Temporal Changes of Inflammatory Cytokine Profiles in Epithelial Lining Fluid in a Canine Lung Transplant Model

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Objectives: Ischemia-reperfusion injury resulting in post-transplant lung dysfunction involves a complicated network of cytokines that has yet to be fully investigated. We analyzed temporal changes in cytokine levels in epithelial lining fluid (ELF) from the distal airways of a canine lung transplantation model.
Methods: Ischemic time was set to 18 hours to enhance lung injury in a left single-lung transplantation model. ELF was collected via bronchoscopic microsampling, a minimally-invasive technique allowing repeated sampling, hourly up to 5 hours after reperfusion started. We compared levels of pro-inflammatory cytokines in ELF with those at baseline (time zero), and with a sham-operated control group.
Results: Concentrations of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) significantly increased immediately after the start of lung reperfusion in the transplant group relative to the sham group ($P < 0.005$ and $P < 0.05$, respectively); both were sustained through the 5 hours. Interferon gamma (IFN-γ levels were significantly reduced 3 h after reperfusion started ($P < 0.05$).
Conclusions: We found time- and cytokine-dependent changes in TNF-α, IL-6, and IFN-γ in ELF from the lung transplantation model. These specific changes in the cytokine profile may relate to the mechanism underlying post-transplant lung dysfunction.

Key words: Bronchoscopic microsampling, ELF, Ischemia-reperfusion injury, Lung transplantation, Distal lung airways

INTRODUCTION

Bronchoscopic microsampling (BMS) was developed as a minimally invasive alternative to bronchoalveolar lavage (BAL), utilizing probes made from urethane that absorb epithelial lining fluid (ELF) in the distal airways [1, 2]. BMS has successfully identified the pathophysiology underlying cases of acute respiratory distress syndrome, acute or chronic lung injury, infectious diseases, and malignant lung tumors [3–6]. We have recently used BMS in a canine model to demonstrate that temporal changes in TNF-α correspond with ischemia-reperfusion lung injury, while simultaneously validating its safety and effectiveness in transplanted lungs for the first time [7].

Despite considerable recent progress in lung transplant management, the outcome is still frequently unsatisfactory because of ischemia-reperfusion lung injury, acute or chronic tissue rejection, or infection [8–11]. It is well-known that transbronchial lung biopsy (TBLB) and fluid collection via BAL can induce or exacerbate existing lung injuries, especially after transplant, so neither technique is suitable for frequent monitoring of lung condition or serial ELF collections after lung transplantation. Furthermore, the significant dilution of BAL samples resulting from lavage can significantly hamper the detection threshold and accuracy of biomarker measurements.

A key mechanism underlying post-transplant lung dysfunction resulting from ischemia-reperfusion injury involves a complicated network of cytokines and chemokines [12, 13]. One of these, tumor necrosis factor alpha (TNF-α) is an upstream regulator of the network; it alters the expression of cell adhesion molecules and inflammatory mediators including interleukins (IL)-1, IL-6, IL-8, and IL-10, as well as interferon (IFN)-γ. However, few studies have performed serial molecular biological measurements to determine the pathological changes in this network that cause lung dysfunction after transplantation [14].

In the present experiment, we used BMS to collect ELF samples repeatedly over time in a canine lung transplant model, and examined the time-dependent changes in ELF concentrations of pro-inflammatory cytokines associated with ischemia-reperfusion lung injury as a step towards clinical use [12, 15] (TNF-α, IL-1β, IL-6, and IFN-γ).

MATERIALS AND METHODS

The study protocol was approved by the Animal Experimentation Committee of Tokai University (protocol No. 173054), and all animals used in this study received humane care in compliance with *The Guide for the Care and Use of Laboratory Animals* published by

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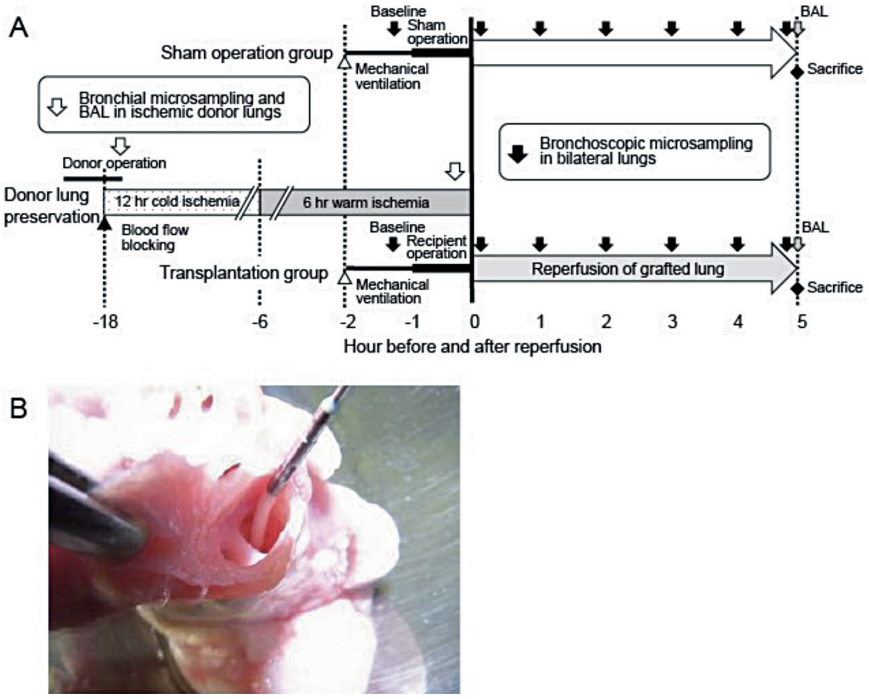

Fig. 1 Experimental protocol and photograph of epithelial lining fluid (ELF) collection using a bronchoscopic microsampling (BMS) probe on a donor lung (A) Schematized representation of the time course of interventions and samplings in the present protocol. (B) The BMS probe has a polyethylene outer sheath and a 1.8-mm diameter and 30-mm long inner probe with a polyurethane absorptive tip at the distal extremity. A BMS probe is advanced into the distal airway until slight resistance is perceived. The inner probe is left in place for 10 seconds to absorb ELF. The same procedure was done for in vivo collection of ELF in the grafted lungs through the forceps channel of the bronchoscope.

the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Animals

Ten beagle dogs (CLEA Japan, Inc., Tokyo, Japan) weighing 10-12 kg were used. The dogs were kept on a 12-h light/dark cycle with free access to food and water. Four weight-matched pairs were assigned to the transplant groups, and two dogs comprised the sham-operated control group (see Fig. 1A).

