

Partial Liquid Ventilation does not Affect BALF TNF- α , MIP-2, CINC-1 Concentrations, or CD11b Cell Surface Expression, but does Increase Macrophage Proportion among BALF Cells in the Acute Phase of Rat LPS-Induced Lung Injury

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To elucidate the mechanism of anti-inflammatory effect of partial liquid ventilation (PLV), cytokine concentration, surface CD11b, and macrophage expression were investigated in BALF. The 30-minutes group was treated with gas ventilation (GV) for 30 minutes after intratracheal LPS administration. The GV group was prepared in the same manner as the 30-minutes group, then the GV was continued for the following 2 hours. The PLV group was treated in the same manner as the 30-minutes group, and then received PLV with perflubron for the following 2 hours. Animals were euthanized to receive BAL. The PLV group showed a tendency to have a higher concentration than the GV group of TNF- α , MIP-2, and CINC-1 as measured by ELISA, although there were no significant differences. The ratio of expressions of CD11b and macrophages to total leukocytes were determined by flow-cytometry. There were no significant differences in the ratio of CD11b-positive expression to acquired cells (GV: $63.6 \pm 8.4\%$, PLV: $60.5 \pm 5.4\%$, $P = 0.73$). However, the proportion of macrophages was significantly increased (GV: 5.6 ± 1.5 , PLV: 14.0 ± 1.3 , $P = 0.006$). These results suggest that the anti-inflammatory effect of PLV is not caused by the change in CD11b expression, and that PLV affects the proportion of macrophage among BALF cells.

Key words: partial liquid ventilation, anti-inflammatory effect, BAL, cytokine, flow cytometry

Partial liquid ventilation (PLV) with perfluorocarbon (PFC) has been investigated as a treatment for acute respiratory distress syndrome (ARDS) in both the experimental and clinical settings. It is evident that PLV improves gas exchange and pulmonary compliance in experimental animals [1-6]. These beneficial features have also been corroborated in a clinical

setting [1, 7-13]. Several *in vitro* studies have been reported. PFC exposed to alveolar macrophages produced less hydrogen peroxide [14]. Lipopolysaccharide (LPS)-induced tumor necrosis factor- α (TNF- α), interleukin-1, and interleukin-6 productions were inhibited in the PFC-exposed human macrophages [15]. Neutrophils exposed to PFC *in vitro* produce less hydrogen peroxide, and have a lower chemotactic response [16].

Based on the findings in these previous studies, PLV apparently has some anti-inflammatory effect. However, the mechanisms of this effect remain unknown. Although

we consider it not necessary to prove the anti-inflammatory effect of PLV, we do need to investigate the mechanisms of the anti-inflammatory effect of PLV. We considered the following four hypotheses as mechanisms of PLV. First, contact with PFC may affect inflammatory cells such as neutrophils and macrophages that have already been activated and migrated into alveoli to change their function. Second, PFC may act as a physical barrier, lining the surface of alveolar epithelial cells then preventing the influx of inflammatory cells or chemical mediators from interstitial space into the alveoli. Third, PFC may also cover the surface of inflammatory cells that were activated and migrated into alveoli as a physical barrier, blocking further interaction for these cells. Fourth, PFC administered into the airway may lavage inflammatory chemical mediators such as cytokines from the distal airways, then remove them from the lung by suction that occurs during PLV, resulting in a lung-protective effect. Subsequently, we investigated CD11b cell surface marker up-regulation to verify these hypotheses.

Neutrophil-mediated tissue injury is a common mechanism underlying the development of organ dysfunction during acute lung injury including ARDS. A CD11b surface marker plays an important role for neutrophils when it is up-regulated, allowing firm adhesion with intercellular adhesion molecule-1 on the endothelial surface, after which the migration or infiltration of neutrophils occurs. The neutrophils that have infiltrated and accumulated in the lungs release reactive oxygen species or proteases, then cause micro-endothelial injury and lung edema characterized by hyperpermeability. Will the contact with PFC change the surface marker up-regulation for neutrophils migrating into alveoli *in vivo*? If some difference were observed in the cell surface marker up-regulation between the GV and the PLV groups, we might hypothesize that the PLV affect on neutrophils that have already activated and migrated into the alveolar space is to inactivate their function. In that event, we could simply focus on the first and the third hypotheses as the mechanisms of the anti-inflammatory effect. In addition, we investigated the macrophage population among bronchoalveolar lavage (BAL) cells that is said to produce cytokines and chemokines. To our knowledge, there have been no previous reports on the number of macrophages in PLV. We undertook to study the cytokine concentration in bronchoalveolar lavage fluid (BALF) to confirm that cytokine concentration is attenuated in PLV. We

