Original Article

Prolonged warm ischemia exacerbated acute rejection after lung transplantation from donation after cardiac death in a mouse

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Meeting presentation: Presented at the 36th annual meeting and scientific sessions of the International Society for Heart and Lung Transplantation, Washington, DC, USA, April, 2016

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Keywords: lung transplantation; rejection; organ preservation; donation after cardiac death; brain dead donor

Abstract

Objective: In lung transplantation (LTx) from donation after cardiac death (DCD), the donor lungs are inevitably exposed to warm ischemic time (WIT) between the cardiac arrest and the initiation of cold preservation. We conducted this study to examine the effect of prolonged WIT on lung allograft rejection in a murine model of LTx from DCD.

Methods: Allogeneic BALB/c→ B6 LTx from DCD was performed with a WIT of 15 minutes (WIT15 group, n = 5) or 60 minutes (WIT60 group, n = 5). Recipients were immunosuppressed by perioperative costimulatory blockade. The lung allografts were analyzed by histology and flow cytometry on day 7 after the LTx.

Results: Histologically, the rejection grade in the WIT60 group was significantly higher than that in the WIT15 group (3.4 ± 0.4 vs. 2.2 ± 0.2, $P = 0.0278$). Moreover, the intragraft CD8+ to CD4+ T cell ratio in the WIT60 group was significantly higher than that in the WIT15 group (2.3 ± 0.12 vs. 1.2 ± 0.11, $P < 0.0001$).

Conclusions: Prolonged WIT could exacerbate the severity of lung allograft rejection after LTx from DCD. Minimization of the WIT could improve the outcomes after LTx from DCD.

Introduction

The problem of donor organ shortage persists in lung transplantation (LTx). In an attempt to resolve this problem, LTx from donation after cardiac death (DCD) began to be performed in addition to LTx from donation after brain death (DBD) [1, 2], and the number of LTx from DCD has been increasing. Different from the case in LTx from DBD, in LTx from DCD, the donor lungs are inevitably exposed to a warm ischemic time (WIT) between the cardiac arrest and the initiation of cold preservation. Prolonged WIT of donor lungs, which can occur in actual clinical situations, has been shown to increase the risk of primary graft dysfunction due to ischemia-reperfusion injury in animal models [3-8]. It has been reported that ischemia-reperfusion injury caused by prolongation of the cold ischemic time of donor lungs from 1 hour to 18 hours could contribute to abrogation of lung allograft acceptance induced by perioperative double costimulatory blockade with anti-CD40 ligand and CTLA4Ig and exacerbation of lung allograft rejection in a mouse model of orthotopic lung transplantation [9, 10]. In contrast to the aforementioned effect of prolonged cold ischemia of the donor lungs in LTx, the effect of prolonged warm ischemia of the donor lungs on the lung allograft acceptance after LTx from DCD remains unclear. The aim of this study was to assess, in a mouse model of orthotopic lung transplantation, the effect of prolonged warm ischemia of the donor lungs on the severity of the lung allograft rejection after LTx from DCD.

Methods

Animals

BALB/c and C57BL/6J (B6) mice were purchased from Charles River Laboratories Japan, Inc. Male mice weighing 25-30 g were used for both the donors and recipients in the

mouse LTx. All the allogeneic mouse LTx procedures were performed from the BALB/c → B6 strains of mice. This experimental protocol was approved by the Animal Care and Use Committee of Okayama University (OKU-2014172).

