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Adipose-derived mesenchymal stem cells attenuate rejection in a rat lung transplantation model

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ABSTRACT

Background: Immunosuppression following lung transplantation is a key aspect to the graft's survival. However, the well-known complications that are caused by immunosuppressive regimens present an opportunity to study ways to minimize the usage of these drugs. Recently, a promising discovery has been made pertaining to the immunomodulatory effects of adipose tissue–derived mesenchymal stem cells (ADMSCs) through their secretion of hepatocyte growth factor. In the hopes of mitigating the adverse effects of standard immunosuppressive regimens, our study aims to investigate the effects of ADMSCs on the immune response utilizing a rat lung transplantation model.

Methods: Each rat's own ADMSCs were intravenously administered immediately after orthotopic left lung transplantation. The experimental subjects were divided into four groups: 1) control group (group C) was administered no treatment following transplantation; 2) ADMSC group (group A), administered a single intravenous injection of ADMSCs following transplantation; 3) tacrolimus group (group T), administered tacrolimus (0.5 mg/kg) every 24 h following transplantation; and 4) ADMSC and tacrolimus group (AT group) administered a single intravenous injection of ADMSCs in combination with tacrolimus every 24 h following transplantation.

Results: The histologically proven rejection grade in group AT was significantly lower than that in group T. The serum levels of hepatocyte growth factor and the expression of cMet in group AT accompanied by low CD40 expression were also significantly higher than those of the lung grafts of group T.

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Conclusions: These results suggest that co-administration of ADMSCs with tacrolimus is a beneficial therapeutic approach in lung transplantation.

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Introduction

The only definitive therapy for many diffuse chronic lung diseases is a lung transplantation.[1,2] Although short- and mid-term survival of recipients has improved with the development and use of immunosuppressive drugs,[3] the long-term use of immunosuppressive drugs is associated with various complications including infection, nephrotoxicity, diabetes, and solid organ tumors.[2,4,5] Therefore, establishment of a less intense immunosuppression regimen should be attempted.

Bone marrow–derived mesenchymal stem cell (BMMSC) and adipose tissue–derived mesenchymal stem cell (ADMSC) are both mesoderm-derived cells that are able to differentiate into multilineage connective tissue. ADMSCs, which are a source of mesenchymal stem cells (MSCs), have recently received much attention due to the fact that adipose tissue contains a much higher number of MSCs than bone marrow.[6-9] Recent evidence has also shown that ADMSCs display a much higher effect on modulating the immune response than BMMSCs.[10] In addition, animal and clinical studies have shown that ADMSCs exert immunosuppressive effects in organ transplantation models or immunomodulatory effects in various autoimmune disease models.[10-21] Moreover, taking into consideration that adipose tissue is very accessible, highly abundant, and also a reproducible source of MSCs, ADMSCs hold great potential in the field of clinical transplantation.

ADMSCs have also been shown to secrete various immunomodulating molecules including hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and interleukin-10 (IL-10).[12] HGF, which is a multifunctional protein with mitogenic, antiapoptotic, and immunomodulatory effects, has a beneficial role in lung disorders.[12,22-26] In addition, ADMSCs also produce a higher amount of HGF than BMMSC.[8] Recent reports have shown that HGF secreted from ADMSCs could ameliorate pulmonary emphysema[22] and reduce pulmonary ischemic reperfusion injury[27] in rat models. On the other hand, administration of HGF combined with low-dose tacrolimus prolonged graft survival in liver, renal, and heart transplantation models.[28-30]

cMet, the receptor for HGF, has been found to be expressed by dendritic cells in lung bronchus-associated lymphoid tissue (BALT). In contrast, it has been shown that T cells do not express cMet. Treatment with HGF both *in vitro* and *in vivo* potently suppressed the capacity of dendritic cells to present antigen.[25] Furthermore, many *in vivo* studies have demonstrated that ADMSCs can alter the differentiation of dendritic cells, as well as suppress T-cell proliferation.[11,31,32] Considering these results, rather than administer HGF itself, we planned to study the efficacy of administration of ADMSCs, which might possibly secrete HGF, on immune responses in a lung transplantation model.

The purpose of this study was to survey the effects that ADMSCs have on immune reactivity in a rat lung transplantation model. To our knowledge, this is the first demonstration of the immunosuppressive effects of autologous ADMSC administration utilizing this model.

