Table 3—*Pulmonary Function, Radiologic Appearance, Serum Biomarkers, Hematologic Indexes, and BALF Cell Findings in Patients With PAP in FR and AT Groups at the End of GM-CSF Inhalation Treatment and Before the 30-Mo Observation*

Measure	FR		AT		*P* Value
	No.	Mean ± SE or Median (IQR)	No.	Mean ± SE or Median (IQR)	
Pulmonary function					
VC, % predicted	23	93.4 ± 3.0	12	74.2 ± 4.2	.0007[a]
FVC, % predicted	23	80.5 ± 3.3	12	72.2 ± 4.5	.0025[a]
FEV_1/FVC	23	85.6 ± 1.6	12	84.7 ± 2.2	.73[a]
DLCO, % predicted	23	68.4 ± 3.4	11	46.8 ± 4.7	.0006[a]
HRCT scan scores[b]					
Upper lung region	23	2 (2-3)	12	3.5 (2-4)	.036[c]
Middle lung region	23	3 (2-3)	12	4 (2.25-4.75)	.023[c]
Lower lung region	23	2 (2-3)	12	4 (2.25-5)	.0039[c]
Serum biomarkers of PAP					
LDH, IU/L	23	242 ± 13	12	308 ± 18	.0064[a]
CEA, ng/mL	23	2.7 ± 0.6	12	5.7 ± 0.8	.0075[a]
KL-6, U/L	23	3,675 ± 735	12	6,565 ± 1,017	.028[a]
SP-A, ng/mL	23	80 ± 12	12	131 ± 16	.015[a]
SP-D, ng/mL	23	170 ± 34	12	304 ± 47	.027[a]
Hematologic indexes					
WBC count, cells/μL	23	5,213 ± 306	12	5,797 ± 424	.27[a]
Neutrophils, cells/μL	22	2,961 ± 205	12	3,026 ± 277	.85[a]
Monocytes, cells/μL	22	320 ± 30	12	338 ± 41	.74[a]
Lymphocytes, cells/μL	22	1,755 ± 131	12	2,153 ± 177	.080[a]
Eosinophils, cells/μL	22	145 ± 40	12	233 ± 55	.20[a]
Basophils, cells/μL	22	27.4 ± 5.9	12	43.7 ± 8.4	.12[a]
Hemoglobin, g/dL	23	14.8 ± 1.3	12	14.4 ± 1.4	.52[a]
Platelets, × 10^3 cells/μL	23	214 ± 9.0	12	235 ± 12	.17[a]
BALF cell classification, %					
Alveolar macrophages	13	67 ± 4.1	5	58 ± 6.7	.28[a]
Neutrophils	13	6.6 ± 2.2	5	7.4 ± 3.5	.86[a]
Eosinophils	13	0.90 ± 0.46	5	0.82 ± 0.75	.93[a]
Lymphocytes	13	25.6 ± 4.8	5	33.2 ± 7.7	.41[a]

See Table 1 and 2 legends for expansion of abbreviations.

[a]Calculated using Student *t* test.

[b]Described previously,[18] left lung.

[c]Calculated using the Wilcoxon's rank sum test.

reported that nine of 21 patients (43%) required WLL. In a retrospective study of 12 patients who underwent aerosolized GM-CSF therapy, Wylam et al[17] reported that five of 11 responders had recurrence of disease. In four of five patients, the mean time to relapse was 6.3 months and ranged from 5.5 to 12 months.[15] It is notable that the dose of GM-CSF used in their study was twice that used in our study, although the prognosis of our cases was comparable to that of their study.

PAP is often described as a lung disorder with restrictive physiology. In the present study, 18 of 35 patients were in the normal range (≤ 80) in %FVC, whereas the other 17 patients were mildly to moderately restricted, which was comparable to previous studies.[24] Seymour et al[25] investigated 14 patients who underwent subcutaneous GM-CSF administration and suggested that higher VC before treatment was one marker to define responsiveness to GM-CSF therapy. In the present study, VC did not correlate with responsiveness to GM-CSF therapy, but it showed significant association with the requirement for additional treatment. Although limited by the small number of cases, the subgroup analyses suggested that VC is an independent factor from age, sex, baseline Pao_2, change in A-aDO_2, and baseline levels of serum markers, including anti-GM-CSF-Ab. However, there is a possibility that some clinical variables might be intrinsically related to VC. The physicians' decision for retreatment might be influenced by such clinical markers. Notably, a recent study of a series of patients with PAP followed in a reference center reported that the need for lavage was significantly associated with FVC.[26]

Reduction of VC might be due to two different factors: accumulation of surfactant-derived materials in the alveolar space and fibrotic changes of lung tissue. In a study of a quantitative CT scan analysis of patients with PAP who underwent WLL and showed improvements in %DLCO and %FVC, Perez et al[27] demonstrated that there was a reduction in lung weight

following lavage, which correlated with the dry weight of the lavage effluent. The study demonstrated a shift in the regional lung inflation toward more inflated lung with a corresponding increase in the mean lung inflation. Surfactant accumulation might be associated with an elevated ventilation-perfusion mismatch and disproportionately impaired DLCO in patients with aPAP.[2] Seymour et al[25] demonstrated serum levels of SP-A correlated with VC in 14 patients at baseline. The present study also showed that serum levels of SP-A correlated with VC at baseline as well as after treatment. However, requirement of additional therapy was not significantly associated with SP-A at baseline. Surfactant materials might be easily redistributed in alveolar spaces and may not be related to the impairment of lung tissue that might lead to additional treatment.

The other factor, fibrotic changes of lung tissue, might be maintained even after GM-CSF therapy or WLL. Pulmonary fibrosis has been reported to be associated with PAP, and exposure to oxygen or repeated WLL have been suggested as potential contributors to fibrosis. Although irreversible scarring of the lung is rarely associated with PAP, a small fraction of patients with PAP demonstrated substantially impaired %VC and rather poor prognosis. To investigate this possibility, two radiologists reevaluated baseline CT scans of 32 of the 35 participants for findings other than PAP without knowing the study results regarding responsiveness and prognosis of the GM-CSF inhalation. They only pointed out traction bronchiectasis in one patient (responder, FR), bronchiectasis in one patient (responder, FR), and multiple bullae in one patient (responder, AT). Thus, we failed to find any significant association between fibrotic change in CT scan and requirement of additional treatments. In the present study, the mean %VC levels of patients in the FR group improved from 85.9% to 93.4%, whereas those of patients in the AT group changed from 71.6% to 74.2%. The difference in improvement between the groups might be associated with the balance of surfactant accumulation and lung fibrosis in the lungs of patients.

For future studies, it would be useful to explore novel treatment regimens for patients with moderately impaired VC. As shown in this study, inhaled GM-CSF therapy did not change serum levels of anti-GM-CSF-Ab. However, the BALF titers of anti-GM-CSF-Ab were reduced in responders, which was likely due to the improved clearance in alveolar spaces. The future treatments might include a combination of GM-CSF inhalation with WLL to improve the environment of airway/alveolar spaces or with administration of rituximab to reduce the systemic production of anti-GM-CSF-Ab.

In conclusion, this study demonstrated that VC might be clinically useful in predicting the need for additional therapy in patients with aPAP who were treated with inhaled GM-CSF therapy. We believe this study contributes to improving the quality of life and treatments for patients with aPAP.

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Additional information: The e-Figures and e-Table can be found in the "Supplemental Materials" area of the online article.

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Effect of Lung Volume on Airway Luminal Area Assessed by Computed Tomography in Chronic Obstructive Pulmonary Disease

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Abstract

Background: Although airway luminal area (Ai) is affected by lung volume (LV), how is not precisely understood. We hypothesized that the effect of LV on Ai would differ by airway generation, lung lobe, and chronic obstructive pulmonary disease (COPD) severity.

Methods: Sixty-seven subjects (15 at risk, 18, 20, and 14 for COPD stages 1, 2, and 3) underwent pulmonary function tests and computed tomography scans at full inspiration and expiration (at functional residual capacity). LV and eight selected identical airways were measured in the right lung. Ai was measured at the mid-portion of the 3rd, the segmental bronchus, to 6th generation of the airways, leading to 32 measurements per subject.

Results: The ratio of expiratory to inspiratory LV (LV E/I ratio) and Ai (Ai E/I ratio) was defined for evaluation of changes. The LV E/I ratio increased as COPD severity progressed. As the LV E/I ratio was smaller, the Ai E/I ratio was smaller at any generation among the subjects. Overall, the Ai E/I ratios were significantly smaller at the 5th (61.5%) and 6th generations (63.4%) and than at the 3rd generation (73.6%, $p<0.001$ for each), and also significantly lower in the lower lobe than in the upper or middle lobe ($p<0.001$ for each). And, the Ai E/I ratio decreased as COPD severity progressed only when the ratio was corrected by the LV E/I ratio (at risk v.s.stage3 $p<0.001$, stage1 v.s.stage3 $p<0.05$).

Conclusions: From full inspiration to expiration, the airway luminal area shrinks more at the distal airways compared with the proximal airways and in the lower lobe compared with the other lobes. Generally, the airways shrink more as COPD severity progresses, but this phenomenon becomes apparent only when lung volume change from inspiration to expiration is taken into account.

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Introduction

In bronchial asthma and chronic obstructive pulmonary disease (COPD), computed tomography has been used extensively to evaluate airway remodeling in recent years.[1–7] COPD is characterized by small airway remodeling and emphysema. Airway remodeling is pathologically described as increased airway smooth muscle mass and subepithelial thickening of the basement membrane in bronchial asthma.[8,9] Thus, airway remodeling is estimated by measuring airway wall thickness and/or airway wall area corrected by airway size and/or total wall area of airways. However, validation of the measurement of such parameters has been challenged and questioned from a technical aspect, particularly when airway size is smaller.[10,11]

Another parameter of airway dimension that can be obtained from CT data is airway luminal area (Ai), along with airway caliber. This parameter may not be suitable for an assessment of airway remodeling because airway size is changeable according to lung volume and very likely affected by the pressure balance between inside and outside the airway wall.[12] This pressure balance may be particularly important in smaller airways that lack cartilage in their walls. In other words, both intra-airway pressure determined by breathing pattern and the elastic recoil pressure of the surrounding tissue[12] would affect Ai in vivo.

