Original Article

Timing of Mesenchymal Stromal Cell Therapy Defines its Immunosuppressive Effects in a Rat Lung Transplantation Model

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Yukinori Tanoue[1], Tomoshi Tsuchiya[2], Takuro Miyazaki[1], Mayumi Iwatake[1], Hironosuke Watanabe[1], Hiroshi Yukawa[3], Kazuhide Sato[3], Go Hatachi[1], Koichiro Shimoyama[1], Keitaro Matsumoto[1], Ryoichiro Doi[1], Koichi Tomoshige[1], and Takeshi Nagayasu[1]

Abstract

Cell therapy using mesenchymal stromal cells (MSCs) is being studied for its immunosuppressive effects. In organ transplantation, the amount of MSCs that accumulate in transplanted organs and other organs may differ depending on administration timing, which may impact their immunosuppressive effects. *In vitro*, adipose-derived mesenchymal stem cells (ADMSCs) suppress lymphocyte activation under cell-to-cell contact conditions. However, *in vivo*, it is controversial whether ADMSCs are more effective in accumulating in transplanted organs or in secondary lymphoid organs. Herein, we aimed to investigate whether the timing of ADMSC administration affects its immunosuppression ability in a rat lung transplantation model. In the transplantation study, rats were intramuscularly administered half the usual dose of tacrolimus (0.5 mg/kg) every 24 h after lung transplantation. ADMSCs (1×10^6) were administered via the jugular vein before (PreTx) or after (PostTx) transplantation. Cell tracking using quantum dots was performed. ADMSCs accumulated predominantly in the lung and liver; fewer ADMSCs were distributed in the grafted lung in the PreTx group than in the PostTx group. The rejection rate was remarkably low in the ADMSC-administered groups, particularly in the PostTx group. Serum tumor necrosis factor-α (TNF-α), interferon-γ, and interleukin (IL)-6 levels showed a greater tendency to decrease in the PreTx group than in the PostTx group. The proportion of regulatory T cells in the grafted lung 10 days after transplantation was higher in the PostTx group than in the PreTx group. PostTx administration suppresses rejection better than PreTx administration, possibly due to regulatory T cell induction by ADMSCs accumulated in the transplanted lungs, suggesting a mechanism different from that in heart or kidney transplantation that PreTx administration is more effective than PostTx administration. These results could help establish cell therapy using MSCs in lung transplantation.

Keywords

cell therapy, adipose-derived mesenchymal stromal cell (ADMSC), immunosuppression, lung transplantation, mesenchymal stromal cell (MSC), regulatory T cell

Introduction

Organ transplantation is a curative treatment for diseases associated with chronic organ failure and is beneficial for patients with intractable diseases[1,2]. Lung transplantation is indicated for refractory chronic lung disease. However, transplantation requires the long-term use of immunosuppressants to avoid rejection[2,3] due to the high antigenicity of the lung; nevertheless, rejection cannot be completely ruled out[3,4]. Long-term immunosuppressant use causes side effects, such as renal dysfunction, infections, and secondary cancer development, which impair patient quality of life after transplantation[3–6]; therefore, treatments should be developed to facilitate dosage reduction or discontinuation of immunosuppressants.

Mesenchymal stromal cells (MSCs) are used in regenerative medicine; however, they exhibit immunosuppressive properties[7–9]. The immunosuppressive characteristics of MSCs have been explored in organ transplantation and autoimmune disease treatment[10–14] and their clinical application is being investigated. However, the use of adipose-derived mesenchymal stem cells(ADMSCs) in lung transplantation has been delayed, as an optimal protocol for stem cell therapy in

lung transplantation has not yet been established. *In vitro* experiments have confirmed that the immunosuppressive effect of ADMSCs is similar to that of bone marrow–derived MSCs; however, ADMSCs release more immunosuppressive cytokines, such as interleukin (IL)-10, IL-6, and transforming growth factor-β[7,15–18]. A rat lung transplantation study reported a reduced rejection rate in the ADMSC-administered group, highlighting the immunosuppressive effect of ADMSCs *in vivo*[19]. In addition, since ADMSCs can be collected and cultured from fat on the body surface, they provide a relatively easy approach for procuring stem cells. However, fat harvesting requires surgery under anesthesia, and caution is required because it involves risks such as bleeding, pain, infection, and fat embolism[7,16–18].

With regard to the biodistribution of administered MSCs, theoretically, MSCs administered via the jugular vein pass through the lungs before distributing to other organs. In animal studies on kidney and heart transplantation, MSC administration before transplantation resulted in MSC accumulation in secondary lymphoid tissues, such as the spleen and lymph nodes, and more immunosuppressive effects than MSC administration after transplantation[20-22]. However, rejection or tolerance in lung transplantation is not dependent on secondary lymphoid organs, unlike that observed in heart and skin transplantation[23,24]. Moreover, a consensus has not yet been reached on whether MSC administration is more beneficial before or after organ transplantation[22,25–32].

Accordingly, herein, we aimed to investigate the immunosuppressive effects of ADMSCs administered at different times in a rat lung transplantation model.

Materials and Methods

Animals

Inbred male 8- to 15-week-old rats were used for experiments. Three allogeneic rats were housed in individual cages and used in experiments after 1 week of housing. For lung transplantation, male Brown Norway rats (MHC haplotype: $RN1^n$, weight: 200–250 g; $n = 50$) were used as donors and male Lewis rats (MHC haplotype: $RN1^l$, weight: 250–300 g; $n = 55$) were used as recipients (Charles River Laboratories Japan, Yokohama, Japan). All animal experiments were conducted following the Nagasaki University Institutional Animal Care and Use Committee guidelines and were approved by the ethics committee of Nagasaki University (Approval Number: 1911261580-7).

ADMSC Preparation and Characterization

Allogenic ADMSCs were derived from Lewis rats as previously described[19] (see the Supplemental Materials and Methods for details). Cells were then seeded in a 100-mm dish (Thermo Fisher Scientific, Waltham, MA, USA) and cultured in a CO_2 incubator at 37°C. Cells were passaged until 90% confluent, and 2–3 passage cells were used for all *in vitro* and *in vivo* experiments. The population doubling time of ADMSCs was 12.5 h. The phenotypic characteristics of the prepared ADMSCs were assessed by flow cytometry, as previously reported[19]. The positive markers used were CD105 (BD Biosciences, Franklin Lakes, NJ, USA; 11-298-C025), CD73 (BD Biosciences; 551123), and CD90 (BD Biosciences; 554897), and the negative markers were CD45 (BD Biosciences; 561867), CD11b (BD Biosciences; 561691), CD31 (BD Biosciences; 555027), and CD34 (Santa Cruz Biotechnology, Dallas, Texas, USA; sc-7324) (Fig. 1A). We also evaluated ADMSCs at confluence in a multi-lineage differentiation assay. Differentiation was induced by culturing ADMSCs in osteogenic differentiation medium (PromoCell GmbH, Heidelberg, Germany; C-28013), chondrogenic differentiation medium (PromoCell GmbH; C-28012), or adipogenic differentiation medium (PromoCell GmbH; C-28016) for 14–28 days. Differentiation into each lineage was confirmed using the following stains: alizarin red S for osteogenic differentiation, Alcian blue for chondrogenic differentiation, and oil red O for adipogenic differentiation (Fig. 1B).