Lung procurement

Donor dogs were intubated using an endotracheal tube (8-mm inner diameter) after being anesthetized with an intramuscular injection of medetomidine 20 μg/kg plus midazolam 0.3 mg/kg. Mechanical ventilation was maintained with a 25-ml/kg tidal volume at a rate of 15 cycles per minute, a positive end-expiratory pressure (PEEP) of 5.0 cm H_2O, and a fraction of inspired oxygen (FiO_2) of 1.0 under 3-5% sevoflurane inhalation and intravenous injection of rocuronium bromide (0.4 mg/kg/hour). A median sternotomy was performed. The pulmonary artery was cannulated after intravenous administration of 5000 IU heparin. The lungs were flushed with 1000 ml of ET-Kyoto solution (Otsuka Pharmaceutical Factory Inc., Tokushima, Japan), an extracellular preservation solution used for clinical lung transplantation [16], at a temperature of 4℃ and pressure of 30 cm H_2O, with continued ventilation. After the donor lungs were semi-inflated to within 20 cm H_2O with 100% oxygen, the heart and lungs were harvested *en bloc*. They were then soaked in the preservation solution and stored at 4℃ for 12 h, and thereafter at 20℃ for 6 h to enhance ischemia-reperfusion lung injury (Fig. 1A).

Cytokine measurements in the ischemic donor lungs

Cytokine expression in the non-reperfused donor ischemic lungs was determined via BMS. Before and after the 18 h ischemia procedure (see **Lung procurement**), ELF was collected using BMS in the preserved donor right lobes. After the donor lungs were harvested, the medial lobe was immediately separated from the other lungs at the hilar region and the BMS probe introduced from the stump of the right medial lobe bronchus; ELF was absorbed from the distal bronchus (Fig. 1B). BAL fluid was collected after intrabronchial injection of 50 ml of normal saline in the same lobes. ELF and BAL fluid were also collected in the donor right cranial lobes after the same 18 h ischemia treatment. Samples were stored at -80℃ for later cytokine concentration measurements.

Recipient surgery

Left single lung transplantation was performed as previously described [7]. Recipient dogs were anesthetized, maintained, and ventilated in the same manner as the donors. Peak inspiratory pressure was monitored via the tracheal tube. For each recipient, a Swan-Ganz catheter (F7; Edwards Lifesciences, Irvine, CA) was placed in the left main pulmonary artery, accessed

from the right femoral vein, to measure pulmonary arterial pressure (PAP). A femoral arterial line was inserted to measure arterial pressure and arterial blood gasses. Baseline physiological indices and blood gas analysis data were obtained before the sham or transplant procedure. Following a left pneumonectomy via a left posterolateral thoracotomy, the left atrium, the left main bronchus, and the left pulmonary artery were anastomosed, and the left lung was transplanted [17]. The implantation time was set at 60 min. Blood flow and ventilation to the transplanted lungs were reestablished after the 18 h ischemia treatment. Thereafter, the chest was closed, and general anesthesia was maintained for 5 h. During this time, physiological data such as systolic arterial blood pressure, heart rate, mean PAP, and peak airway pressure were recorded, and arterial blood samples were collected for blood gas analysis (Fig. 1A). Sham-operated dogs underwent dissection of the left bronchi, pulmonary arteries and veins alone, under the same conditions as the transplant group.

Serial ELF collection via BMS

The BMS probe (Olympus Corporation, Tokyo, Japan) consists of a polyethylene outer sheath and a 1.8-mm diameter, 30-mm long inner probe with an absorbent polyurethane tip at the distal extremity capable of absorbing up to 20 μL of liquid. Serial ELF collection using BMS probes was done as described previously [7]. An airway connector (Bodai Bronch-Safe, Double Swivel for Bronchoscopy; Sontek Medical Inc., Hingham, MA) was attached to the endotracheal tube forming an air-tight seal around the bronchoscope for continuous ventilation and PEEP maintenance during BMS. After introducing the flexible fiber optic bronchoscope (6-mm diameter) via an endotracheal tube, ELF was collected by introducing the BMS probe through the forceps channel, and advancing it into the distal airway until slight resistance was met. The probe insertion into the segmental or subsegmental bronchi of the caudal lobes minimized the risk of damaging the anastomotic region of the main bronchus in the transplanted lungs. For ELF collection in the contralateral lungs and sham-operated dogs, the BMS probes were similarly introduced to the caudal lobes. The inner probe was left in place for 10 seconds before it was withdrawn via the outer sheath. After withdrawal, each wet probe tip was weighed, the ELF was extracted with 1 ml of saline, and each probe was dried and weighed again. The amount of ELF collected was calculated as the difference between wet and dry weights. The extracted sample was spun at 3000 rpm for 15 minutes, and the supernatant was stored at -80℃. ELF was collected from the grafted left caudal lung and the right contralateral lung just after reperfusion, and every hour thereafter until 5 h after the start of reperfusion. Before left pneumonectomy, ELF samples were collected from the recipients' original left and the right lungs to obtain baseline data (Fig. 1A).

BAL fluid collection

Animals were sacrificed, and the bilateral lungs were removed 5 h after the start of reperfusion. BAL samples were collected from the transplanted left cranial lobes and the contralateral right cranial lobes after intrabronchial injection of 50 ml of saline. The BAL fluid was spun at 3000 rpm for 15 minutes, and the supernatant was stored at -80℃.

Measurements of TNF-α, IL-1β, IL-6, and IFN-γ

The concentrations of canine TNF-α, IL-1β, IL-6, and IFN-γ in ELF and BAL fluid were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine® ELISA; R&D Systems, Minneapolis, MN). Assuming that 1 ml = 1000 μg, the weights of the BMS samples were converted to volumes, and the concentrations of cytokines in ELF (in pg/ml) were calculated as the concentrations in 1 ml of saline 1 mg/wet weight (mg) – dry weight (mg).