also tried to manifest the effect of PLV in the early phase of inflammation after lung injury was induced in the lungs. For this purpose, we chose two C-X-C chemokines, macrophage inflammatory protein (MIP) -2 and cytokine-induced neutrophil chemoattractant (CINC) -1, which shows its peak 90 to 120 minutes after lung injury, neither of which had yet been investigated. We adopted a rat model of acute lung injury by intratracheal LPS administration, which induces rapid lung injury [17], so that the peak time of TNF- α , MIP-2 and CINC-1 elevations could be easily assumed.

Materials and Methods

Animal model. Male Wistar pathogen-free rats weighing 250 – 300 g (283.2 ± 9.8 g, Japan SLC, Hamamatsu, Japan) were anesthetized with 50 mg/kg of intraperitoneal sodium pentobarbital. An open tracheostomy was performed and a 14-gauge angiocatheter (Becton-Dickinson, CA, USA), which was connected to a three-way stopcock with a side adapter tube, was secured.

The rats were ventilated with a volume-controlled mechanical ventilator (Shinano seisakusho, model SN-480-7), with tidal volume = 10 ml/kg, $FiO_2 = 1.0$, and respiratory rate = 30 breath/min. Pancronium bromide 0.1 mg was injected in the femoral muscle every hour. After 15 minutes of gas ventilation, 10 mg/kg of LPS (from *Escherichia coli* Serotype 0111:B4, Sigma Chemical Co., St. Louis, MO, USA) dissolved in 1 ml/kg of saline was administered intratracheally. All rats then received gas ventilation for the next initial 30 minutes.

Group 1 (Negative control, n = 9): Rats were euthanized at 30 minutes after LPS administration. This group, sacrificed prior to initiation of the 2-hour experimental period, served as a “negative” control to identify the degree of inflammatory response. Group 2 (gas ventilation: GV, n = 11): GV continued to receive LPS for two more hours (2 hours and 30 minutes in total). No airway suction was performed. Group 3 (partial liquid ventilation: PLV, n = 11): Animals received administration of 8 ml/kg of Perflubron (Green Cross Co., Osaka, Japan) as PFC over 3 minutes through the side port of the three-way stopcock 30 minutes after LPS administration. Just after the administration of perflubron, the side port of the stopcock was opened once to the outside air then closed again. This was to remove the extra air in the ventilator circuit, in order to prevent ventilator-associated

lung injury that might have occurred in response to high tidal volume caused by the sum of the gas ventilator tidal volume and perflubron. PLV was then continued for 2 hours. No additional perflubron was administered and no suction from the lungs was performed during PLV. All animals underwent euthanasia at completion of the preparation by intraperitoneal pentobarbital overdose. Subsequent exsanguination was performed by incision of the abdominal aorta.

Bronchoalveolar Lavage (BAL). Immediately after euthanasia and exsanguination, 8-ml aliquots of phosphate-buffered saline (PBS, pH 7.4) were slowly infused in the lungs through the tracheostomy then withdrawn gently. No drainage or suction of perflubron or exudate in the airway was done before this lavage. This lavage was repeated four times using the same syringe. The collected lavage fluid was stored in a 50-ml tube on ice. The fluid was centrifuged at $400 \times g$ for 5 minutes within several hours, and the cell-free supernatants were stored in microcentrifuge tubes at -80 centigrade in a refrigerator for subsequent cytokine analysis. The pellet was re-suspended in 10 mL of PBS. Contaminating erythrocytes were lysed with 1 mL of NH_4Cl solution for 5 minutes and washed with PBS. After washing twice with PBS, the pellet was re-suspended in 10 mL of RPMI-1640 (Nissho 87-640-0, Osaka, Japan) with 1% fetal bovine serum. Total leukocyte cell count was performed by Toluidine blue-O stain. Cell differentiation analysis was not performed.