However, the unique characteristics of Ai may be advantageous when examining the relationship between airway dimension and pulmonary function. Furthermore, there are some technical advantages in the measurement of Ai compared with airway wall

parameters. Its assessment appears to be technically more reliable and reproducible, because the inner edge of the airway wall can be much more easily delineated than the outer edge, which would be mandatory for airway wall assessment. We often encounter serious difficulties in defining the outer edge of airways due to attachment of lung tissue and vessels, leading to potential measurement error. Indeed, our previous study showed that FEV1 % predicted is more closely correlated with Ai than airway wall parameters such as % airway wall area in patients with COPD.[5] Furthermore, we demonstrated, using the parameter of Ai, in another study that we could quantitatively evaluate the magnitude of bronchodilation at the 3rd to 6th generations of airways separately, which was induced by inhaled tiotropium in patients with COPD. This approach would open the new arena because it enables us to look at any geographical difference in the effect of bronchodilation which conventional pulmonary function tests would never elucidate.[13] On the other hand, lung volume intuitively affects the size of airway, so that an assessment of Ai must be interpreted with caution when we attempt to compare Ai at different time points.

In this study, using our proprietary software, we evaluated the effect of lung volume on Ai by comparing the CT data taken at full inspiration and at expiration in COPD patients. The goal of the study was to examine the effect of lung volume change on Ai in a quantitative manner. Ederle JR et.al.[14] and Yamashiro et.al.[15] have reported positive correlations between the changes in lung volume and the changes in size of the central airways from inspiration to expiration. In this study, we attempted to extend their observations to the more distal airways and hypothesized that the effect of lung volume on Ai might differ by airway generation, lung lobe, and/or spirometric COPD severity.

Methods

Subjects

The subjects were 61 male and 6 female patients with clinically diagnosed COPD who participated in the Hokkaido COPD cohort study[16,17] and agreed to have CT scans twice on one occasion. Based on the post-bronchodilator FEV1 (forced expiratory volume in 1 sec) data, the patients were diagnosed according to the GOLD criteria updated 2003[18] as: COPD at risk, 15 patients; Stage 1, 18 patients; Stage 2, 20 patients; and Stage 3, 14 patients. There were no marked physical differences, such as height and body weight, among the groups.

Study protocol

All subjects were patients who participated in Hokkaido University Hospital. They underwent CT scans and lung function tests on a single day, except for some who attended twice within an interval of ≤1 week. Prior to the CT scans, the subjects were carefully instructed by a radiologist how to hold their breath by recorded voice instructions at deep inspiration and at relaxed expiration. This study was conducted in accordance with the amended Declaration of Helsinki. The Health Authority Research Ethics Committee of Hokkaido University School of Medicine approved the protocol as part of the Hokkaido COPD cohort study, and written, informed consent was obtained from all patients.

Pulmonary function tests

A rolling seal type of spirometer CHESTAC-33 (CHEST M.I., Inc., Tokyo, Japan) was used. The results of pulmonary function tests met the requirements of the Japanese Respiratory Society guideline,[19] which are similar to those of the American Thoracic Society (ATS). Acceptable maneuvers were defined as those with peak expiratory flow within 10% of the maximum observed, a rapid start, absence of major flow fluctuations, and adequate expiration time. Reproducible maneuvers agreed within 200 mL of the larger FEV1. The FEV1 and forced vital capacity (FVC) values taken to characterize each participant were the maximum results obtained from acceptable maneuvers. Forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC) were expressed as percentages of predicted values according to the prediction equations of the Japanese Respiratory Society. Lung volumes (total lung capacity (TLC), functional residual capacity (FRC), and residual volume (RV)) were measured by the helium closed circuit method. Lung volumes were expressed as percentages of predicted values according to the prediction equations of Nishida.[20]

CT Data scanning and image analysis

CT scans were performed using a multidetector-row spiral CT scanner with four detector arrays (SOMATOME plus Volume Zoom; Siemens, Berlin, Germany). CT scans were acquired with the following parameters: 120–140 kVp, 75–350 mA, 4 detector ×1 mm collimation, 1.25 mm thickness and helical pitch 7, reconstruction filter, kernel B30f, FOV 280–340×280–340 mm. In this study, the entire lung of each patient was scanned in the supine position. All CT raw data sets were reconstructed to voxel data using both soft-tissue and bone algorithms. The length of the 1-voxel side was 0.625 mm or around this value. Raw data was transferred to the workstation, and then reconstructed into three-dimensional chest images (Virtual place Fujin raijin 310, AZE Ltd., Tokyo, Japan). The detailed process of CT data acquisition and reconstruction has been described previously.[13,21,22] A segmental bronchus is first defined as the 3rd generation of bronchi, after which one proceeds peripherally, using the longitudinal image and the short axis image simultaneously and searching for any bifurcation around the entire circumference.

At each bifurcation, in general, one bronchus was randomly selected. If the image of the bronchus was poor or it was obstructed, then the other bronchus, up to the 6th generation, was selected. It was possible to compare the same sites of identical bronchi in two respiratory phases in a given subject because we use two screens that allow simultaneous assessment of dual images of inspiration and expiration.

Total lung volume (LV) on CT measurement was also calculated using the same software. In short, the whole lung containing airways (A) was extracted from the 3D image of the thorax, resulting in deletion of the heart and major vessels in the lungs. Then, the bronchial skeleton (B) was extracted from the whole lung, resulting in the lung consisting of parenchyma without either major vessels or proximal bronchial trees. LV was defined as (A)–(B).

Data analysis

Eight bronchi were selected in the right lung: apical (B1), posterior (B2), and anterior (B3) of the upper lobe; lateral (B4) and medial (B5) of the middle lobe; and anterior basal (B8), lateral basal (B9), and posterior basal (B10) of the lower lobe. Then, Ai was measured at the midpoint between bifurcations, from the 3rd to 6th generation of each airway, leading to a total of 32 measurement sites per subject; the averages per generation and per lobe were calculated for the analysis. The ratio of expiratory to inspiratory LV (LV E/I ratio) and Ai (Ai E/I ratio) were defined for evaluation of changes in LV and Ai. If the LV E/I ratio was 70%, this means that the subject exhaled 30% of the inspiratory LV during expiration. To evaluate the effect of lung volume on Ai according to COPD severity, we examined the Ai E/I ratio itself

and also the Ai E/I ratio corrected by lung volume change from inspiration to expiration in each subject, that is, the Ai E/I ratio divided by LV E/I ratio. This is because LV E/I ratio was highly variable according to COPD severity. All measurements were performed by one of the authors (K.K.), who was blinded to all other subject information.

Statistical analysis

All statistical computations were performed with a statistical software package(JMP for Windows, version 8 and RX 64 3.0.0). Results are expressed as means6 SD for the subjects' characteristics and the results of pulmonary function tests and as means6 SEM for comparison of means of any CT parameters. Linear regression analysis was used to evaluate the relationship between LV data at expiration measured by CT and FRC values physiologically measured and the relationship between Ai and LV changes from the inspiratory to the expiratory phase. One way analysis of variance of LV E/I ratio and Ai E/I ratio among GOLD stage was done, using Tukey's honestly significant difference test. Freidman test was used for the comparison of Ai E/I ratio for the generation and for the lobe. A value of $p<0.05$ was considered significant.

Results

The patients' characteristics and the results of pulmonary function testing are shown in Tables 1 and 2.

LV measurements

It was presumed that the lung volumes at full inspiration and expiration on CT would be highly varied among the subjects because they were COPD patients with various degrees of airflow limitation. Therefore, the lung volume at expiration, which was calculated by CT data, was first compared with the level of FRC, which was measured by the helium closed circuit method. As expected, the lung volumes measured by the two methods were well-correlated ($R = 0.83$, $p<0.001$; Figure 1), which indicated that the lung volume at expiration when CT was taken would roughly represent the FRC level of the subjects. The LV E/I ratio was then calculated from CT data. The LV E/I ratio increased as the COPD stage progressed: 46.2%6 4.3%(SEM) in the subjects at risk, 50.5%6 2.3% at Stage 1, 56.6%6 2.8% at Stage 2, and 72.7%6 2.1% at Stage 3 ($p<0.001$ at Stage 3 compared with the other Stages; Figure 2, Table 3).

Ai measurements

The hypothesis that the Ai E/I ratio would differ by the generation of the airways and/or by the lobe where the airways were located was then examined, using the data from all subjects. The mean Ai E/I ratio was 73.6%6 1.3% (SEM) at the 3rd, 65.7%6 1.5% at the 4th, 61.5%6 1.5% at the 5th, and 63.4%6 1.4% at the 6th generation. Thus, the mean Ai E/I ratios of the distal airways at the 5th and 6th generations were significantly smaller than those of the proximal airways at the 3rd and 4th generations ($p<0.001$ for each) (Figure 3, Table 3). The same parameters were then examined by lobe. The mean Ai E/I ratio was significantly smaller in the lower lobe than in the upper or middle lobes ($p<0.001$) (Figure 4, Table 3). There were no statistically significant differences in the mean Ai E/I ratio at any of the 3rd to 6th generations of the airways according to the spirometric COPD stage (Figure 5a, Table 3); however, if the Ai E/I ratio was corrected by the LV E/I ratio in each subject, it became smaller as the spirometric COPD severity progressed. (Figure 5b) This is because the LV E/I ratio was larger (less volume change from inspiration to expiration) as the COPD stage progressed, so that the magnitude of Ai change was seemingly smaller. In other words, the airways actually shrink more in advanced COPD from inspiration to expiration if corrected by

Table 1. Characteristics of the subjects.

Subjects	Median	Range	Mean	SD
Age (yr)	71	48–85	68	8
Height (cm)	164	149–176	164	6
Weight (kg)	61	42–92	63	11
Smoking (pack-years)	57	21–174	65	30

The subjects were 61 males and 61 females.
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Table 2. Results of pulmonary function tests.