Histopathology and immunohistochemistry

The transplanted left caudal lobes and the contralateral right caudal lobes were used for histopathology. They were inflated with 10% buffered formalin at a pressure of 10 cm H_2O for fixation, and then embedded in paraffin. Embedded lungs were cut into 4 μ m-thick sections and stained with hematoxylin and eosin. The paraffin-embedded organs were also processed for immunohistochemical analysis with an anti-TNF-α antibody (ab6671; Abcam, Inc., Cambridge, MA) and an anti-IL-6 antibody (ab6672; Abcam, Inc., Cambridge, MA). Tissue was blocked with 10% serum for 20 minutes at room temperature, and antigen retrieval was performed by heating the tissue sections in citrate buffer (pH 6.0). Samples were incubated with primary antibody for 30 minutes at room temperature. A biotin-conjugated goat anti-rabbit IgG polyclonal antibody (1:2000 dilution) was used as the secondary antibody.

Statistical analyses

The data are presented as means ± standard deviations. Changes of values from baseline or differences between groups were compared using the Student's paired or unpaired *t*-test. Longitudinal changes of cytokine concentrations were compared between groups using repeated-measures analysis of variance (ANOVA). A P value < 0.05 was considered statistically significant. The data were analyzed using StatView-J 5.0 (Abacus Concepts Inc., Berkeley, CA).

RESULTS

Physiological indices and blood gas analysis after lung reperfusion

Physiological measurements and blood gas analyses were done serially in the canine lung transplantation model, and the values taken 5 hours after the start of lung reperfusion are shown in Table 1. In the sham-operated group, PaO_2 was stable at around 400 mmHg throughout the experiment using ventilator settings of FiO_2 1.0 and PEEP 5-cm H_2O, which is similar to the previous canine studies [18, 19]. The PaO_2 of the lung transplant group was 167 ± 85 mmHg and significantly lowered compared with the sham operated group, which indicated severe ischemia-reperfusion lung injury ($P < 0.05$; Table 1). Peak airway pressure was significantly elevated in the transplanted group compared to the sham operated group ($P < 0.05$; Table 1). Physiological indices were stable during throughout the ELF collection period.

Table 1 Physiological measurements and blood gas analyses during ischemia-reperfusion lung injury after canine lung transplantation.

Group	Systolic arterial pressure (mmHg)	Heart rate (/min)	Mean pulmonary arterial pressure (mmHg)	Peak airway pressure (cm H_2O)	PaO_2 (mmHg)	$PaCO_2$ (mmHg)	pH
Transplant (n=4)	83 ± 19	120 ± 11	25 ± 11	17.0 ± 1.0*	167 ± 85*	53 ± 21	7.22 ± 0.10
Sham (n=2)	107 ± 21	99 ± 27	19 ± 0	15.0 ± 0	403 ± 105	41 ± 10	7.32 ± 0.11

Date are shown as mean ± SD. *P < 0.05, compared with the sham group, Student's unpaired *t*-test.

Table 2 Amount of epithelial lining fluid (ELF) collected by bronchoscopic microsampling (BMS) technique according to the groups in a canine lung transplant model.

	Donor lung		Transplant group		Sham-operated group	
	Before ischemia	After ischemia	Transplanted lung	Contralateral lung	Left lung	Right lung
Amount of collected ELF (μg)	2.8 ± 3.1	8.2 ± 10.2	5.0 ± 4.8*	5.9 ± 5.0#	1.9 ± 1.8	2.7 ± 1.9

Date are shown as mean ± SD. *P < 0.05, compared with the left lungs of the sham group; #P < 0.05, compared with the bilateral lungs of the sham group, Student's unpaired *t*-test.

Table 3 Cytokine concentrations in bronchoalveolar lavage (BAL) fluid before and after 12-hour cold and 6-hour warm ischemic insults in the canine lung transplant model.

Cytokines (pg/ml)	BAL fluid (n = 4)	
	Before ischemia	After ischemia
TNF-α	0.90 ± 1.51	0.40 ± 0.47
IL-1β	5.4 ± 6.3	6.9 ± 8.2
IL-6	0.0 ± 0.0	0.01 ± 0.01
IFN-γ	0.19 ± 0.25	0.14 ± 0.17

IL-1b: Interleukin-1β, IL-6: Interleukin-6, IFN-γ: Interferon-γ, TNF-α: Tumor necrosis factor-α. Data are shown as mean ± SD

ELF volume collected using BMS

The volumes of ELF collected in donor lungs before and after ischemia, transplanted lungs, and sham-operated lungs are shown in Table 2. There was no significant difference in the amount of collected ELF between before and after ischemia (Table 2). The ELF amount collected from the grafted lungs was significantly greater than that collected in the left lungs of the sham-operated group. Moreover, significantly more ELF was collected from the contralateral (non-transplanted) lungs of the transplanted group than from the left or right lungs of the sham-operated group ($P < 0.05$, both; Table 2).

Cytokine concentrations in the ELF and BAL fluid before vs. after ischemia in donor lungs

Concentrations of four inflammatory cytokines were measured in samples of BAL fluid and ELF collected from ex vivo donor lungs before and after ischemia (see **Lung procurement** in **Materials and Methods**). Cytokine concentrations were mostly below the detection limits in BAL fluid from the donor lungs before and after ischemia (Table 3). Temporal changes of cytokine concentrations in ELF from individual donor lungs are shown in Fig. 2. The IL-1β concentrations were significantly lower after ischemia in all four donor lungs (before, 3043 ± 1603 pg/ml; after