Lung Transplantation and ADMSC Administration

Left lung transplantation in rats was performed using the cuff technique (see the Supplemental Materials and Methods for details)[33,34]. For ADMSC administration, a 1-cm-long

[1] Division of Surgery Oncology, Department of Surgery, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan
[2] Department of Thoracic Surgery, Faculty of Medicine, Academic Assembly, University of Toyama, Toyama, Japan
[3] Division of Quantum Science, Technology, and Quantum Life Science, Institute of Nano-Life-Systems, Institutes of Innovation for Future Society, Nagoya University, Nagoya, Japan

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Corresponding Authors:

Tomoshi Tsuchiya, Department of Thoracic Surgery, Faculty of Medicine, Academic Assembly, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan.
Email: tsuchiya@med.u-toyama.ac.jp

Takeshi Nagayasu, Division of Surgery Oncology, Department of Surgery, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.
Email: nagayasu@nagasaki-u.ac.jp

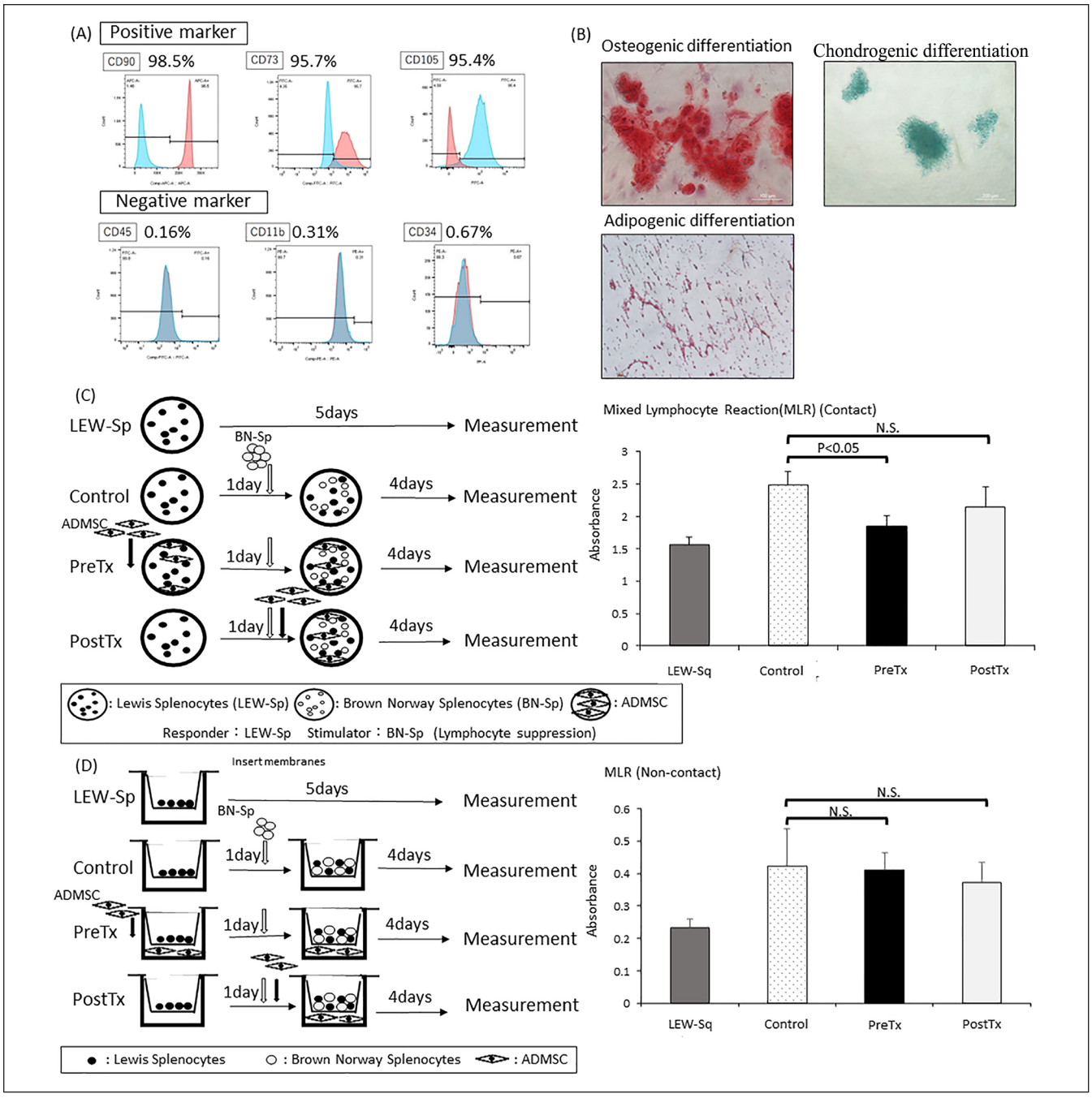

Figure 1. Validation of ADMSC surface markers and induction of differentiation. (A) Expression of MSC positive markers CD90, CD73, and CD105 and negative markers CD45, CD11b, and CD34 using flow cytometry. (B) Results of inducing differentiation of ADMSCs into osteogenesis, chondrogenesis, and adipogenesis using a differentiation-inducing medium. Evaluation of direct and indirect immunosuppressive effects of ADMSCs *in vitro* using MLRs. (C) Schematic and time-course of the contact-MLR experiments. BrdU-incorporated LEW-Sps were measured as responders, and mitomycin C–treated BN-Sps were used as stimulators. LEW-Sp group: LEW-Sps only; control group: BN-Sps added to LEW-Sps; PreTx group: BN-Sps added to co-cultured LEW-Sps and ADMSCs; PostTx group: BN-Sps added, followed by ADMSC addition, to LEW-Sps. The absorbance of BrdU was measured 4 days after the addition of BN-Sps. (D) Schematic and time-course of the non-contact-MLR experiment performed using insert membranes. ADMSCs: adipose-derived mesenchymal stem cells; MSC: mesenchymal stromal cells; MLR: mixed lymphocyte reactions; BrdU: bromodeoxyuridine; LEW: Lewis; Sp: splenocytes; BN: Brown Norway; PreTx: pre-transplantation; PostTx: post-transplantation.

skin incision was made in the neck, the jugular vein was detached, and intravenous administration was performed using a 26-gauge catheter. From the first day following transplantation, 0.5 mg/kg tacrolimus was administered intramuscularly every 24 h until euthanasia 3, 4, or 10 days after transplantation.

Experimental Design

The experimental design was developed based on the timings of ADMSC administration and treatments rather than transplantation. Rats were divided into the following groups: control group, which did not receive any treatment other than lung transplantation; tacrolimus group, which received an intramuscular tacrolimus injection every 24 h after lung transplantation; pre-transplantation administration (PreTx) group, in which a jugular administration of 1×10^6 ADMSCs was performed 24 h before lung transplantation and tacrolimus was intramuscularly injected every 24 h after lung transplantation; and post-transplantation administration (PostTx) group, in which a jugular administration of 1×10^6 ADMSCs was performed immediately after lung transplantation and tacrolimus was intramuscularly injected every 24 h after lung transplantation. Recipient rats in each group were euthanized by carbon dioxide inhalation on days 3 ($n = 4$/group), 7 ($n = 4$/group), and 10 ($n = 4$/group) after transplantation, and the grafted lungs, blood, spleens, and livers were recovered and used for further experiments. Only in models evaluating ADMSC biodistribution after lung transplantation, rats were euthanized on day 1 post-transplant to examine early post-transplant biodistribution. Lewis rats were euthanized on days 1, 2, 3, 4, and 7 after administration of ADMSCs to assess their distribution in the body.