Orthotopic vascularized aerated lung transplantation of the mouse

Orthotopic vascularized aerated LTx of the mouse was performed as previously described [11]. The donor mice were anesthetized by intraperitoneal injection of ketamine (0.1 mg/g) and xylazine (0.01 mg/g) and ventilated with a mixture of halothane and oxygen. A median laparosternotomy was performed, heparinization was performed by intravenous injection of 100 IU/body, and cardiac arrest was induced by intravenous injection of potassium chloride at 4 mg/body. According to the length of the WIT, the donor mice were divided into two groups: the group with a short WIT of 15 minutes (WIT15 group) and the group with the prolonged WIT of 60 minutes (WIT60 group) (Fig. 1). After the induction of cardiac arrest, the ventilated donor mice were left at room temperature for 15 minutes in the WIT15 group, and for 60 minutes in the WIT60 group. At the end of the WIT, the donor lungs were flushed through the main pulmonary artery with 2 mL of 4°C low-potassium dextran glucose solution and harvested. Orthotopic vascularized single left LTx from the DCD was performed in both the groups of allogeneic BALB/c → B6 mice after the specified WIT (n = 5 in each group). The recipient BALB/c → B6 mice were perioperatively treated with 250 μg of anti-CD40 ligand and 200 μg of CTLA4Ig, to prevent lung allograft rejection and promote lung allograft acceptance [10]. On day 7 after the LTx, the lung allografts were examined histologically, and the intragraft lymphocytic infiltrates were analyzed by flow cytometry.

Histopathological evaluation

The recipient mice were sacrificed on day 7 after the LTx. The lower part of the left lung of each recipient was fixed in 10% formaldehyde, sectioned and stained with hematoxylin and eosin (H–E). Grading of the severity of rejection was performed by two blinded pathologists (T.O. and A.M.) using the standard criteria published by the International Society for Heart and Lung Transplantation for acute rejection: grade 0 = no changes; grade 1 = minimal changes; grade 2 = mild changes; grade 3 = moderate changes; grade 4 = severe changes [12].

Flow-cytometric analysis

Flow-cytometric analysis was performed of the lung graft tissue harvested on day 7 after the LTx in both the groups. The upper part of the left lung of each recipient was digested in RPMI 1640 solution containing 1 mg/ml of collagenase (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and 5 U/ml of DNAse (Qiagen, Hilden, Germany) at 37°C for 60 minutes. The digested lung tissue was passed through a 70-μm cell strainer and treated with ACK lysing buffer (Thermo Fisher Scientific, Waltham, MA, USA). T lymphocyte infiltration into the lung grafts was assessed by staining with fluorochrome-labeled anti-CD90.2, anti-CD4, and anti-CD8a antibodies (BD Japan, Tokyo, Japan). Cells were acquired on the MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) and data analysis was performed with the FlowJo software (BD Biosciences, San Jose, CA, USA).

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6.00 (GraphPad Software, La Jolla California USA). All values are expressed as the means ± standard error of the mean. Student's t test was used to compare the rejection grades and CD8+ to CD4+ T cell ratios. Differences were considered significant at $P < 0.05$.

Results

Histopathology of the immunosuppressed lung allografts

The left lung grafts in the recipient mice of the allogeneic BALB/c → B6 LTx immunosuppressed by perioperative costimulatory blockade with MR1 and CTLA4-Ig showed an almost normal appearance on gross examination on day 7 after the LTx in the WIT15 group (Fig. 2a); on the other hand, atelectasis and edema were observed in the WIT60 group (Fig. 2b). Histopathologically, the transplanted lungs in the immunosuppressed recipient mice of the allogeneic BALB/c → B6 LTx showed minimal mononuclear cell infiltration in the perivascular area on day 7 after the LTx in the WIT15 group, suggesting acute rejection of minimal severity (Fig. 3a); on the other hand, in the WIT60 group, the transplanted lungs in the immunosuppressed recipient mice showed extensive mononuclear cell infiltration not only in the perivascular area, but also in the interstitial spaces, suggestive of severe acute rejection (Fig. 3b). The mean rejection grade in the WIT60 group was significantly higher than that in the WIT15 group (3.4 ± 0.4 vs. 2.2 ± 0.2, $P = 0.0278$) (Fig. 4).

Flow-cytometric analysis of the immunosuppressed lung allografts

In the WIT15 group, the number of CD8+ T-cells was slightly higher than the number of

CD4+ T-cells (Fig. 5a), and the intragraft CD8+ to CD4+ T cell ratio was 1.2 ± 0.11. In contrast, in the WIT60 group, CD8+ T cells significantly outnumbered the CD4+ T cells (Fig. 5b), and the intragraft CD8+ to CD4+ T cell ratio was 2.3 ± 0.12. Accordingly, the intragraft CD8+ to CD4+ T cell ratio in the WIT60 group was significantly higher than that in the WIT15 group ($P < 0.0001$) (Fig. 6).