Stages	at risk	1	2	3	all
N	15	18	20	14	67
postFEV1[L]	2.676 0.47	2.676 0.54	1.696 0.39	1.046 0.17	2.046 0.79
post%FEV1[%]	96.16 11.1	93.26 11.3	64.06 8.8	38.46 6.1	73.76 24.5
postFEV1/FVC[%]	74.46 2.6	62.26 5.7	51.26 9.5	34.76 6.1	55.96 15.3
TLC[L]	5.716 0.79	6.516 1.15	5.946 0.61	6.756 1.08	6.216 0.99
FRC[L]	3.306 0.69	3.896 0.75	3.706 0.60	4.846 0.89	3.906 0.89
RV[L]	2.136 0.48	2.376 0.45	2.656 0.49	3.876 0.76	2.716 0.82
RV/TLC[%]	35.36 3.2	37.86 5.8	44.86 1.2	57.16 5.5	43.36 9.5

Definition of abbreviations: FVC = forced vital capacity, FEV1 = forced expiratory volume 1s, post = post-inhalation of bronchodilator inhalation (mean6 SD).
doi:10.1371/journal.pone.0090040.t002

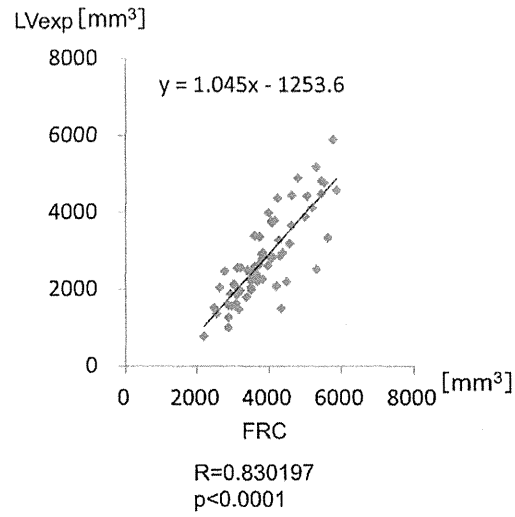

Figure 1. Comparison of the lung volume at expiration with the level of FRC. Comparison of the lung volume at expiration, which was calculated by CT data, with the level of FRC, which was measured by the helium closed circuit method. The lung volumes measured by the two methods are well-correlated ($R = 0.83$, $p<0.001$). LV exp: lung volume at expiration.
doi:10.1371/journal.pone.0090040.g001

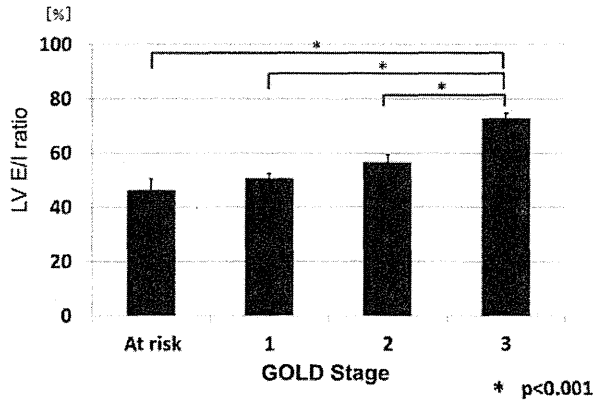

Figure 2. LV E/I ratio among the subjects. The LV E/I ratios were 46.2%6 4.3% in subjects at risk, 50.5%6 2.3% at Stage 1, 56.6%6 2.8% at Stage 2, and 72.7%6 2.1% at Stage 3 ($p<0.001$ at Stage 3 compared with other Stages). Expiration levels differed depending on the severity of airflow limitation.
doi:10.1371/journal.pone.0090040.g002

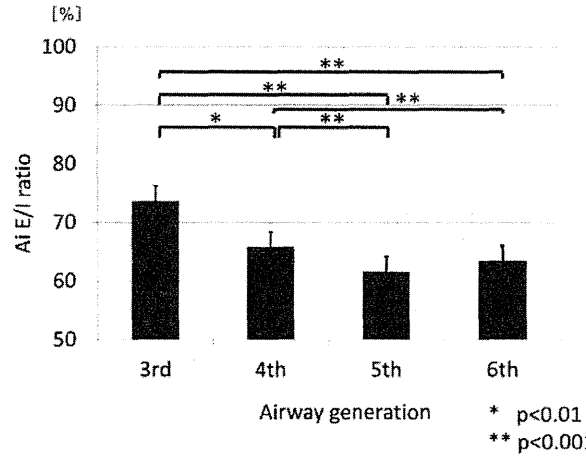

Figure 3. Ai E/I ratio from the 3rd to the 6th generation. The mean values of Ai E/I ratio of the distal airways at the 5th and 6th generations were significantly smaller than those of the proximal airways at the 3rd and 4th generations ($p<0.001$).
doi:10.1371/journal.pone.0090040.g003

volume change; however, it was likely to be masked by the smaller change in lung volume without such correction.

Correlations between changes in LV and Ai

The relationship between the LV E/I ratio and the Ai E/I ratio at the 3rd to 6th generation of the airways was next examined. Figure 6 demonstrated the relationship of the two variables, not in a single subject, but among the subjects who exhibited variable levels of LV E/I ratio based on COPD stages. The results clearly indicated that Ai E/I ratios were significantly smaller as the LV E/I ratios were smaller at any generation of the airways.

Discussion

In this study, we first confirmed that the lung volume at expiration measured by CT was significantly well-correlated with the FRC level physiologically measured on the same day. This is very important for this study because the level of expiration could be highly variable in COPD. We then demonstrated that the change in Ai from deep inspiration to expiration differed significantly according to airway generation and also according to the lobe of the lung where the airways are located. In other words, the airway caliber shrinks more at the distal airways than the proximal airways in the 3rd to 6th generations and in the lower lobe compared with the upper or middle lobe when the subjects exhale from full inspiration to expiration. These data clearly indicate that we must always consider lung volume not only when assessing emphysema by lung densitometry,[23–29] but also when assessing Ai for comparison in any observational cohort studies and/or with any pharmacological interventions. Additionally, we found that there were significant correlations between the Ai E/I ratios and the LV E/I ratios at any of the 3rd to 6th generation of the airways among the subjects who exhibited variable levels of LV E/I ratio, depending on spirometric COPD stages. Finally, we demonstrated that the airways shrink more as COPD severity

Table 3. Results of Lung Volume and Airway luminal area measurements.

LV E/I ratio (*p<0.01v.s. at risk)					
Stages	**at risk**	**1**	**2**	**3**	**all**
LV E/I ratio	46.26 4.3	50.56 2.0*	56.66 2.8*	72.76 2.1*	56.06 1.8
Ai E/I ratio (*p<0.01, **p<0.001 v.s. 3rd generation, †p<0.01, ††p<0.001 v.s. 4th generation)					
Stages	**at risk**	**1**	**2**	**3**	**all**
3rd generation	73.06 3.3	73.86 2.6	74.26 2.1	79.86 2.5	75.06 1.3
4th generation	62.16 3.6	65.86 3.2	68.96 2.7	70.56 3.6	66.96 1.6**
5th generation	55.26 3.6**	57.96 3.0**†	61.66 2.5**†	64.56 3.9*	60.06 1.6**††
6th generation	54.76 3.2**	59.76 3.2**†	60.56 2.6**	62.86 2.6**	59.56 1.5**††
	Upper Lobe		**Middle Lobe**		**Lower Lobe**
all	74.56 1.4[1]		74.06 1.4[1]		58.76 1.7

[1]p<0.001v.s. lower lobe.
Definition of abbreviations: LV= lung volume, Ai=airway luminal area, LV E/I ratio= The ratio of expiratory to inspiratory LV, Ai E/I ratio= The ratio of expiratory to inspiratory Ai (mean6 SEM).
doi:10.1371/journal.pone.0090040.t003

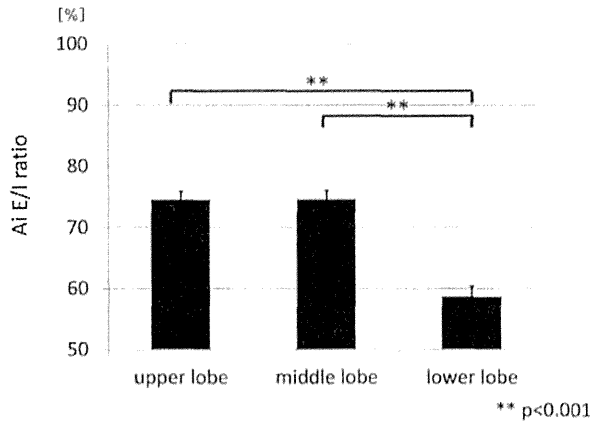

Figure 4. Ai E/I ratio of upper, middle and lower lobes. The mean Ai E/I ratio was significantly smaller in the lower lobe than in both the upper or middle lobes ($p<0.001$).
doi:10.1371/journal.pone.0090040.g004

progresses, but this phenomenon becomes apparent only when lung volume change from full inspiration to expiration is taken into account.

It is important to note that the CT scans were conducted while the subjects were breath-holding both at full inspiration and at relaxed expiration. Several points must be considered when interpreting the present data. Firstly, dynamic Ai changes during breathing were not observed. Either inspiration or expiration may lead to dynamic pressure changes both inside and outside of the airway wall, thus potentially causing dynamic Ai changes during breathing. This may be particularly important when considering the effects of airway generation and spirometric COPD stage on Ai. Secondly, the level of expiration might vary in any individual even in the same clinical setting. In this study, prior to CT scans being taken, the subjects were carefully instructed by a radiologist how to hold their breath by recorded voice instructions at deep inspiration and at relaxed expiration. There was a significant and good correlation between LV at expiration assessed by CT and FRC measured by the helium closed circuit method, thus indicating that LV at expiration when CT scans were taken roughly represented the level of FRC in this study.

We have demonstrated that there were significant correlations between Ai E/I ratio and LV E/I ratio from the 3rd to the 6th generation in Figure 6, which extended the results of the previous studies showing the positive correlations between Ai E/I ratio and LV E/I ratio of the central airways.[14,15] However, it must be noted that each dot represents the data of individual subject in Figure 6 and thus the relation between two variables indicates the relationship, not in an individual, but among the subjects whose expiration levels were so different. That might be the reason why the trend line does not go through the point (100%, 100%), which should be the case in an individual data. Quite interestingly and importantly, the slope of correlation coefficients between LV E/I ratio and Ai E/I ratio appears to get more flat as the airways go from the 3rd to the 6th generation. This fascinating phenomenon may indicate the influence on COPD (the degree of airflow limitation) may be different, in reality, in the airway generations in terms of the effect of lung volume change on Ai.