Mixed Lymphocyte Reaction

Two mixed lymphocyte reactions (MLRs) were performed to verify the difference in the immunosuppressive effect of ADMSCs depending on administration timing *in vitro*[29]. Contact-MLR simulates the transplanted donor lung, while non-contact-MLR simulates secondary lymphoid tissues, such as the spleen. Lewis rat splenocytes (LEW-Sps) were used as response cells, and Brown Norway rat splenocytes (BN-Sps), pretreated with 25 μg/ml mitomycin C (Merck, Darmstadt, Germany) at 37°C for 30 min, were used as stimulating cells. LEW-Sps (1×10^5) were cultured in 96-well plates in RPMI-1640 with L-Glutamine and Phenol Red (FUJIFILM Wako, Osaka, Japan), 10% fetal bovine serum, 1% streptomycin, and 1% amphotericin B in a CO_2 incubator at 37°C, and 1 day later, BN-Sps (1×10^5) were added and co-cultured for 4 days. To examine the difference in the immunosuppressive effect of different timings of the co-culture of ADMSCs (1×10^5) *in vitro*, the CytoSelect™ BrdU Cell Proliferation ELISA Kit (Cell Biolabs, Inc., San Diego, CA, USA) was used to measure T-cell proliferation, as T-cell activation is inhibited due to the immunosuppressive effects of ADMSCs[29]. Bromodeoxyuridine (BrdU) (10 μl of 0.1 mM BrdU/well) was added 5 days after transplantation, and the absorbance was measured at 450 nm (Multiskan FC; Thermo Fisher Scientific). For the analysis of the immunosuppressive effects of ADMSCs, Lewis ADMSCs (1×10^5) were co-cultured before or after the co-culture of LEW-Sps and BN-Sps. The group without ADMSC co-culture was used as the control. The different ADMSC-treated groups were as follows (Fig. 1A): PreTx model of MLR, in which ADMSCs (1×10^5) and LEW-Sps were co-cultured 1 d before BN-Sp addition, and PostTx model of MLR, in which ADMSCs (1×10^5) were co-cultured at the same time as BN-Sps. BrdU assay was similarly performed. To analyze the indirect effect of ADMSCs, a non-contact culture model was also created by co-culturing LEW-Sq and BN-Sq on insert membranes (Transwell®, Corning Inc., Corning, NY, USA) and culturing ADMSCs on wells (Fig. 1B).

ADMSC Labeling

To determine the distribution of the administered ADMSCs in the body, cells were labeled with quantum dots (QDs) as previously reported[35,36] (see the Supplemental Materials and Methods for details). Next, labeled ADMSCs were administered to recipient rats as described above. In the PreTx and PostTx models, QD-transduced ADMSCs (1×10^6) were administered via the jugular vein 24 h before lung transplantation and immediately after lung transplantation, respectively. Rats in both groups were euthanized 1 day after transplantation, and the collected tissue samples were imaged by an *in vivo* imaging system (Summit Pharmaceuticals International Corporation, Tokyo, Japan) to measure the absorbance of each tissue (Fig. 2).

Histological Study

Four recipient rats from each group were euthanized on days 3, 7, and 10 after transplantation (Fig. 3A). All allografts were collected, and phosphate-buffered saline (-) was refluxed from the pulmonary artery. The collected grafts were fixed with 4% paraformaldehyde, embedded in paraffin, sliced to approximately 5-μm thickness, and stained with hematoxylin–eosin. Histological findings were graded blindly by assessing acute rejection and airway inflammation according to the International Society for Heart and Lung Transplantation classification[37] (Fig. 3B). Acute rejection was classified as follows: grade 0, no evidence of mononuclear cell infiltration; grade 1, minimum; grade 2, mild; grade 3, moderate; and grade 4, severe. Airway inflammation in the narrow airways was classified as follows: grade 0, without inflammation; grade 1, low grade; and grade 2, high grade. The scores obtained for both criteria were added to obtain a unique rejection score.

Immunohistochemistry

Immunohistochemistry was performed as previously described (see the Supplemental Materials and Methods for

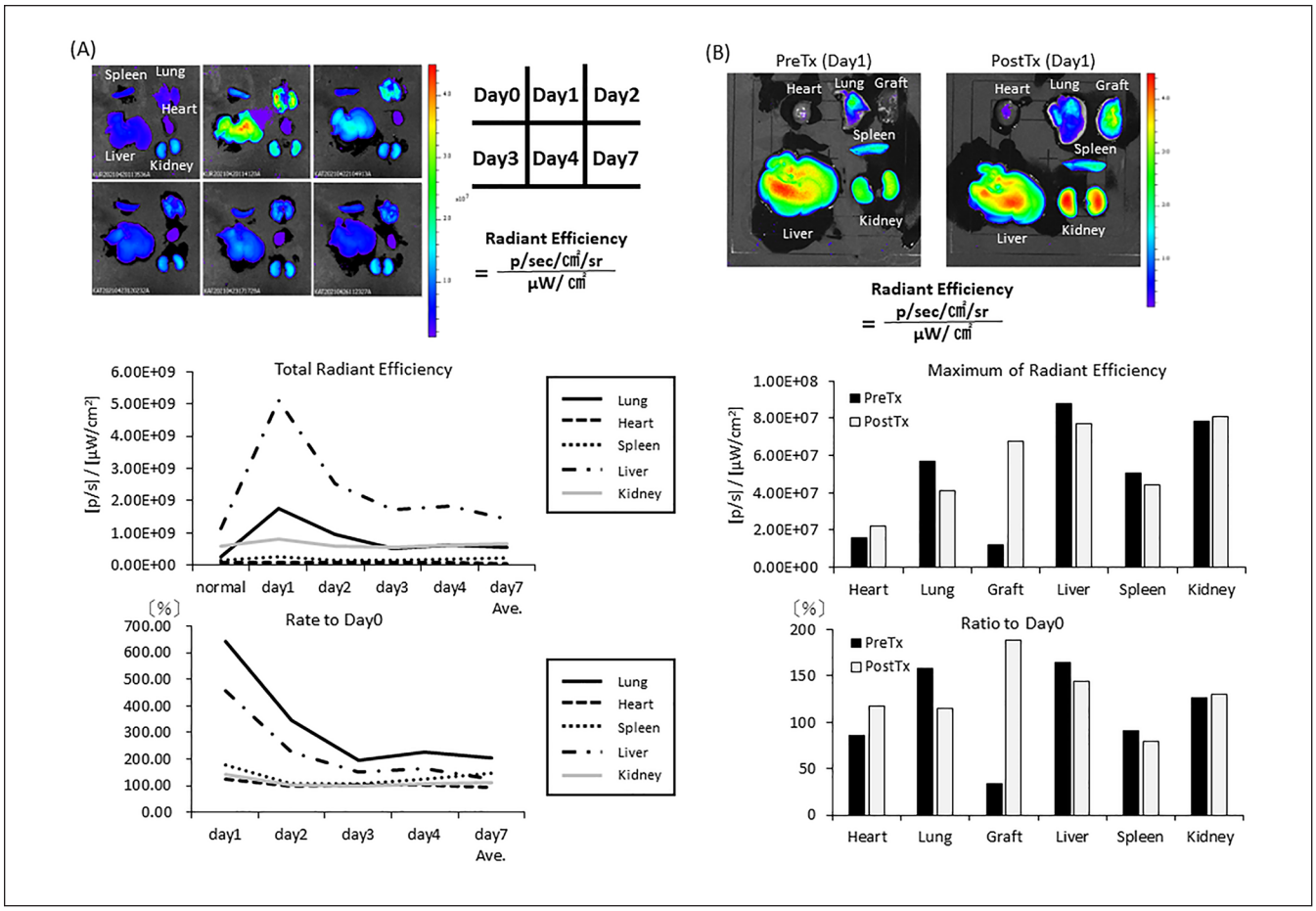

Figure 2. Distribution of ADMSCs after jugular vein administration in a rat lung transplantation model. (A) Distribution of QD-labeled ADMSCs (1×10^6) after jugular vein administration. (B) Distribution of ADMSCs after pre-/post-transplantation administration on day 1 after lung transplantation. ADMSCs: adipose-derived mesenchymal stem cells; QD: quantum dot; PreTx: pre-transplantation; PostTx: post-transplantation.

details) using mouse anti-CD68 (ab31630, 1:500; Abcam, Cambridge, UK), rabbit anti-CD163 (ab182422, 1:500; Abcam), rabbit anti-CC chemokine receptor 7 (CCR7; ab32527, 1:100; Abcam), mouse anti-Fox-P3 antibody (ab22510, 1:50; Abcam), rabbit anti-IL-17A (ab214588, 1:200; Abcam), and rabbit anti-CD4 (ab237722, 1:100; Abcam)[38,39].

