

Reduction of donor mononuclear phagocytes with clodronate-liposome during ex vivo lung perfusion attenuates ischemia-reperfusion injury



Keiji Yamanashi, MD, Akihiro Ohsumi, MD, PhD, Hiromi Oda, MD, PhD, Satona Tanaka, MD, PhD, Yoshito Yamada, MD, PhD, Daisuke Nakajima, MD, PhD, and Hiroshi Date, MD, PhD

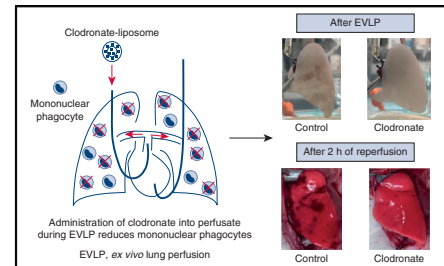
ABSTRACT

Objectives: Clodronate-liposome is used for depleting mononuclear phagocytes associated with ischemia-reperfusion injury. We hypothesized that administration of clodronate-liposome into the perfusate during ex vivo lung perfusion could reduce mononuclear phagocytes and attenuate ischemia-reperfusion injury.

Methods: First, the number of mononuclear phagocytes in flushed grafts (minimum cold ischemic time, 6-hour cold ischemic time, 15-hour cold ischemic time, and 18-hour cold ischemic time; $n = 6$ each) was determined using flow cytometry. Second, grafts (15-hour cold ischemic time) were allocated to control or clodronate ($n = 5$ each). In the clodronate group, clodronate-liposome is administered into the perfusate. After 4 hours of ex vivo lung perfusion, the number of mononuclear phagocytes in the perfusate and lung tissues was measured. Third, grafts (15-hour cold ischemic time) were allocated to control or clodronate ($n = 6$ each). After 4 hours of ex vivo lung perfusion, the left lungs were transplanted and reperfused for 2 hours. Lung function was evaluated, and samples were analyzed.

Results: First, mononuclear phagocytes remain in flushed grafts after prolonged cold ischemia. Second, the number of mononuclear phagocytes in lung tissues after ex vivo lung perfusion was significantly reduced in the clodronate group ($P = .008$). Third, lung compliance and vascular resistance during ex vivo lung perfusion were significantly improved in the clodronate group ($P < .001$ for both). Blood oxygenation and pulmonary edema were significantly improved in the clodronate group after 2 hours of reperfusion ($P = .015$ and $P = .026$, respectively). Histological findings showed reduced lung injury in the clodronate group ($P = .013$).

Conclusions: Administration of clodronate-liposome into the perfusate during ex vivo lung perfusion resulted in a significant reduction of mononuclear phagocytes in donor lungs, leading to attenuation of ischemia-reperfusion injury. (J Thorac Cardiovasc Surg 2023;165:e181-203)



Reduction of MPs during EVLP attenuated IRI.

CENTRAL MESSAGE

Administration of clodronate-liposome into the perfusate during EVLP significantly reduced MPs in donor lungs and attenuated IRI.

PERSPECTIVE

The true potential of EVLP lies in reconditioning donor lungs. Administration of clodronate-liposome into the perfusate during EVLP resulted in the graft function improvement and attenuation of graft injury post-transplantation. These findings suggest the protective effects of clodronate-liposome against IRI and support further investigation into its role in clinical practice.

From the Department of Thoracic Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan.

This work was supported by the Japan Society for the Promotion of Science KAKENHI (19K09302) and SPIRITS 2021 from Kyoto University.

The experimental protocol was approved by the Ethics Committee of the Graduate School of Medicine at Kyoto University (date: April 1, 2018, reference number: Med Kyo 20294).

Received for publication July 2, 2022; revisions received Sept 28, 2022; accepted for publication Oct 13, 2022; available ahead of print Oct 22, 2022.

Address for reprints: Akihiro Ohsumi, MD, PhD, Department of Thoracic Surgery, Kyoto University Hospital, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto, 606-8507, Japan (E-mail: akohsumi@kuhp.kyoto-u.ac.jp).

0022-5223/\$36.00

Copyright © 2022 by The American Association for Thoracic Surgery

<https://doi.org/10.1016/j.jtcvs.2022.10.022>

Lung transplantation (LTx) is recognized as an effective treatment for patients with end-stage lung diseases worldwide.¹ Primary graft dysfunction (PGD) represents the predominant cause of short-term mortality after LTx and chronic lung allograft dysfunction.^{2,3} Because a major cause of PGD is pulmonary ischemia-reperfusion injury (IRI), its attenuation might reduce PGD incidence and severity.⁴

Despite flushing with preservation solution during donor procurement, donor lungs still contain leukocytes.^{5,6} Among leukocyte subtypes, mononuclear phagocytes (MPs) are considered the main cause of IRI through the release of cytokines and chemokines, leading some studies

Abbreviations and Acronyms

CIT	= cold ischemic time
EVLP	= ex vivo lung perfusion
IL	= interleukin
IRI	= ischemia-reperfusion injury
LTx	= lung transplantation
MP	= mononuclear phagocyte
PGD	= primary graft dysfunction

to suggest that the reduction of donor MPs might attenuate IRI.^{5,7-9} Notably, MPs comprise monocytes, macrophages, and dendritic cells.¹⁰ Some drugs such as clodronate-liposome,⁵ anti-CCR2 antibody,⁷ and diphtheria toxin¹¹ have been used to deplete MPs (except for dendritic cells). The use of clodronate-liposome is one of the most promising approaches for depleting monocytes and macrophages in animal models,^{5,12} because anti-CCR2 antibody depletes a limited subset of MPs⁷ and diphtheria toxin could exert adverse effects such as local inflammation.¹¹

Previous studies have described direct injection of clodronate-liposome into animals 1 day before experiments^{5,13}; however, this administration also affects other organs. Ex vivo lung perfusion (EVLP) has been applied in patients worldwide for the pretransplant assessment of lung quality.¹⁴ The true potential of EVLP lies not only in evaluating the quality of donor grafts but also in reconditioning them before LTx without affecting other organs.^{15,16}

This study examined the hypothesis that administering clodronate-liposome into the perfusate during EVLP can reduce the number of MPs in donor lungs and attenuate IRI.

MATERIALS AND METHODS**Ethical Statement**

Male Lewis rats (aged 10-11 weeks; weight, 260-300 g) were purchased from Japan SLC. The experimental protocol was approved by the Ethics Committee of the Graduate School of Medicine at Kyoto University (date: April 1, 2018; reference number: Med Kyo 20294). All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals, as prepared by the National Institutes of Health (Publication No. 86-23, revised 1996).

Flow Cytometry

Flow cytometry was used to evaluate the distribution of leukocyte subtypes in right lung grafts after cold storage and perfusate during EVLP. Isolated cells were washed, and red blood cell lysis was performed using FACSlyse (BD Biosciences). Thereafter, cells were stained with the following antibodies: anti-CD45, anti-CD11b, anti-CD3, anti-CD4, anti-CD8, anti-CD45RA, and anti-CD11a (BD Biosciences). Cells were counted (cells/mL) on a cell counter (Countess automated cell counter; Thermo Fisher Scientific) and analyzed using a multiparameter flow cytometer (LSRFortessa; BD Biosciences). Flow cytometry data were analyzed using FlowJo software (v10; FlowJo LLC), the gating strategy of which was designed according to the manufacturer's manual.¹⁷ A

representative gating strategy to evaluate leukocyte populations in the right lung is shown in [Figure E1](#).

Experiment I: Retention of Mononuclear Phagocytes in Flushed Donor Lungs After Prolonged Cold Ischemia

Only flushed grafts were investigated in this study. The amounts of MPs and other leukocyte subtypes in flushed grafts at each cold ischemic time (CIT) were measured using flow cytometry.

Donor procurement and study groups. Details of the donor procurement procedure have been reported previously¹⁸ and are provided in the [Appendix E1](#). Lung grafts were allocated to 4 groups: Min-CIT, 6h-CIT, 15h-CIT, and 18h-CIT. In the Min-CIT group, CIT was approximately 20 minutes in almost all cases.

Experiment II: Reduction of Mononuclear Phagocytes by Clodronate-Liposome During Ex Vivo Lung Perfusion

The reduction in the number of MPs by clodronate-liposome during EVLP was assessed.

Ex vivo lung perfusion procedure and study groups. Details of the EVLP procedure have been reported previously¹⁹ and are provided in the [Appendix E1](#). The circuit was primed with 120 mL Steen solution (XVIVO Perfusion AB) supplemented with 800 USP units of sodium heparin and 40 mg methylprednisolone (Pfizer Ltd). Because rat donor grafts have been shown to maintain a stable lung function during 4 hours of EVLP up to 12h-CIT,¹⁹ CIT for the comparison was set to 15 hours in this study. Lung grafts were allocated to 3 groups: Min-CIT, control (15h-CIT), and clodronate (15h-CIT with clodronate-liposome). Three milliliters clodronate-liposome (FormuMax Scientific, Inc, Sunnyvale, Calif) was administered into the perfusate immediately before perfusion in the clodronate group, whereas nothing was administered in the control group.

Wet-to-dry weight ratio, vascular endothelial cadherin staining, zonula Occludens-1 staining, and inflammatory markers. To evaluate endothelial and epithelial cell integrity, lung tissues were stained using anti-vascular endothelial-cadherin and anti-zonula occludens-1 tight junction protein antibodies. Details of these procedures have been reported previously¹⁹ and are provided in the [Appendix E1](#).

Experiment III: Attenuation of Ischemia-Reperfusion Injury in a Rat Lung Transplantation Model

The attenuation of IRI by administration of clodronate-liposome during EVLP was evaluated.

Lung transplantation procedure after ex vivo lung perfusion and study groups.

Donor lungs after EVLP were stored at 4°C in Perfadex (XVIVO Perfusion) until implantation. Left LTx was performed, as previously described,¹⁸ with the details of the procedure provided in the [Appendix E1](#). Lung grafts were allocated to the control (15h-CIT) and clodronate (15h-CIT with clodronate-liposome) groups.

Histologic examination and lung injury scoring. The formalin-fixed, paraffin-embedded lower part of left lung tissues was sectioned and stained using standard hematoxylin-eosin staining. Lung injury was semiquantitatively scored under the supervision of a pulmonary pathologist. Each collected section was scored by assessing air space hemorrhage (ie, presence of red blood cells in alveoli), vascular congestion (>75% of alveolar septum occupied by red blood cells), edema/fibrin in alveoli, and the presence of infiltrating white blood cells as previously described.¹⁸ These criteria were graded on a scale ranging from normal

appearance (0%), mild (<10%), moderate (10%-50%), or severe (>50%) abnormalities and scored from 0 to 3, respectively.

Myeloperoxidase staining. To evaluate neutrophil infiltration, lung tissues were stained using anti-myeloperoxidase antibodies. Details of this procedure are provided in the [Appendix E1](#).

Statistical Analysis

Continuous values are expressed as the median (interquartile range) or mean $\pm$ standard deviation. The Mann–Whitney *U* test was used to compare differences between 2 groups, whereas the Kruskal–Wallis test was used to compare differences among more than 2 groups. As repeated measures analysis of variance was used to compare the mean scores of subjects during 3 or more time points between 2 groups,^{20,21} the data obtained during EVLP were analyzed using this analysis. All data were processed and analyzed using the statistical software R version 3.3.2 (R Foundation for Statistical Computing). All *P* values were 2-tailed.

RESULTS

Experiment I

Twenty-four rats were allocated to 4 groups, and their weights were similar ($n = 6$ each; $P = .55$; [Table E1](#)).

Distribution of live leukocyte subtypes in donor grafts after cold ischemia. The number of live leukocytes in the right lungs after donor procurement tended to decrease in the 15h-CIT and 18h-CIT groups compared with that in the Min-CIT group ($P = .18$ and $.24$, respectively; [Figure E2](#)). The number of live MPs remained unchanged in the 6h-CIT, 15h-CIT, and 18h-CIT groups compared with that in the Min-CIT group ($P = .13$, 1.00 , and $.94$, respectively; [Figure 1, A](#)), whereas that of other leukocyte subtypes tended to decrease ([Figure E3, A-D](#)). Additionally, the percentage of live MPs increased significantly in the 6h-CIT, 15h-CIT, and 18h-CIT groups compared with that in the Min-CIT group ($P = .002$, $.002$, and $.002$, respectively; [Figure 1, B](#)), whereas that of other leukocyte subtypes decreased significantly ([Figure E4, A-D](#)).