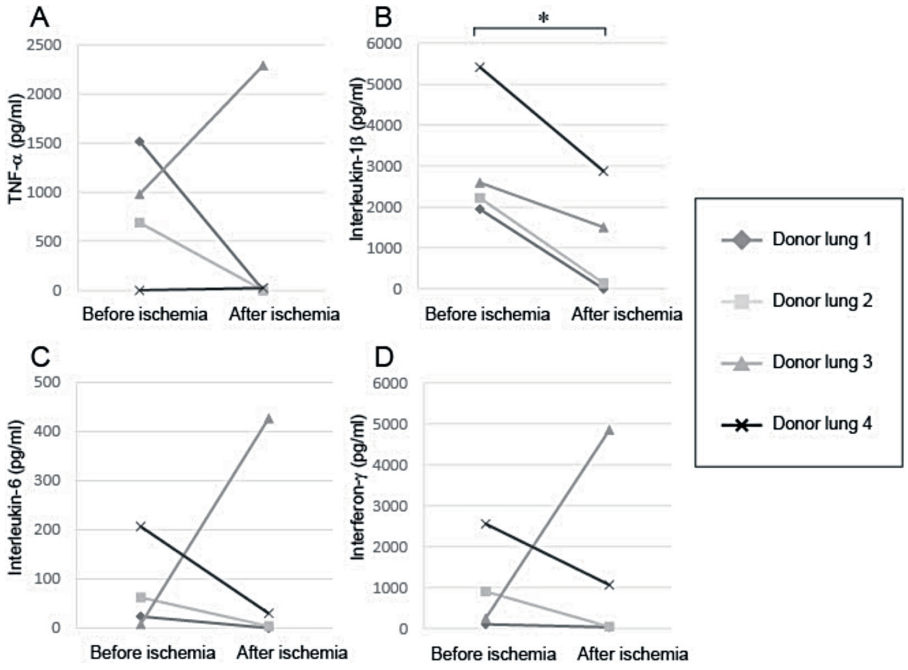

Fig. 2 Changes of cytokine concentrations in epithelial lining fluid (ELF) from individual donor lungs before and after 12 h of cold ischemia and 6 h of warm ischemia
(A) tumor necrosis factor (TNF)-*α*, (B) interleukin (IL)-1, (C) IL-6, and (D) interferon (IFN)-*γ*. $^{*}P < 0.05$, compared between before and after the ischemia time by the Student's paired *t*-test.

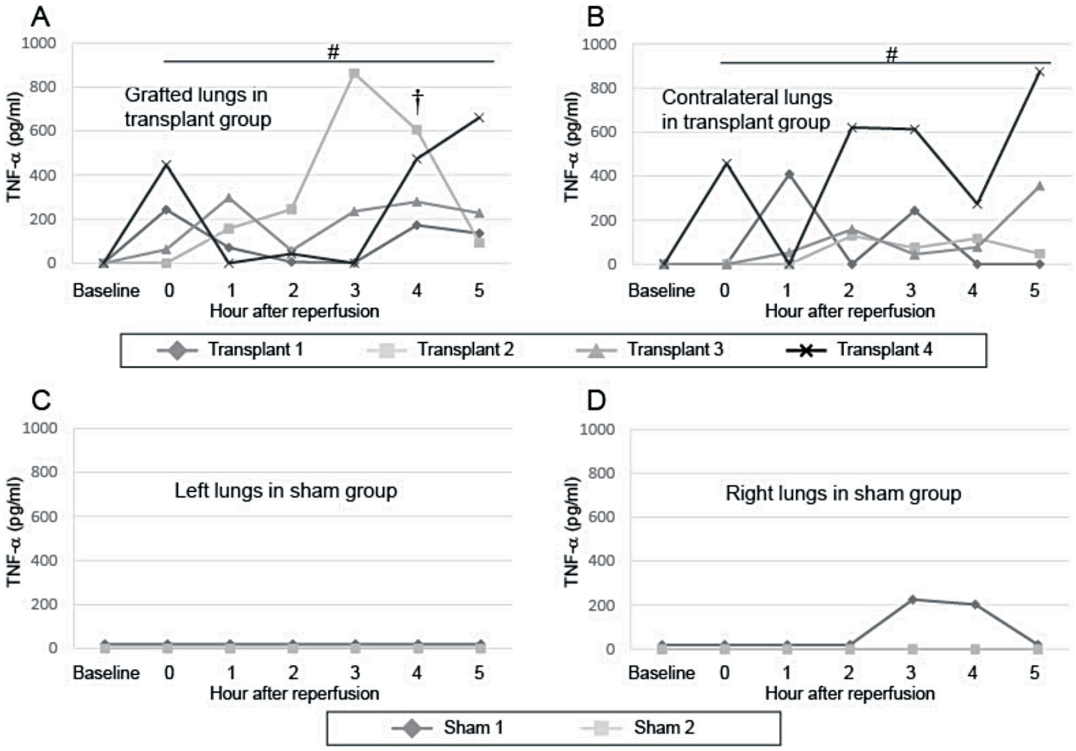

Fig. 3 Temporal changes in tumor necrosis factor (TNF)-*α* concentrations in epithelial lining fluid (ELF) after lung transplantation
(A) The graft lungs and (B) contralateral lungs of the lung transplant groups, and (C) the left and (D) the right lungs of the sham-operated groups. $^{\dagger}P < 0.05$, compared with the baseline, Student's paired *t*-test. $^{\#}P < 0.05$, longitudinal difference between groups by repeated-measure analysis of variance.

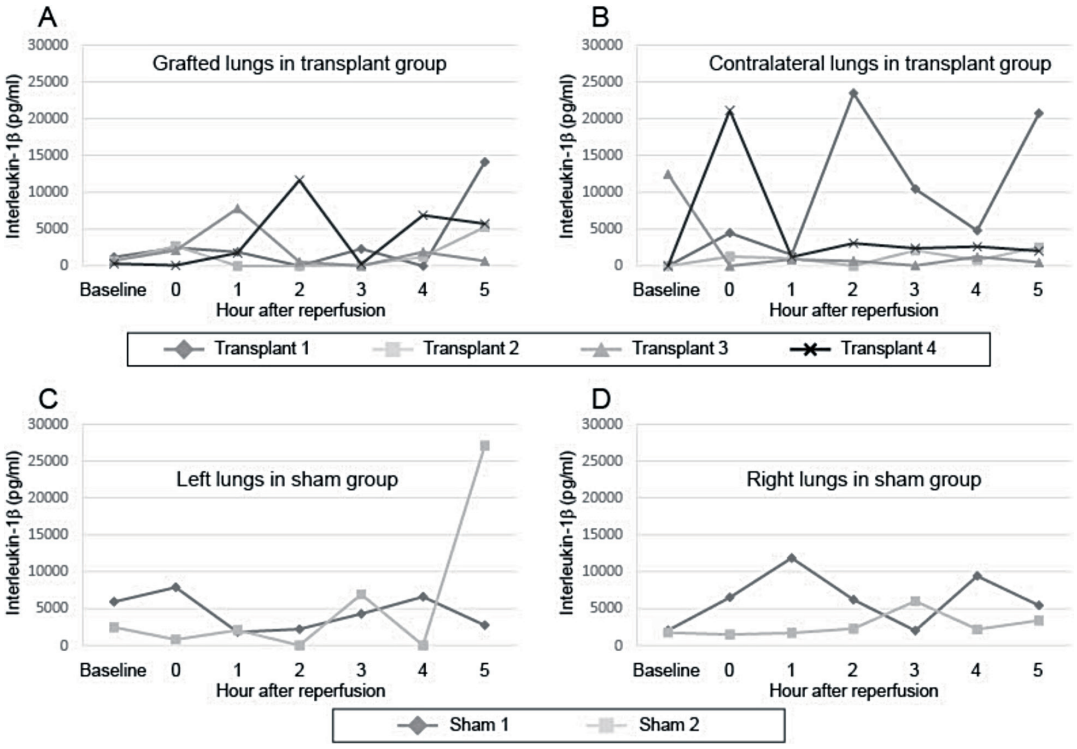

Fig. 4 Temporal changes in interleukin (IL)-1β concentrations in epithelial lining fluid (ELF) after lung transplantation
(A) The graft lungs and (B) contralateral lungs of the lung transplant groups, and (C) the left and (D) the right lungs of the sham-operated groups.