Enzyme-Linked Immunosorbent Assay

The serum levels of inflammatory and anti-inflammatory cytokines [IL-2, IL-4, IL-6, IL-17A, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and IL-10] were evaluated via an enzyme-linked immunosorbent assay (ELISA) as per the MILLIPLEX MultiAnalyte Profiling Rat Cytokine/Chemokine panel (Merck) (see the Supplemental Materials and Methods for details). We collected 7 ml whole blood from the inferior vena cava of recipient rats 3, 7, and 10 days after transplantation. The entire plate was read using a multiplex plate reader (Multiskan JX, 51118230C; Thermo Fisher Scientific), using the manufacturer's software.

Flow Cytometry Analysis

To assess whether MSC infusions were associated with recipient regulatory T cell (Treg) proliferation, flow cytometry was performed to analyze the proportion of CD4+CD25+ forkhead box protein 3 (Foxp3)+T cells in the peripheral blood, spleen, and grafted lung (see the Supplemental Materials and Methods for details)[26,38]. For Foxp3 staining, the Anti-Mouse/Rat Foxp3 Staining Set PE (eBioscience, San Diego, CA, USA) was used according to the manufacturer's protocol. Cells were stained with fluorescein isothiocyanate-conjugated anti-rat CD4 (OX35; eBioscience), allophycocyanin-conjugated anti-rat CD25 monoclonal antibodies (OX39; eBioscience), and phycoerythrin-conjugated anti-rat Foxp3 antibody (FJK-16s; eBioscience). The proportion of stained cells was evaluated using flow cytometry (BD FACSCanto™ II; BD BioSciences).

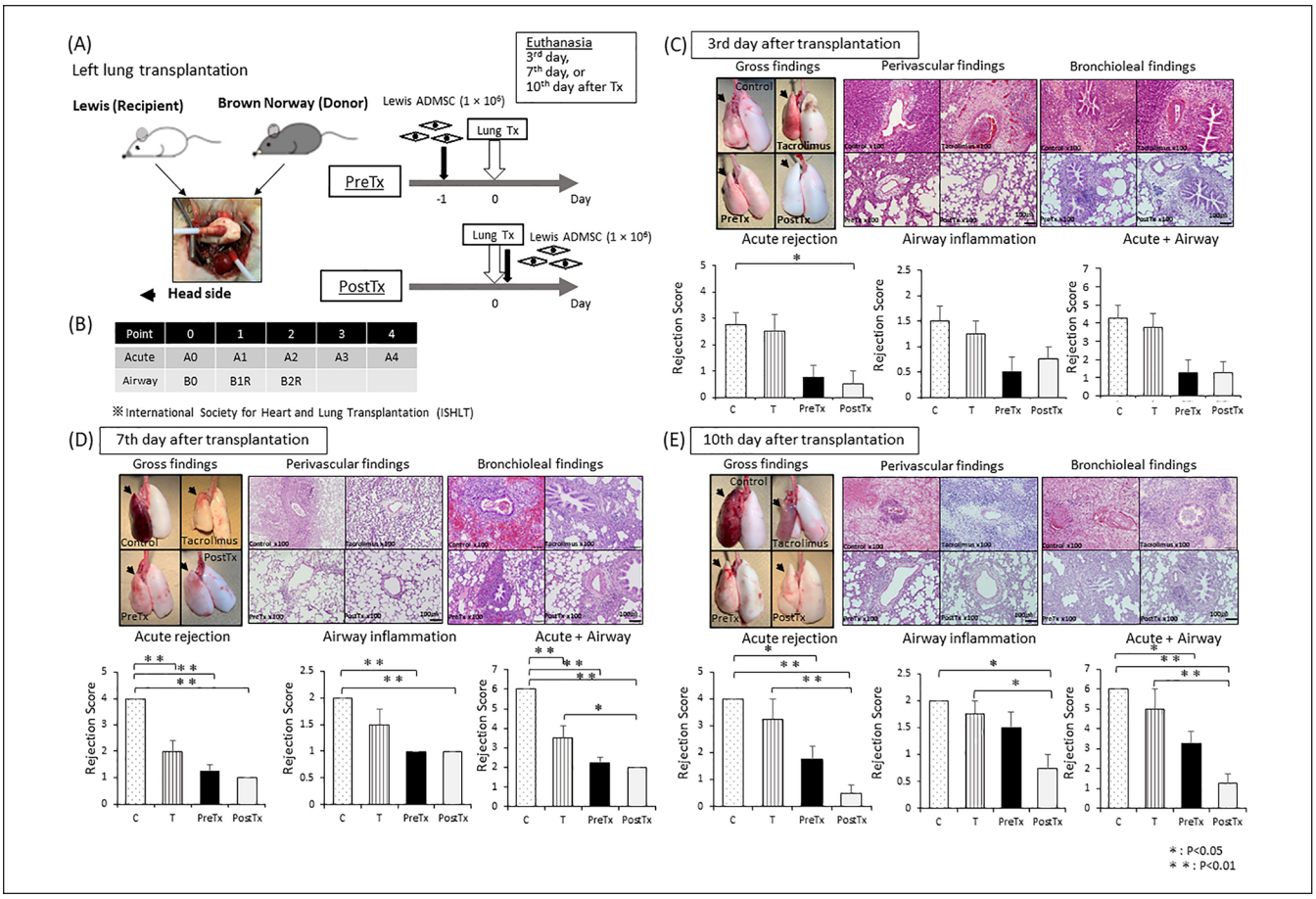

Figure 3. Gross and histological findings of the grafted lung after lung transplantation. (A) Schematic and time-course of the transplantation experiments. The left lung of Brown Norway rats was transplanted into Lewis rats, and ADMSCs of Lewis rats were administered via the jugular vein before or after lung transplantation. (B) Acute rejection and peri-airway rejection findings and rejection score based on the International Society for Heart and Lung Transplantation classification. (C–E) Gross and histological findings of grafted lungs and rejection score on days 3 (C), 7 (D), and 10 (E) after transplantation (n = 4/group). ADMSC: adipose-derived mesenchymal stem cell; C: control; T: tacrolimus; PreTx: pre-transplantation; PostTx: post-transplantation. P values are denoted as follows: *$P \leq 0.05$; **$P \leq 0.01$.

Statistical Analysis

The Kruskal–Wallis test and the Mann–Whitney U test, using JMP software (version 10.0.2), were used to compare the absorbance, rejection score, positive cells, ELISA measurements, and percentage of Foxp3-positive cells between groups. P values < 0.05 were considered significant.

Results

ADMSCs Suppress Lymphocyte Activation in MLR

Surface marker and differentiation induction experiments were performed to confirm the characterization of ADMSCs. Flow cytometry confirmed the positive expression of the MSC positive markers CD90, CD73, and CD105, and negative expression of the negative markers CD45, CD11b, and CD34 (Fig. 1A). In addition, differentiation into three lineages (osteogenesis, chondrogenesis, and adipogenesis) was observed (Fig. 1B). These ADMSCs were used in the following experiments. To simulate the effects of ADMSCs in the transplantation models, we performed MLR with ADMSCs and splenocytes using both contact-MLR and non-contact-MLR models. In contact-MLR, BrdU absorbance decreased in both PreTx and PostTx groups, particularly in the PreTx group (Fig. 1C, Control vs PreTx, $P = 0.029$), indicating that ADMSC administration suppressed T-cell activation in the PreTx group. Co-administration of donor splenocytes and ADMSCs resulted in a limited efficacy of ADMSCs in the PostTx group. Only MSCs added the day before the start of the MLR caused inhibition under cell-contact conditions, whereas MSCs added together with the donor splenocytes did not inhibit T-cell proliferation. In contrast, in the non-contact-MLR, no decrease in BrdU absorbance was observed in both PreTx and PostTx groups (Fig. 1D).