All animals received humane care in compliance with the "Principles of Laboratory Animal Care" included in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Science and published by the National Institute of Health (National Institute of Health publication No. 85-23, revised 1985), and also with the "Guidelines for Animal Experiments of the Okayama University Medical School".

Measurement of $\text{TNF-}\alpha$, MIP-2 , and CINC-1 . BAL total protein concentration was measured by the Bradford method [18]. The concentrations of $\text{TNF-}\alpha$, MIP-2 , and CINC-1 in the BALF supernatant were quantitated by Enzyme-linked immunosorbent assay (ELISA). A rat $\text{TNF-}\alpha$ ELISA Kit #KRC3010SB (BioSource International, Inc., CA, USA), Rat MIP-2 ELISA Kit #KRC1022 (BioSource International, Inc., CA, USA), and Rat GRO/CINC measuring Kit-IBL (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan) were used for the assays. Samples

were diluted with PBS within an appropriate range to achieve the same dilution ratio, because the concentrations of the samples were out of range of measurement with the kit. The absorbance at 450 nm wavelength was measured by a microplate reader Immuno-mini NJ-2300 (System Instruments Co., Ltd., Tokyo, Japan). All samples were analyzed in duplicate using a blinded method.

CD45 , CD11b , and macrophage expression. BAL cells were harvested and analyzed for the expression of CD11b and for macrophage surface marker by flow cytometry within the same day. A suspension containing about 1×10^6 of cells was used for each flow cytometric analysis. The suspension was mixed with $1 \mu\text{g}$ of FITC-conjugated mouse anti-rat CD11b monoclonal antibody (mAb, clone OX-42). One μg of FITC-conjugated mouse anti-Rat IgG_{2a} isotypic control mAb (clone R35-95) was added into another tube containing a suspension of cells from the same sample. These samples were incubated for 20 minutes at room temperature. After washing 3 times with PBS, CD11b receptor expression was analyzed by a flow cytometer FACS Calibur (Becton Dickinson, CA, USA). Ten thousand individual cells were captured in the cytometer using a neutrophil gate set for both forward scatter and side scatter. Fluorescence was measured using a FL-1 detector. The total number of CD11b positive cells was determined by assessing positive cells of isotypic control mAb (Fig. 1). The ratio of cells positive for CD11b expression to total cells was then compared between the two groups of GV and PLV.

For macrophage identification, R-PE-conjugated mouse anti-rat macrophage subset mAb (clone HIS36) or R-PE-conjugated mouse anti-Rat $\text{IgG}_1 \kappa$, isotypic control mAb (clone R3-34) was used for analysis. A FL-2 detector was used for PE-conjugated mAbs, and no gate was used for this measurement of anti-rat macrophage subset mAb expression. The positive ratio of macrophage expression to total cells was determined using the same marker in a histogram that excluded positive cells of the isotypic control mAb (Fig. 2).

All mAbs were purchased from PharMingen (CA, USA) via Fujisawa Pharmaceutical Co., Ltd. (Tokyo, Japan).

Statistical analysis. Data are shown as mean \pm SEM. Statistical tests were carried out between the experimental groups of GV and PLV using the Mann-Whitney U test. Statistical significance was