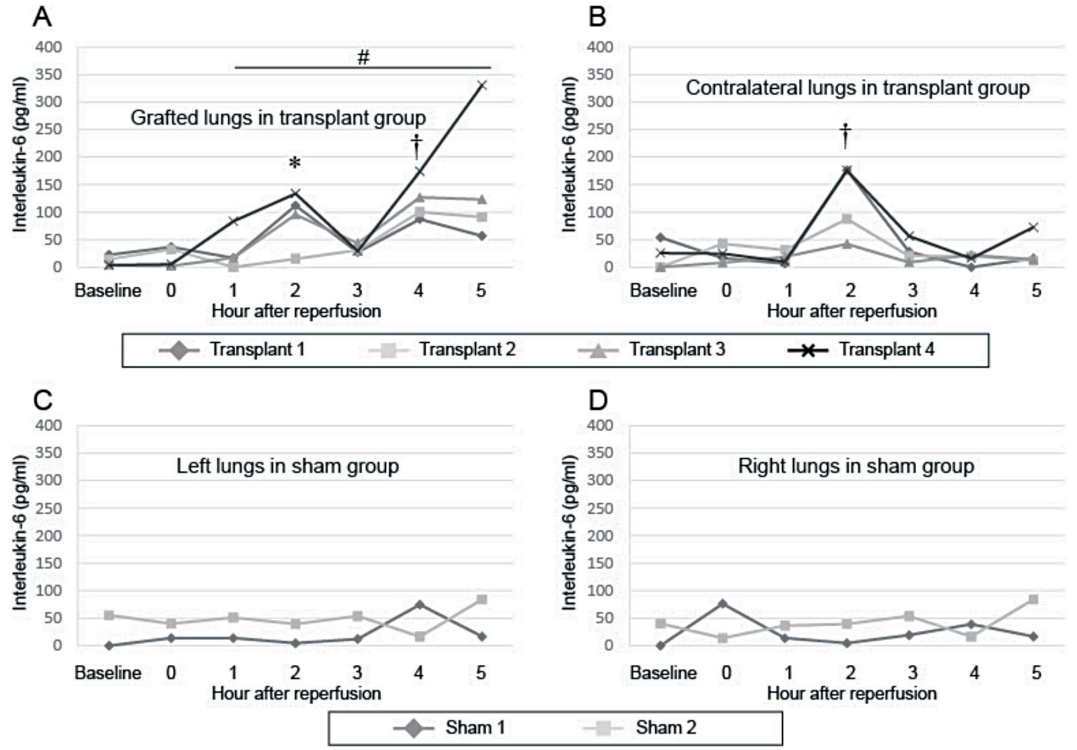

Fig. 5 Temporal changes in interleukin (IL)-6 concentrations in epithelial lining fluid (ELF) after lung transplantation
(A) The graft lungs and (B) contralateral lungs of the lung transplant groups, and (C) the left and (D) the right lungs of the sham-operated groups. $^{*}P < 0.05$, difference between groups at each time point by Student's unpaired *t*-test. $^{\dagger}P < 0.05$, compared with the baseline, Student's paired *t*-test. $^{\#}P < 0.05$, longitudinal difference between groups by repeated-measure analysis of variance.

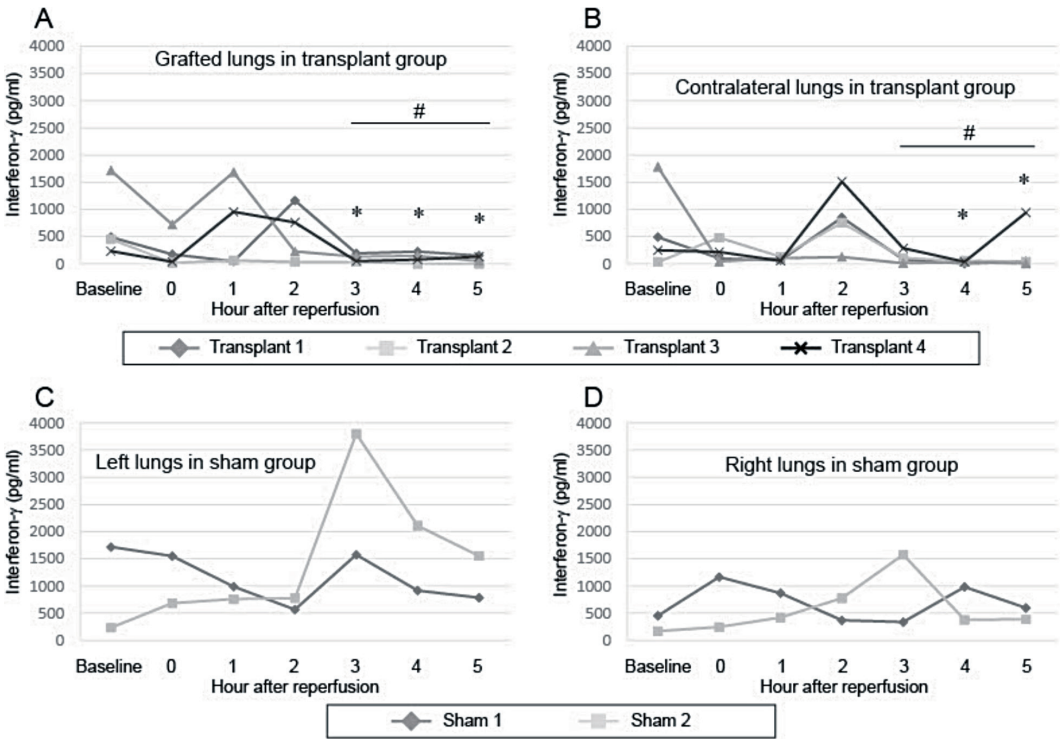

Fig. 6 Temporal changes in interferon (IFN) -γ concentrations in epithelial lining fluid (ELF) after lung transplantation
(A) The graft lungs and (B) contralateral lungs of the lung transplant groups, and (C) the left and (D) the right lungs of the sham-operated groups. $^{*}P < 0.05$, difference between groups at each time point by Student's unpaired *t*-test. $^{\#}P < 0.05$, longitudinal difference between groups by repeated-measure analysis of variance.