Materials and methods

Animals

Adult male inbred rats, 8-12 weeks old, were used for all the experiments. Lung transplantations were performed using Brown Norway rats (BN, $RT1A^n$, and $RT1B^n$) weighing 240-280 gram donors, and Lewis rats ($RT1A^1$, $RT1B^1$) weighing 280-320 gram recipients (Charles River Japan, Yokohama, Japan). All animal care was in compliance with the guidelines of the Institutional Animal Care and Use Committee of Nagasaki University.

Experimental design

The experimental subjects were divided into four groups: Control group (group C) was administered no treatment following transplantation ($n = 17$); ADMSC group (group A), administered a single intravenous injection of ADMSCs (autologous, 1.0×10^6) following transplantation ($n = 17$); tacrolimus group (group T), administered tacrolimus (0.5 mg/kg) intramuscularly every 24 h following transplantation ($n = 17$); and ADMSC and tacrolimus group (AT group) administered a single intravenous injection of ADMSCs (autologous 1.0×10^6) in combination with intramuscular administration of tacrolimus (0.5 mg/kg) every 24 h following transplantation ($n = 17$). Both AT and T groups were treated with the same dose of tacrolimus. We have previously shown that 0.5 mg/kg of tacrolimus without other therapy lead to mild to severe rejection.[33] Therefore, we employed this dose in our present study. No corticosteroids were given. In each group, rats were euthanized at each time point of postoperative day (POD) 1 ($n = 5$), 3 ($n = 5$), and 7 ($n = 7$).

Preparation of ADMSCs

Lewis rats were anesthetized with an intraperitoneal injection of 90 mg/kg of ketamine hydrochloride (Ketalar; Daiichi Sankyo, Tokyo, Japan) and 10 mg/kg of xylazine (Ceractal Bayer, Osaka, Japan), and abdominal adipose tissue was isolated. The animals were allowed to recover from anesthesia after tissue isolation. The adipose tissue was digested with 0.001% collagenase (Celease; Cytori, Tokyo Japan) at 37°C for 30 min. After several cycles of shaking and centrifugation, the specimen was filtered through a nylon mesh. The mesenchymal cells were separated by centrifugation, and then resuspended in OriCell Mesenchymal Stem Cell Growth Medium (DS Pharma Biomedical, Osaka, Japan). The cells were cultured in 100-mm dishes for 14 to 18 days until they reached

confluence. The primary cells and the passaged cells were used in subsequent *in vivo* and *in vitro* experiments after they reached confluence.

Phenotypic characterization and multilineage differentiation of ADMSCs

Stromal cells derived from adipose tissue were labeled with fluorescence-labeled antibodies for positive or negative ADMSC markers or the isotype control (all from BD Biosciences) for 20–30 min on ice. The primary cells that had reached confluence were used for a multilineage differentiation assay. Differentiation was induced by culture for 14 to 28 days in either Mesenchymal Stem Cell Osteogenic, Chondrogenic, or Adipogenic Differentiation Medium with the appropriate supplements (DS Pharma Biomedical). Differentiation into each lineage was then confirmed by staining as follows: Alizarin red S staining for osteogenic differentiation, Alcian blue staining for chondrogenic differentiation, and Oil red O staining for adipogenic differentiation.

Lung transplantation and administration of ADMSCs

Orthotopic left lung transplantation was performed using the cuff technique, as previously described with partial modification.[33,34] Briefly, the donors were anesthetized and intubated orally. The setting of mechanical ventilation was 10 mL/kg of tidal volume and 90 breaths/min. The maintenance of anesthesia was obtained with an inhalation of isoflurane (Isoflu; DS Pharma Animal Health, Osaka, Japan). The donor procedure was begun with median sternotomy. After intravenous injection of heparin (1000 units/kg), the left lung was harvested. The cold ischemic time was 15 min. Recipients were anesthetized in the same manner. Left thoracotomy was performed in the fifth intercostal space in the lateral position, and the hilum of the left lung was isolated. After clamping, the left pulmonary artery, vein, and main bronchus were anastomosed using the cuff technique with a 16-gauge catheter, 1.5 mm in length, and 7-0 polypropylene suture. After the lung transplantation, the recipients of group AT were administered auto cell transplantation of ADMSCs dissolved in phosphate buffered saline via the left jugular vein using a heparin-coated 24-gauge catheter. All other animals received phosphate buffered saline via the same manner. No additives were used in the injection media.

Histological study

All allografts were harvested and fixed in 10% buffered formalin. The specimens were embedded in paraffin, sliced into approximately 5 μm-thick slices, and hematoxylin-eosin stained sections were graded in a blinded fashion according to the International Society for Heart & Lung Transplantation classification. In brief, the grading scale was as follows: grade 0, without evidence of mononuclear cell infiltration; grade 1 (minimal), grade 2 (mild), grade 3 (moderate), and grade 4 (severe).