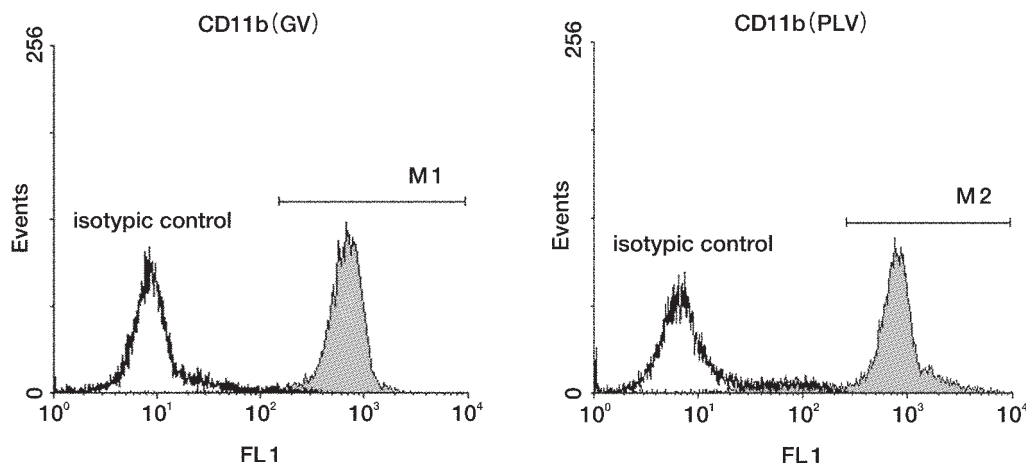


Fig. 1 Examples of flow cytometry histograms are shown. Individual cells ($n = 10000$) were captured into a cytometer using a neutrophil gate. Fluorescence of FITC was measured using a FL-1 detector. In the GV sample (left), the ratio of cells positive for CD11b expression was determined using a marker (M1) in the histogram that excluded 99 % of the positive cells of the isotypic control. The same procedure was performed on the PLV sample (right) using marker (M2). These markers were determined for each sample, and the ratio of cells positive for CD11b to total cell number was calculated.

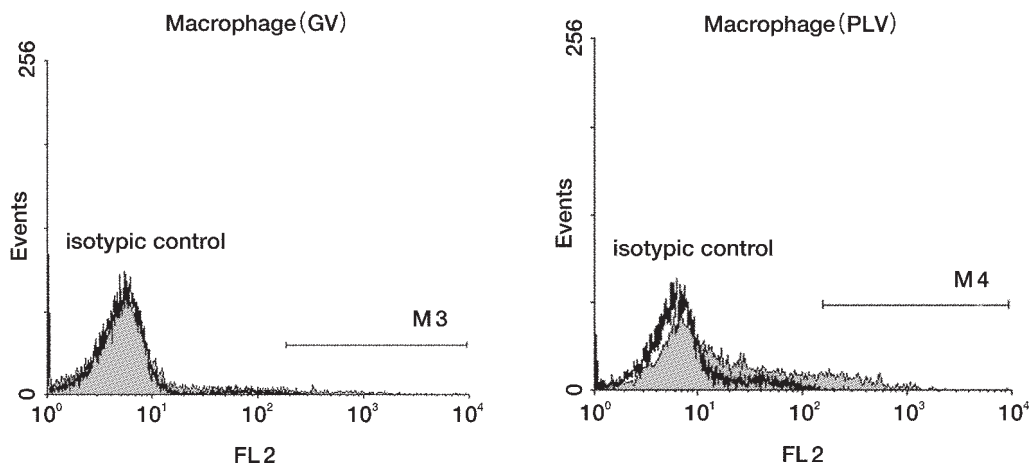


Fig. 2 Examples of flow cytometry histograms are shown. Individual cells ($n = 10000$) were captured into a cytometer. Fluorescence of PE was measured using a FL-2 detector. In the GV sample (left), the ratio of positive macrophage expression was determined using a marker (M3) in the histogram that excluded 99 % of the positive cells of the isotypic control. The same procedure was performed on the PLV sample (right) using marker (M4). These markers were determined for each sample, and ratio of cells showing positive anti-macrophage mAb expression to total cell number was calculated.

accepted at $P < 0.05$.

Results

All animals survived until the end of the experiment.
BAL recovery volume. Bronchoalveolar lavage

fluid (BALF) volumes for the three groups were as follows: negative control (LPS 30 min): 31.9 ± 0.1 ml, GV: 30 ± 0.6 ml, PLV: 30.2 ± 0.3 ml. No significant differences in BAL recovery volume were observed between GV and PLV groups.

BALF supernatant analysis. BALF super-

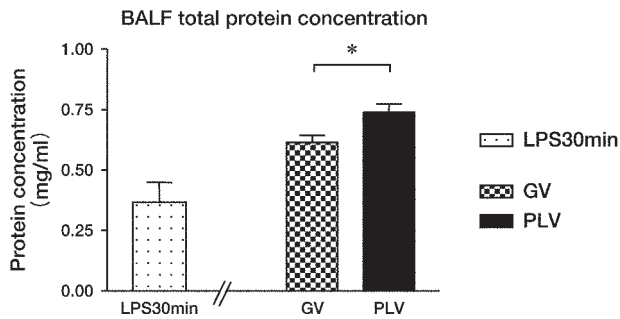


Fig. 3 BALF total protein concentration per 1 ml of BALF is shown. BALF total protein concentrations were measured by the Bradford method. Although there was no difference in BALF recovery volume among the 3 groups, protein concentrations were standardized by the volume here. The PLV group demonstrated a significantly higher concentration than that in the GV group (LPS 30 min: 0.37 ± 0.08 , GV: 0.60 ± 0.03 vs. PLV: 0.74 ± 0.03 , $P = 0.02$).

