutiere and Mitzner (48) have recently demonstrated that intact mouse lungs can be temporarily inflated with pressures >60 cmH2O, relating to a VT of 60–70 ml/kg, without reaching a traditionally defined total lung capacity (i.e., plateau of the pressure volume curve) or producing apparent morphological damage. Attempts to directly compare the absolute values of “injurious” VT between mice and other species, particularly humans, may be misleading due to the much larger compliance of the mouse respiratory system at high lung volumes.

It is important to note that the PMN populations obtained in this study may differ slightly from cell populations studied in previous literature of PMN sequestration (6, 10, 16, 24–26, 29). Pulmonary arterial perfusion was performed before lung removal, which we found to produce greater consistency than without perfusion, enabling sensitive detection of relatively small changes in PMN numbers. The poor reproducibility in nonperfused lungs is likely to originate from the influence of freely moving, nonadherent leukocytes in the residual blood within the lung, not only in the capillaries but also in larger vessels, which may produce greater variability in PMN counts between mice. In contrast to studies using intravital microscopy (16, 25, 26, 29) or morphometric analysis whereby the lung has been excised with the major vessels ligated to minimize blood loss (6, 10, 24), our method aims to minimize nonadherent PMN from the studied PMN population.

In summary, the present study offers insights into physiological and molecular mechanisms by which pure mechanical stress applied to the lung can lead to PMN recruitment in VILI. We demonstrated for the first time that enhanced pulmonary PMN sequestration is evident at an early stage of VILI, preceding substantial development of physiological signs of lung injury. Furthermore, our results provide evidence that this early PMN sequestration is associated with prolonged stiffening of circulating PMN and mediated by L-selectin interactions, whereas the sequestration process is independent of CD18 integrin-mediated interactions or raised alveolar pressure during mechanical ventilation. Further studies are required to elucidate the complete picture of specific mechanisms involved in PMN sequestration and migration during VILI, which may lead to new therapeutic strategies targeting PMN recruitment.

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REFERENCES