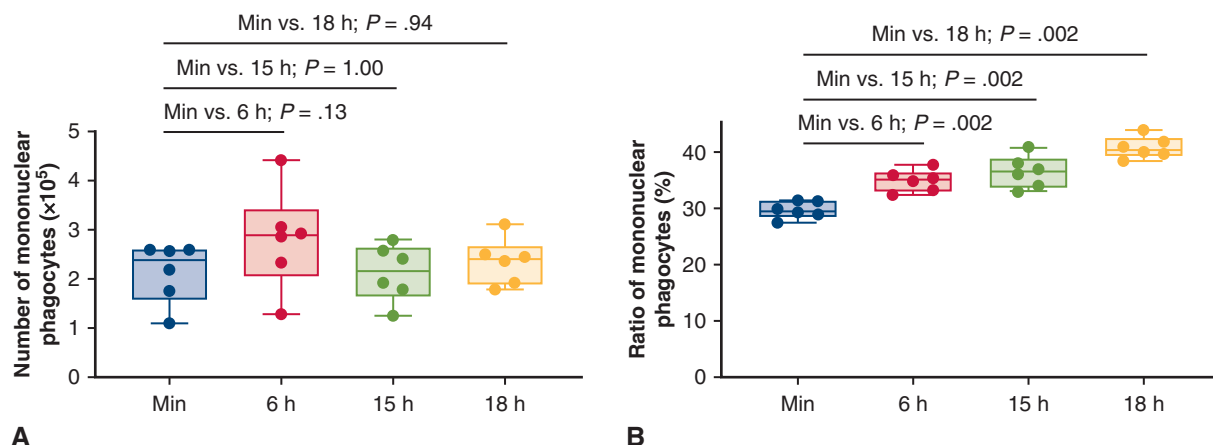


FIGURE 1. The isolated cells in the right lungs were counted using a cell counter and the number (A) of MPs at each CIT was calculated according to the ratio (MPs/leukocytes; B) measured using a multiparameter flow cytometer. Box-and-whisker plots are displayed with the middle line representing median, lower, and upper borders representing 25th and 75th percentiles, and the lower and upper whiskers representing the minimum and maximum, respectively. *P* values were calculated using the Mann–Whitney *U* test.

Experiment II

The weights of animals were similar among the 3 groups ($P = .92$; [Table E2](#)).

Fourteen rats were randomly allocated to 3 groups: Min-CIT ($n = 3$), control (15h-CIT; $n = 6$), and clodronate (15h-CIT with clodronate-liposome; $n = 5$). One of 6 grafts in the control group was lost due to severe pulmonary edema after 150 minutes of perfusion (excluded from analyses), whereas all 5 grafts in the clodronate group were perfused for 4 hours. Data on physiological parameters during EVLP are shown in [Figure E5, A-J](#).

Distribution of live leukocyte subtypes in the perfusate during ex vivo lung perfusion and donor grafts after ex vivo lung perfusion. The number of live leukocytes in the right lungs after EVLP decreased significantly in the clodronate group compared with that in the control group ($P = .008$; [Table E2](#)). Specifically, the number of live MPs in the perfusate was low throughout the process of EVLP, and both the number and percentage were significantly lower in the clodronate group relative to those in the control group ($P = .023$ and $.034$; [Figure 2, A and B](#), respectively). Furthermore, no marked differences were detected in the number and percentage of other leukocyte subtypes between the 2 groups ([Figure E6, A-H](#)).

Likewise, the number and percentage of MPs in the right lungs after EVLP were significantly lower in the clodronate group compared with those in the control group ($P = .008$ and $.016$; [Figure 2, C and D](#), respectively). However, no marked differences were detected in the number and percentage of other leukocyte subtypes between the 2 groups ([Figure E7, A-H](#)).

Assessment of endothelial and epithelial cell integrity. The expression of vascular endothelial-cadherin after 4 hours of EVLP remained stable in the clodronate group,

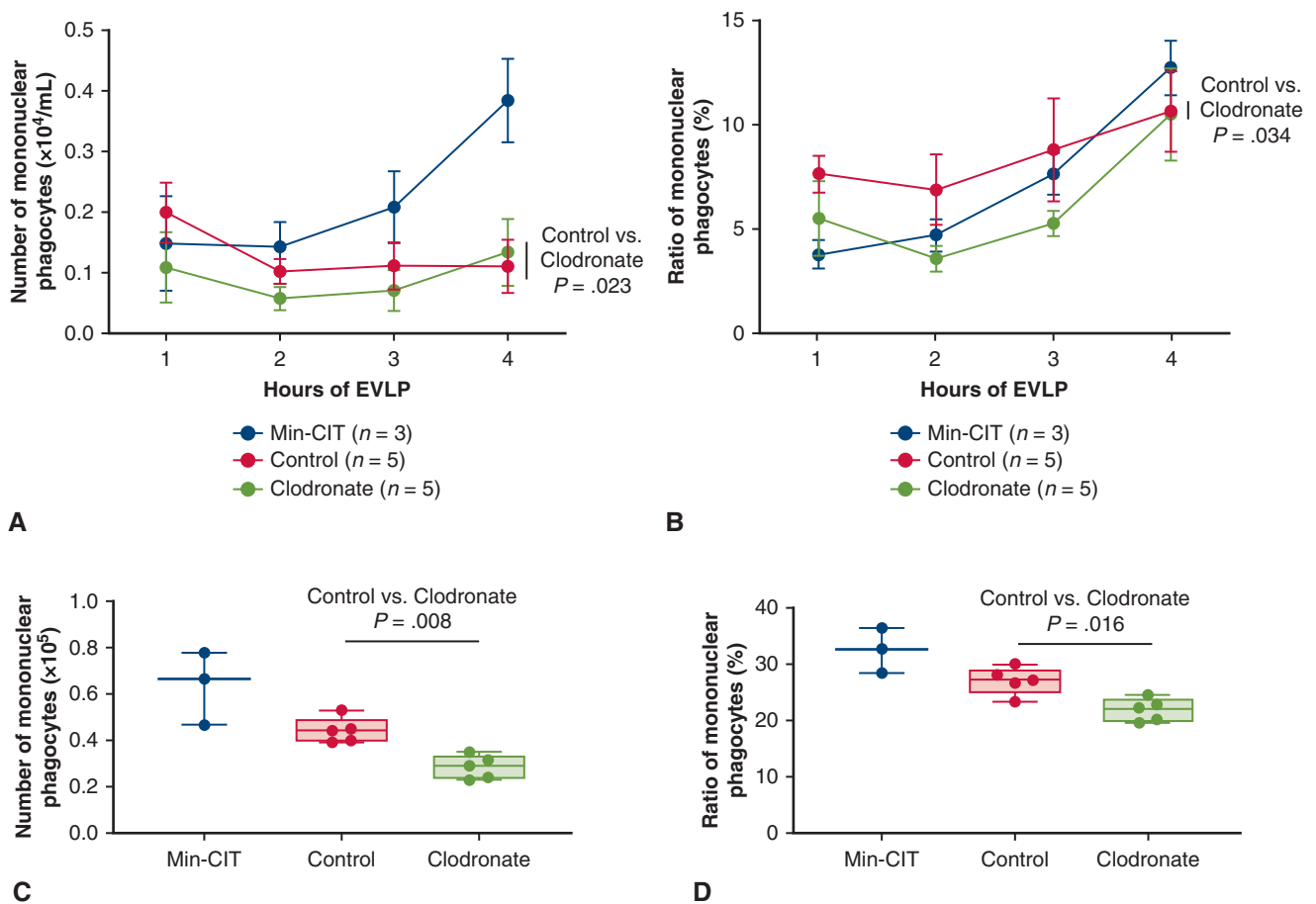


FIGURE 2. The isolated cells in the perfusate and right lungs were counted using a cell counter and the number of MPs was calculated according to the ratio (MPs/leukocytes) measured using a multiparameter flow cytometer. Both the number (A) and percentage (B) of MPs in the perfusate were significantly lower in the clodronate group relative to those in the control group ($P = .023$ and $P = .034$, respectively). Both the number (C) and percentage (D) of MPs in the right lungs were significantly lower in the clodronate group compared with those in the control group ($P = .008$ and $P = .016$, respectively). Box-and-whisker plots are displayed with the middle line representing median, lower, and upper borders representing 25th and 75th percentiles, and the lower and upper whiskers representing the minimum and maximum, respectively. P values were calculated using repeated-measures analysis of variance (ANCOVA) or the Mann-Whitney U test. CIT, Cold ischemic time; EVLP, ex vivo lung perfusion.

whereas it was reduced in the control group (Figure 3, A). Likewise, zonula occludens-1 staining was preserved in the clodronate group, whereas loss of epithelial tight junctions was detected in the control group (Figure 3, B).

Inflammatory markers. Levels of interleukin (IL)-1 β in lung tissues tended to be lower in the clodronate group than in the control group; however, the difference was not statistically significant ($P = .22$; Table 1). Moreover, there was no significant difference in levels of CXCL2 in lung tissues between the 2 groups ($P = .69$; Table E3).

Experiment III

The weights of animals, second CIT (from the end of EVLP to implantation), and warm ischemic time (implantation time) were similar between the 2 groups (Table E4).

Lung function during EVLP. Fourteen rats were randomly allocated to 2 groups: control (15h-CIT; $n = 8$)

and clodronate (15h-CIT with clodronate-liposome; $n = 6$). Two of 8 grafts in the control group were lost due to severe pulmonary edema after 135 minutes and 180 minutes of perfusion (excluded from analyses), whereas all 6 grafts in the clodronate group were perfused for 4 hours. Additionally, lung compliance was significantly higher and vascular resistance was significantly lower in the clodronate group than in the control group ($P < .001$ and $.001$; Figure 4, A and B, respectively). Moreover, the PaO₂/FiO₂ ratio was significantly higher in the clodronate group than in the control group ($P = .004$; Figure 4, C), whereas no significant difference in PaCO₂ was observed between the 2 groups ($P = .31$; Figure 4, D). Data on other physiological parameters during EVLP are shown in Figure E8, A-E. Additionally, the appearance of the left lungs after EVLP tended to be better in the clodronate group (Video 1) than in the control group (Video 2).

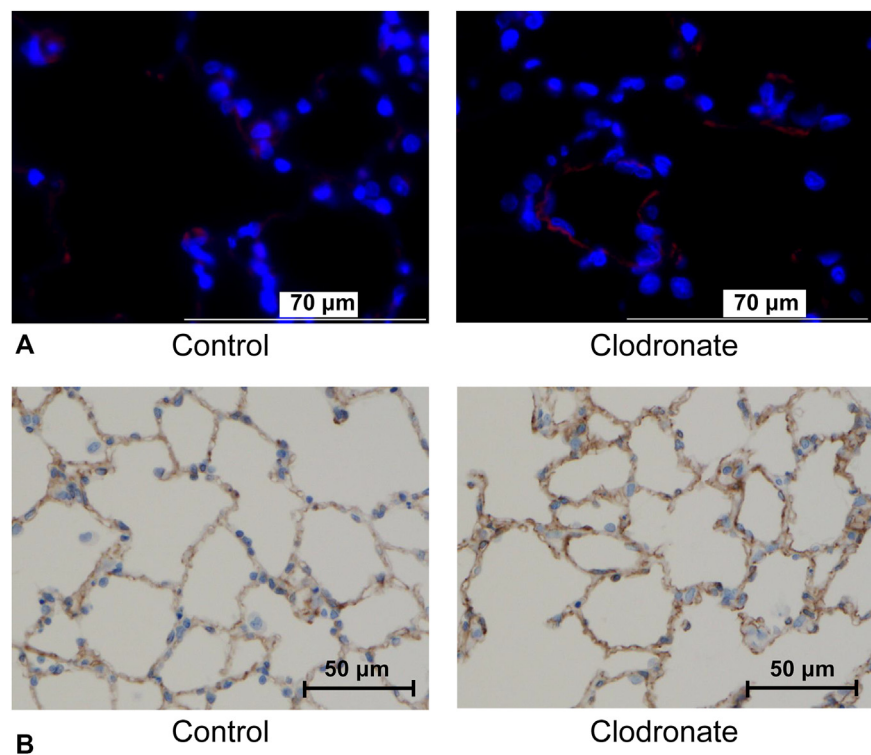


FIGURE 3. A, Staining with vascular endothelial-cadherin, a marker of endothelial cell integrity (blue, cells stained with 4',6-diamidino-2-phenylindole; and red, cells stained with vascular endothelial-cadherin). B, Staining with zonula occludens-1, a marker of tight junction integrity.