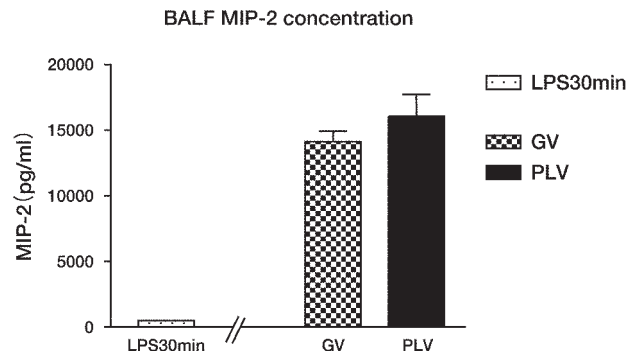


Fig. 5 BALF supernatant MIP-2 concentration is shown. When compared with the negative control group of LPS 30 min, the GV and PLV groups demonstrated significant increases in MIP-2. The PLV group seemed to have a higher concentration than that in the GV group, though the difference between the GV and the PLV groups was not significant (LPS 30 min: 398.8 ± 64.7 , GV: 14071.5 ± 809.9 vs. PLV: 16030.3 ± 1663.9 , $P = 0.64$).

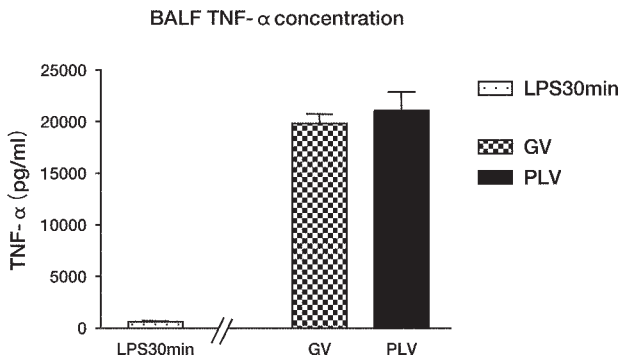


Fig. 4 BALF supernatant TNF- α concentration is shown. BALF TNF- α levels at 30 min after LPS administration in GV and PLV animals are demonstrated. The response of TNF- α in BALF supernatant was unclear at 30 min after LPS intratracheal administration. After an additional 2 h of GV or PLV, TNF- α concentration increased markedly. There was no statistical difference in TNF- α concentration between the GV and PLV groups (LPS30 min: 637.55 ± 70.0 , GV: 19808.5 ± 922.9 vs. PLV: 21062.8 ± 1816.3 , $P = 1.00$).

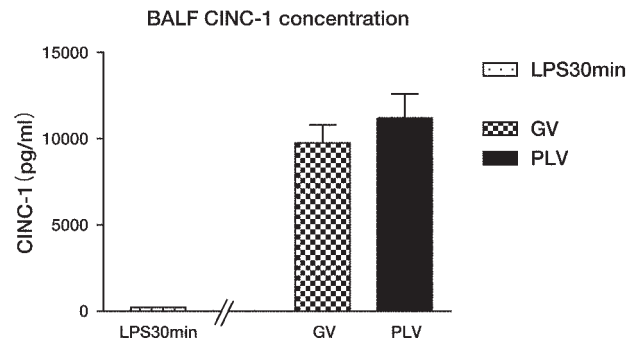


Fig. 6 BALF supernatant CINC-1 is shown. As was the case for TNF- α and MIP-2, the concentration of CINC-1 demonstrated a significant increase in both the GV and PLV group in comparison with levels in the negative control group. No significant difference was observed between the GV and the PLV groups (LPS 30 min: 192.6 ± 41.9 , GV: 9728.2 ± 1047.4 vs. PLV: 11177.1 ± 1411.5 , $P = 0.6$).