Discussion

In this study, we demonstrated that the B6 recipients, immunosuppressed by perioperative costimulatory blockade, of BALB/c lung grafts exposed to a prolonged WIT of 60 minutes, showed significantly more severe rejection after LTx from DCD than those exposed to a short WIT of 15 minutes. These results suggest that prolongation of the WIT of the donor lungs from 15 to 60 minutes exacerbates the severity of acute rejection after LTx from DCD. Because acute rejection has been shown to be associated with the development of chronic lung allograft dysfunction [13], in clinical settings, minimization of the WIT after cardiac arrest might contribute to an improvement of the long-term survival after LTx from DCD.

Recently, LTx from DCD has been aggressively performed as a solution to the donor organ shortage, especially in high-volume centers [14-16]. LTx from DCD has been shown to provide similar outcomes to LTx from DBD, including in terms of the incidence of primary graft dysfunction, frequency of acute rejection, and the 1-year survival rate [17-20]. The reported WIT, which is defined as the interval from the circulatory arrest to the start of cold preservation of the donor lungs, from one clinical series of LTx from DCD was approximately 30 minutes [21]. Moreover, most LTx centers have protocols with a maximum tolerable length of 1 hour of initial WIT, based on reports from animal studies

that the lung can tolerate no more than 1 hour of exposure to warm ischemia, especially from the point of view of the incidence of primary graft dysfunction after LTx from DCD [3-8]. The agonal period, which is defined as the interval between withdrawal of life-sustaining therapy and cardiac arrest, is one of the major issues in LTx from DCD and might be more important than the WIT [22]; however, to simplify the study design, we focused on the WIT in LTx from DCD in this study. Although the setting of the WIT was limited to two points in this study, our results suggest that the minimization of the WIT could be desirable even in the context of lung allograft rejection. Conversely, enhanced immunosuppression might be required when the WIT is prolonged beyond one hour in LTx after DCD.

In this study, to examine the effect of extended WIT on lung allograft rejection after LTx from DCD, we modified an established murine model of LTx that has been shown to reproduce lung allograft acceptance induced by perioperative costimulatory blockade with MR-1 and CTLA4Ig [9]. Different from the histological findings of ischemia-reperfusion injury after LTx, which is represented by diffuse infiltration of the lung parenchyma by macrophages and neutrophils as well as interstitial edema with thickened alveolar walls, our results showed the accumulation of mononuclear cells specifically around vessels and bronchi, suggesting lung allograft rejection after LTx from DCD [23]. Consistent with previous reports [9-11], we observed a CD8+ T cell/CD4+ T cell ratio of greater than 2:1 in the rejected lung, which is similar to the ratio seen in the bronchoalveolar lavage fluid of human lung transplants in the early phase after LTx [24]. Our murine model that showed exacerbated lung allograft rejection after LTx from DCD when the WIT was prolonged to 1 hour could serve as a useful model to examine the mechanism of lung allograft rejection triggered by warm ischemia.

Ischemia-reperfusion injury with prolongation of the cold ischemic time of donor lungs from 1 hour to 18 hours could cause lung allograft rejection through linkage between innate and adaptive immune responses after LTx [9]. Recently, a cold ischemic time of 18 hours after cardiac arrest was shown to have a similar pathophysiological effect on ischemia-reperfusion injury after LTx from DCD to that of a WIT of 3 hours in a rat model [25]. Therefore, we speculated that the enhanced ischemia-reperfusion injury caused by prolongation of the WIT to up to 1 hour could exacerbate the severity of the lung allograft rejection. Further study is required to elucidate the detailed mechanism.

In conclusion, prolonged warm ischemia worsened the severity of acute rejection after LTx from DCD in a mouse model of orthotopic LTx. Minimization of the WIT after cardiac arrest could improve the long-term outcomes after LTx from DCD.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research, Grant no. 24791461, from the Japan Society for the Promotion of Science and JSPS Fujita Memorial Fund for Medical Research.

Compliance with ethical standards

Conflict of interest: Yutaka Hirano and his co-authors have no conflicts of interest.