ADMSC Distribution After Jugular Vein Administration

We successfully detected QD-labeled ADMSCs using *in vivo* imaging. Under normal conditions, the administered ADMSCs were distributed in the lungs and liver on day 1 and thereafter accumulated in the spleen and kidneys (Fig. 2A). In the lung transplant model, ADMSCs accumulated in the liver, right lung, kidney, and spleen in the PreTx group, and almost no uptake was observed in the grafted lungs (Fig. 2B). However, high accumulation of QDs was observed in the grafted lungs in the PostTx group. ADMSC accumulation in each organ except for the grafted lungs was almost the same as that in the PreTx group (Fig. 2B).

Gross and Histological Findings

Gross findings of the transplanted donor lungs in the control group included a liver-like dark red color 7 days after transplantation, indicating that the transplanted lung had failed with acute resection and atelectasis of the grafted lung. In the tacrolimus group (only immunosuppressant), atelectasis was observed in the grafted lungs 10 days after transplantation. However, in the PreTx and PostTx groups, few changes in the visceral pleura were observed. In the PreTx group, only mild pleural redness was observed 10 days after transplantation (Fig. 3C–E).

Grade 4 rejection[37], with marked mononuclear cell infiltration around blood vessels and the bronchi, was observed in the control group from day 3 after transplantation; alveolar structure destruction with pulmonary edema and alveolar hemorrhage were also observed, and these findings persisted after 3 days. Similarly, in the tacrolimus group, mononuclear cell infiltration around the blood vessels and bronchi was remarkable, and grade 3–4 rejection was observed[37]. However, in the PreTx and PostTx groups, mononuclear cell infiltration around the bronchi and alveoli was mild with no observable lung tissue destruction (Fig. 3C–E). Rejection scores were considerably lower in the PreTx and PostTx groups than in the control and tacrolimus groups at every time point. Moreover, the rejection score was relatively lower in the PostTx group than in the PreTx group on day 10 after transplantation (Fig. 3E; PreTx vs PostTx, $P = 0.173$).

Immunohistochemistry of Immunocompetent Cells in the Transplanted Lungs

The number of CD68-positive cells, a pan-macrophage marker, was significantly lower in the PreTx and PostTx groups than in the control and tacrolimus groups on day 3 after transplantation (Fig. 4A; control vs PreTx, $P = 0.039$; tacrolimus vs PreTx, $P = 0.027$; control vs PostTx, $P = 0.042$; tacrolimus vs PostTx, $P = 0.029$). However, on days 7 and 10 after transplantation, no significant difference was observed between the groups (Fig. 4A). The number of CCR7-positive cells, a marker for M1 macrophages[39,40], was significantly low in the PreTx group (control vs PreTx, $P = 0.037$); it also tended to be low in the PostTx group on day 10 after transplantation (Fig. 4B). In addition, high expression of CD163, a marker for M2 macrophages[39,40], was observed in the PreTx and PostTx groups on day 3 after transplantation (Fig. 4C). These results indicate that ADMSCs induce macrophage polarity from the inflammatory M1 type to the immunosuppressive M2 type. The expression of Foxp3, a marker for Tregs[4], was relatively high in the PreTx and PostTx groups on days 7 and 10 after transplantation; however, no significant difference was observed (Fig. 4D).

We observed that the number of IL-17A-positive cells, a marker for T helper 17 cells[41], was significantly lower in the PostTx group than in the control and tacrolimus groups on day 3 after transplantation (Fig. 4E; control vs PostTx, $P = 0.037$; tacrolimus vs PostTx, $P = 0.009$). No significant difference was observed between the PreTx and PostTx groups on days 7 and 10 after transplantation. Immunostaining of CD4-positive T cells showed no significant difference in the number of positive cells in each group on days 3, 5, and 7 after transplantation (Fig. 4F). The ratio of Foxp3-positive cell counts to CD4-positive cell counts was evaluated in each group on days 3, 5, and 7 after transplantation. Although no significant difference was detected, a relatively high ratio of Foxp3-positive cells/CD4-positive cells was observed in the PostTx group on day 10 after transplantation (Fig. 4G).

ELISA

We measured serum cytokine levels of recipient rats 3, 7, and 10 days after transplantation. The levels of inflammatory cytokines IFN-γ, IL-2, IL-4, IL-6, TNF-α, and IL-17A were the highest on day 7 after transplantation in all groups and gradually decreased thereafter (Fig. 5). The highest inflammatory cytokine level was relatively low in the control group. Because the control group exhibited strong rejection with blood vessel occlusion, the release of cytokines into the peripheral blood was difficult. Thus, although rejection was severe in both control and tacrolimus groups, the tacrolimus group exhibited inflammatory cytokine levels. On day 7 after transplantation, the IFN-γ, IL-2, IL-4, IL-6, TNF-α, and IL-17A levels tended to be lower in the PreTx group than in the tacrolimus and PostTx groups (Fig. 5).

Flow Cytometry Analysis

In preliminary experiments, we evaluated the proportion of Tregs in the peripheral blood mononuclear cells (PBMCs), spleen, and grafted lungs in the PostTx group on days 3, 5, and 10 after transplantation. We observed no difference in the proportion of Tregs in the PBMCs and spleen at each time point, but the proportion of Tregs in the grafted lungs increased considerably on day 10 after transplantation (data