Lung function after lung transplantation. All donor lungs were transplanted successfully. Notably, the PaO₂/FiO₂ ratio of the blood from the left pulmonary vein was significantly higher in the clodronate group than in the control group (362.0 [223.3-425.3] mm Hg vs 141.5 [75.5-205.5] mm Hg, *P* = .015; [Figure 5, A](#)), whereas the PaCO₂ level was significantly lower in the clodronate group than in the control group (22.4 [18.4-25.9] mm Hg vs 28.7 [25.6-34.9] mm Hg, *P* = .041; [Figure 5, B](#)). Additionally, the wet-to-dry weight ratio after 2 hours of reperfusion was significantly lower in the clodronate group than in the control group (6.7 [5.6-7.2] vs 7.5 [7.1-7.8], *P* = .026; [Figure 5, C](#)). Moreover, graft appearance after 2 hours of reperfusion tended to be better in the clodronate group ([Video 3](#)) relative to that in the control group ([Video 4](#)).

Histologic examination and neutrophil infiltration in lung tissues. Representative histological images of the lungs are shown in [Figure 6, A](#). The histological scores of acute lung injury were significantly lower in the clodronate group than in the control group (4.5 [4.0-5.5] vs 8.0 [6.5-8.0], *P* = .013; [Figure 6, B](#)). Representative images of immunohistochemical staining of myeloperoxidase are shown in [Figure 6, C](#). The number of neutrophils in each high-power field was significantly lower in the clodronate group than in the control group (27.8 [26.0-40.8] vs 51.6 [43.7-57.5], *P* = .015; [Figure 6, D](#)).

Inflammatory markers. Levels of IL-1β in lung tissues tended to be lower in the clodronate group than in the control group; however, the difference was not statistically significant (*P* = .18; [Table 1](#)). Moreover, there were no

TABLE 1. Levels of interleukin-1β in each experiment

Experiment II	Min-CIT (n = 3)	Control (n = 5)	Clodronate (n = 5)	<i>P</i> value
Left lung tissue after 4 h of EVLP, pg/mg protein	507.5 (407.2-547.8)	1021.2 (1014.2-1088.2)	848.4 (771.1-959.8)	.22
Experiment III	Control (n = 6)	Clodronate (n = 6)		<i>P</i> value
Perfusate after 4 h of EVLP, pg/mL	NA	NA		NA
Plasma after 2 h of reperfusion, pg/mL	53.8 (22.2-86.4)	50.2 (27.4-100.1)		.94
Left lung tissue after 2 h of reperfusion, pg/mg protein	2406.4 (2016.5-2700.9)	2028.8 (1720.5-2366.7)		.18

Data are presented as the median (interquartile range). *P* values represent differences between the control and clodronate groups using the Mann–Whitney *U* test. CIT, Cold ischemic time; EVLP, ex vivo lung perfusion; NA, not applicable.

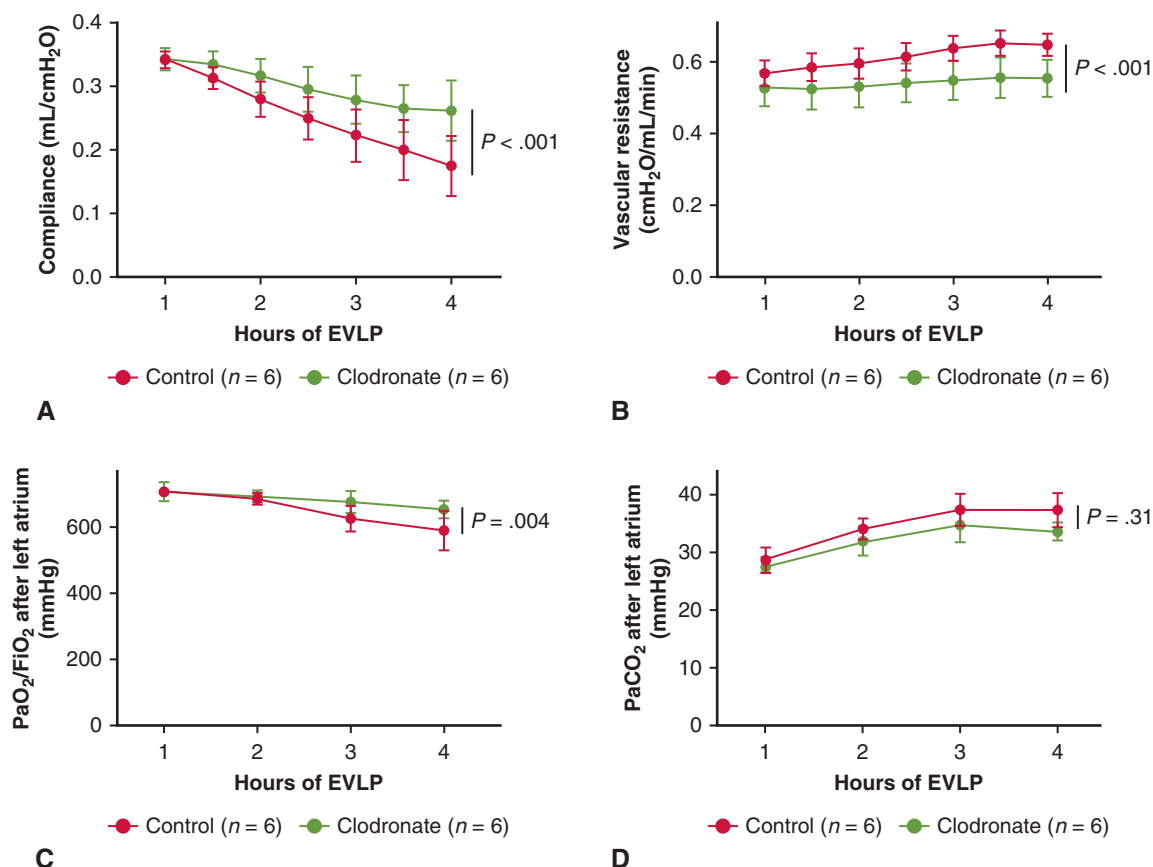


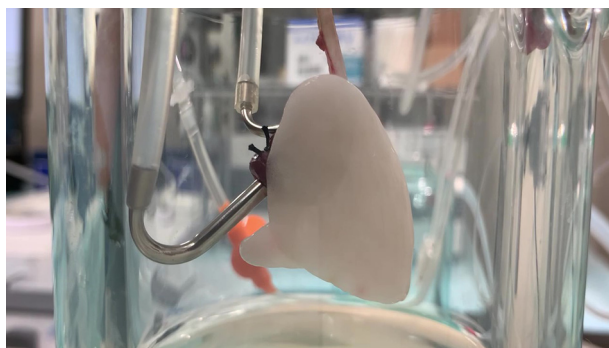
FIGURE 4. Compliance (A), vascular resistance (B), PaO₂/FiO₂ ratio (C), and PaCO₂ level (D) during EVLP in experiment III. *P* values were calculated using repeated-measures ANOVA. EVLP, Ex vivo lung perfusion; PaO₂/FiO₂, partial pressure of arterial oxygen/fraction of inspired oxygen; PaCO₂, partial pressure of arterial carbon dioxide.

significant differences in levels of CXCL2 in samples between the 2 groups (Table E3).

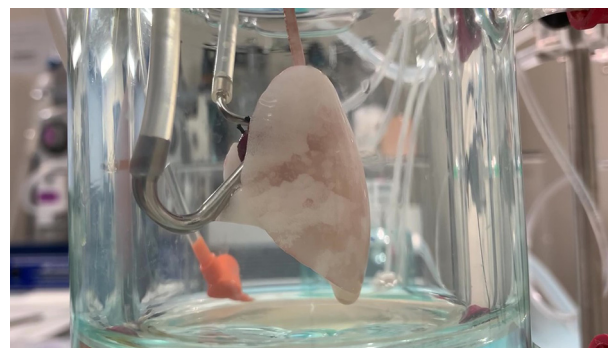
DISCUSSION

In this study, we demonstrated that live MPs remain in flushed donor lungs after prolonged cold ischemia, although

the number of other leukocyte subtypes tended to decrease. Moreover, the administration of clodronate-liposome into the perfusate during EVLP significantly reduced the remaining live MPs. Graft function maintenance (Figure 7) and decreased lung injury in histologic findings were also observed after LTx with this treatment, indicating the



VIDEO 1. Appearance of the left lung after EVLP in the clodronate group. EVLP, Ex vivo lung perfusion. Video available at: [https://www.jtcvs.org/article/S0022-5223\(22\)01141-2/fulltext](https://www.jtcvs.org/article/S0022-5223(22)01141-2/fulltext).



VIDEO 2. Appearance of the left lung after EVLP in the control group. EVLP, Ex vivo lung perfusion. Video available at: [https://www.jtcvs.org/article/S0022-5223\(22\)01141-2/fulltext](https://www.jtcvs.org/article/S0022-5223(22)01141-2/fulltext).

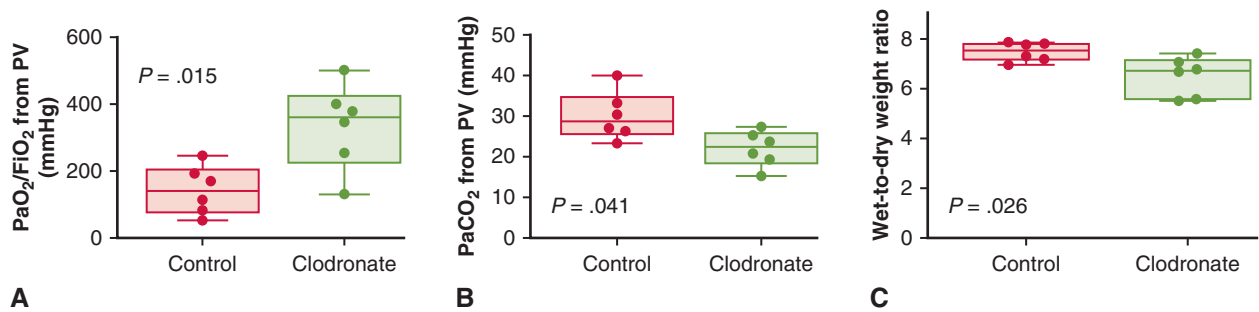


FIGURE 5. The $\text{PaO}_2/\text{FiO}_2$ ratio (A) and PaCO_2 level (B) from the left pulmonary vein after 2 hours of reperfusion. C, The wet-to-dry weight ratio after 2 hours of reperfusion. Box-and-whisker plots are displayed with the middle line representing median, lower, and upper borders representing 25th and 75th percentiles, and the lower and upper whiskers representing the minimum and maximum, respectively. P values were calculated using the Mann–Whitney U test. $\text{PaO}_2/\text{FiO}_2$, Partial pressure of arterial oxygen/fraction of inspired oxygen; PaCO_2 , partial pressure of arterial carbon dioxide.

protective effects of clodronate-liposome against IRI after prolonged CIT (Figure 8).

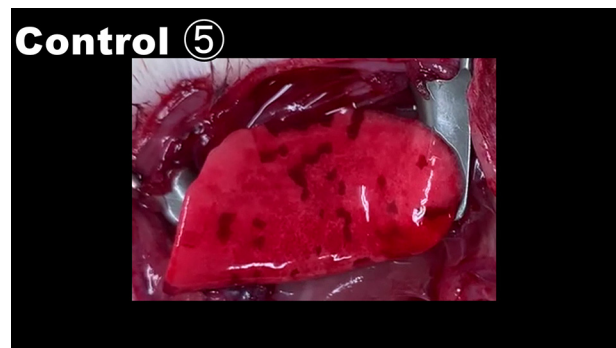
After flushing with preservation solution, most granulocytes are removed from human donor lungs, and the remaining major leukocyte subtypes include lymphocytes and MPs.²² Because most MPs are distributed throughout the interstitial spaces and line the alveolar walls,²² their complete removal by flushing might be difficult. Moreover, it remains unclear whether these MPs survive in donor lungs after prolonged cold ischemia. Generally, the half-lives of leukocytes differ depending on their subtype.^{23–25} MPs mainly comprise monocytes and macrophages.¹⁰ Monocytes have half-lives of more than 2 days,²⁶ whereas macrophages have 3 weeks according to in vivo mouse models.²⁷ In the present study, the number of live MPs remained unchanged, whereas their percentages increased significantly during 18 hours of ex vivo cold storage, suggesting that MPs survived in donor lungs up to 18 hours of cold ischemia, even in the preservation solution. Therefore, MPs, which are associated with the IRI process, may serve as good targets for attenuating IRI.^{5,7–9}

To the best of our knowledge, there have been no reports on the distribution of MPs during EVLP. In the present model, the amounts of circulating MPs remained low throughout EVLP, with many of them remaining in donor lungs at the end of EVLP. Because small amounts of MPs come out of donor lungs during EVLP, removing MPs from the circuit remains difficult, even using a type of leukocyte filter.²⁸ Therefore, in the present study, clodronate-liposome was administered during EVLP, which resulted in a significant reduction of MPs in donor lungs.