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Figure Legends

Fig. 1. Experimental design. In both groups, allogeneic BALB/c → B6 mouse lung transplantation (LTx) was performed. After the cardiac arrest, the ventilated donor BALB/c mice were left at room temperature for 15 minutes in the WIT15 group and for 60 minutes in the WIT60 group, and the donor lungs were harvested. Orthotopic vascularized single left LTx was performed, and the B6 recipients received perioperative costimulatory blockade with a combination of MR1 and CTLA4-Ig. The lung grafts were assessed on day 7 after the LTx.

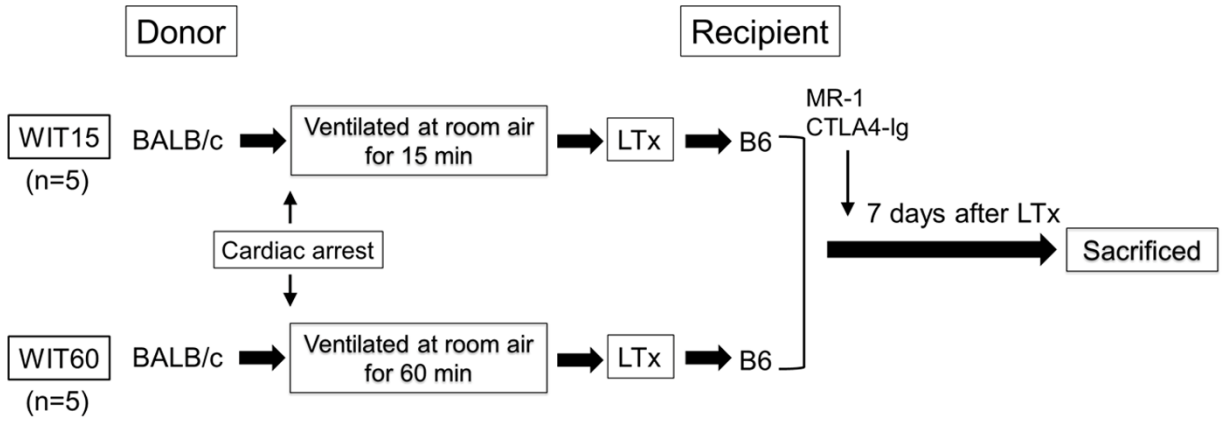

Fig. 2. Representative macroscopic findings of the harvested lungs on day 7 after lung transplantation in the group with a warm ischemic time (WIT) of 15 minutes (WIT15 group) (a) and the group with a WIT of 60 minutes (WIT60 group) (b).