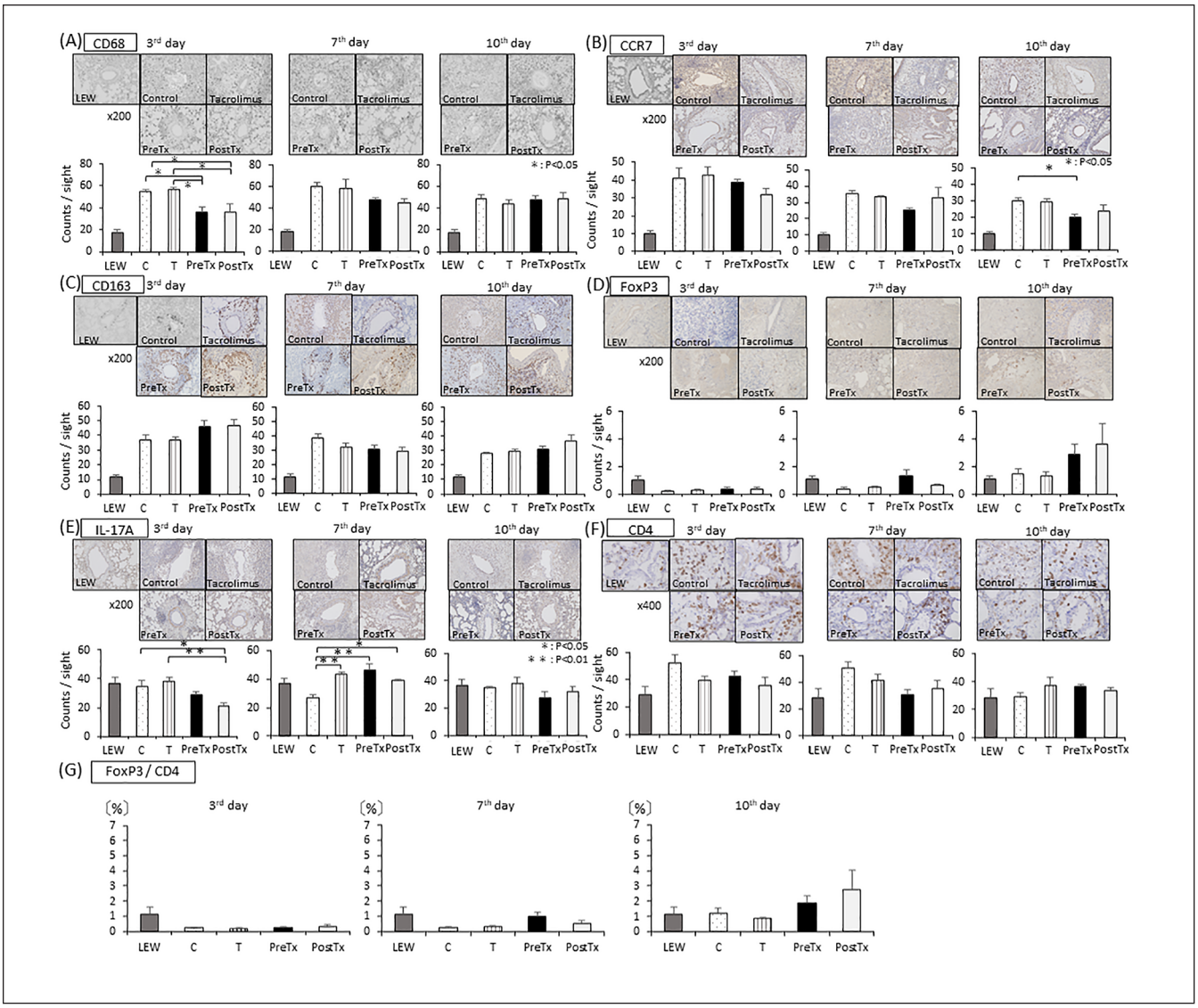

Figure 4. Immunostaining of grafted lung tissue on days 3, 7, and 10 after lung transplantation. (A) CD68 immunostaining of grafted lung samples. (B) CCR7 immunostaining of grafted lung samples. (C) CD163 immunostaining of grafted lung samples. (D) Foxp3 immunostaining of grafted lung samples. (E) IL-17A immunostaining of grafted lung samples. (F) CD4 immunostaining of grafted lung samples. (G) Percentage of Foxp3-positive cells among CD4-positive cells. Positive cells were counted in five randomly selected high-magnification microscopic fields (×400; 0.0625 mm^2) and averaged [Foxp3 tissue only, 10 high-magnification microscopic fields of view (×200; 0.125 mm^2)]. LEW: Lewis; C: control; T: tacrolimus; PreTx: pre-transplantation; PostTx: post-transplantation; CCR7: CC chemokine receptor 7; IL-17A: interleukin 17A; Foxp3: forkhead box protein 3.

not shown). Therefore, further analyses were done using the samples obtained on day 10 after transplantation. The Tregs are expressed as percentage of CD4+CD25+ T cells. The proportion of Tregs in the spleen was slightly higher in the PreTx and PostTx groups than in the control and tacrolimus groups (Fig. 6A). The proportion of Tregs did not differ between the PostTx and PreTx groups in both PBMCs and the spleen (Fig. 6A, B). However, the proportion of Tregs in the grafted lungs was significantly higher in the PostTx group than in the PreTx group (Fig. 6C, PreTx vs PostTx, $P = 0.030$).

Discussion

In MSC-based cell therapy for organ transplantation, which aims to suppress immune responses, administration timing is crucial as it may influence whether the administered cells enter the transplanted organ. Herein, we observed a reduced transplant rejection rate and a higher immunosuppressive effect of ADMSCs in the PostTx group than in the PreTx group; these results are different from those obtained previously in heart and kidney transplantation models[20–22]. Histological analysis revealed suppressed lymphocyte infiltration in the PostTx group compared with that in the PreTx

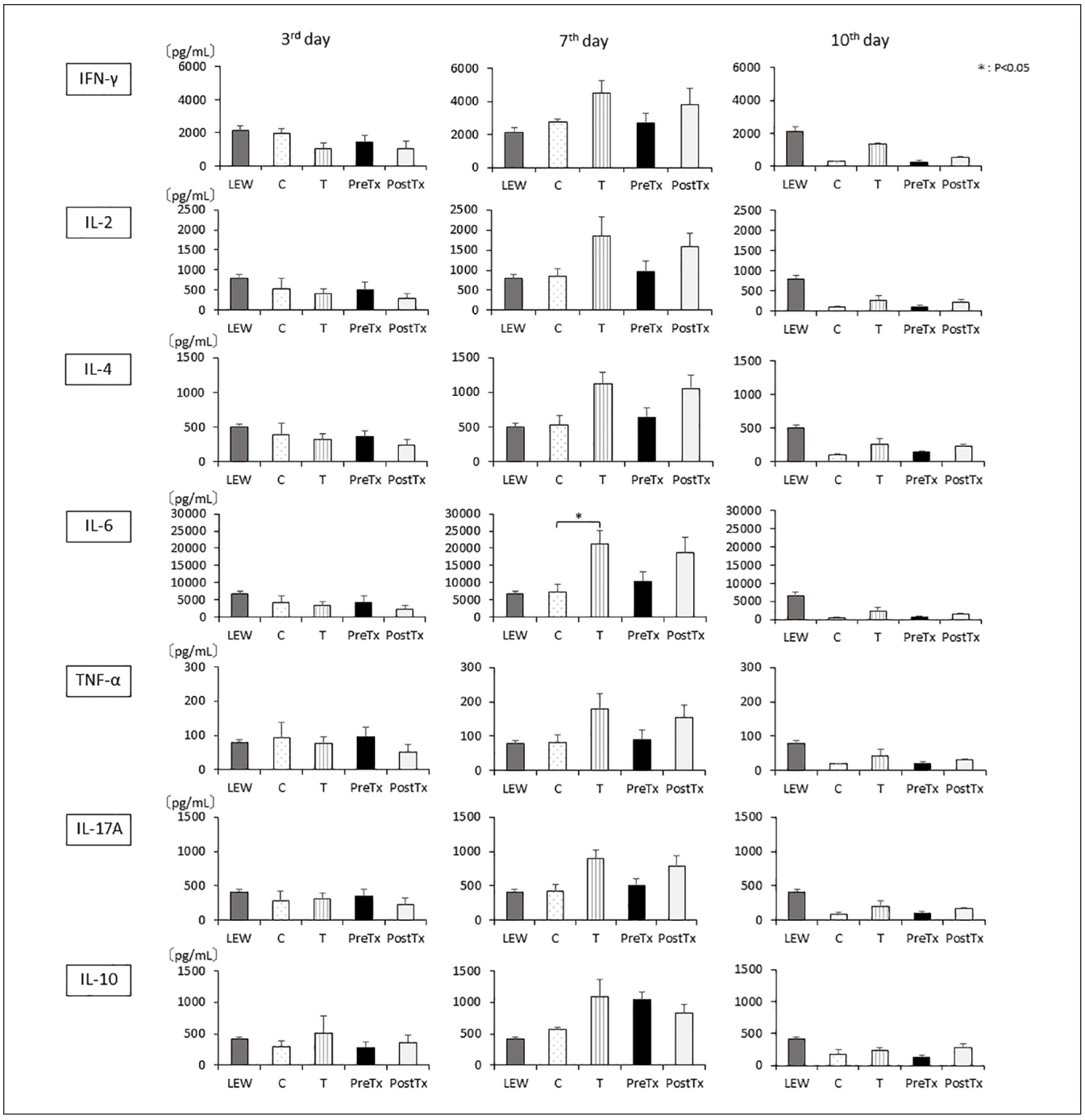

Figure 5. Serum cytokine levels of recipient rats on days 3, 7, and 10 after lung transplantation. LEW indicates the serum of untreated Lewis rats without transplantation. LEW: Lewis; PreTx: pre-transplantation; PostTx: post-transplantation; C: control; T: tacrolimus; IFN-γ: interferon-γ; IL: interleukin; TNF-α: tumor necrosis factor-α; ELISA: enzyme-linked immunosorbent assay.