nant total protein per unit BALF volume was assessed. Compared to the 30-minute ventilation group, PLV demonstrated about twice as much protein, and the GV group showed significantly lower total protein concentration than that in the PLV group (LPS 30 min: 0.37 ± 0.08 , GV: 0.60 ± 0.03 vs. PLV: 0.74 ± 0.03 , $P = 0.02$) (Fig. 3). Compared with the negative control group, the GV and PLV groups demonstrated a significant increase in TNF- α (LPS 30 min: 637.5 ± 70.0 , GV: 19808.5 ± 922.9 vs. PLV: 21062.8 ± 1816.3 , $P = 1.00$)

(Fig. 4). The PLV group showed a tendency to have a higher TNF- α value than that in the GV group, though no statistical difference between two groups could be identified. The same tendency for higher TNF- α was observed in both MIP-2 (LPS 30 min: 398.8 ± 64.7 , GV: 14071.5 ± 809.9 vs. PLV: 16030.3 ± 1663.9 , $P = 0.64$) (Fig. 5) and CINC-1 (LPS 30 min: 192.6 ± 41.9 , GV: 9728.2 ± 1047.4 vs. PLV: 11177.1 ± 1411.5 , $P = 0.6$) (Fig. 6).

Flow cytometric analysis. The number of

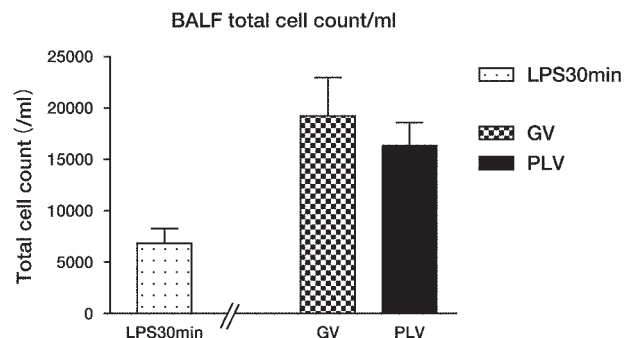


Fig. 7 Total cell count per 1 ml of BALF, measured by Toluidine-blue O stain, is shown.

Total cell number increased during 2 h of GV or PLV. The PLV group demonstrated a trend to have fewer cells, though there were no statistical differences between the GV and PLV groups (LPS 30 min: 6825.0 ± 1435.7 , GV: 19269.0 ± 3751.6 vs. PLV: 16368.0 ± 2258.8 , $P = 0.76$).

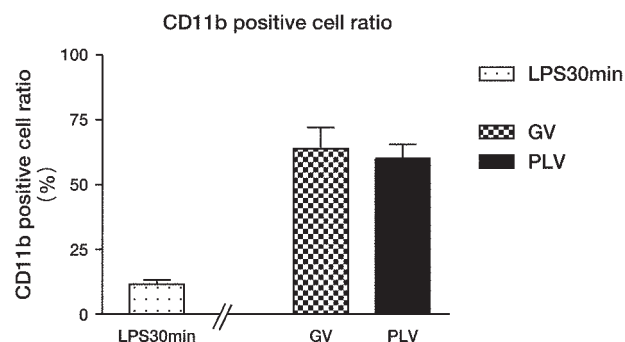


Fig. 8 CD11b surface expression is shown. CD11b expression is demonstrated as a ratio. The ratio of anti rat CD11b mAb positive cells to total acquired cells (1×10^4 cells) was calculated. Both the GV and the PLV groups showed marked increases in CD11b expression in comparison with the negative control. However, there was no statistical difference between the GV and PLV groups (LPS 30 min: 11.2 ± 1.6 , GV: 63.6 ± 8.4 vs. PLV: 60.5 ± 5.4 , $P = 0.73$).

total cells per ml in the BALF pellet was counted. The total cell counts in both the GV and the PLV groups increased to twice that of the control group (LPS 30 min) during two hours, though no statistical differences were found between GV and PLV (LPS 30 min: 6825.0 ± 1435.7 , GV: 19269.0 ± 3751.6 vs. PLV: 16368.0 ± 2258.8 , $P = 0.76$) (Fig. 7).