Clodronate-liposome is a formulation of liposomes encapsulating clodronate molecules. MPs ingest clodronate-liposome via endocytosis, resulting in their clodronate-induced apoptosis.^{29,30} Notably, adverse effects of clodronate-liposome were not observed in a clinical study.³¹ Administration of clodronate-liposome in some animal models has demonstrated promising results for the depletion of MPs^{5,12,13}; however, the present study is the first to specifically administer clodronate-liposome only to the target organ during EVLP and reduce the number of MPs before transplantation. In a previous in vivo study,⁵ clodronate-liposome was injected into mice and approximately one-



VIDEO 3. Appearance of the left lung after 2 hours of reperfusion in the clodronate group (A) and control group. EVLP, Ex vivo lung perfusion. Video available at: [https://www.jtcvs.org/article/S0022-5223\(22\)01141-2/fulltext](https://www.jtcvs.org/article/S0022-5223(22)01141-2/fulltext).



VIDEO 4. Appearance of the left lung after 2 hours of reperfusion in the control group. EVLP, Ex vivo lung perfusion. Video available at: [https://www.jtcvs.org/article/S0022-5223\(22\)01141-2/fulltext](https://www.jtcvs.org/article/S0022-5223(22)01141-2/fulltext).

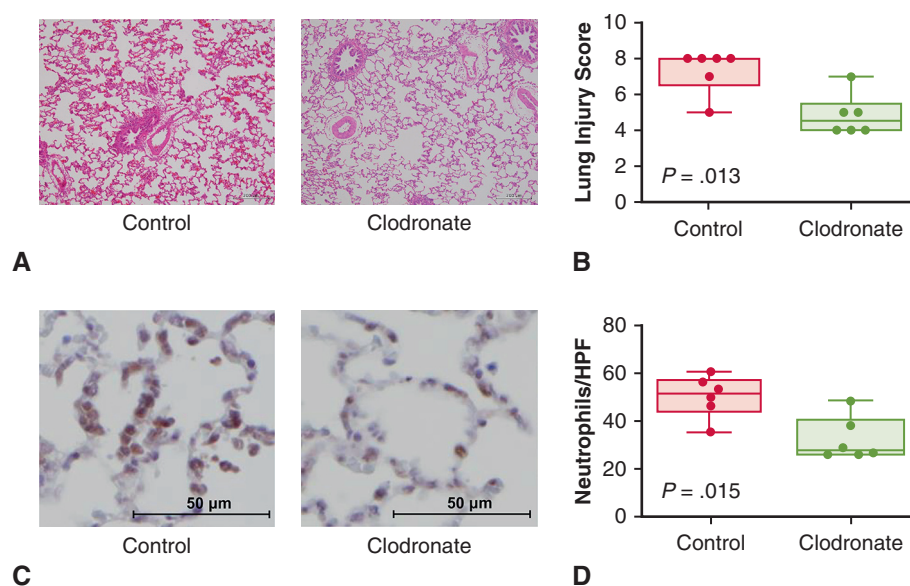


FIGURE 6. Histologic evaluation using the lung injury score. A, Representative histological findings in the 2 groups. B, The lung injury score in the clodronate group was significantly lower than in the control group ($P = .013$). C, Neutrophils in lung tissues after reperfusion were stained with myeloperoxidase (brown; original magnification, $\times 400$). D, The average number of neutrophils in each high-power field was significantly lower in the clodronate group than in the control group ($P = .015$). Box-and-whisker plots are displayed with the middle line representing median, lower, and upper borders representing 25th and 75th percentiles, and the lower and upper whiskers representing the minimum and maximum, respectively. P values were calculated using the Mann-Whitney U test. HPF, High-power field.

third of the targeted MPs in the grafts were depleted, which is consistent with our ex vivo results, indicating ex vivo administration of clodronate-liposome might serve as an in vivo treatment. Moreover, there are some advantages of ex vivo administration such as reconditioning grafts without affecting other organs and real-time observation of the graft function compared with in vivo administration. This treatment preserved graft function presumably due to the protective effects of clodronate-liposome against endothelial and epithelial

injuries after perfusion. Regarding the potential mechanism of these protective effects, Kurihara and colleagues⁷ reported that IL- 1β released by MPs induced the opening of tight junctions. Because IL- 1β in the lung tissue after EVLP tended to decrease with this treatment, the cell integrity might be maintained.

Previous studies demonstrated MPs as key facilitators of neutrophil migration³² by mediating their extravasation into pulmonary interstitial spaces after reperfusion by releasing

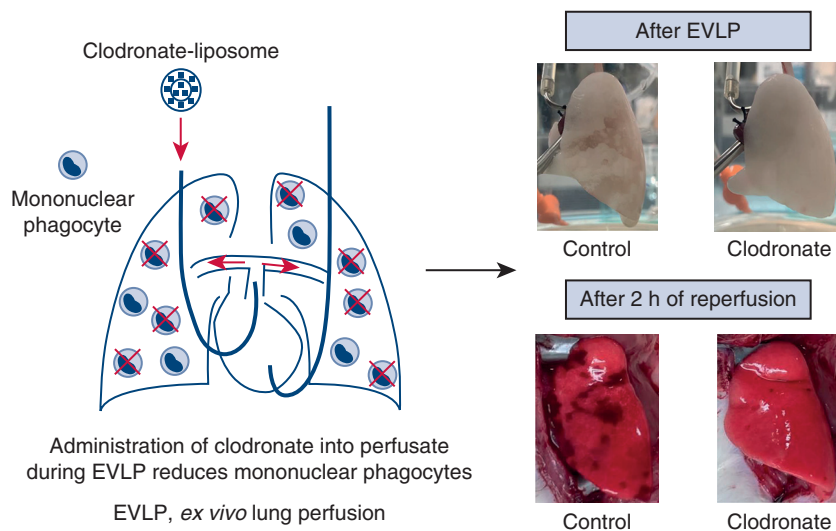


FIGURE 7. Administration of clodronate-liposome into the perfusate during EVLP significantly reduced MPs in donor lungs and attenuated IRI. EVLP, Ex vivo lung perfusion.

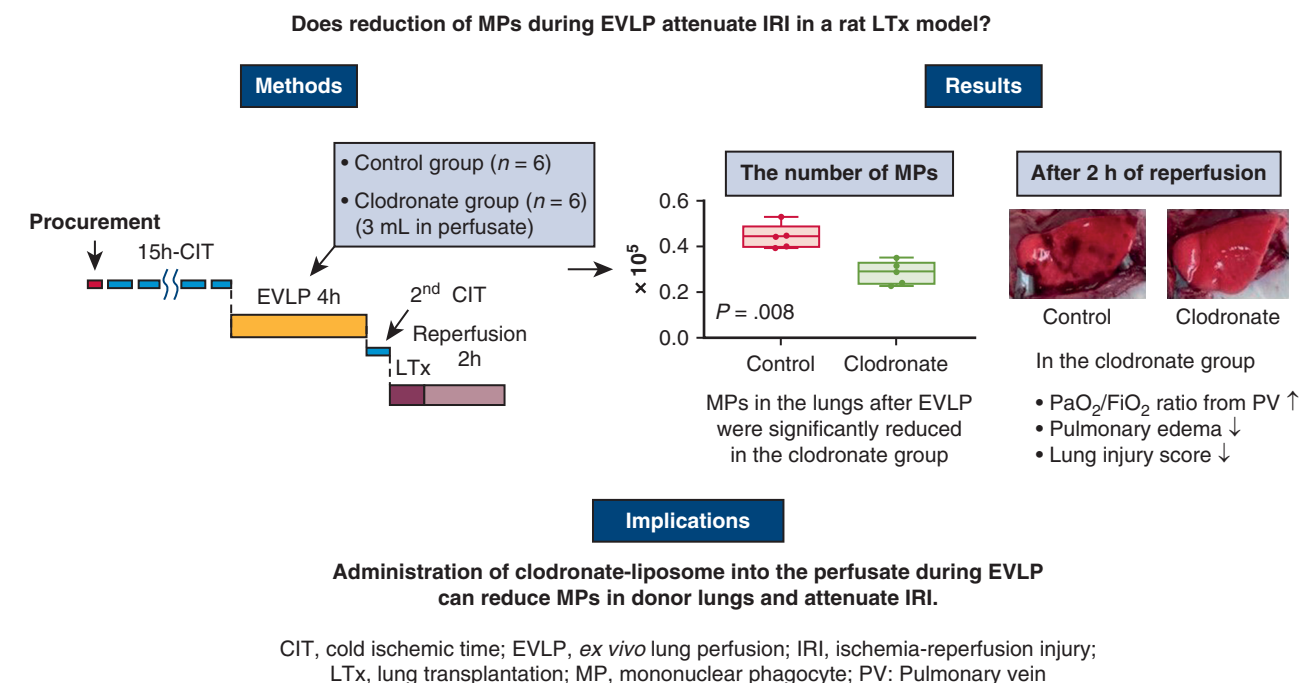


FIGURE 8. Administration of clodronate-liposome into the perfusate during EVLP resulted in effective reduction of MPs and attenuation of IRI. *Box-and-whisker plots* are displayed with the *middle line* representing median, *lower*, and *upper borders* representing 25th and 75th percentiles, and the *lower* and *upper whiskers* representing the minimum and maximum, respectively. *P* values were calculated using the Mann–Whitney *U* test. *MP*, Mononuclear phagocyte; *EVLP*, ex vivo lung perfusion; *IRI*, ischemia-reperfusion injury; *LTx*, lung transplantation; *CIT*, cold ischemic time; PaO_2/FiO_2 , partial pressure of arterial oxygen/fraction of inspired oxygen.

IL-1 β .^{7,33} Given that neutrophil infiltration into lung tissues is associated with IRI,^{4,34} IL-1 β appears to play an important role in the IRI process via MPs. In this study, IL-1 β levels in lung tissues tended to decrease after reperfusion in the clodronate group, which might be related to this hypothesis. Application of this procedure might consequently reduce neutrophil infiltration into lung tissues, thereby leading to the attenuation of IRI.

Moreover, Stone and colleagues³⁵ indicated that the removal of donor passenger leukocytes using EVLP leads to a reduction of graft immunogenicity and allorecognition, associated with acute rejection. Particularly, because MPs play a central role in facilitating the immunogenicity of pulmonary allografts,⁶ a reduction of MPs might have potentially mitigated alloimmune response leading to the attenuation of acute rejection in this study.

Study Limitations

This study has limitations. First, the number of animals in both groups was kept relatively small to avoid additional animal deaths from the viewpoint of animal welfare. Second, monocytes or macrophages were not distinguished from other MPs during flow cytometric analysis due to the lack of well-established methods for distinguishing

subpopulations of MPs in a rat model.¹⁰ Third, the optimal timing of drug administration during EVLP was not investigated. As some studies potentially imply time-dependent effects of clodronate-liposome,^{5,13} clodronate-liposome was administered before perfusion to achieve the maximum effect in this study. Although clodronate-liposome was used safely in a pilot study of 10 patients with rheumatoid arthritis,³¹ large-animal EVLP models are needed to further demonstrate the present findings before applying these procedures for human EVLP.

CONCLUSIONS

These data suggested that live MPs remain in flushed donor lungs after prolonged cold ischemia. Moreover, administration of clodronate-liposome into the perfusate during EVLP significantly reduced these MPs in donor lungs and attenuated IRI.

Conflict of Interest Statement

The authors reported no conflicts of interest.

The *Journal* policy requires editors and reviewers to disclose conflicts of interest and to decline handling or reviewing manuscripts for which they may have a conflict

of interest. The editors and reviewers of this article have no conflicts of interest.