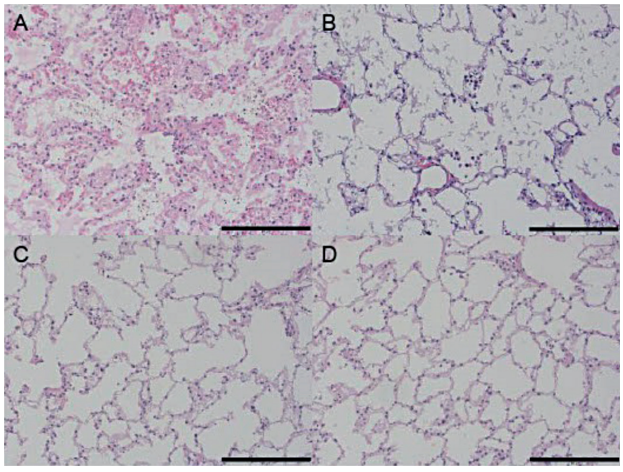

Fig. 7 Representative histological sections of canine lungs following lung transplantation after 18 hours of ischemia and 5 hours of reperfusion
(A) The graft lung. (B) The contralateral lung of the lung transplant group. (C) The left lung of the sham-operated group. (D) The contralateral lung of the sham operation group. Bars = 200 μm. Hematoxylin and eosin stain.

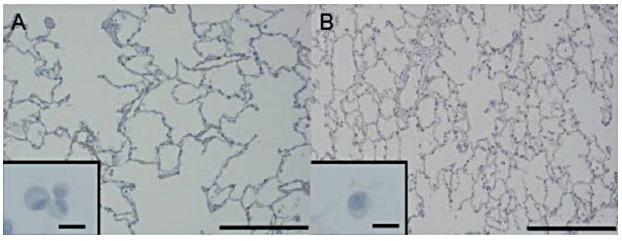

Fig. 8 Representative immunohistochemical labeling for tumor necrosis factor (TNF)-α and interleukin (IL)-6 on non-reperfused canine lungs after 12-hour cold and 6-hour warm ischemic insults.
(A) Anti-TNF-α immunolabeling. (B) Anti-IL-6 immunolabeling. Scale bars = 200 μm. Representative inflammatory cells are shown in the insets. Scale bars = 5 μm.

Table 4 Cytokine concentrations in bronchoalveolar lavage fluid after lung transplantation in the canine model.

Cytokines (pg/ml)	Transplant group (n = 4)		Sham-operated group (n = 2)	
	Transplanted lung	Contralateral lung	Left lung	Right lung
TNF-α	0.24 ± 0.43	0.64 ± 0.43	3.18 ± 4.46	0.80 ± 1.12
IL-1β	585 ± 1096	113 ± 158	68 ± 67	154 ± 196
IL-6	1.12 ± 0.83	0.54 ± 0.15	0.18 ± 0.10	0.16 ± 0.19
IFN-γ	0.32 ± 0.23	0.40 ± 0.17	0.44 ± 0.12	0.47 ± 0.39

IL-1β: Interleukin-1β, IL-6: Interleukin-6, IFN-γ: Interferon-γ, TNF-α: Tumor necrosis factor-α. Date are shown as mean ± SD

1128 ± 1348 pg/ml; $P < 0.05$) according to Student's paired *t*-test (Fig. 2A). The other measured cytokines were lower after ischemia in three of the donor lungs; however, in one donor lung they were elevated. None of the differences in these cytokines were significant, however.

Temporal changes of ELF cytokine concentrations after lung transplantation

ELF was collected hourly in the distal airways of both the grafted and the contralateral lung, and temporal changes of ELF cytokine concentrations in the individual dogs were shown and compared with the sham-operated group.

As shown in Fig. 3, the ELF TNF-α concentration in the grafted lung was significantly elevated over baseline at the 4 h time point ($P < 0.005$); however, TNF-α concentrations in the sham-operated group were mostly too low to detect at all time points. Furthermore, in a longitudinal comparison between the 0 to 5 h time points, there were significant differences in the ELF TNF-α concentrations between the grafted and sham-operated left lungs, as well as between the contralateral lungs of the two groups, when compared using a repeated measures ANOVA ($P < 0.005$, and $P < 0.05$, respectively).

No significant changes in IL-1β concentrations in ELF were found for any time points in either the grafted or contralateral lungs (Fig. 4). In addition, there were no significant differences between the transplanted and the sham-operated groups.

ELF IL-6 concentrations were increased after the start of reperfusion in the grafted lungs, including a significant elevation at the 4 h time point ($P < 0.05$, Fig. 5A). In addition, there was a significant elevation in the contralateral lungs at the 2 h time point ($P < 0.05$, Fig. 5B). In the longitudinal comparison between the 1 to 5 h time points, a significant increase in IL-6 levels was detected in the grafted lungs relative to the sham-operated lungs ($P < 0.05$, Fig. 5A & C), although there was no significant difference between the contralateral lungs of the two groups (Fig. 5B & D).

IFN-γ concentrations in ELF from the grafted lungs were significantly lower than those in the sham-operated lungs from the 3 h time point through the 5 h time point when tested by either Student's unpaired *t*-test or repeated measures ANOVA ($P < 0.05$ each, Fig. 6A & C), although no significant differences were detected between the baseline and any other time point. The concentrations in ELF from the contralateral lungs was also significantly different between groups at the 3, 4, and 5 h time points. ($P < 0.05$, each, Fig. 6B & D).

Cytokine concentrations in BAL fluid after lung transplantation

Concentrations of TNF-α, IL-6, IL-1β and IFN-γ in BAL fluid collected in the grafted lungs after 5 hours of reperfusion were very low, and mostly below the detection threshold (Table 4). Concentrations of all tested cytokines in BAL fluid collected from the contralateral lungs after 5 hours of reperfusion were also very low (Table 4).

Histopathology in grafted lungs

Severe lung injury was confirmed on histologic examination in the grafted lungs of the lung transplant group. Extensive alveolar hemorrhage, infiltration of inflammatory cells, and interstitial thickening were observed (Fig. 7A). In addition, there was marked inflammatory infiltration in the contralateral lungs of the lung transplant group (Fig. 7B). By contrast, there were no significant findings in the bilateral lungs of the sham-operated group (Fig. 7C & D).