The effect of inspiration level on the CT assessment of pulmonary emphysema severity has been studied extensively.[23–29] On the other hand, attention has been paid only recently to the assessment of airway dimensions at inspiration and

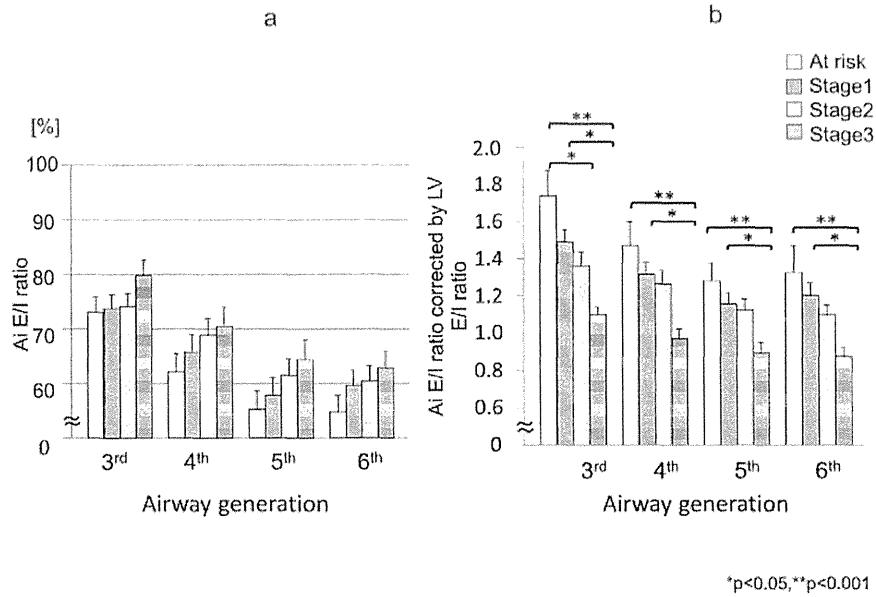

Figure 5. (**a**) *Ai E/I ratio from the 3rd to the 6th generation compared according to the spirometric COPD stage. There were no significant* differences in the Ai E/I ratio at any of the 3rd to 6th generations of the airways when compared according to the spirometric COPD stage. Rather, Ai E/I ratio of more severe COPD subjects tended to be higher comparing with mild COPD subjects. (**b**) Ai E/I ratio corrected by lung volume change from inspiration to expiration (LV E/I ratio) from the 3rd to the 6th generation compared according to the spirometric COPD stage. Ai E/I ratio corrected by lung volume change from inspiration to expiration (LV E/I ratio) was significantly different among the groups according to COPD severity.
doi:10.1371/journal.pone.0090040.g005

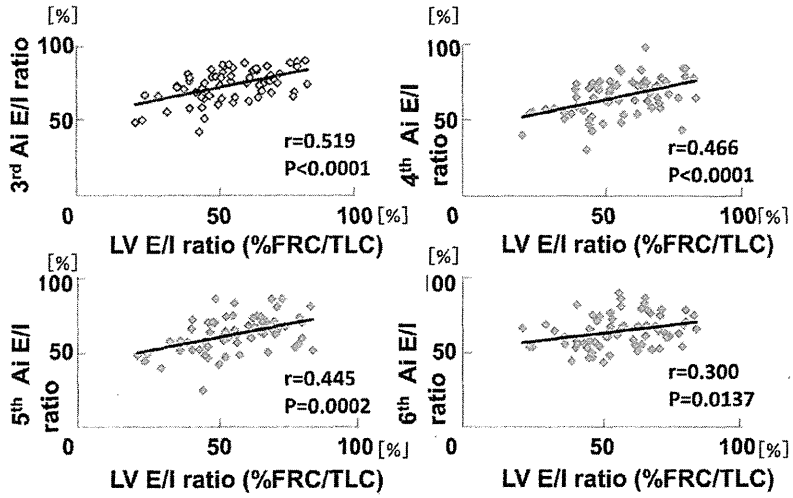

Figure 6. Comparisons of Ai E/I ratio with LV E/I ratio(%FRC/TLC) from the 3rd to the 6th generation. There were significant positive correlations between the ratio of Ai and that of LV at any generation. X-axis indicates % FRC/TLC expressed as LV E/I ratio, and Y-axis indicates Ai E/I ratio. Each dot represents the data of individual subject and thus the relation between two variables indicates the relationship, not in an individual, but among the subjects, whose % FRC/TLC was so variable, dependent on COPD stages.
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expiration. Matsuoka et al.[30] demonstrated that the severity of airflow limitation assessed by pulmonary function tests was better correlated with airway caliber at expiration compared with at inspiration at the 3rd to 5th generations of three bronchi in 50 subjects with COPD, whose spirometric data was similar to those of our current study. In this study, correlation coefficients between FEV1 % predicted and the mean Ai at the 3rd to the 6th generation were 0.385 to 0.439 at inspiration ($p<0.01$, Figure S1) and 0.280 to 0.318 at expiration ($p<0.05$, Figure S2), which seems to be opposite to the results of Matsuoka et al since the correlation between FEV1 % predicted and Ai was apparently better at inspiration rather than at expiration in the current study. The Ai E/I ratios in their study were much smaller, 63%6 13% (mean6 SD), 60%6 19%, and 45%6 15% at the 3rd, 4th, and 5th generation, compared with 73.6%6 1.3% (mean6 SEM), 65.7%6 1.5%, and 61.5%6 1.5%, respectively, in the current study. These marked differences existed despite similar spirometric data on average in the two study populations. Therefore, we speculate that the subjects were forced to exhale deeper in their study at expiration, particularly in more severe COPD, when expiratory CT was taken. Anyhow, when we take expiratory CT scans, the level of expiration would be vitally important for later analysis of airway calibers and even comparison could not be possible between the studies unless expiration level was carefully monitored. More recently, Bakker et al.[31] reported that the airway luminal areas of the 3rd generation of the right apical and bilateral basal segmental bronchi were actually dependent on inspiration level in 44 subjects with COPD with alpha-1 antitrypsin deficiency, and the distensibility, defined as the difference in airway luminal area from FRC to TLC levels divided by the corresponding lung volume change, was different between the upper lobe and lower lobe, which is concordant with the present result. In the current study, their observations were further extended, as more accurate figures on the effect of lung volume from TLC to FRC on airway luminal area were provided per airway generation. The current study suggests that lung volume particularly at relaxed expiration varies highly among subjects, so that the effect of lung volume should be carefully monitored in such studies that deal with airway calibers and/or luminal area.

In contrast with examining the effect of lung volume on Ai of the airways in this study, the concept of airway distensibility has long been explored, in asthma[32–34] and/or COPD research, from the standpoint of airway remodeling. Brown et al.[32] failed to demonstrate a defect in the distensibility of the asthmatic airways; Castagnaro et al. and Johns et al. reported that airway distensibility might be less in bronchial asthma patients than in healthy controls.[33,34] Airway distensibility has recently been examined in COPD patients. Scichilone et al. reported that loss of the effect of deep inspiration is strongly associated by COPD severity.[35] Diaz et al.[36] hypothesized that the airway caliber would be affected by the extent of emphysema and examined the distensibility, defined as the ratio of absolute change in airway inner diameter to the cube root of absolute change in lung volume from relaxed exhalation to full inflation (D d/^{3}!D LV). They found that airway distensibility was smaller in those with emphysema-predominant COPD compared with those with airway-predominant COPD. They speculated that airway-parenchymal interdependence might be impaired in emphysema-predominant COPD, thus reducing airway distensibility. Distensibility was not examined in the current study as the interest was in the effect of the change in lung volume on Ai from inspiration to expiration.

There were a couple of limitations in this study. First, since the subjects were mostly male, a potential sex-related bias was not explored. Second, lung volume was measured as a whole, but not per lobe. The finding in this study that the airway shrinks more in the lower lobe compared with the upper or middle lobe may simply reflect that the change in lung volume differs depending on lobe when the subjects exhale. Finally, since only one bronchus was randomly selected at each bifurcation, one cannot be sure that this reflects the whole picture of all airways. It is highly likely that the effect of lung volume on airway luminal area may differ depending on the nature of airway inflammation and remodeling; thus, heterogeneity must be taken into account.

In conclusion, we, in the present study, quantitatively and precisely examined the effect of lung volume change on airway

luminal area in patients with COPD. In particular, we demonstrated that the lung volume effect on the Ai E/I ratio from full inspiration to relaxed expiration is greater at the distal airways and in the lower lobe of the lung in a given subject. Finally, we demonstrated that the airways shrink more as COPD severity progresses, but this phenomenon becomes apparent only when the Ai E/I ratio is corrected by lung volume change from full inspiration to expiration.

Supporting Information

Figure S1 Relationship of pulmonary function parameter (FEV1 %predicted) with airway luminal area (Ai) at full inspiration. The relationships of FEV1 %predicted with the mean Ai at the 3rd to the 6th generations in all subjects at full inspiration are shown. See text if one wishes to know how the mean Ai at each generation was calculated and how CT was taken at full inspiration and expiration. There were significant correlations between FEV1 %predicted and the mean Ai at the 3^{rd} to the 6^{th} generations at any generation.
(TIF)

Figure S2 **Relationship of pulmonary function parameter (FEV1 %predicted) with airway luminal area (Ai) at expiration.** The relationships of FEV1 %predicted with the mean Ai at the 3rd to the 6th generations in all subjects at expiration (at functional residual capacity) are shown. Although statistically significant at any generation, the correlation coefficients were evidently better for the data obtained at full inspiration compared with those at expiration.
(TIF)

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Author Contributions

Conceived and designed the experiments: KK KS HM MH MN. Performed the experiments: KK. Analyzed the data: KK KS. Contributed reagents/materials/analysis tools: KK KS HM MH KN SK MN. Wrote the paper: KK KS MN.

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Relationship between neutrophil influx and oxidative stress in alveolar space in lipopolysaccharide-induced lung injury

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ABSTRACT

We intratracheally administered lipopolysaccharide (LPS) to ICR mice and then collected BAL fluid and lung tissue to determine whether levels of neutrophils and/or myeloperoxidase (MPO) in bronchoalveolar lavage (BAL) fluid reflect lung tissue damage. Robust neutrophil accumulation into the alveolar space and lung tissue were almost completely abolished at seven days along with oxidative stress markers in the lung. However, lung injury scores and lung wet/dry ratios, as well as MPO and oxidative stress markers in BAL fluid were significantly increased at five and seven days after LPS administration. At later time points, BAL neutrophils generated more MPO activity and ROS than those harvested sooner after LPS administration. Although elevated neutrophil levels in BAL fluid reflected oxidative stress in the lungs, MPO might serve as a useful marker to evaluate damage sustained by epithelial cells over the long term.

1. Introduction

Acute respiratory distress syndrome (ARDS) is a type of acute diffuse, inflammatory lung injury that leads to increased pulmonary vascular permeability, increased lung weight, and loss of aerated lung tissue. The clinical hallmarks are hypoxemia and bilateral radiographic opacities associated with increased venous admixture, increased physiological dead space and decreased lung compliance (Force et al., 2012). Acute respiratory distress syndrome is a frequent complication among critically ill patients and it is responsible for high morbidity and mortality rates (Lesur et al., 1999; Ware and Matthay, 2000). Treatment of the underlying disease and supportive care using the "lung protective" strategies of mechanical ventilation and prone positioning, contribute to successful clinical outcomes (TARDS Network, 2000; Guerin et al., 2013). However, specific therapies have not been established and once the cascade of events leading to ARDS has been initiated, the condition becomes much less amenable to specific treatment.