References

- Chambers DC, Cherikh WS, Goldfarb SB, Hayes D Jr, Kucheryavaya AY, Toll AE, et al. The International Thoracic Organ Transplant Registry of the International Society for Heart and Lung Transplantation: thirty-fifth adult lung and heart-lung transplant report-2018; focus theme: multiorgan transplantation. *J Heart Lung Transplant*. 2018;37:1169-83.
- Bharat A, Kreisel D. Immunopathogenesis of primary graft dysfunction after lung transplantation. *Ann Thorac Surg*. 2018;105:671-4.
- Huang HJ, Yusen RD, Meyers BF, Walter MJ, Mohanakumar T, Patterson GA, et al. Late primary graft dysfunction after lung transplantation and bronchiolitis obliterans syndrome. *Am J Transplant*. 2008;8:2454-62.
- Chen-Yoshikawa TF. Ischemia-reperfusion injury in lung transplantation. *Cells*. 2021;10:1333.
- Tatham KC, O'Dea KP, Romano R, Donaldson HE, Wakabayashi K, Patel BV, et al. Intravascular donor monocytes play a central role in lung transplant ischaemia-reperfusion injury. *Thorax*. 2018;73:350-60.
- Stone JP, Sevenoaks H, Sjöberg T, Steen S, Yonan N, Fildes JE. Mechanical removal of dendritic cell-generating non-classical monocytes via ex vivo lung perfusion. *J Heart Lung Transplant*. 2014;33:864-9.
- Kurihara C, Lecuona E, Wu Q, Yang W, Núñez-Santana FL, Akbarpour M, et al. Crosstalk between nonclassical monocytes and alveolar macrophages mediates transplant ischemia-reperfusion injury through classical monocyte recruitment. *JCI Insight*. 2021;6:e147282.
- Tsushima Y, Jang JH, Yamada Y, Schwendener R, Suzuki K, Weder W, et al. The depletion of donor macrophages reduces ischaemia-reperfusion injury after mouse lung transplantation. *Eur J Cardiothorac Surg*. 2014;45:703-9.
- Zhao M, Fernandez LG, Doctor A, Sharma AK, Zarbock A, Tribble CG, et al. Alveolar macrophage activation is a key initiation signal for acute lung ischemia-reperfusion injury. *Am J Physiol Lung Cell Mol Physiol*. 2006;291:L1018-26.
- Hume DA, Caruso M, Keshvari S, Patkar OL, Sehgal A, Bush SJ, et al. The mononuclear phagocyte system of the rat. *J Immunol*. 2021;206:2251-63.
- Ferenbach DA, Sheldrake TA, Dhaliwal K, Kipari TM, Marson LP, Kluth DC, et al. Macrophage/monocyte depletion by clodronate, but not diphtheria toxin, improves renal ischemia/reperfusion injury in mice. *Kidney Int*. 2012;82:928-33.
- Yang Q, Wang Y, Pei G, Deng X, Jiang H, Wu J, et al. Bone marrow-derived Ly6C(-) macrophages promote ischemia-induced chronic kidney disease. *Cell Death Dis*. 2019;10:291.
- Zheng Z, Chiu S, Akbarpour M, Sun H, Reyfman PA, Anekalla KR, et al. Donor pulmonary intravascular nonclassical monocytes recruit recipient neutrophils and mediate primary lung allograft dysfunction. *Sci Transl Med*. 2017;9:eal4508.
- Cypel M, Yeung JC, Liu M, Anraku M, Chen F, Karolak W, et al. Normothermic ex vivo lung perfusion in clinical lung transplantation. *N Engl J Med*. 2011;364:1431-40.
- Nakajima D, Liu M, Ohsumi A, Kalaf R, Iskender I, Hsin M, et al. Lung lavage and surfactant replacement during ex vivo lung perfusion for treatment of gastric acid aspiration-induced donor lung injury. *J Heart Lung Transplant*. 2017;36:577-85.
- Cypel M, Liu M, Rubacha M, Yeung JC, Hirayama S, Anraku M, et al. Functional repair of human donor lungs by IL-10 gene therapy. *Sci Transl Med*. 2009;1:4ra9.
- Rat immunology from genes to proteins to cells. Accessed March 10, 2022. <https://dokument.pub/dl/rat-immunology-from-genes-to-proteins-to-cells-flip-book-pdf>
- Ohsumi A, Marseu K, Slinger P, McRae K, Kim H, Guan Z, et al. Sevoflurane attenuates ischemia-reperfusion injury in a rat lung transplantation model. *Ann Thorac Surg*. 2017;103:1578-86.
- Ohsumi A, Kanou T, Ali A, Guan Z, Hwang DM, Waddell TK, et al. A method for translational rat ex vivo lung perfusion experimentation. *Am J Physiol Lung Cell Mol Physiol*. 2020;319:L61-70.
- Burki S, Noda K, Philips BJ, Velayutham M, Shiva S, Sanchez PG, et al. Impact of triptolide during ex vivo lung perfusion on grafts after transplantation in a rat model. *J Thorac Cardiovasc Surg*. 2020 [In press].
- Kayawake H, Chen-Yoshikawa TF, Saito M, Yamagishi H, Yoshizawa A, Hirano SI, et al. Protective effects of a hydrogen-rich preservation solution in a canine lung transplantation model. *Ann Thorac Surg*. 2021;111:246-52.
- Richter N, Raddatz G, Steinhoff G, Schäfers HJ, Schlitt HJ. Transmission of donor lymphocytes in clinical lung transplantation. *Transpl Int*. 1994;7:414-9.
- Basu S, Hodgson G, Katz M, Dunn AR. Evaluation of role of G-CSF in the production, survival, and release of neutrophils from bone marrow into circulation. *Blood*. 2002;100:854-61.
- Suratt BT, Young SK, Lieber J, Nick JA, Henson PM, Worthen GS. Neutrophil maturation and activation determine anatomic site of clearance from circulation. *Am J Physiol Lung Cell Mol Physiol*. 2001;281:L913-21.
- Buckton KE, Brown WM, Smith PG. Lymphocyte survival in men treated with x-rays for ankylosing spondylitis. *Nature*. 1967;214:470-3.
- Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M, et al. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity*. 2013;38:79-91.
- Jaensson E, Uronen-Hansson H, Pabst O, Eksteen B, Tian J, Coombes JL, et al. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. *J Exp Med*. 2008;205:2139-49.
- Luc JGY, Aboelnazar NS, Himmatt S, Hatami S, Haromy A, Matsumura N, et al. A leukocyte filter does not provide further benefit during ex vivo lung perfusion. *ASAIO J*. 2017;63:672-8.
- Poole JA, Gleason AM, Bauer C, West WW, Alexis N, van Rooijen N, et al. CD11c(+)CD11b(+) cells are critical for organic dust-elicited murine lung inflammation. *Am J Respir Cell Mol Biol*. 2012;47:652-9.
- Cho SW, Soki FN, Koh AJ, Eber MR, Entezami P, Park SI, et al. Osteal macrophages support physiologic skeletal remodeling and anabolic actions of parathyroid hormone in bone. *Proc Natl Acad Sci U S A*. 2014;111:1545-50.
- Barrera P, Blom A, van Lent PL, van Bloois L, Beijnen JH, van Rooijen N, et al. Synovial macrophage depletion with clodronate-containing liposomes in rheumatoid arthritis. *Arthritis Rheum*. 2000;43:1951-9.
- Maus UA, Waelsch K, Kuziel WA, Delbeck T, Mack M, Blackwell TS, et al. Monocytes are potent facilitators of alveolar neutrophil emigration during lung inflammation: role of the CCL2-CCR2 axis. *J Immunol*. 2003;170:3273-8.
- Hsiao HM, Fernandez R, Tanaka S, Li W, Spahn JH, Chiu S, et al. Spleen-derived classical monocytes mediate lung ischemia-reperfusion injury through IL-1 β . *J Clin Invest*. 2018;128:2833-47.
- Kreisel D, Sugimoto S, Zhu J, Nava R, Li W, Okazaki M, et al. Emergency granulopoiesis promotes neutrophil-dendritic cell encounters that prevent mouse lung allograft acceptance. *Blood*. 2011;118:6172-82.
- Stone JP, Critchley WR, Major T, Rajan G, Risnes I, Scott H, et al. Altered immunogenicity of donor lungs via removal of passenger leukocytes using ex vivo lung perfusion. *Am J Transplant*. 2016;16:33-43.

Key Words: clodronate-liposome, ex vivo lung perfusion, ischemia-reperfusion injury, lung transplantation, mononuclear phagocyte

APPENDIX E1. SUPPLEMENTARY MATERIALS AND METHODS

Donor Procurement

All donor rats were intraperitoneally injected with 60 mg/kg ketamine (Daiichi Sankyo Ltd) and 7.5 mg/kg xylazine (Bayer Yakuhin Ltd) to induce anesthesia. After being fully anesthetized, the animals were orotracheally intubated with a 14-gauge intravenous catheter, with the tracheal tube connected to a ventilator (Model SN-480-7; Shinano Seisakusyo). Rats received volume-controlled ventilation at a rate of 60 breaths/min with a tidal volume of 10 mL/kg, an inspired oxygen fraction of 0.5, and a positive end-expiratory pressure of 2 cm H₂O. Subsequently, a median laparotomy was performed, and 400 USP units of sodium heparin were injected into the inferior vena cava. Thereafter, lungs were flushed with 20 mL of a low potassium dextran glucose preservation solution (Perfadex; XVIVO Perfusion), followed by the clamping of the trachea at the end of inspiration. Both heart and lung blocks were procured and stored in Perfadex at 4 °C until further use.

Ex Vivo Lung Perfusion Procedure

EVLP was performed using Isolated Perfused Lung System for Rat and Guinea Pig (IPL-2; Harvard Apparatus; Hugo Sachs Elektronik). Briefly, the heart-lung block was applied to the IPL-2 system. During EVLP, pulmonary artery pressure, peak airway pressure, and airway flow were continuously monitored, whereas dynamic lung compliance and vascular resistance were analyzed and recorded in real time. Physiological data were gathered every 30 minutes. At 5 minutes before these assessments, the lungs were recruited to prevent pulmonary atelectasis. Perfusate samples were taken every 1 hour and used for the evaluation of pH, PaO₂, PaCO₂, lactate, and electrolytes. After the final assessment, the perfusion was stopped, and the trachea was clamped at an inflated state.

Lung Transplant Procedure

All recipient rats were anesthetized with an intraperitoneal injection of 60 mg/kg ketamine and 7.5 mg/kg xylazine. After being fully anesthetized, the animals were orotracheally intubated with a 14-gauge intravenous catheter, with the tracheal tube connected to a ventilator (Model SN-480-7; Shinano Seisakusyo), and maintained on 2.0% isoflurane (Pfizer Ltd). Rats were ventilated under the following conditions: tidal volume, 10 mL/kg; respiratory rate, 60 breaths/min; positive end-expiratory end pressure, 2 cmH₂O; and FiO₂, 1.0. Donor lung cuffs were placed into the corresponding recipient structures through each ventral incision, and anastomoses were secured using 7-0 polypropylene ties. Recipient rats were ventilated and reperfused for 2 hours, and 0.3 mL saline was administered via subcutaneous injection every 30 minutes for volume supplement. Blood samples for blood gas analyses were taken from the

left pulmonary vein using a 27-gauge needle directed toward the transplanted lungs. All rats were euthanized by exsanguination, and lungs were removed for further examination.

Wet-to-Dry Weight Ratio

The upper part of the left lung tissue was used to calculate the wet-to-dry weight ratio. The wet weight was measured first, after which the tissue was dried for 24 hours at 100 °C before measuring the dry weight. The wet-to-dry weight ratio was calculated by dividing the wet by the dry weight.

Vascular Endothelial Cadherin Staining

For vascular endothelial-cadherin immunofluorescence staining, formalin-fixed paraffin-embedded lung tissue sections were stained using an anti-vascular endothelial cadherin antibody (1:20; Thermo Fisher Scientific), as previously described.^{E1} After the addition of primary antibodies and fluorescence tagging using secondary antibodies, each slide was imaged under a confocal fluorescence microscope (BZ-X810 All-in-One; KEYENCE).

Zonula Occludens-1 Staining

For zonula occludens-1 immunohistochemical staining, formalin-fixed paraffin-embedded lung tissue sections were stained using an anti-zonula occludens-1 tight junction protein antibody (1:1000; Abcam), as previously described.^{E1,E2}

Myeloperoxidase Staining

For myeloperoxidase immunohistochemical staining to evaluate neutrophil infiltration in the lungs, formalin-fixed paraffin-embedded lung tissue sections were stained using an anti-myeloperoxidase antibody (1:150; Abcam), as previously described.^{E3} The number of neutrophils was expressed as the average number of extravascular neutrophils in 8 randomly selected high-power fields per section at a magnification of 400×.

Cytokines

Plasma (obtained by centrifugation of blood at 6000 rpm for 5 minutes), perfusate, and the middle part of left lung tissues were collected and stored at -80 °C until use. The same quantities of lung tissue (25 mg) were homogenized with 800 μL radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors and then centrifuged (10,000×g for 20 minutes) to obtain lung tissue lysates for enzyme-linked immunosorbent assay. The concentrations of interleukin-1β (R&D Systems) and CXCL2 (R&D Systems) in the plasma, perfusate, and lung tissue lysates were measured using an enzyme-linked immunosorbent assay kit according to the manufacturer's instructions. Respective concentration levels in tissue

lysates were measured using a protein assay kit (Bio-Rad) and normalized to 1 mg of protein in the lung.