group. However, PreTx administration tended to suppress inflammatory cytokine expression compared with PostTx administration on day 7 after transplantation, suggesting that PreTx administration induced a systemic immunosuppressive environment. Immunostaining analysis revealed an increase in Treg proportion in the grafted lungs on day 10 after transplantation in the PostTx group. Flow cytometry analysis revealed that the proportion of Tregs did not differ between the PostTx and PreTx groups in both PBMCs and the spleen; however, in the grafted lungs, it was significantly higher in the PostTx group than in the PreTx group, which could explain the reduced acute rejection rate observed in the PostTx group.

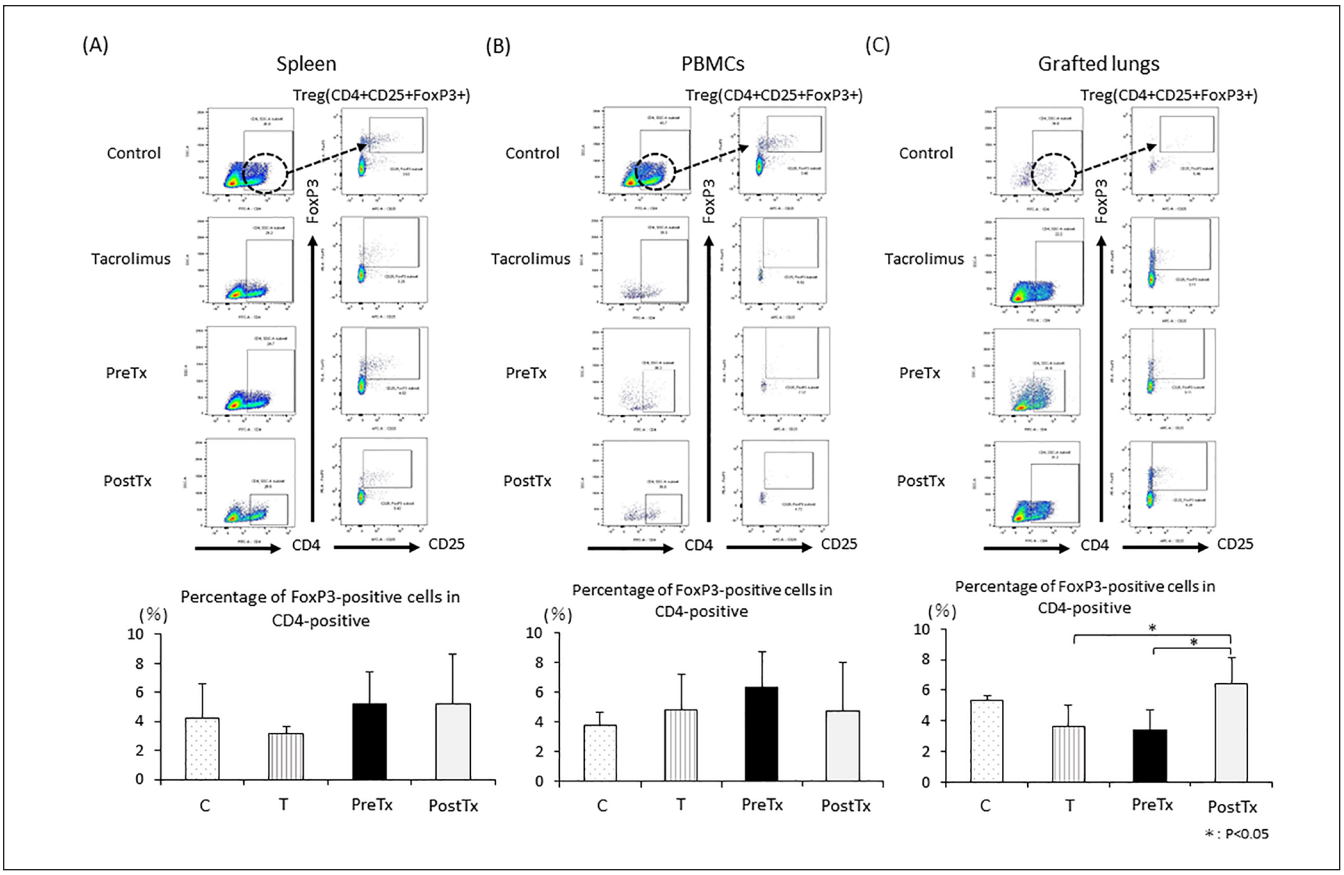

Figure 6. Proportion of Foxp3-positive cells in the spleen, PBMCs, and grafted lungs of recipients on day 10 after lung transplantation as per flow cytometry analysis (n = 4/group). (A) The proportion of Foxp3-positive cells in the spleen. (B) The proportion of Foxp3-positive cells in PBMCs. (C) The proportion of Foxp3-positive cells in the grafted lungs. Foxp3: forkhead box protein 3; PBMCs: peripheral blood mononuclear cells; PreTx: pre-transplantation; PostTx: post-transplantation; C: control; T: tacrolimus.

In vitro MLR experiments showed that only ADMSCs added the day before the start of the MLR showed inhibitory effects under cell-contact conditions, whereas ADMSCs added together with the donor splenocytes did not inhibit T-cell proliferation. It has been shown that the immunosuppressive effect of MSCs is weakened on polarized T cells[41–44]. Therefore, to exert beneficial ADMSC immunosuppressive effects, it is preferable to add ADMSCs before T-cell polarity changes.

Notably, we elucidated that immediate ADMSC administration after transplantation induces local immunosuppression, which is more important than systemic immunosuppression via secondary lymphoid tissues, in the case of lung transplantation. Although previous evidence has indicated that the main mechanism of ADMSC-based immunosuppression occurs via soluble mediators from the ADMSCs[19,21,42,44], our *in vitro* MLR study showed that ADMSCs exhibited immunomodulatory abilities only in a cell-to-cell contact state. In an *in vivo* situation, the mediator is diluted by the surrounding blood and lymph fluid. Therefore, we consider that cell-to-cell proximity allows for exposure to higher concentrations of the mediator, which may result in higher efficacy.

In the PostTx group, administered ADMSCs accumulated more in the transplanted lung than in the recipient lung, suggesting that ADMSCs could suppress immunoreactions in the grafted lungs. However, in the PreTx group, the transplanted lungs did not contain ADMSCs. The administered ADMSCs accumulated in the lungs, liver, kidneys, and secondary lymphoid tissues, including the spleen, of recipient rats. The indirect immunosuppressive effect of ADMSCs might be too weak to control severe immunoreaction on site. Therefore, given the importance of cell-to-cell proximity for the immunosuppressive effect of ADMSCs, the presence of ADMSCs in the lungs might be more crucial than their presence in the secondary lymphoid tissues.