Reactive oxygen species (ROS) such as superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and hypochlorous acid (HOCl) play central roles in the pathogenesis of acute lung injury (Haegens et al., 2009; Tate and Repine, 1983). Endothelial or epithelial cells express several antioxidants such as superoxide dismutase, catalase and glutathione peroxidase to neutralize free radicals and counteract the detrimental effects of ROS (Fink, 2002). However, ROS generated by phagocytes during the acute inflammatory response overwhelm these antioxidants and lead to cell and lung damage. Neutrophils that have high oxidant-generating capacity migrate into the alveolar space where they degranulate and release proteins from azurophilic granules into phagolysosomes (Nauseef, 2001). Bronchoalveolar lavage (BAL) is a diagnostic method of sampling cells in the airway-alveolar space and soluble substances in the extracellular lining. The number of neutrophils in BAL fluid is robustly increased in ARDS, and the ratios (%) of neutrophils are markers of disease activity (Steinberg and Hudson, 1994). The time course of transpulmonary polymorphonuclear leukocyte migration has been investigated (Hirano, 1997; Reutershan et al., 2005). However, whether or not inflammatory cells, especially neutrophils that presently serve as clinical markers in BAL fluid, reflect the extent of damage in lung tissues remains obscure.

The short biological half-life of ROS renders them difficult to measure directly in biological materials from the lungs of patients with ARDS, and reports describing increased ROS activity in ARDS are scant (Baldwin et al., 1986). Alternatively, the oxidative modification of ROS targets such as proteins, lipids, and antioxidants are regarded as useful markers with which to indirectly reflect oxidative stress. Levels of protein carbonyls, myeloperoxidase (MPO), thiobarbituric acid-reactive substances (TBARS), lipid oxidation products and oxidized glutathione are elevated in BAL fluid from patients with ARDS (Bunnell and Pacht, 1993; Winterbourn et al., 2000). Whether or not oxidative stress markers exactly reflect lung

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oxidative stress in patients with ARDS is unknown. These oxidative markers have been evaluated in animal models of ARDS to determine the amount of oxidative stress in the lungs. However, few studies have investigated the same oxidative stress markers both in BAL fluid and in lung tissue (Bergeron et al., 1998).

The present study investigated whether or not neutrophils and MPO in BAL fluid can reflect oxidative stress or epithelial damage in the lungs of a mouse model of LPS-induced lung injury. We compared the kinetics of various oxidative stress markers with neutrophil accumulation and MPO activities in BAL fluid and tissues from mouse lungs with lipopolysaccharide (LPS)-induced lung injury. We also examined the ROS-producing potential of neutrophils harvested from BAL at various intervals after the intratracheal instillation of LPS to produce ROS. Not only a higher ratio of neutrophils but also an increase in MPO activity in BAL fluid suggested the existence of epithelial cell damage and oxidative stress both in BAL fluid and in the lungs with LPS-induced lung injury. Thus, MPO might be a useful marker to evaluate long term damage sustained by epithelial cells.

2. Materials and methods

2.1. Animals

Nine-week-old male ICR mice purchased from Japan Clea (Tokyo, Japan) were housed in plastic chambers with free access to food and water. None of the mice had gross pathological lesions. The Ethics Committee for Animal Research at Hokkaido University School of Medicine approved the experimental protocols.

2.2. Mouse model of LPS-induced lung injury

Saline (50 μL) containing 200 μg of LPS (Sigma Chemical Co., St. Louis, MO, USA) was intratracheally administered to mice anesthetized with a mixture of ketamine and xylazine as described (Betsuyaku et al., 1999; Ito et al., 2009). Age-matched, untreated healthy mice served as controls.

2.3. BAL and tissue measurements

2.3.1. Wet/dry weight ratio

The wet lungs of mice from which BAL had not been collected were weighed immediately after dissection, dried at 37 °C for 72 h, and then weighed once again to determine the wet/dry (W/D) weight ratio.

2.3.2. Lung histopathology

Paraffin-embedded lung sections were stained with hematoxylin and eosin for assessment by light microscopy. Lung damage was graded from 0 (normal) to 4 (severe) based on the criteria of interstitial inflammation, neutrophil infiltration, congestion and edema (Michetti et al., 2003). Lung damage was scored by adding the individual scores for each category and the score for each mouse was calculated as the mean of four lung sections.

2.3.3. BAL and sampling of mouse lung tissues

Mice were killed by CO_2 narcosis at 1, 3, 5, 7 and 14 days after LPS injection ($n = 5$–6 per time point) and BAL was collected using three 0.6-mL injections of saline through a tracheal cannula. Red blood cells in BAL fluid samples were disrupted using red blood cell lysis buffer (Sigma) and then total numbers of cells were counted using a hemocytometer. Cell differentials in BAL fluid were examined in Cytospin preparations stained with Diff-Quik reagent (Sysmex International Reagents, Kobe, Japan). After BAL fluid was collected, the lungs were inflated with 50% (v/v) Tissue-Tek OCT (Sakura Finetek USA, Torrance, CA, USA) in RNase-free phosphate-buffered saline (PBS) containing 10% sucrose and stored at −80 °C as described (Suzuki et al., 2008).

2.3.4. Immunohistochemical evaluation of neutrophils in lungs

Lung sections were immunostained for Gr-1 as described (Moriyama et al., 2010). Briefly, non-specific binding was blocked for 30 min using 5% (v/v) normal rabbit serum in PBS. Neutrophils were detected using a polyclonal rat anti Ly-6G (Gr-1) monoclonal antibody (BD Biosciences, San Jose, CA, USA) followed by anti-rat IgG-horseradish peroxidase-conjugated secondary antibody (DakoCytomation, Glostrup, Denmark). Labeling was visualized using diaminobenzidine as the chromogen (Vector Laboratories). Gr-1-positive cells were counted in five random fields per section of 5–6 grafts per group, and then the ratio (%) of total cells per high-power field was calculated.

2.3.5. Assay of MPO activity

We spectrophotometrically assayed MPO activity in BAL fluid and lung tissues as described (Haslam and Baughman, 1999). Briefly, BAL fluid (25 μL) or lung homogenate was reacted with H_2O_2 (0.0005%) in the presence of o-dianisidine dihydrochloride (0.167 mg/mL) for 30 min and changes in absorbance at 450 nm were measured. Protein concentrations of tissue extracts were determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA).

2.3.6. Assessment of carbonylated protein in BAL fluid

The carbonylation of proteins in BAL fluid was measured by Western blotting as described (Nagai et al., 2006, 2008). Briefly, raw BAL fluid (16 μL) was derivatized with dinitrophenylhydrazine (DNP) using the OxyBlot Protein Oxidation Detection Kit (Chemicon International, Temecula, CA, USA) and resolved by electrophoresis on 10% SDS-polyacrylamide gels. Proteins were Western blotted with anti-DNP antibody and band intensity was calculated using NIH Image software (version 1.62). The intensity of the 68-kDa band corresponding to carbonylated albumin on each blot is shown as arbitrary units (AU).

2.3.7. Total protein assay

Total protein concentration in BAL fluid was quantified using the bicinchoninic acid microassay method (Pierce Chemical).

2.3.8. Measurement of LPO, GSH and GSSG in BAL fluid

Levels of LPO, GSH and GSSG in BAL fluid were measured using kits according to the manufacturer's protocols (Cayman Chemical, Ann Arbor, MI, USA).

2.3.9. Measurement of protein carbonyl contents of the lung

Protein carbonyl contents in lung homogenates were determined using a protein carbonyl assay kit (Cayman Chemical), according to the manufacturer's instructions.

2.3.10. Immunohistochemical evaluation of 4 hydroxy-2-nonenal modified proteins (4-HNE)

Frozen sections cut at 4 μm were fixed in 4% paraformaldehyde for 10 min and then immunostained using the Vectastain ABC-AP Kit (Vector Laboratories, Burlingame, CA, USA) with rabbit anti-4-HNE (Alpha Diagnostic, San Antonio, TX, USA) antibody. Non-specific binding was blocked for 1 h using 5% goat serum diluted in PBS at room temperature, and then the sections were incubated in primary antibody (diluted 1:3000) at room temperature for 30 min. Biotinylated universal secondary antibody and Elite ABC reagent were applied at room temperature for 30 min. The sections were washed with Tris-buffered saline containing 0.05% Tween 20 (Sigma) and then alkaline phosphatase substrate was

used as chromogen (Vector Laboratories). Staining of 4-HNE was quantified in images captured in a blinded fashion using NIH Image software. The lower threshold of detection was established using lungs that had not been exposed to LPS and then the overall area of the alveolar wall was measured. The intensity of 4-HNE positive areas was captured from five random fields per graft section and calculated as the ratio (%) of all alveolar wall areas per high-power field.

2.3.11. Evaluation of intracellular ROS generation in alveolar neutrophils from lungs of mice instilled with LPS

Cells from BAL fluid were resuspended in PBS and loaded with 10 μm aminophenyl fluorescein (APF) (Sekisui Medical, Tokyo, Japan) by incubation at room temperature in a humidified atmosphere with 5% CO_2 in 96-well black tissue culture plates (BD BioCoat, Tokyo, Japan). Fluorescence intensity per neutrophil was measured using a microplate fluorescence reader at 495 (excitation) and 520 (emission) nm after incubation for 30 min and MPO activity in lysed cells from BAL fluid was also evaluated as described above.

2.3.12. Flow cytometry

Cells collected from BAL fluid were resuspended in Hanks' balanced salt solution (HBSS) containing 0.1% BSA. Nonspecific binding was blocked by incubating the cells in HBSS supplemented with anti-mouse CD16/32 antibodies (BD Biosciences) for 20 min at 4 °C. The cells were stained with 10 μM APF and anti-mouse Ly-6G and Ly-6 C (Gr-1) conjugated to phycoerythrin (BD Biosciences) for 20 min, at 4 °C, washed twice and then analyzed by flow cytometry using a FACSCalibur (BD Biosciences). Ten minutes before FACS analysis, 7-AAD (BD Biosciences) was added to exclude dead cells. The ratio of neutrophils expressing ROS to total neutrophils was calculated as the ratio of double positive to Gr-1-positive cells.

2.4. Data presentation and statistical analysis

All data are shown as means ± standard error (SE). Differences between groups were analyzed using an unpaired *t*-test. More than two means were compared using Dunnett's method. All data were analyzed using StatView J 5.0 software (SAS Institute, Inc., Cary, NC, USA). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Neutrophilic inflammation after intratracheal LPS instillation

We assessed the kinetics of inflammatory cells in BAL fluid after LPS instillation. Inflammatory cells consisting mainly of neutrophils accumulated in the lungs of mice that were intratracheally administered with LPS (Fig. 1A and B). Neutrophilia was evident in BAL fluid at day one, peaked on day five and then significantly decreased at seven days after LPS administration. Macrophage counts gradually increased for up to 5 days after LPS administration and returned to baseline levels by 14 days thereafter (Fig. 1C).