E-References

- E1. Ohsumi A, Kanou T, Ali A, Guan Z, Hwang DM, Waddell TK, et al. A method for translational rat *ex vivo* lung perfusion experimentation. *Am J Physiol Lung Cell Mol Physiol*. 2020;319:L61-70.
- E2. Cypel M, Liu M, Rubacha M, Yeung JC, Hirayama S, Anraku M, et al. Functional repair of human donor lungs by IL-10 gene therapy. *Sci Transl Med*. 2009;1:4ra9.
- E3. Oda H, Tanaka S, Shinohara M, Morimura Y, Yokoyama Y, Kayawake H, et al. Specialized proresolving lipid mediators agonistic to formyl peptide receptor type 2 attenuate ischemia-reperfusion injury in rat lung. *Transplantation*. 2022;106:1159-69.

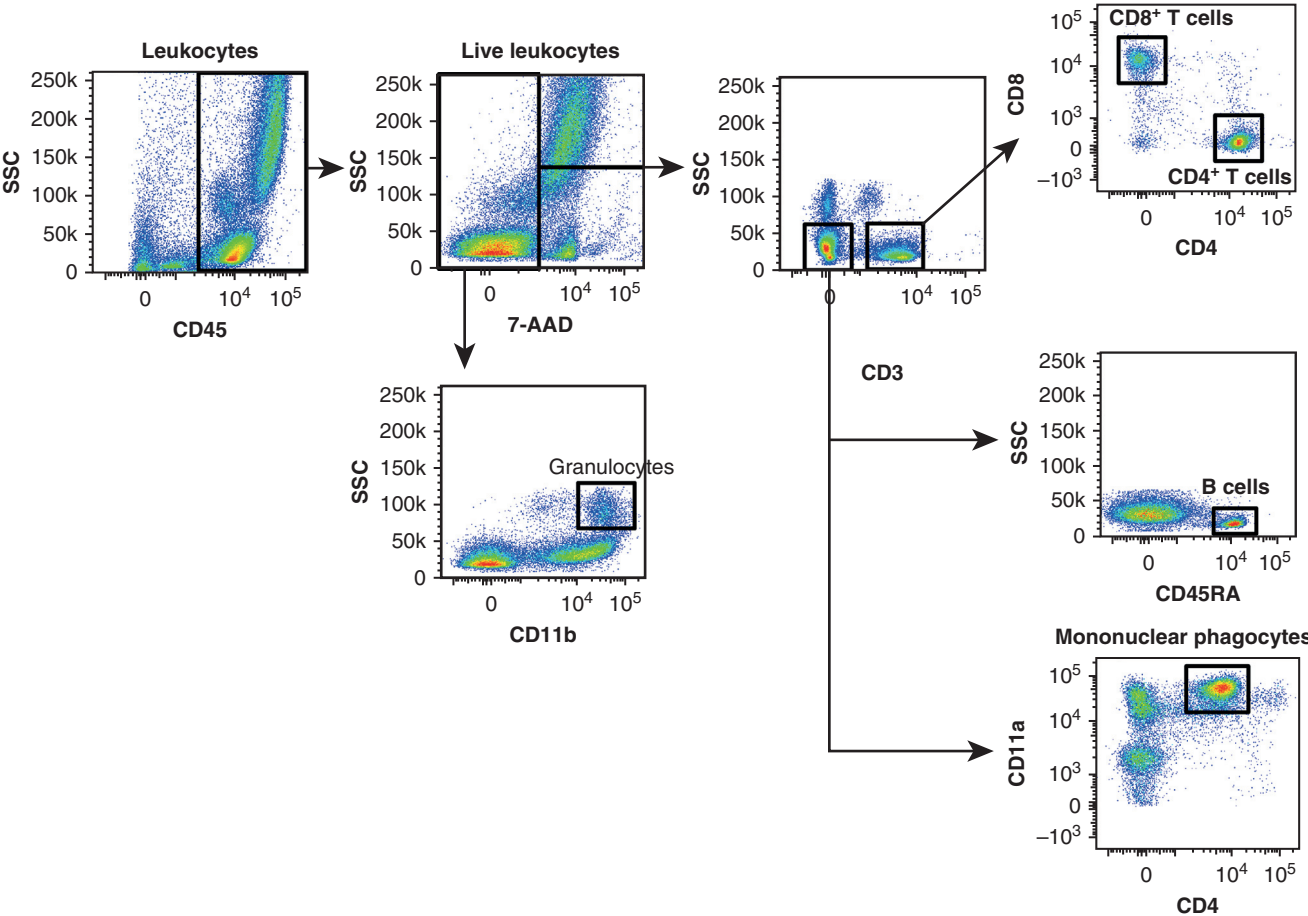


FIGURE E1. Representative gating strategy for the evaluation of leukocyte populations. After including only CD45⁺ cells and excluding dead cells using 7-aminoactinomycin D, granulocytes were selected on the side scatter plot using the expression of CD11b. CD4⁺ T cells, CD8⁺ T cells, B cells, and MPs were selected using antibodies against CD3, CD4, CD8, CD45RA, and CD11a. SSC, Side scatter; CD, cluster of differentiation; AAD, aminoactinomycin D.

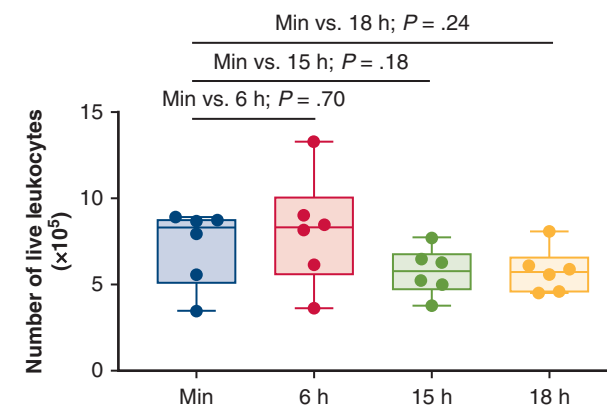


FIGURE E2. The number of live leukocytes in the right lungs at each CIT. Box-and-whisker plots are displayed with the middle line representing median, lower, and upper borders representing 25th and 75th percentiles, and the lower and upper whiskers representing the minimum and maximum, respectively. P values were calculated using the Mann–Whitney U test.

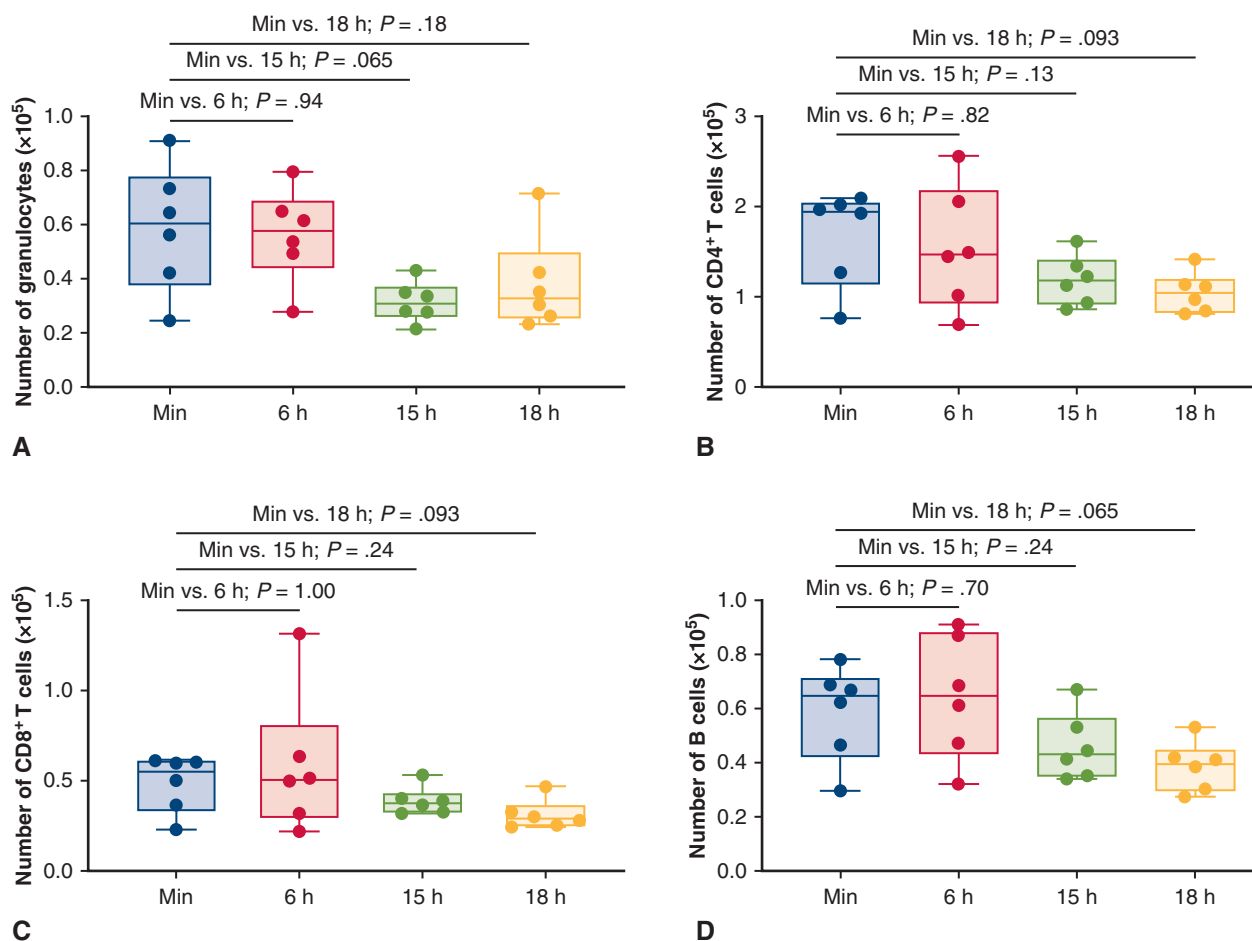


FIGURE E3. The number of live leukocyte subtypes at each CIT. (A) granulocytes, (B) CD4⁺ T cells, (C) CD8⁺ T cells, and (D) B cells. Box-and-whisker plots are displayed with the middle line representing median, lower, and upper borders representing 25th and 75th percentiles, and the lower and upper whiskers representing the minimum and maximum, respectively. P values were calculated using the Mann–Whitney U test. CD, Cluster of differentiation.

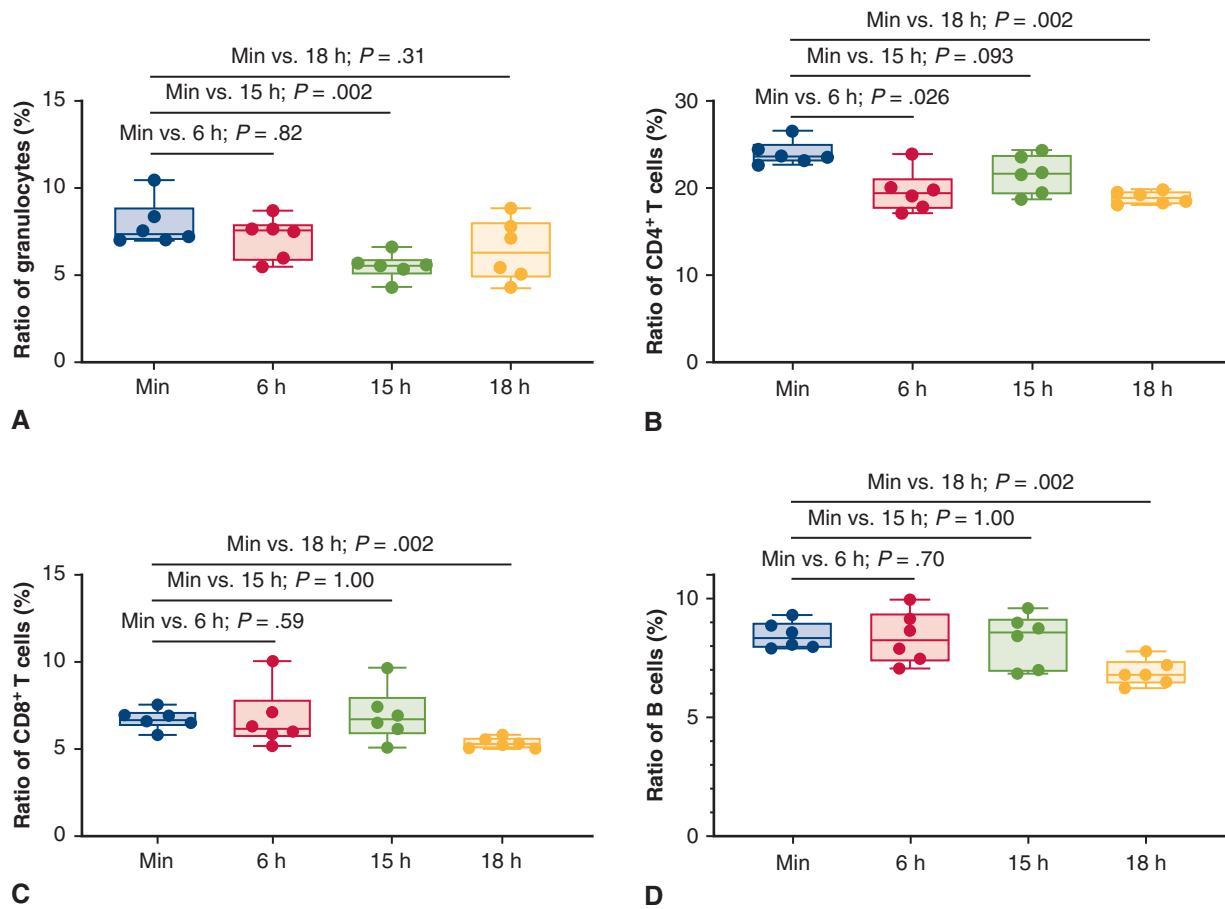


FIGURE E4. The percentages (leukocyte subtypes/leukocytes) of granulocytes (A), CD4⁺ T cells (B), CD8⁺ T cells (C), and B cells (D) at each CIT. Box-and-whisker plots are displayed with the middle line representing median, lower, and upper borders representing 25th and 75th percentiles, and the lower and upper whiskers representing the minimum and maximum, respectively. *P* values were calculated using the Mann–Whitney *U* test. *CD*, Cluster of differentiation.