On day 7 after transplantation, serum levels of inflammatory cytokines, including IFN-γ, IL-2, IL-4, IL-6, TNF-α, and IL-17A, tended to be lower and IL-10 levels tended to be higher in the PreTx group than in the PostTx group, suggesting that systemic immunosuppression was higher in the PreTx group than in the PostTx group. It is interesting that the serum levels of inflammatory cytokines were not high in the control group. This is possibly due to the blood vessel occlusion that

occurred with severe rejection. Immunohistochemistry and flow cytometry analyses revealed that the number of Tregs in the grafted lungs was higher in the PostTx group than in the PreTx group; this is consistent with a previous study on an islet transplantation model[38], which reported that graft rejection was lower and immunosuppressive effects higher when the number of Tregs increased locally within the graft than when that number increased systemically.

The administered MSCs migrate to organs damaged by reperfusion injury[8,9,42,45]. The MSC distribution in the body depends on the time of administration[20,42,43] and is related to their immunomodulatory ability. Therefore, administration timing is a key factor for MSC-based cell therapy. The ideal timing of cell therapy may differ based on the tissue or organ involved in the transplant. In a kidney transplantation model[20], the group receiving MSCs 1 day or 7 days before transplantation showed a more intense accumulation of MSCs in the spleen and exhibited a lower rejection rate than the group receiving MSCs 2 days after transplantation, in which MSCs accumulated intensively in the graft. That study[20] concluded that local MSCs induce organ dysfunction and a systemic effect was more important in the case of kidney transplantation. Similarly, in a heart transplant study[21], the group administered MSCs 4 days before transplantation exhibited a lower rejection rate than the group administered MSCs on the day of transplantation or 3 days after transplantation. The systemic effect of MSCs is more potent than its local effect in the kidney and heart because, as observed herein, ADMSC accumulation in the heart and kidney was considerably lower than that in the lungs and liver, which indicates that cell-to-cell interactions cannot be triggered. In the kidney and heart transplantation models, even when MSCs are administered immediately post-transplantation, only a small number of MSCs are incorporated into the graft, and most of them are distributed throughout the body.

Moreover, although ADMSC administration post-transplantation is more effective than that pre-transplantation, Luz-Crawford et al.[46] reported that the immunosuppressive effect of MSCs is not exerted on T cells, including mature Th1 cells, after their polarity is determined[41-43]. We hypothesized that the increase in Tregs induced by MSCs decreases with time after transplantation[41,42,47]. Because MSCs were administered 2 days after transplantation in the kidney transplantation model, T-cell polarization might have occurred; thus, the immunomodulatory ability of MSCs to mature into Tregs might have been reduced[20]. Therefore, MSC administration immediately after transplantation (i-PostTx) could maximize their efficacy to a greater extent than late administration, as T-cell polarization would not have occurred yet.

Patients who have undergone transplantation are immunocompromised due to lifelong immunosuppressant administration, which causes infection and malignancy, thus reducing quality of life and survival. Moreover, immunosuppressants cause several adverse effects. Therefore, the reduction of immunosuppressant administration is crucial for the success of organ transplantation. Previously, we reported that MSC-based cell therapy exhibited additive immunosuppressive effects with the immunosuppressant tacrolimus[19]. Thus, in clinical application, the use of ADMSCs might reduce the dose of immunosuppressants. A clinical study of patients with systemic lupus erythematosus[48] reported that the combined use of MSCs with steroids and cyclophosphamide in immunosuppressive therapy reduced the dose of immunosuppressive agents to a minimum. They also reported that MSC administration suppressed adverse events, such as infectious diseases and renal dysfunction. By optimizing a therapeutic protocol for ADMSC administration, the dose of immunosuppressive agents and the occurrence of adverse events could be reduced.

This study had several limitations. First, in the PreTx treatment in this study, ADMSCs were administered on the day before transplantation, and the left lung with accumulated ADMSCs was removed. The reduced amount of ADMSCs in the body may have reduced the immunosuppressive effect in the PreTx group. However, the biodistribution results of the present study showed that most ADMSCs accumulated in the liver, and the accumulation in the lungs was approximately one-third of that in the liver (Figure 2A). The calculated radiant efficiency showed that the amount of ADMSCs that accumulated in the bilateral lungs was estimated to be approximately 20% of the total accumulation in a normal rat, suggesting that 80% of the administrated ADMSCs were present in the body on day 1 after transplantation in the PreTx group. In addition, in the case of kidney transplantation[20], although some accumulated ADMSCs were removed with the kidneys, PreTx MSC administration still showed higher immunosuppressive effects than PostTx administration. In the case of heart transplantation, in which single and double doses of ADMSCs were administered, the single dose exerted an immunosuppressive effect similar to that of the double dose[22]. As the accumulation tendencies of administrated MSCs differ between the organs, it is extremely difficult to evaluate the impact of the cells lost during the left pneumonectomy.

Second, in this study, recipient-derived ADMSCs were administered in the PreTx treatment in the same way as in the PostTx treatment. In our previous study[49], which compared syngeneic and allogenic ADMSCs in PostTx administration, significant differences were not observed between cells with different origins. Therefore, we selected syngeneic ADMSCs in this study. However, according to a previous study, in a heart transplantation model, administration of donor-derived ADMSCs had a higher immunosuppressive effect than that of recipient-derived ADMSCs in PreTx administration[22]. As there is a possibility that the origin of the ADMSCs could explain why we observed superior results when injecting the ADMSCs in the PostTx group, further studies, including studies using lung transplantation models, are necessary to evaluate the effects of allogenic ADMSCs between PreTx and PostTx administration

conditions. Third, the pre-transplantation administration time was set to the day before transplantation. Because lung transplantation in Japan involves a high proportion of brain-dead lung donors[50], the recovery and culture of recipient ADMSCs might be difficult in the case of a brain-dead donor. Fourth, the observation period after transplantation was short; thus, the long-term effect of MSCs could not be examined. In QD-based cell tracking, most administered MSCs might have disappeared 7 days after administration; thus, repeated administration might be ideal for ensuring continuous immunosuppressive effects. Whether subsequent additional doses will be required to maintain the immunosuppressive effects of the MSCs should be verified in a long-term model. In addition, it would be interesting to study whether ADMSC treatment in either PreTx or PostTx treatment groups is capable of generating regulatory cell populations in secondary lymphatic organs or the lungs when restimulated with an antigen, thus examining the long-term *in vivo* effects. Furthermore, MSCs exert immunomodulatory effects on B cells[51]; thus, future studies on this model should analyze the effects of MSCs on B cells, which will clarify the involvement of various other cells and the immunomodulatory ability of MSCs for organ transplantation.

In summary, because the distribution of ADMSCs in the body differs depending on the timing of administration, it is crucial to select the timing appropriately to effectively increase the number of Tregs according to organ characteristics. Herein, ADMSCs administered immediately after lung transplantation accumulated in the grafted lungs and reduced rejection rates. The graft-accumulated ADMSCs might act directly on immature T cells and increase the number of Tregs in the graft, which would exert an immunomodulatory effect. This study provides insights for establishing an optimal dosage protocol. Nevertheless, future studies should investigate the frequency of administration of PreTx and i-PostTx using a long-term graft survival model.

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Author Contributions

YT, TT, and TM participated in research design. MI, HY, and KS participated in the statistical analysis plan and conducted statistical analyses. HW, GH, and KS performed the research. RD and KT participated in the interpretation of the results. YT drafted the original manuscript. KM and TN supervised the study. All authors reviewed the manuscript draft and revised its intellectual content critically. All authors approved the final version of the manuscript to be published.

Availability of Data and Material

Data will be made available by the corresponding author upon reasonable request.

Ethical Approval

All animal experiments were conducted following the Nagasaki University Institutional Animal Care and Use Committee guidelines and were approved by the ethics committee of Nagasaki University (Approval Number: 1911261580-7).

Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

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ORCID iDs

Yukinori Tanoue https://orcid.org/0009-0001-7672-3526
Koichiro Shimoyama https://orcid.org/0000-0002-0561-4082

Supplemental Material

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