Immunohistochemical staining of TNF-α and IL-6 in non-perfused donor lungs after ischemia

Immunohistochemical staining showed no significant TNF-α expression in the capillary endothelial cells or inflammatory cells in the donor lungs after the 18 hours of ischemia treatment (Fig. 8A). In addition, there was no significant IL-6 expression in the ischemic donor lungs (Fig. 8B).

Immunohistochemical staining of TNF-α and IL-6 in grafted lungs

Immunohistochemical staining showed significant TNF-α expression in the alveolar macrophages and the capillary endothelial cells of the grafted lungs after 18 hours of ischemia and 5 hours reperfusion, whereas TNF-α expression in the sham-operated group was not detectable (Fig. 9). TNF-α expression was also observed in alveolar macrophages in the contralateral lungs of

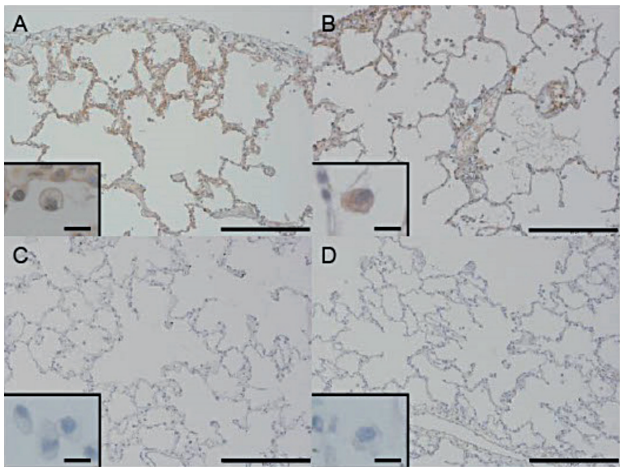

Fig. 9 Immunohistochemistry for tumor necrosis factor (TNF)-α on the transplanted canine lungs after 5 hours of reperfusion
(A) The graft lung. (B) The contralateral lung of the lung transplant group. (C) The left lung of the sham-operated group. (D) The contralateral lung of the sham-operated group. Scale bars = 200 μm. Arrows indicate alveolar macrophages. Representative macrophages of each group are shown in the insets. Scale bars = 5 μm.

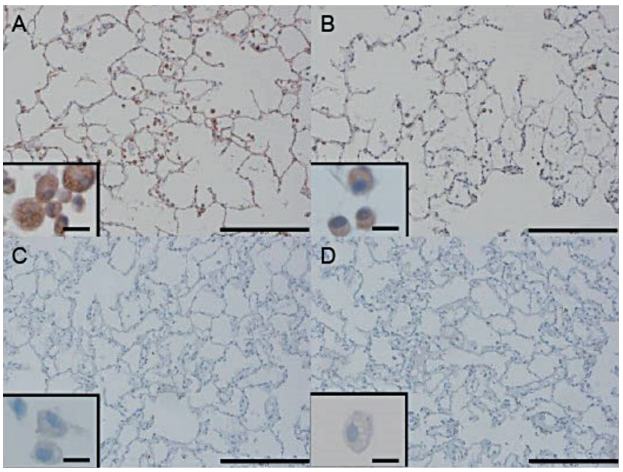

Fig. 10 Immunohistochemistry for interleukin (IL)-6 on the transplanted canine lungs after 18 hours of ischemia and 5 hours of reperfusion
(A) The graft lung. (B) The contralateral lung of the lung transplant group. (C) The left lung of the sham-operated group. (D) The contralateral lung of the sham-operated group. Scale bars = 200 μm. Arrows indicate alveolar macrophages. Representative macrophages of each group are shown in the insets. Scale bars = 5 μm.

the transplantation group, but not in the alveolar macrophages of the sham-operated group. Significant IL-6 expression was immunohistochemically observed in the lymphocytes and macrophages infiltrating the alveoli of the grafted lungs after 5 hours reperfusion following 18 hours of ischemia, but not in the sham-operated group (Fig. 10). IL-6 expression was also observed in infiltrating cells in the alveoli in the contralateral lungs of the transplantation group.

DISCUSSION

Pro-inflammatory cytokines have been shown to play a critical role in ischemia-reperfusion lung injury after lung transplantation [20]. However, the underlying mechanisms and exact roles of the individual cytokines have yet to be investigated in detail. In the present study in a canine lung transplant model, ELF was collected in the distal airways of grafted lungs from each animal using serial BMS collection without sacrifice, and the temporal changes of pro-inflammatory cytokines in ELF were elucidated for the first time. The time-dependent changes of ELF cytokine concentrations during reperfusion varied by cytokine. TNF-α and IL-6 concentrations in ELF significantly increased over 5 hours after the start of reperfusion, while ELF IFN-γ concentration was significantly reduced after 3 h of reperfusion in the grafted lungs. These specific cytokine profile changes in ELF may underlie the mechanisms of ischemia-reperfusion lung injury after lung transplantation.

We have recently reported that BMS is a safe and efficient technique for serial ELF collection in a canine model of lung transplantation [7]. It permits the collection of multiple samples over a significant duration without causing adverse effects. BMS is minimally invasive and could satisfy the ethical mandate of reducing the number of animals sacrificed to study the mechanisms of pulmonary disorders after lung transplantation. Because BAL and other current techniques carry a risk of injury, BMS would be useful for ELF collection in lung transplant patients; having validated the safety of the technique with this study, our future plans include adopting BMS in the clinical setting.

As mentioned above, the temporal changes of cytokine levels in ELF were dependent on the specific cytokine. For example, the changes in IL-1β were not significant throughout the 5 hour-long experimental period. On the other hand, IL-6 and TNF-α concentrations in ELF significantly increased after reperfusion had begun, suggesting they were associated with ischemia-reperfusion lung injury. Notably, these two cytokine concentrations also increased in the contralateral lungs of the transplanted group. We found that IL-6 and TNF-α expression were strongly localized to alveolar macrophages or lymphocytes in the alveoli of lungs. This suggests a mechanism in which the cytokines are released by the inflammatory cells in the alveoli, then diffuse or are transported out to terminal bronchioles; from the bronchioles, they are finally drained to the distal airways. Prolonged local accumulation of pro-inflammatory cytokines injures the lung and its vasculature, causing hyper-permeability of pulmonary vessels and subsequent pulmonary edema. It should be noted that the capillary endothelial cells in the grafted lungs demonstrated anti-TNF-α immunolabeling, suggesting they may be an alternative source of the cytokine. Although the significance of this is unclear, it is possible that the extra TNF-α could create a situation where the grafted lung is more vulnerable to injury than the contralateral lung.