The percentage of cells positive for CD11b surface expression among 10000 cells is demonstrated (LPS 30 min: 11.2 ± 1.6 , GV: 63.6 ± 8.4 vs. PLV: 60.5 ± 5.4 ,

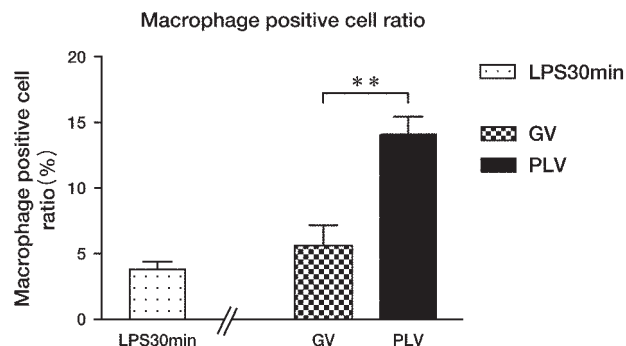


Fig. 9 An anti-macrophage subset expression is shown. The anti-macrophage subset expression is demonstrated as a ratio. The ratio of anti-macrophage mAb positive cells among total acquired cells (1×10^4 cells) was calculated. The proportion of macrophage expression was significantly higher in the PLV group than in the GV group (LPS 30 min: 3.7 ± 0.5 , GV: 5.6 ± 1.5 vs. PLV: 14.0 ± 1.3 , $P = 0.006$).

$P = 0.73$) (Fig. 8). Both the GV and PLV groups showed marked increases in the CD11b expression ratio when compared to the negative control group (30-minute group). However, no significant difference in CD11b expression between the GV and PLV groups was identified.

The percentage of cells positive for anti-rat macrophage subset mAb surface expression is demonstrated (LPS 30 min: 3.7 ± 0.5 , GV: 5.6 ± 1.5 vs. PLV: 14.0 ± 1.3 , $P = 0.006$) (Fig. 9). The positive ratio of the anti-rat macrophage mAb expression in the PLV group was significantly greater than that of the GV groups.

Discussion

Two rat C-X-C chemokines, MIP-2 and CINC, are generated mainly by macrophages in response to TNF- α [19, 20]. It is reported that both MIP-2 and CINC result in neutrophil activation and migration [21-23]. Subsequent neutrophil accumulation and resulting lung injury are reduced by administration of anti-MIP-2 and anti-CINC mAb. Several studies have suggested that pulmonary neutrophil accumulation was also decreased in PLV groups in the setting of lung injury [24-29].

In this study, we performed no suction from the airway during GV or PLV. Generally, suction of exudate from the lung is performed via intratracheal tube during PLV. A PFC can lavage exudate from the alveoli to the central airway easily because of its large density

(perflubron: 1.98 g/ml) and low surface tension. The elimination of exudate, which is thought to be full of these cytokines and chemokines from the lung, is beneficial to prevent the progression of inflammation. We thought that the suction of exudate from the airway typically performed during PLV might play an important role. At this point, we developed the idea of performing no suction from the airway during PLV, and compared the group in which no suction was performed with the GV group. We supposed that PFC might act as a physical barrier for the alveoli by covering its surface, thereby suppressing the influx of inflammatory mediator or cells into the alveoli as mentioned earlier. The characteristics of inflammatory cells such as neutrophils or macrophages might be changed by contact with PFC. The CD11b cell surface expression would be attenuated as a result. If these suppositions were true, CD11b surface expression would be attenuated by PLV, and the production of cytokine as an inflammatory mediator in the PLV group, even without any airway suction, would be attenuated as a result.

In this study, we found no significant difference in BALF supernatant TNF- α , MIP-2, or CINC-1 concentration between the GV and the PLV groups. The CD11b cell surface expression was up-regulated in BAL during GV and PLV 2.5 hours after intratracheal LPS administration, though no difference between the GV and PLV groups was noted. From these results, we suppose that the anti-inflammatory effect of PLV in terms of cytokine inhibition is caused mainly by the lavage effect in the *in vivo* setting. Actually, TNF- α , MIP-2, and CINC-1 concentrations tended to be higher in the PLV group than in the GV group, although there were no significant differences between these groups. To explain this, we came up with three possible explanations. Weiss *et al.* reported that PFC liquid, when instilled into the lungs intratracheally, might cause transient pulmonary inflammatory response in the rodent model [30]. Given that our measurement was performed in the acute phase, after lung injury had been initiated, this transient inflammation is the first assumption made by way of explanation. The second assumption is that the distribution of PFC in alveoli might have helped to flush more cytokine or chemokine when BAL was performed, even if the cytokine accumulated in the lungs was approximately equal in both GV and PLV groups. The third assumption is that the increased macrophage proportion among BAL cells might be actually associated with increased cytokine and chemokine production.