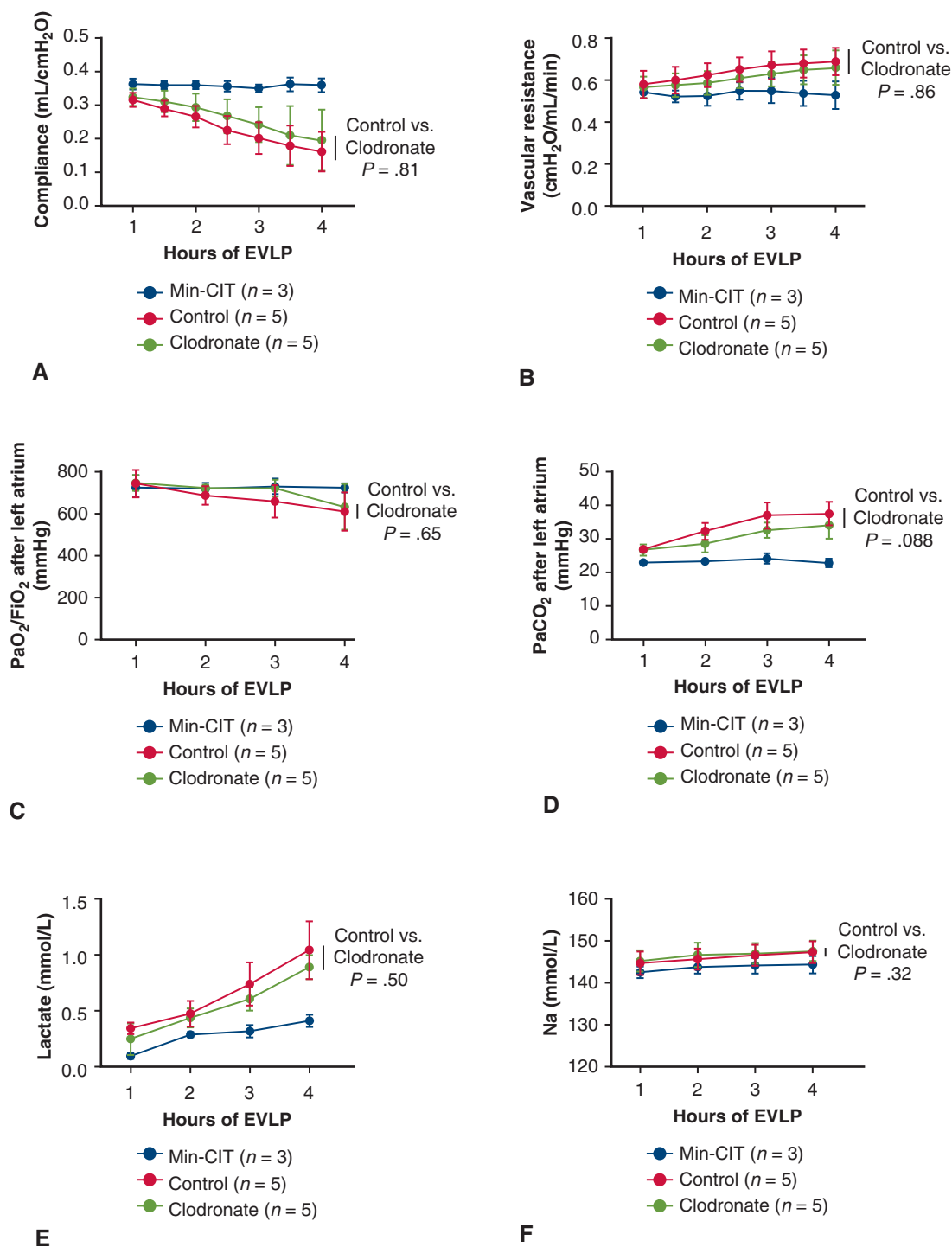


FIGURE E5. A-H, Lung function and blood gas analyses during EVLP in experiment I. J, The wet-to-dry weight ratio after EVLP. Box-and-whisker plots are displayed with the middle line representing median, lower, and upper borders representing 25th and 75th percentiles, and the lower and upper whiskers representing the minimum and maximum, respectively. P values were calculated using repeated measures ANOVA or the Mann-Whitney U test. CIT, Cold ischemic time; PaO₂/FiO₂, partial pressure of arterial oxygen/fraction of inspired oxygen; EVLP, ex vivo lung perfusion; PaCO₂, partial pressure of arterial carbon dioxide.

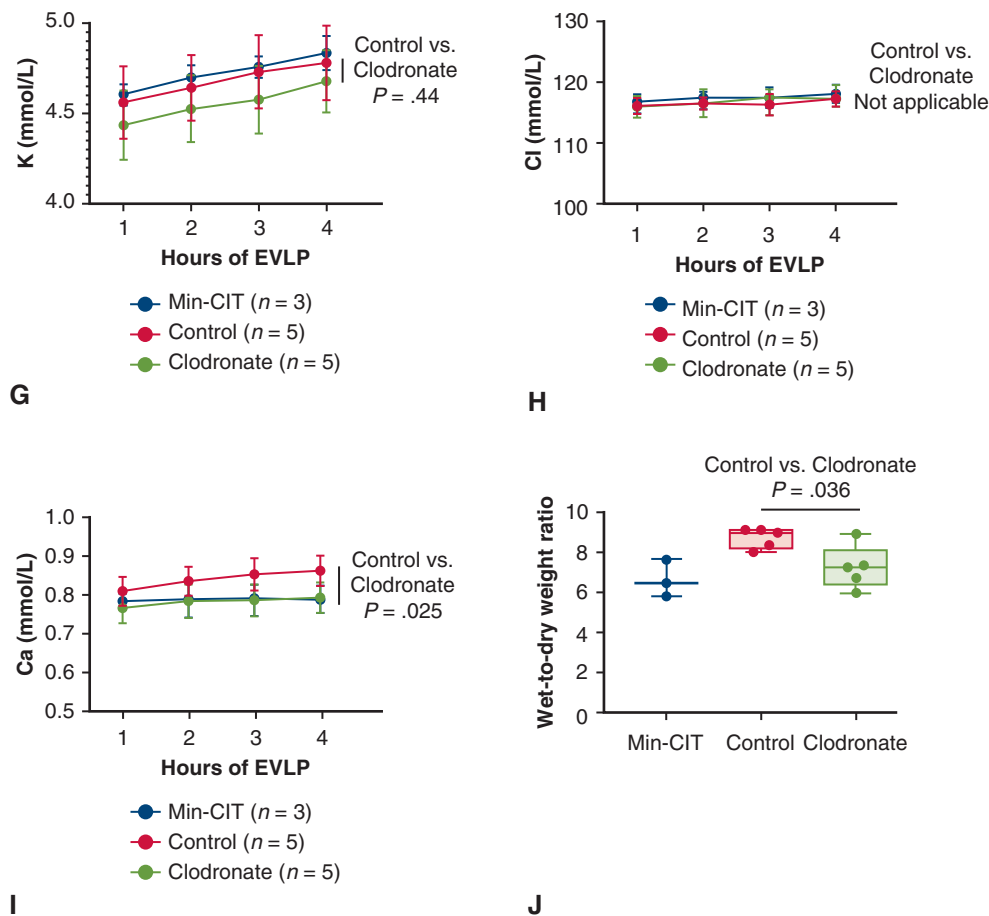


FIGURE E5. (Continued).

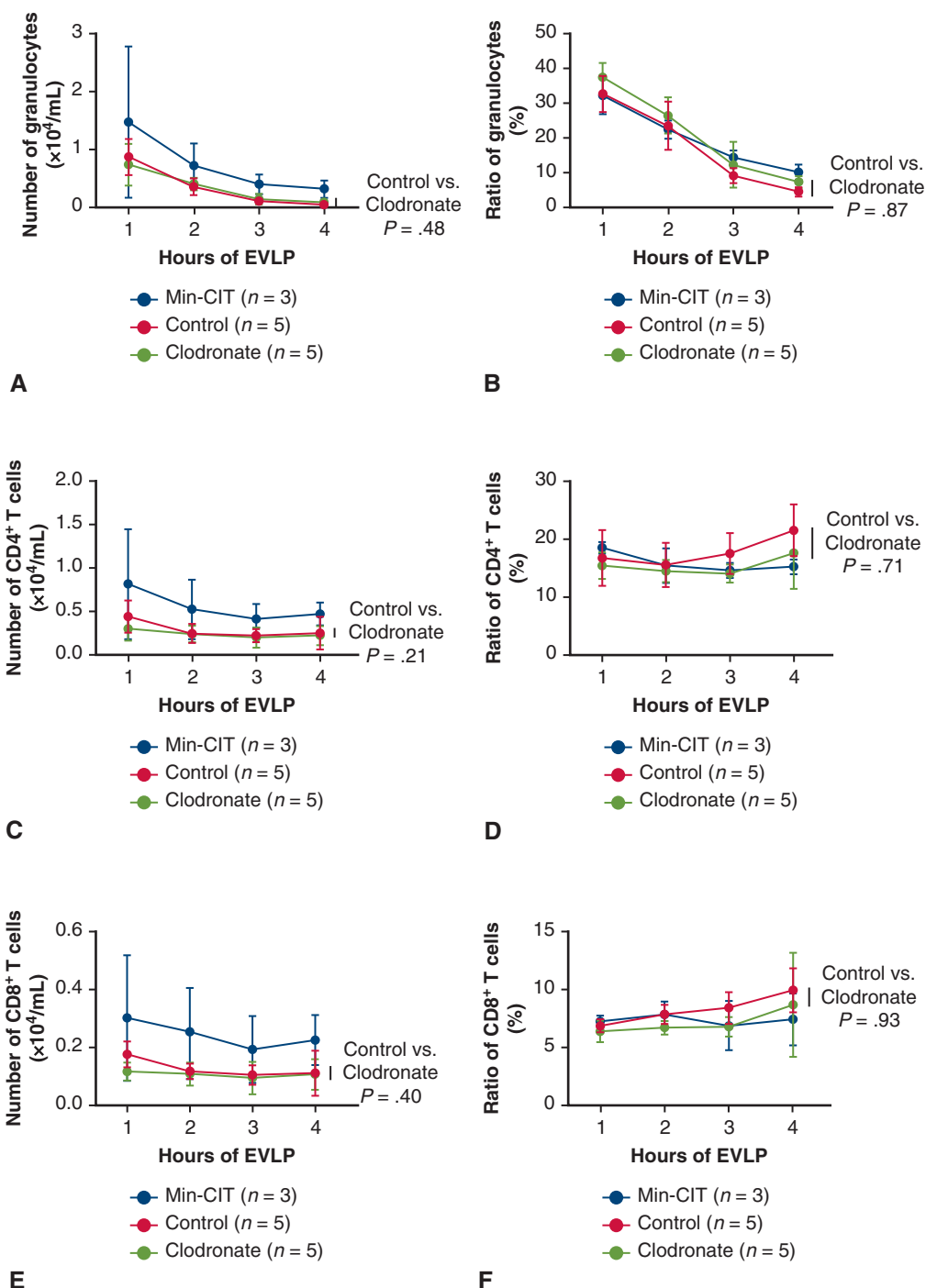


FIGURE E6. A-H, The numbers and percentages (leukocyte subtypes/leukocytes) of granulocytes, CD4⁺ T cells, CD8⁺ T cells, and B cells in the perfusate. P values were calculated using repeated-measures ANOVA. CIT, Cold ischemic time; CD, cluster of differentiation; EVLP, ex vivo lung perfusion.