We believe it significant that the temporal changes in IFN-γ concentrations in the transplanted group were different from the others. IFN-γ concentrations were significantly lower (opposite the cytokine changes) after 3 h of reperfusion in both the grafted and contralateral lungs. Unfortunately, the antibodies available

for immunolabeling did not work against canine IFN-γ, so the source(s) of IFN-γ expression remain to be determined. Investigation of T lymphocytes which is supposed as the main source of IFN-γ and IL-10 which is a suppressant of IFN-γ may clarify the pathway.

Temporal changes in systemic TNF-α, an early-phase cytokine, vary according to the animal species and nature of the insult; they typically increase to a peak within 1–2 hours, and then decline within 3–4 hours of an initial insult such as lipopolysaccharide [21, 22]. Upregulation of TNF-α stimulates production of other pro-inflammatory cytokines in a cascade including IL-1β, IL-6 and IFN-γ, which typically reach peak levels within 2–4 hours, 4–5 hours, or 5–6 hours, respectively. The present findings suggest that, after lung transplantation, ischemia-reperfusion injury results in sustained elevations of TNF-α in the distal airways for an extended period that might disturb the normal activity of the cytokine cascade. For example, de Perrot *et al*. analyzed cytokine concentrations in relation to several factors in clinically-derived samples of lung tissue after 1 or 2 hours of reperfusion during lung transplant operations [20]. They noted that higher IL-8 levels were correlated with early graft dysfunction after lung transplantation [20], supporting the idea of altered cytokine activity described above. Thus, serial collection and analysis of ELF could be used to monitor IL-8 levels in the grafted lungs in the future, should this interleukin be validated as a biomarker for early graft dysfunction. We are shortly planning to measure ELF IL-8 levels in the canine lung transplant model.

Cytokine concentrations in ELF collected from donor lungs after the ischemic insult were decreased relative to those collected before ischemia, with the exception of IL-1β in a single animal. Moreover, there was no significant expression of TNF-α or IL-6 immunoreactivity in the ischemic lungs. This suggests that cytokine expression in inflammatory cells such as macrophages or lymphocytes was suppressed; alternatively, the cytokines may have lost their biological activities in most donor lungs during the long ischemic period in this study. However, given the immediate elevation in TNF-α after the start of reperfusion, there must be some molecular-level changes that induce TNF-α in non-reperfused ischemic lungs that would be the crux of post-transplant ischemia-reperfusion lung injury. In the previous study reported by de Perrot *et al*. at the clinical setting, TNF-α was significantly higher during the ischemic time and decreased after reperfusion in human lung transplantation, which is divergent from our measurement. In the present experiment, the donor lungs were harvested immediately after the start of mechanical ventilation, while the lungs were harvested after a significant time of mechanical ventilation in the clinical lung transplant. It has been reported that mechanical ventilation can cause cytokine upregulation in the healthy lungs [23]. On the other hands, severe ischemia-reperfusion lung injury occurred on all the animals in the present experiment, while the majority of the patients seemed free from severe ischemia-reperfusion injury in the clinical study. We think these differences brought a different result in the measurement of TNF-α between our study and the clinical study.

TNF-α production in the donor lungs before ischemia time might be due to the procedure of donor procurement including systemic anesthesia, pulmonary arterial perfusion, and surgical manipulation, and the main source might be donor-derived alveolar macrophages. On the other hands, after the start of reperfusion, the main source of cytokines may be recipient-derived macrophages and the other immunocompetent cells, because the blood in the donor lungs was washed out with the perfusate at procurement. However, some remaining donor-derived alveolar macrophages and the capillary endothelial cells of the grafted lungs may also produce cytokines.

In comparing the BAL and BMS techniques, we found that the concentrations of IL-6, IFN-γ, and TNF-α were less than 1% of those in ELF samples; additionally, IL-1β concentrations had large deviations related to the effects of dilution. When they were above the detection threshold, which was not often, cytokine measurements in BAL fluid did not show significant changes, while the counterpart samples of ELF demonstrated dynamic changes in the transplanted group. It may be supposed that BAL fluid reflects the alveolar areas rather than the bronchial areas, whereas ELF collected by BMS represents the bronchial areas. However, we found the most significant TNF-α and IL-6 immunolabeling in the alveolar areas. Therefore, potentially depending on the cytokine or molecular species being studied, BMS analysis of ELF might reflect conditions in both the conducting portion of the airway and the respiratory portion [24].

There are some limitations to the present study. Firstly, we chose a considerably long duration of ischemia to enhance ischemia-reperfusion lung injury and thus enhance cytokine production. However, we noted that in severe ischemia-reperfusion lung injury in lung transplant patients, similar PaO_2 data to ours were gathered (Table 1), suggesting the extent of injury is similar as well. In our future animal or clinical research, we are planning to collect ELF samples from lungs exposed to shorter durations of ischemia. Secondly, the volume of ELF collected using BMS ranged from 1–18 µL, which resulted in large standard deviations in the measurement of cytokines. This is a technical issue, since the probe will not collect a sufficient volume of ELF if it does not achieve adequate contact with the distal bronchial wall within 10 seconds of insertion. This error could be reduced by the use of three probes for each collection point, and using averages values of the triplicate samples. Finally, the sample size of animals was small in the present experiment, especially only two in the sham-operated group. However, the values of PaO_2 measured at FiO_2 1.0 or PaO_2/FiO_2 at PEEP of 4–5 cmH_2O in previous canine studies were similar to or higher than those in the sham-operated group of the present study [18, 19]. Therefore, we think that the sham-operated group was appropriately treated as a control, and the results of data analysis have a certain degree of reliability.

In conclusion, we validated our earlier work demonstrating that BMS is minimally invasive and safe, while still being effective. We identified early and sustained TNF-α and IL-6 elevations, and a concurrent IFN-γ reduction, associated with ischemia-reperfusion lung injury. However, we also noted that cytokines measured in the non-perfused donor lungs were not elevated.

The changes in the lung cytokine profile may be related to post-transplant graft dysfunction.

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DISCLOSURES

The authors have no conflict of interest to disclose.

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