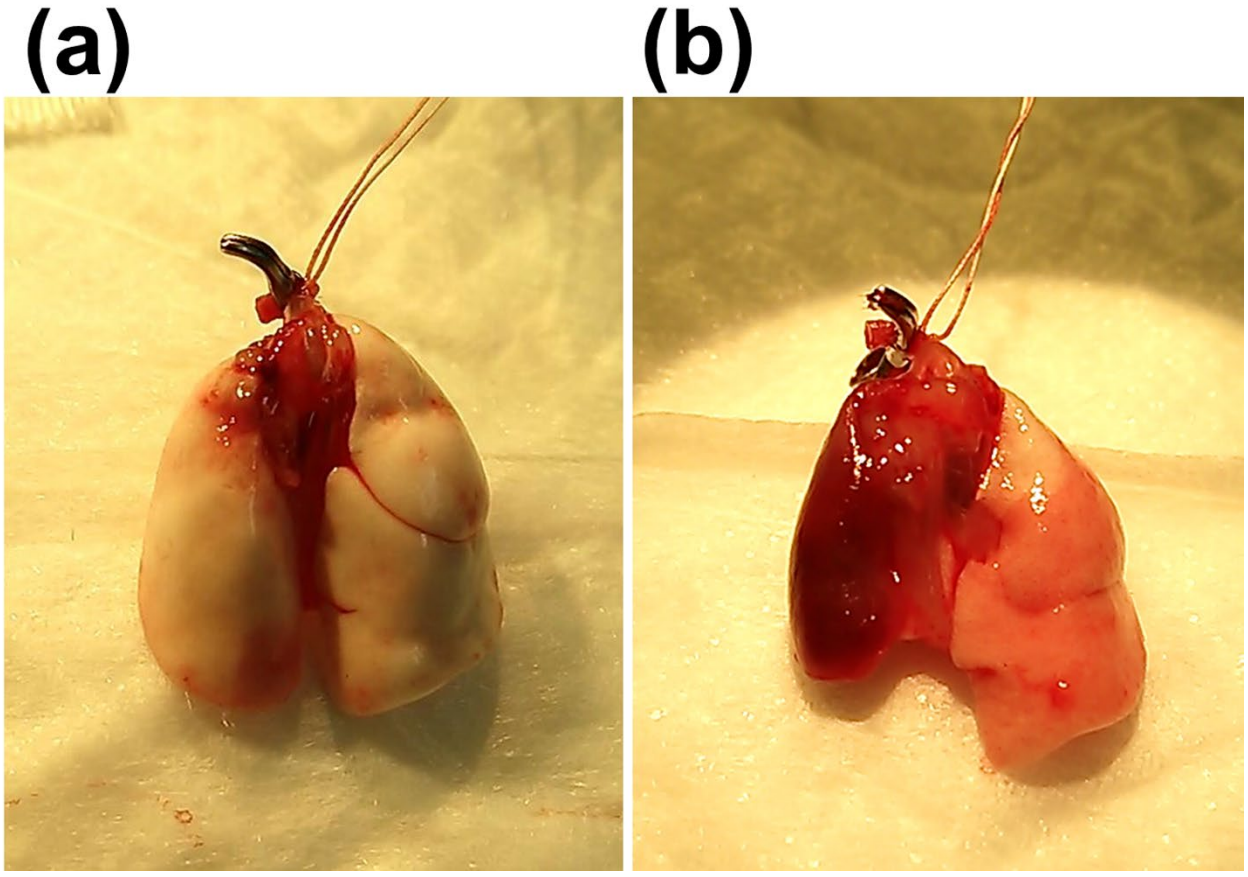

Fig. 3. Representative histological findings of the lung allografts in the group with a warm ischemic time (WIT) of 15 minutes (WIT15 group) (a) and the group with a WIT of 60 minutes (WIT60 group) (b) (hematoxylin-eosin stain, ×200 magnification).