We then immunohistochemically investigated neutrophil accumulation by staining lung sections for Gr-1. The lungs of mice that did not receive LPS contained a few neutrophils. Immunohistochemical staining revealed a remarkable increase in neutrophil influx into the pulmonary interstitium after LPS instillation (Fig. 2A and B). The number of neutrophils in lung tissues peaked at 5 days after LPS administration and returned to control levels two days later. The ratio of neutrophils in BAL fluid significantly correlated with the ratio of Gr-1-positive cells in lung tissue ($r = 0.831$, $p < 0.001$; Fig. 2C). Neutrophils accumulated at equal rates in lung tissues and in BAL fluid.

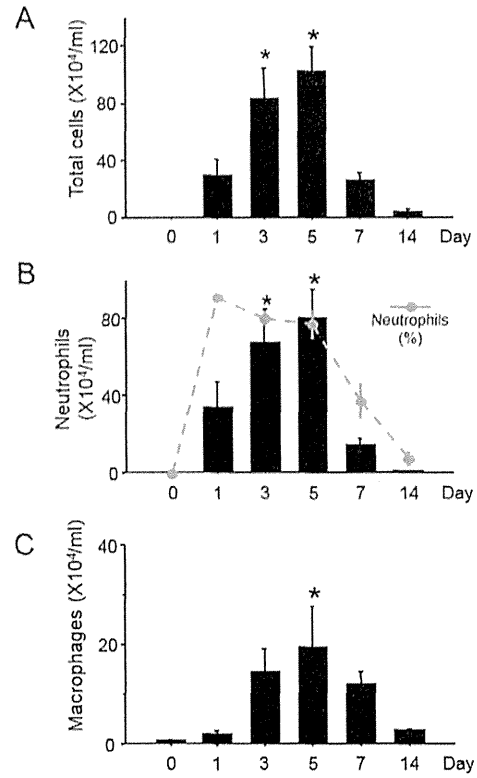

Fig. 1. Kinetics of LPS-induced inflammatory cell accumulation in bronchoalveolar lavage (BAL) fluid. Mice were intratracheally administered with LPS and the cell content in BAL fluid was determined as described in Section 2. Data represent average numbers of total cells (A), neutrophils (B), and macrophages (C) per mL of BAL fluid (±SE) from five to six mice.

3.2. Oxidative stress markers in lung tissues from mice with LPS-induced lung injury

We investigated whether neutrophils in BAL fluid reflect oxidative stress in lung tissues. The carbonylated protein content in lung tissue continued to increase for five days, and then fell to almost baseline levels at seven days after LPS administration (Fig. 3A). We immunohistochemically stained 4 hydroxy-2-nonenal modified proteins (4-HNE) to identify lipid modification caused by oxidative stress. After LPS administration, 4-HNE staining was prominently localized in the alveolar walls (Fig. 3B). Areas of 4-HNE-staining were significantly increased at three and five days after LPS administration and dropped to the control level at seven days thereafter (Fig. 3C). The ratio of neutrophils in BAL fluid closely correlated with levels of oxidative stress markers in the lung (carbonylated protein: $r = 0.830$, $p < 0.001$; 4-HNE: $r = 0.703$, $p < 0.001$; Fig. 3D and E).

3.3. Development of lung injury after intratracheal LPS instillation

We evaluated the degree and duration of lung damage after LPS instillation. An increase in lung injury scores and W/D ratios indicated that lipopolysaccharide caused significant pulmonary damage and edema. Lung injury scores and W/D ratios were both significantly increased at five and seven days after LPS administration and returned to control levels at 14 days thereafter (Fig. 4A–C). These kinetic profiles differed from those in neutrophils in BAL fluid.

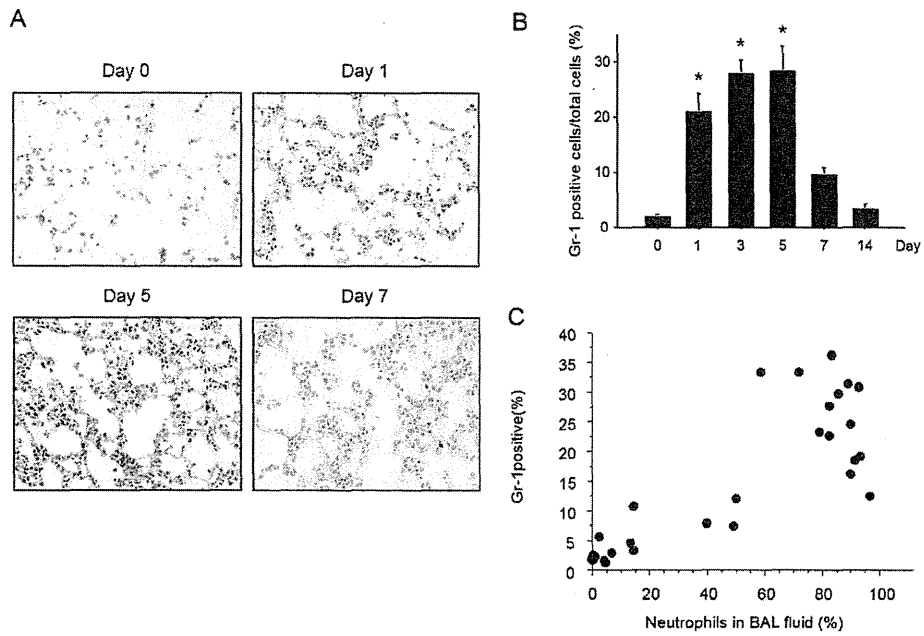

Fig. 2. Kinetics of LPS-induced neutrophil accumulation in lung tissue. (A) Immunohistochemically identified neutrophils in parenchyma of untreated mice (day 0) and at one, five and seven days after LPS administration. Original magnification, ×200. (B) Gr-1-positive cells presented as ratios (%) of total cells per high-power field. (C) Number of neutrophils in BAL fluid correlates with ratio of Gr-1 positive cells in damaged lung tissue ($r = 0.729$, $p < 0.001$).

3.4. Myeloperoxidase activity in lung tissue and BAL fluid

We evaluated the activity of MPO because it is the most popular marker of neutrophil activation (Chooklin et al., 2009). We found that MPO activity in BAL fluid gradually increased for up to seven days after LPS administration, although neutrophils were almost completely undetectable by that time (Fig. 5A). The kinetics of MPO activity in BAL fluid and the process of lung epithelial damage

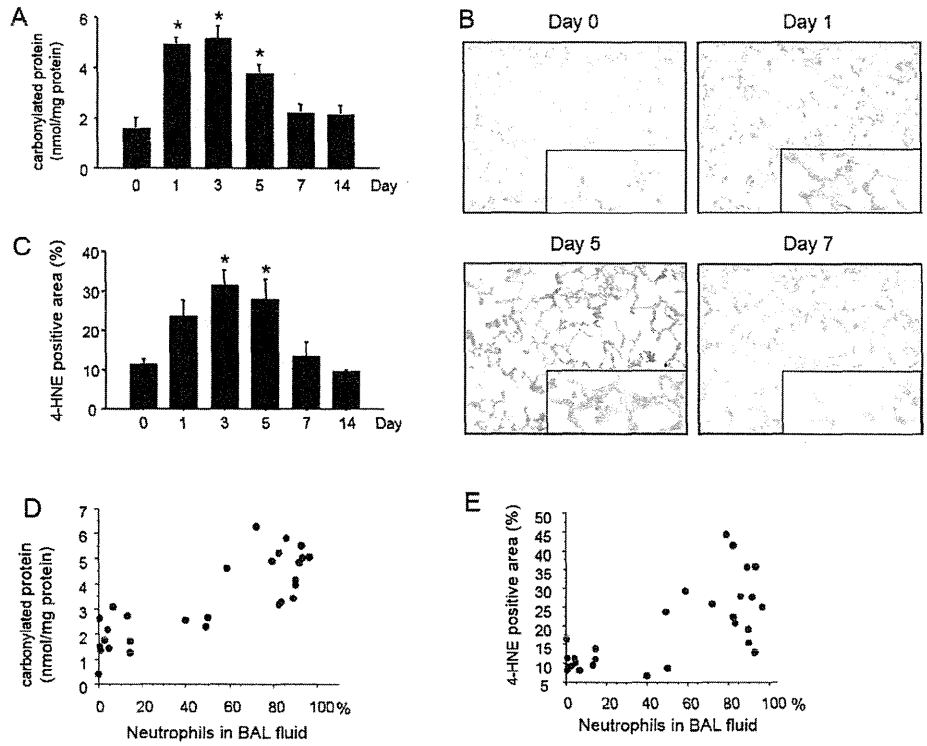

Fig. 3. Oxidative stress markers in lung tissue after LPS administration. Carbonylated protein contents in lung tissues after LPS instillation evaluated for up to 14 days using ELISA (A). Sections of lungs from untreated mice (day 0) and at one, five and seven days after intratracheal administration of LPS stained for 4-HNE (B). Original magnification, ×200 (large panels) and ×400 (insets). Staining intensity was determined as ratio (%) of 4-HNE positive areas (C; $n = 5$–6 per group). *$p < 0.05$ compared with untreated mice. Correlation between ratio of neutrophils in BAL fluid and carbonylated protein content (D; $r = 0.830$, $p < 0.001$) and ratio of 4HNE positive areas (E; $r = 0.703$, $p < 0.001$) in lung tissue.

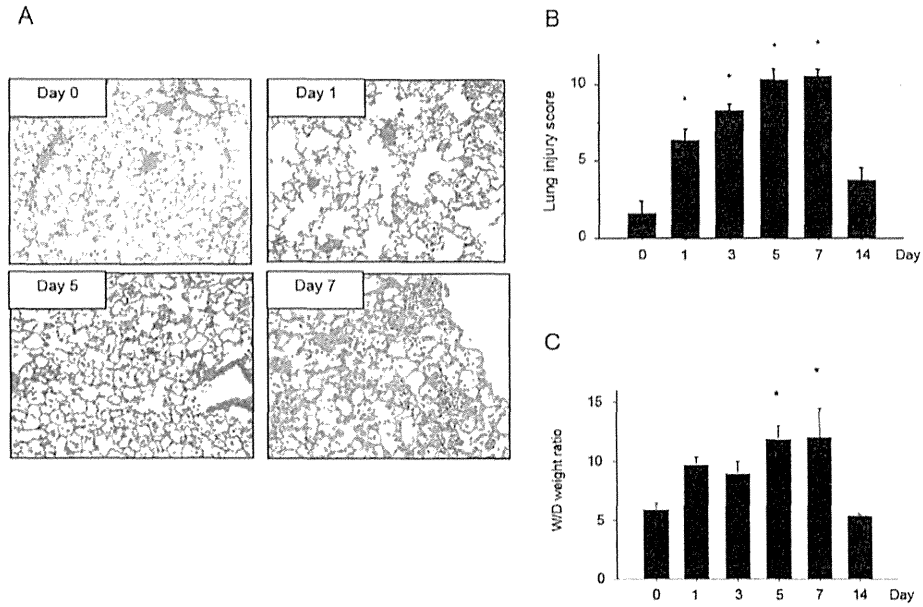

Fig. 4. Lung injury after LPS administration. (A) Representative sections of untreated lungs (day 0) and at one, five and seven days after LPS administration. (B) Histological lung injury scores from tissue sections from mice with (LPS)-induced lung injury stained with hematoxylin and eosin. (C) Lung wet/dry (W/D) ratios (n = 5–6 per group). *$p < 0.05$ compared with untreated mice.

notably occurred in parallel. In contrast, MPO activity in lung homogenates was significantly elevated for one to five days after LPS administration and returned to baseline levels at seven days thereafter in accordance with neutrophil recruitment in lung tissue (Fig. 5B). The relationship between the ratio of neutrophils and MPO activity was more evident in lung tissue ($r = 0.937$, $p < 0.001$; Fig. 5D) than in BAL fluid ($r = 0.564$, $p = 0.0018$; Fig. 5C). These data suggest that MPO activity does not simply reflect the number of neutrophils in BAL fluid.