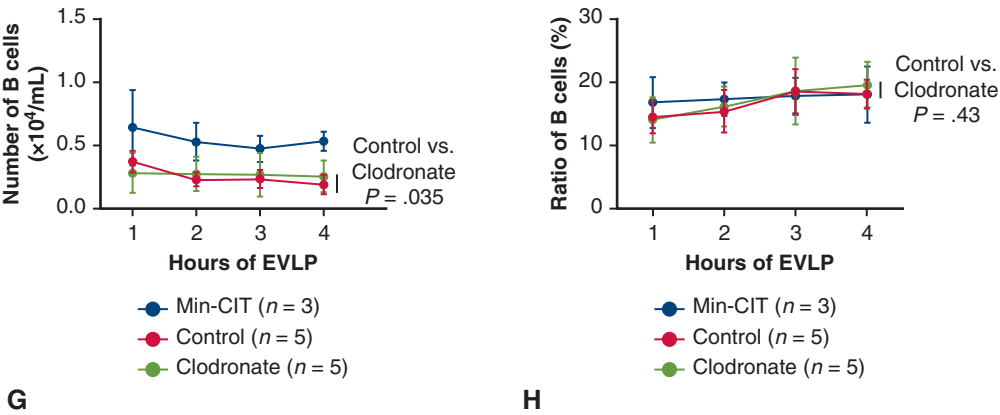


FIGURE E6. (Continued).

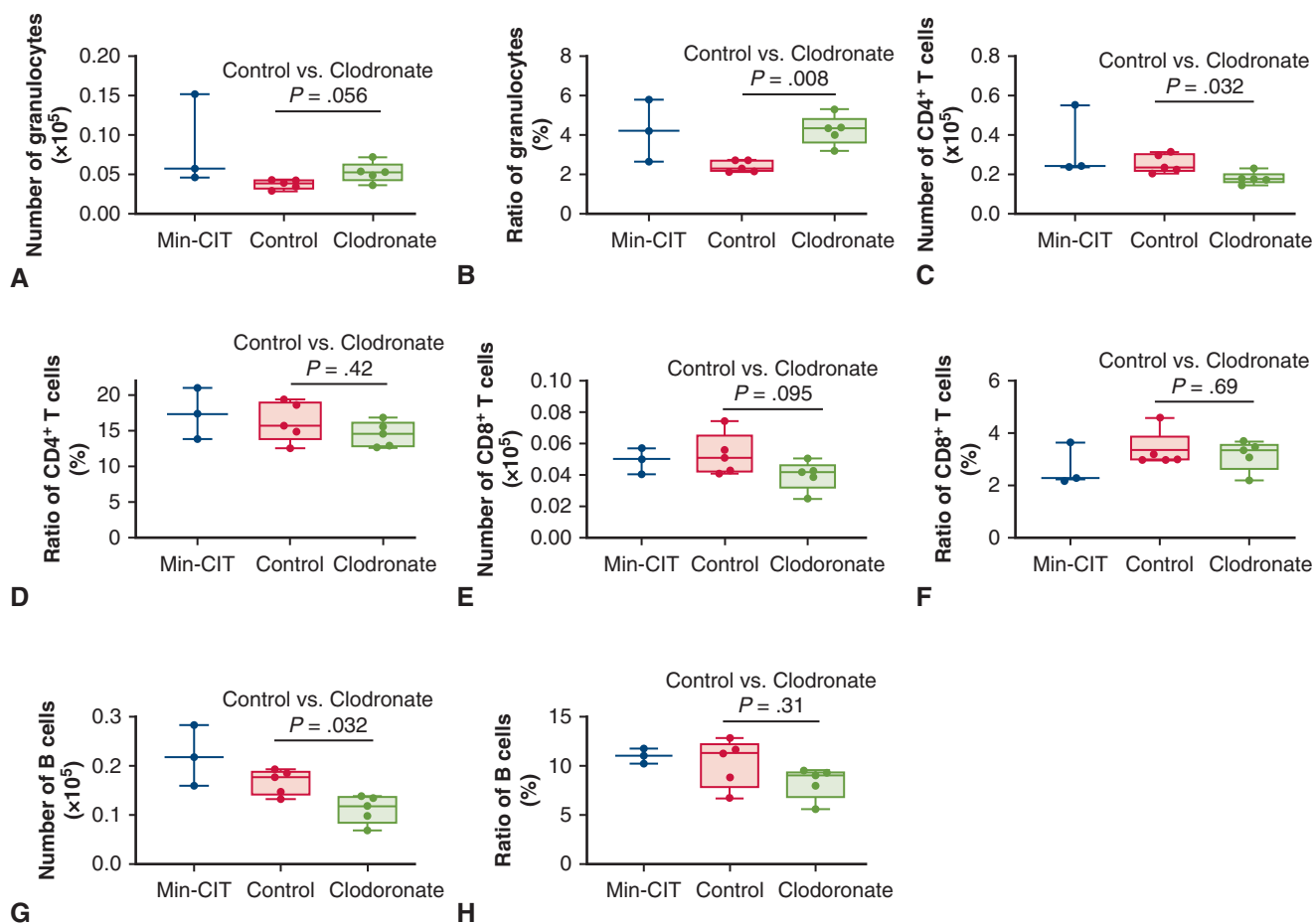


FIGURE E7. A-H, The numbers and percentages (leukocyte subtypes/leukocytes) of granulocytes, CD4⁺ T cells, CD8⁺ T cells, and B cells in the right lung. Box-and-whisker plots are displayed with the middle line representing median, lower, and upper borders representing 25th and 75th percentiles, and the lower and upper whiskers representing the minimum and maximum, respectively. *P* values were calculated using the Mann-Whitney *U* test. CIT, Cold ischemic time; CD, cluster of differentiation.

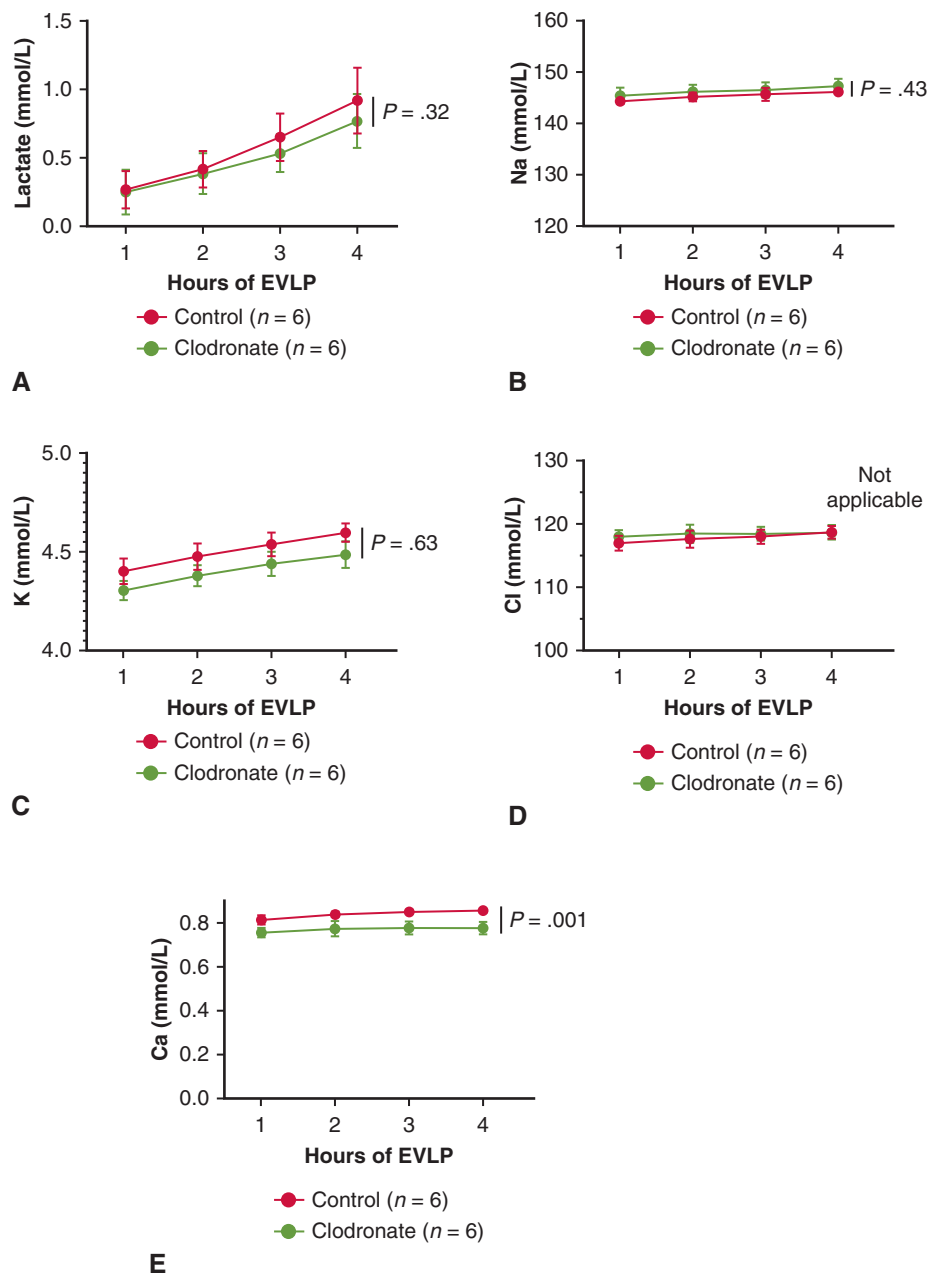


FIGURE E8. Levels of lactate (A), sodium (B), potassium (C), chloride (D), and calcium (E) during EVLP. *P* values were calculated using repeated-measures ANOVA. *EVLP*, Ex vivo lung perfusion.

TABLE E1. Baseline data in experiment I

	Min-CIT (n = 6)	6h-CIT (n = 6)	15h-CIT (n = 6)	18h-CIT (n = 6)	<i>P</i> value
Rat weight (g)	286 (280.5-291.5)	286.5 (281.3-289.5)	281 (280-282)	281 (280-288)	.55

Data are presented as median (interquartile range). *P* values are for the differences among groups using the Kruskal–Wallis test. *CIT*, Cold ischemic time.

TABLE E2. Baseline data in the experiment II

	Min-CIT (n = 3)	Control (n = 5)	Clodronate (n = 5)	<i>P</i> value
Weight (g)	280 (277-280.5)	274 (270-290)	280 (274-282)	.92
Live leukocytes in a right lung after EVLP ($\times 10^5$ cells)	1.8 (1.6-2.3)	1.7 (1.5-1.7)	1.3 (1.2-1.4)	.008*

Data are presented as median (interquartile range). *P* value is for the difference among groups using the Kruskal–Wallis test. *CIT*, Cold ischemic time. *EVLP*, ex vivo lung perfusion. **P* value is for the difference between the control and the clodronate groups using the Mann–Whitney *U* test.

TABLE E3. Levels of CXCL2 in each experiment

Experiment II	Min-CIT (n = 3)	Control (n = 5)	Clodronate (n = 5)	<i>P</i> value
Left lung tissue after 4 h of EVLP, pg/mg protein	464.0 (313.1-556.4)	1866.5 (1786.1-2118.6)	1769.5 (1555.0-2054.5)	.69
Experiment III	Control (n = 6)	Clodronate (n = 6)	<i>P</i> value	
Perfusate after 4 h of EVLP, pg/mL	262.2 (240.5-308.6)	244.3 (226.8-295.8)	.70	
Plasma after 2 h of reperfusion, pg/mL	2114.5 (1607.7-3321.3)	2609.1 (1586.3-3036.8)	1.00	
Left lung tissue after 2 h of reperfusion, pg/mg protein	3164.6 (2402.5-3870.8)	2542.1 (2507.6-3038.1)	.70	

Data are presented as median (interquartile range). *P* values are for the differences between the control and the clodronate groups using the Mann–Whitney *U* test. *CIT*, Cold ischemic time; *EVLP*, ex vivo lung perfusion.

TABLE E4. Baseline data in experiment III

	Control (n = 6)	Clodronate (n = 6)	P value
Donor weight (g)	279 (276.5-281.5)	274 (272.5-277)	.42
Recipient weight (g)	292 (288.5-295.5)	294 (291-294.8)	.94
Second CIT (min)	65.5 (62-72.8)	65 (64.3-65.8)	.81
Warm ischemic time (min)	21.5 (18-24.3)	21 (18-22.5)	.87

Data are presented as median (interquartile range). *P* values are for the differences between groups using the Mann–Whitney *U* test. *CIT*, Cold ischemic time.