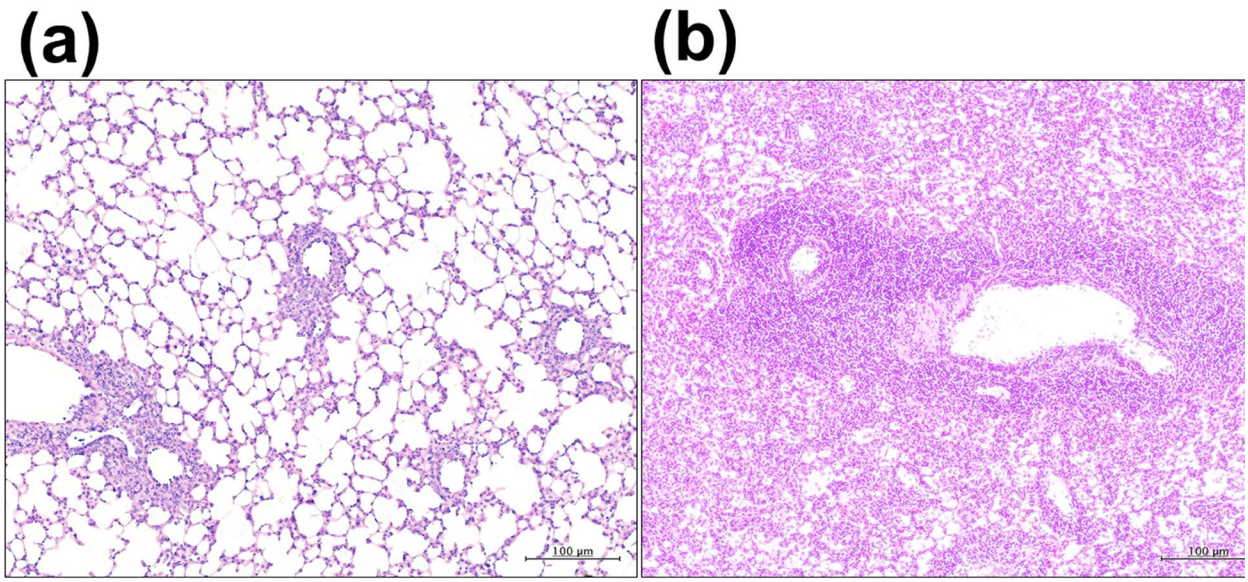

Fig. 4. The rejection severity grade in the group with a warm ischemic time (WIT) of 60 minutes (WIT60 group) was significantly higher than that in WIT15 group (3.4 ± 0.4 vs. 2.2 ± 0.2, *P* = 0.0278).