3.5. *Total protein concentration in BAL fluid*

We estimated alveolar-capillary injury by measuring the total protein content in BAL fluid (Holter et al., 1986). Levels of total

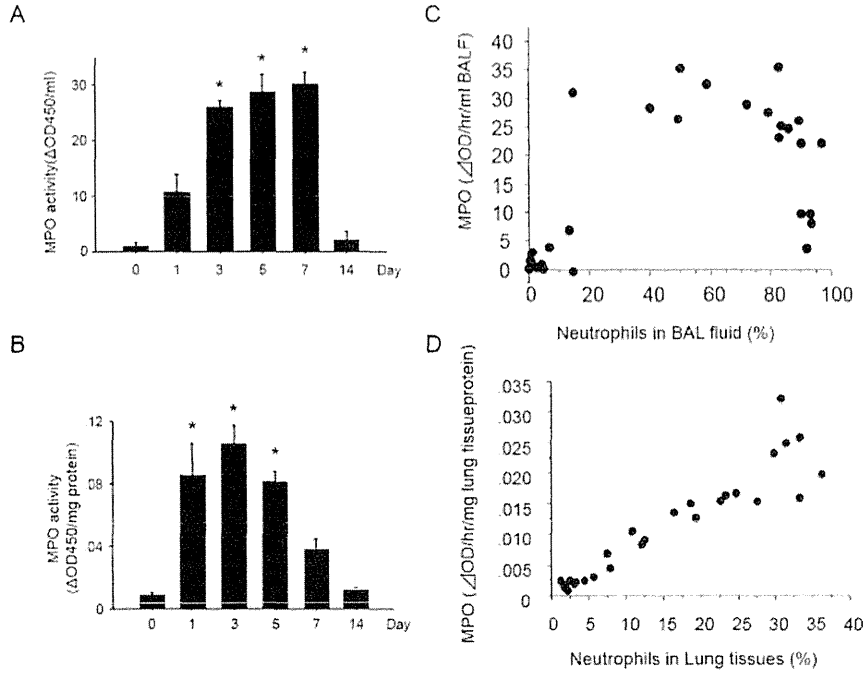

Fig. 5. MPO activity in BAL fluid and lung tissue after LPS administration. Activity of MPO in BAL fluid from mice evaluated for up to 14 days after intratracheal LPS administration (A). Time course of MPO activity in lung tissue (B) (n = 5–6 per group). *$p < 0.05$ compared with untreated mice. Correlation between ratio of neutrophils and MPO activity in BAL fluid (C: $r = 0.564$, $p = 0.0018$) and in damaged lung tissue (D: $r = 0.937$, $p < 0.001$).

protein were significantly elevated at three and seven days after LPS administration (Fig. 6A). The activity of MPO in BAL fluid significantly correlated with levels of total protein ($r=0.682$, $p<0.001$; Fig. 6B).

3.6. Oxidative stress markers in BAL fluid

We compared levels of various oxidative stress markers with neutrophil accumulation in BAL fluid. Levels of carbonylated albumin were significantly increased at five and seven days after LPS administration (Fig. 7A) and closely correlated with those of MPO activity ($r=0.660$, $p<0.001$; Fig. 7B). Levels of LPO (a marker of lipid peroxidation), total glutathione (GSH), a major intracellular antioxidant, and its oxidized form, glutathione disulfide (GSSG), in BAL fluid gradually increased for up to seven days after LPS administration (Fig. 7C–E). These results showed that levels of various oxidative stress markers remained elevated for 7 days in BAL fluid even though the number of neutrophils had decreased by that time in mouse lungs with LPS-induced lung injury. The trends for markers of oxidative stress in BAL fluid and MPO levels were similar to that of lung damage in LPS-induced lung injury. The activity of MPO in BAL fluid also correlated with levels of total GSH ($r=0.615$, $p<0.001$), and LPO ($r=0.550$, $p=0.002$) in BAL fluid. However, relationships between ratios of neutrophils and oxidative stress markers in BAL fluid were less evident (carbonylated albumin: $r=0.403$, $p=0.033$; total GSH: $r=0.233$, $p=0.232$; LPO: $r=0.410$, $p=0.030$). These results indicated that levels of oxidative stress markers in BAL fluid are linked to neutrophil activation.

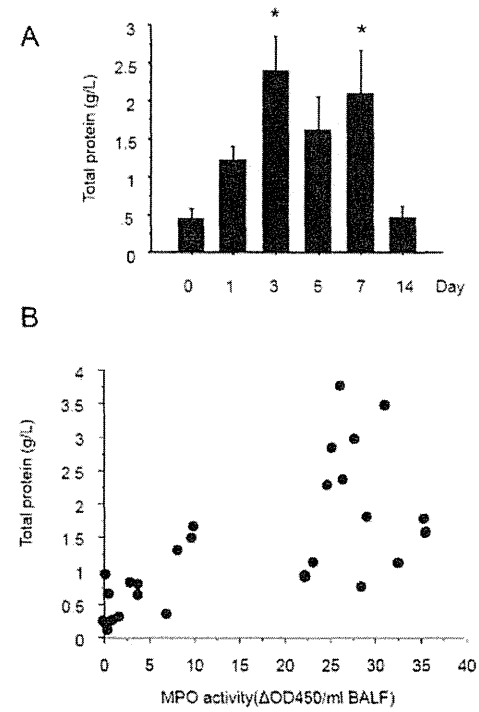

Fig. 6. Total protein concentration in BAL fluid and correlation with MPO activity. Total protein concentration in BAL fluid after intratracheal LPS administration (A). Correlation between MPO activity and total protein concentration in BAL fluid (B; $r=0.682$, $p<0.001$).

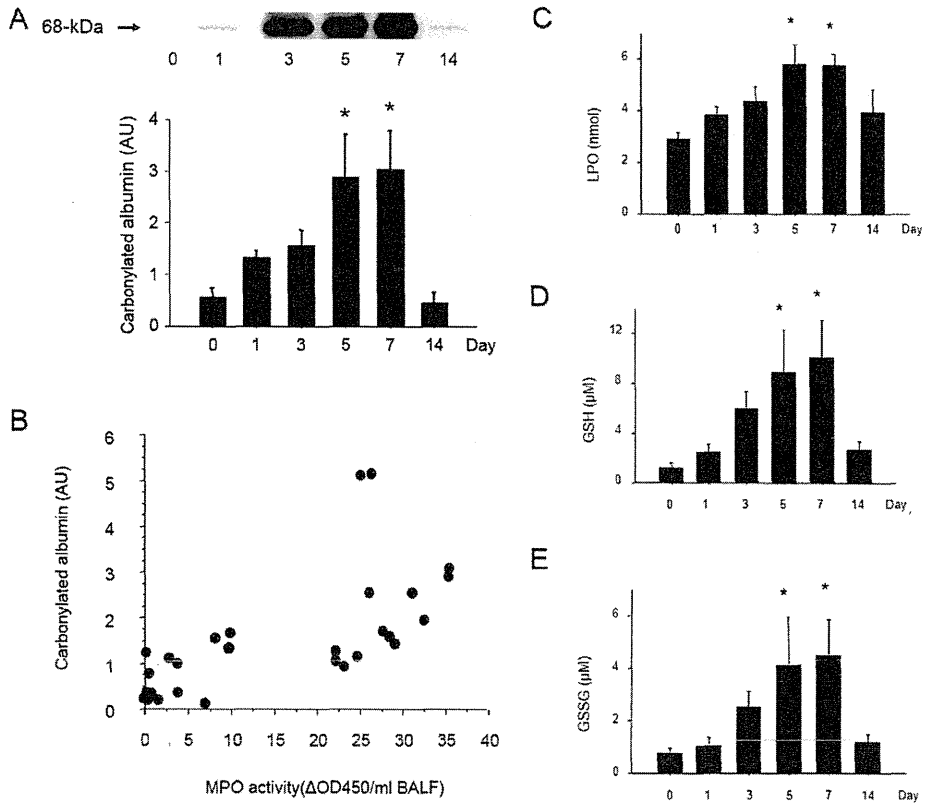

Fig. 7. Oxidative stress markers in BAL fluid after LPS administration. (A) Representative Western blot of 68-kDa carbonylated albumin in BAL fluid and time course of carbonylated albumin in BAL fluid after LPS administration. (B) Activity of MPO correlates with carbonylated albumin in BAL fluid ($r=0.660$, $p<0.001$). Levels of LPO (C), GSH (D) and GSSG (E) at various times up to 14 days measured in BAL fluid ($n=5$–6 per group). *$p<0.05$ compared with untreated mice.

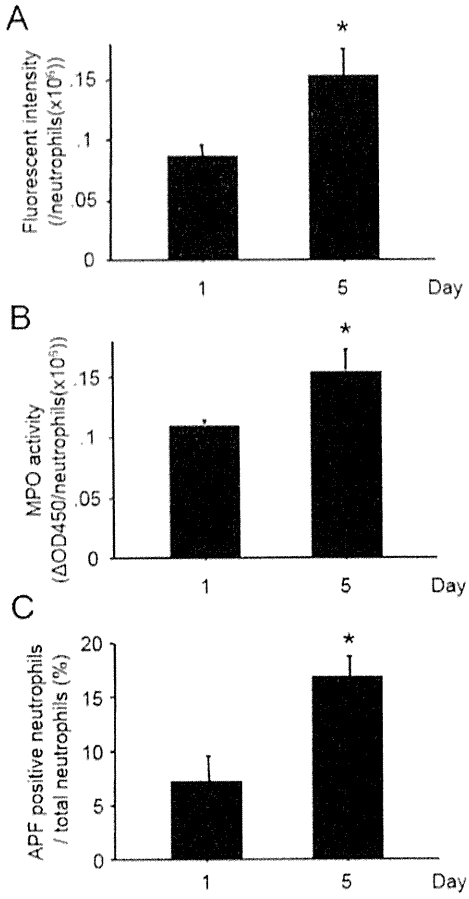

Fig. 8. Comparison of ROS generation and MPO activity in activated neutrophils from mouse lungs at 1 and 5 days after LPS administration. Mice were administered with intratracheal LPS and BAL was collected at one and five days thereafter. Cells from BAL fluid were incubated with APF for 30 min at room temperature and then fluorescence intensity measured at 495/520 nm was calculated per neutrophil (A). Intracellular MPO activity in BAL fluid cells at same time points (B; $n = 5$ per group). Cells in BAL fluid incubated with APF, stained with phycoerythrin (PE)-labeled anti Gr-1 antibody and analyzed by flow cytometry. APFhighGr-1^{high} cells/Gr-1^{high} cells were calculated as ratios of neutrophils expressing ROS to total neutrophils (C; $n = 5$ per group). *$p < 0.05$ compared with cells from BAL fluid collected from mice at one day after LPS instillation.