Several studies have reported reduced production of TNF- α or other cytokines in the *in vivo* experimental setting of PLV. Our result was contrary to these commonly observed results. We speculate that this difference in results can be mainly attributed to the fact that we performed no suction from the airway during PLV. The descriptions of suction performed during PLV are unclear in many reports on PLV. We consider that a detailed description of the airway suction procedure should be noted.

In our results, the proportion of macrophages among BAL cells was significantly increased. It is noted that our result on macrophages did not reflect numbers of actual cells. However, based on the result of the BAL total cell count (that there were no differences between the GV and PLV groups), the actual number of macrophages might be increased in PLV. Van Eeden *et al.*, using a rabbit model, reported the histological finding of fewer alveolar macrophages in the PLV group than in other groups [31]. The reason our results differed from theirs is unknown, though there are some differences in details of the study models such as duration of PLV. We regret that we did not perform a cell differentiation count when getting the BAL samples and conduct a histological analysis.

Our experimental model was the LPS intratracheal administration model. Intratracheal endotoxin administration causes an inflammatory lung injury that results in the rapid recruitment of activated white cells to the lungs and the production of TNF- α and other proinflammatory cytokines [17, 32]. In our preliminary experiment, there were no significant differences in blood pressure or arterial blood gas analysis up to 3 hours after LPS administration. Therefore, we did not perform these measurements to eliminate other factors, such as blood loss, which can influence sensitive cytokine measurements. The concentration of TNF- α , MIP-2, and CINC-1 increased markedly in 2.5 hours after LPS intratracheal administration, but this duration may not be enough to cause changes in these physiological data.

A discussion can be raised at this point about the duration of PLV for 2 hours, and about the timing of initiating PLV at 30 minutes after LPS administration. Apparently, 2-hours duration is not sufficient for inflammation to be completed in the lungs. And it is impossible, in fact, to start PLV 30 minutes after lung injury occurs in actual clinical settings. The aim of this study was to manifest the mechanism underlying the

anti-inflammatory effect of PLV, not to mimic the clinical settings. Thus, we tried to start PLV before completion of inflammation in the lungs, then performed BAL to measure cytokines and chemokines at the peak point of response. Baba *et al.* reported that the inhibitory effect of PLV on IL-8 was more apparent when PFC was added earlier in the *in vitro* setting [33]. And for cytokine and chemokine measurement, it was necessary to obtain samples at the time of peak elevation; that is, at 2 hours.

Our result that CD11b surface expression was not affected by PLV was somewhat surprising to us. Varani *et al.* suggested that PFC reduces neutrophil-mediated injury to epithelial cells only if PFC is present at the time at which the neutrophils are added to the target cells [34]. When the neutrophils are added to epithelial cell monolayers and are stimulated prior to PFC administration, no protective effect for the lungs was present. Thus, as an explanation for our result of CD11b surface expression, the neutrophils might have already been primed by LPS in the initial 30 minutes. Thus, even though PFC was administered into the lungs, the surface expression of CD11b might have already been established, and was therefore not altered by the contact with PFC.

In summary, PLV does not affect CD11b cell surface marker up-regulation when started 30 minutes after LPS intratracheal administration. PLV caused no significant attenuation in the BALF supernatant TNF- α , MIP-2, or CINC-1 concentration when no airway suction was performed during PLV. The proportion of macrophages among BAL cells was increased by PLV. From these results, we conclude that anti-inflammatory effect of PLV is not caused by the change in CD11b expression, and that PLV affects the proportion of macrophages among BAL cells. Our future study will include investigation of the lavage effect of PLV.

Elucidation of the mechanism of PLV's anti-inflammatory effect is important for obtaining maximum benefit of PLV in a clinical setting.

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