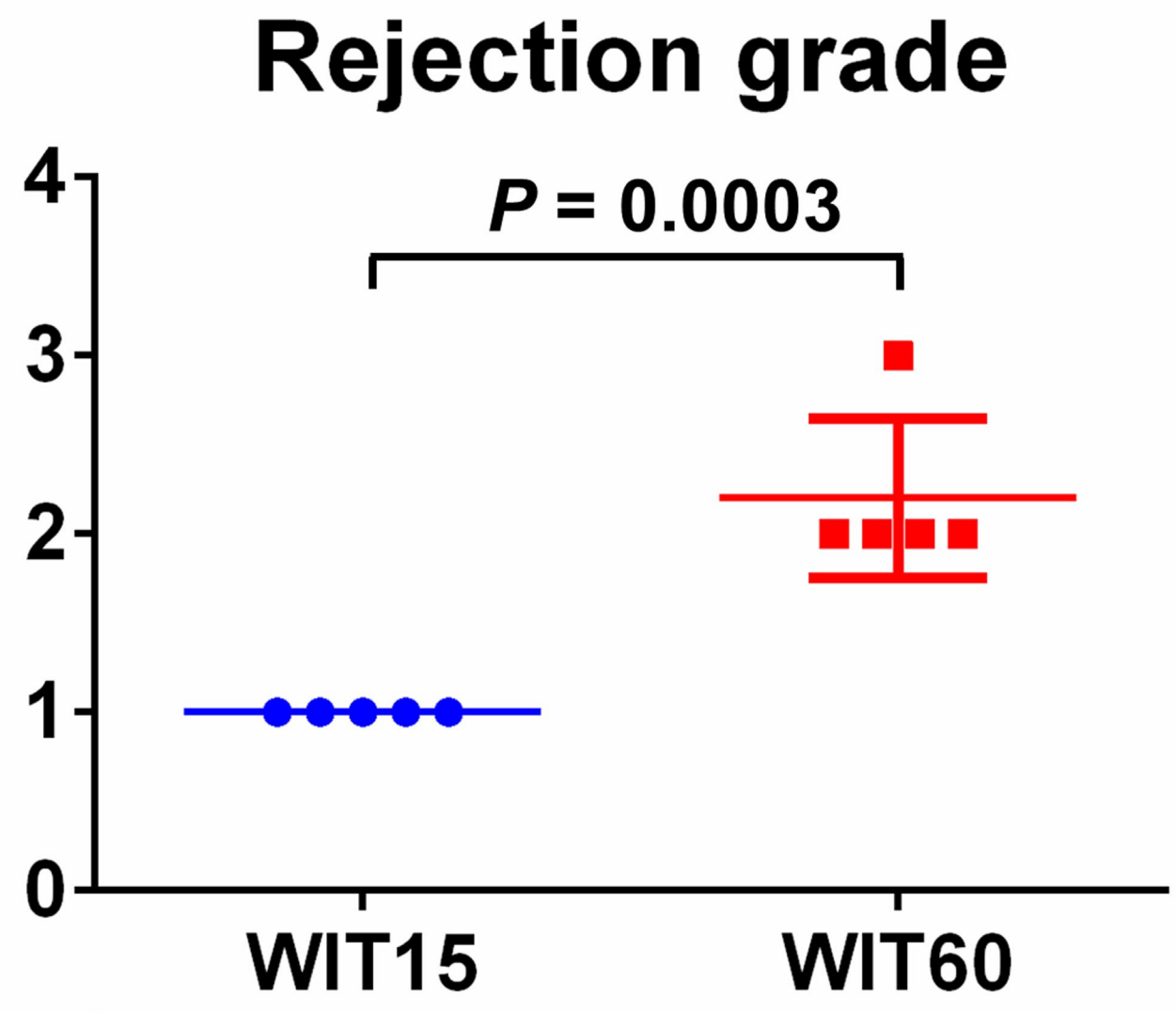

Fig. 5. Representative result of flow-cytometric analysis of the lung allografts. In the group with a warm ischemic time (WIT) of 15 minutes (WIT15 group), the number of CD8+ T-cells was equivalent to that of the CD4+ T-cells (a), whereas in the WIT60 group, the CD8+ T cells outnumbered the CD4+ T cells (b).

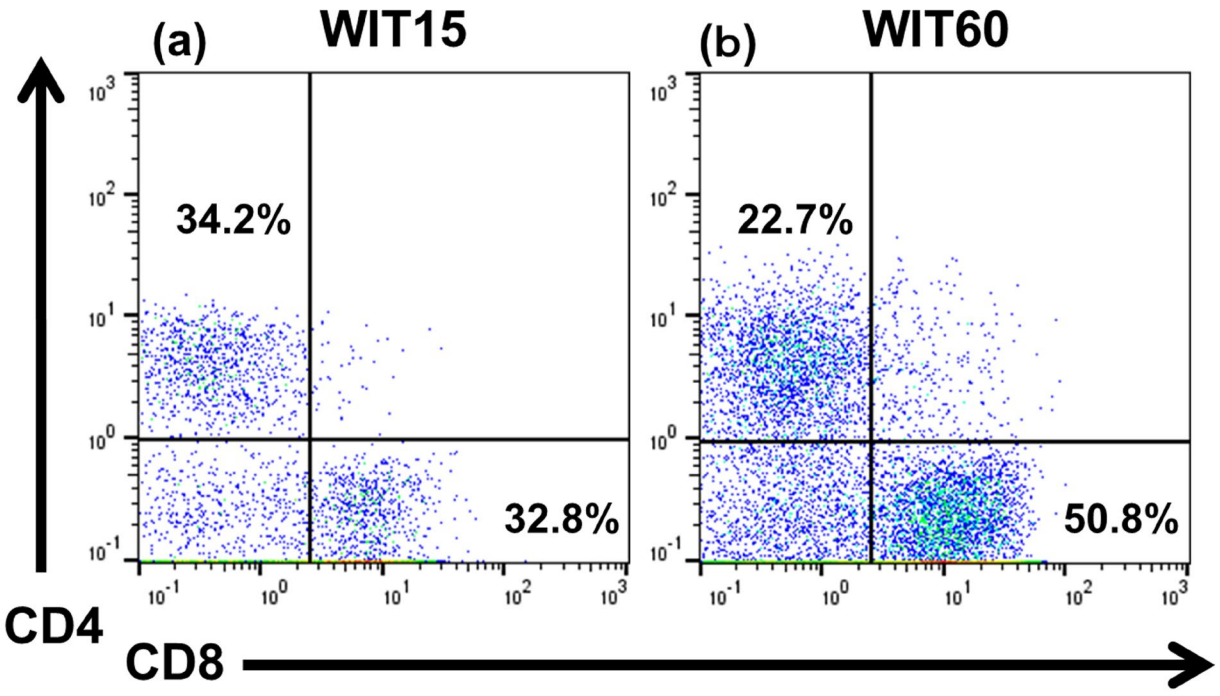

Fig. 6. The intragraft CD8+ to CD4+ T cell ratio in the group with a warm ischemic time (WIT) of 60 minutes (WIT60 group) was significantly higher than that in the WIT15 group (2.3 ± 0.12 vs. 1.2 ± 0.11, $P < 0.0001$).