3.7. *Myeloperoxidase activity and ROS generation in alveolar neutrophils at one and five days after LPS administration*

We speculated that activated neutrophils produce more MPO in the alveolar space at later, than at earlier time points and thus increase the oxidative stress load on epithelial cells and lining fluid. Inflammatory cells were retrieved from the BAL fluid of mice at one and five days after LPS instillation. Cells retrieved from BAL fluid at various time points were incubated with the probe aminophenyl fluorescein (APF) to selectively detect ROS including HOCl that is specifically produced in neutrophils from hydrogen peroxide via the action of MPO. Neutrophils in BAL fluid recovered from mice produced more ROS at five days than at one day after LPS administration when corrected for the number of neutrophils (Fig. 8A). Furthermore, intracellular MPO activity was higher in neutrophils at five days than at one day after LPS administration (Fig. 8B). The expression of ROS probed with APF in neutrophils was further assessed using FACS analysis. Cells isolated from BAL fluid were double-stained with APF and for Gr-1. The ratio of APF-positive cells among Gr-1-positive neutrophils was significantly increased at five days compared with one day after LPS administration (Fig. 8C).

4. Discussion

Calculating the ratios of neutrophils in BAL fluid is a popular method of clinically evaluating lung injury. The present study found that the ratio of neutrophils in BAL fluid reflected elevated oxidative stress markers in damaged mouse lungs. However, the ratio of neutrophils was not always the optimal marker of damage in LPS-induced lung injury because MPO activity in BAL fluid was not always associated with neutrophil accumulation. We also identified a close correlation between carbonylated albumin and MPO in BAL fluid, implying that they interact in the alveolar space. Neutrophils that had accumulated in the alveolar space tended to release far more MPO and ROS at five days after LPS administration than at one day thereafter, which might partly explain the lack of an association among the numbers of neutrophils, MPO activity and oxidative stress, particularly in the alveolar space, at the later stage of inflammation.

Azurophilic granules in neutrophils contain MPO that catalyzes the reaction between H_2O_2 and chloride to yield HOCl, an oxidant that is ~100-fold more reactive than H_2O_2 (Grisham et al., 1990). Levels of MPO activity and of chlorotyrosine formed by the HOCl-dependent chlorination of para-tyrosine are increased in BAL fluid from patients with ARDS (Lamb et al., 1999). Neutrophilic inflammation is prolonged, MPO activity is enhanced and pro-inflammatory cytokines are up-regulated at three days after LPS administration in BAL fluid from elderly compared with young mice (Ito et al., 2007). In addition, neutrophil influx and inflammatory cytokines levels are decreased in BAL fluid collected from MPO knockout mice after LPS administration (Haegens et al., 2009). Although neutrophils produce ROS via other mechanisms including NADPH oxidase, MPO activity itself in the alveolar space might play a potential role in the generation of oxidative stress. Our findings of a significant correlation between MPO activity and carbonylated albumin in BAL fluid are consistent with previous findings (Chooklin et al., 2009).

Protein carbonylation is a popular oxidative modification marker of protein (Chevion et al., 2000). Since levels of carbonylated proteins in BAL fluid from patients with ARDS are elevated (Baldwin et al., 1986; Lenz et al., 1999), we evaluated the major 68-kDa carbonylated protein, albumin, in BAL fluid (Bunnel and Pacht, 1993; Ito et al., 2009). Albumin is important because it has antioxidative properties and thus participates in the first defense against free-radical attack derived from phagocytes to protect epithelial cells, endothelial cells and basement membrane from excessive damage in the alveolar space (Soriani et al., 1994). The antioxidative properties of albumin are attenuated by oxidative modification (Bourdon et al., 1999). The clearance of carbonylated protein could be delayed in the alveolar space due to impaired protein degradation. Proteasomal activity and albumin degradation rates are lower among patients with ARDS than among healthy individuals (Sixt et al., 2009), but the mechanism remains unknown.

Although regarded as a marker of oxidative processes, increases in oxidative stress markers indicate cellular damage and/or impaired functions of target molecules, resulting in subsequent damage (Nys et al., 2005; Song et al., 2010). The oxidation of lipid components of the endothelial or epithelial plasma membrane could facilitate neutrophil recruitment into the lungs, thus facilitating the leakage of chemokines and other chemoattractant molecules into the vascular space (Chow et al., 2003). The highly reactive and specific diffusible end product of lipid peroxidation, 4-HNE, is a second messenger that might function in regulating the expression of protective γ-glutamylcysteine synthetase (γ-GCS) (Rahman and MacNee, 2000). Protein carbonyls are generated on proteins by the addition of lipid peroxidation products (Blakeman et al., 1995). A marker of lipid peroxidation closely correlates with the protein carbonyl content and MPO activity in BAL fluids from

patients with ARDS at risk (Baldwin et al., 1986). Furthermore, the time courses of indicators of oxidative stress and of neutrophil accumulation with a significant decrease in oxygenation index (PaO_2/FiO_2 ratio) have been investigated in an ovine model of acute lung injury and sepsis (Lange et al., 2012). The findings of that study indicated that neutrophil accumulation, oxidative stress in the lung and deteriorated lung function are linked.

The predominant non-protein thiol in cells is GSH and it is vital in the lungs. Increased levels of GSSG in BAL fluid are normally regarded as a marker of oxidative stress in the lungs (Biswas and Rahman, 2009). Total GSH and GSSG were both elevated in BAL fluid from our mouse model. The BAL fluid from patients with ARDS is deficient in total GSH and GSSG is elevated compared with that from normal individuals. This is probably due to the rapid extracellular oxidation of GSH (Winterbourn et al., 2000). The increase of total GSH in BAL fluid, despite being reduced in lung tissues in a mouse model of lung damage caused by oxidative stress, is probably associated with alveolar cell rupture (Sciuto, 1998).

Taken together, these findings indicate that the sustained elevation of these oxidative stress markers might reflect lung damage at a specific time. In fact, our assessment of the W/D ratio and lung histopathology revealed that lung damage was sustained for seven days after LPS administration and that the kinetics of oxidative stress markers were the same in BAL fluid. Our findings are consistent with a recent finding that histological lung damage is not restored while neutrophils are scant in the alveolar space of a mouse model of LPS-induced lung injury (Janssen et al., 2011). Neutrophil influx into BAL collected from patients with lung disease is often regarded as the most sensitive indicator of an inflammatory response (Haslam and Baughman, 1999; Trisolini et al., 2004). We previously reported that levels of the oxidative stress markers, oxidized glutathione and carbonylated albumin in humans do not correlate with the number of inflammatory cells in BAL fluid obtained from older individuals with a long history of smoking cigarettes (Nagai et al., 2006). Our results indicate that oxidative stress markers in BAL fluid might be more a useful marker of ARDS severity than the number of neutrophils.

We compared levels of ROS production and MPO activities of neutrophils harvested from BAL at various intervals after the induction of lung injury by LPS to investigate the mechanism underlying the dissociation between the number of neutrophils and MPO activity in BAL fluid at the resolution stage of the lung injury. Our results implied that accumulated neutrophils in the alveolar space at the later stages are more activated and generate more ROS via the MPO-HOCl pathway (Fig. 8). This might partly explain the sustained elevation of MPO activity and oxidative stress markers in BAL fluid. The mechanism of the chronological upregulation of MPO activity and ROS in neutrophils remains unclear. However, MPO modulates neutrophil intracellular signaling in the vasculature, affects the activation state of neutrophils (El Kebir et al., 2008; Johansson et al., 1997), upregulates surface CD11b expression and evokes MPO release from neutrophils, implying the involvement of autocrine and paracrine mechanisms (Lau et al., 2005).

Another possible explanation for the sustained MPO activity in BAL fluid is that oxidative stress impairs alveolar macrophage function, thus leading to delayed clearance of MPO. Extracts of cigarette smoke induces defective pathogen clearance in murine macrophages via the carbonylation of pseudopodia (Bozinovski et al., 2011) and MPO released from activated neutrophils in the alveolar space is cleared from epithelial lining fluid by alveolar macrophages through a reaction with mannose receptors (Klebanoff, 2005). Reactive oxygen species compartmentalized in the alveolar space might mediate the functional impairment of alveolar macrophages and result in prolonged MPO activation. An MPO inhibitor prevents the progression of emphysema induced by cigarette smoke along with the downregulation of MPO-generated oxidative stress markers (Churg et al., 2011). We speculate that MPO inhibition could also be a useful therapeutic treatment for ARDS.

Although LPS-induced pulmonary inflammation in mice is a popular model of ARDS that is characterized by neutrophil accumulation in the lungs, it does not fully manifest the features of ARDS in humans (Matute-Bello et al., 2011). Nevertheless, intratracheal LPS delivery has led to significant advances in understanding of the fundamental mechanisms that regulate lung injury and resolution. The manifestation of ARDS includes a massive sequestration of neutrophils within the pulmonary microvasculature in response to various stimuli, and a decrease in the number of neutrophils in the lung is considered to play a major role in the resolution of this pathophysiological process (Tsushima et al., 2009). The present study is the first to demonstrate that the association between the number of neutrophils in alveolar space or lung interstitium and the degree of lung damage disappears during the resolution of acute lung injury.

In conclusion, the ratio (%) of neutrophils in BAL fluid reflects oxidative stress in the lung, whereas MPO activity in BAL fluid indicates oxidative stress in BAL fluid as well as epithelial damage in lung tissue. The potential of neutrophils to release ROS and MPO into the alveolar space time-dependently differed from that of the lungs of mice with LPS-induced lung injury. Evaluating not only inflammatory cell differentials, but also MPO activity in BAL fluid from patients with ARDS should help to understand disease status.

Author disclosure statement

No competing financial interests exist.

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