Proliferating cell nuclear antigen staining

Proliferating cell nuclear antigen (PCNA) immunochemical staining was performed using the monoclonal murine antibody, PC10 (M0879 immunoglobulin G2a; DAKO A/S Glostrup, Denmark) at a dilution of 1:400. The signals were visualized with 3,3′-diaminobenzidine, and nuclei were stained with hematoxylin. PCNA positive cells, which showed red nuclei by fast red staining, and negative cells, which showed blue nuclei by hematoxylin staining, were counted in BALT. The percentage of PCNA positive cells were evaluated from 1000 counted cells.

Immunohistochemistry

The 5-μm-thick formalin-fixed and paraffin-embedded lung sections were used for immunochemical staining of cMet with a rabbit antiphosphorylated cMet antibody (#28083; IBL, Gunma, Japan) at a dilution of 1:100.

Frozen samples were also used for immunohistochemistry as previously described with minor modifications.[35] To assess the expression of cMet on dendritic cells in the graft lung, double staining for cMet and OX-62 was performed, using a polyclonal rabbit anti-cMet antibody (SP260; Santa Cruz Biotechnology, Dallas, TX) and a monoclonal mouse anti-integrin alpha E antibody (OX-62; Abcam, Tokyo, Japan). In addition, a polyclonal anti-rat CD40 antibody (Novus Biologicals, Littleton, CO) was used to assess CD40 activation in the lung graft.

Enzyme-linked immunosorbent assay

HGF was measured with Rat HGF EIA Kit (#8157; Institute of Immunology, Tokyo, Japan). According to the assay protocol, samples were measured in duplicate. Rat IL-10 was measured using the Rat IL-10 ELISA Kit (670.070.096; Diaclone, Besancon, France) in the same manner.

Western blotting

Samples were homogenized using a previously described protocol with partial modification.[36] Blots were incubated with anti-cMet antibody (Tyr1349) (#3133S; Cell Signaling Technology, Tokyo, Japan). Anti-α/β-tubulin antibody (#2148; Cell Signaling Technology) was used as the control.

Transduction of quantum dots into ADMSCs

An inorganic probe, quantum dots (QDs) consist of CdSe/ZnS-core/shell semiconductor nanocrystals.[37,38] The transduction of QDs with octa-arginine peptide (R8) has been used for labeling ADMSCs and maintains stem cell potency with low cytotoxicity as previously described.[37,38] Cultured ADMSCs were incubated with the R8-QDs complex (2 nM). The ADMSCs were administered to the lung-transplanted recipients together with tacrolimus on the same day on which QD transduction was confirmed. These rats were sacrificed 7 days after transplantation, and major organs including the graft and native lungs were harvested. The collected tissues were

fixed in 10% buffered formalin, embedded in paraffin, and observed by fluorescent microscopy.

Statistical analysis

For comparisons of the rejection grade among groups, the Kruskal–Wallis test was performed to determine differences between median values with standard deviation. If a difference was significant, the Steel-Dwass test was performed. The Mann–Whitey U test was used to analyze results of the PCNA index and enzyme-linked immunosorbent assay. A P value $<$ 0.05 was considered significant. JMP software (version 10.0.2) was used for the statistical tests.

Results

Characterization of ADMSCs

Freshly isolated stromal cells from the abdominal subcutaneous adipose tissue of Lewis rats showed fibroblast-like morphology, adhesion to wells and good expansion in culture.

Furthermore, the cells expressed ADSMC marker, CD44, CD73, and CD90 and the absence of CD11 b/c, CD31, C34, and CD45, as reported previously[26] (Fig. 1A and B).*In vitro* tests using the appropriate culture conditions and supplements confirmed multilineage differentiation of ADMSCs toward osteogenic, adipogenic, and chondrogenic lineages (Fig. 1C). Based on those results, we determined that these stromal cells have characteristics of ADMSCs.

Histopathological evaluation after lung transplantation

All animals tolerated our immunosuppression regimen well, although mild diarrhea was observed in both T and AT groups, possibly as an adverse reaction to tacrolimus.

Various grades of rejection were first observed among the groups on POD 7 after the lung transplantation. Hematoxylin-eosin staining demonstrated grade 4 rejection with extensive injury to pulmonary tissue in all transplanted lungs of group C (no treatment) as well as group A (ADMSC administration alone). In transplanted lungs, mild to moderate rejection was observed in group T (tacrolimus treatment alone) (rejection score, 2.1 ± 0.87). Notably, transplanted lungs in group AT

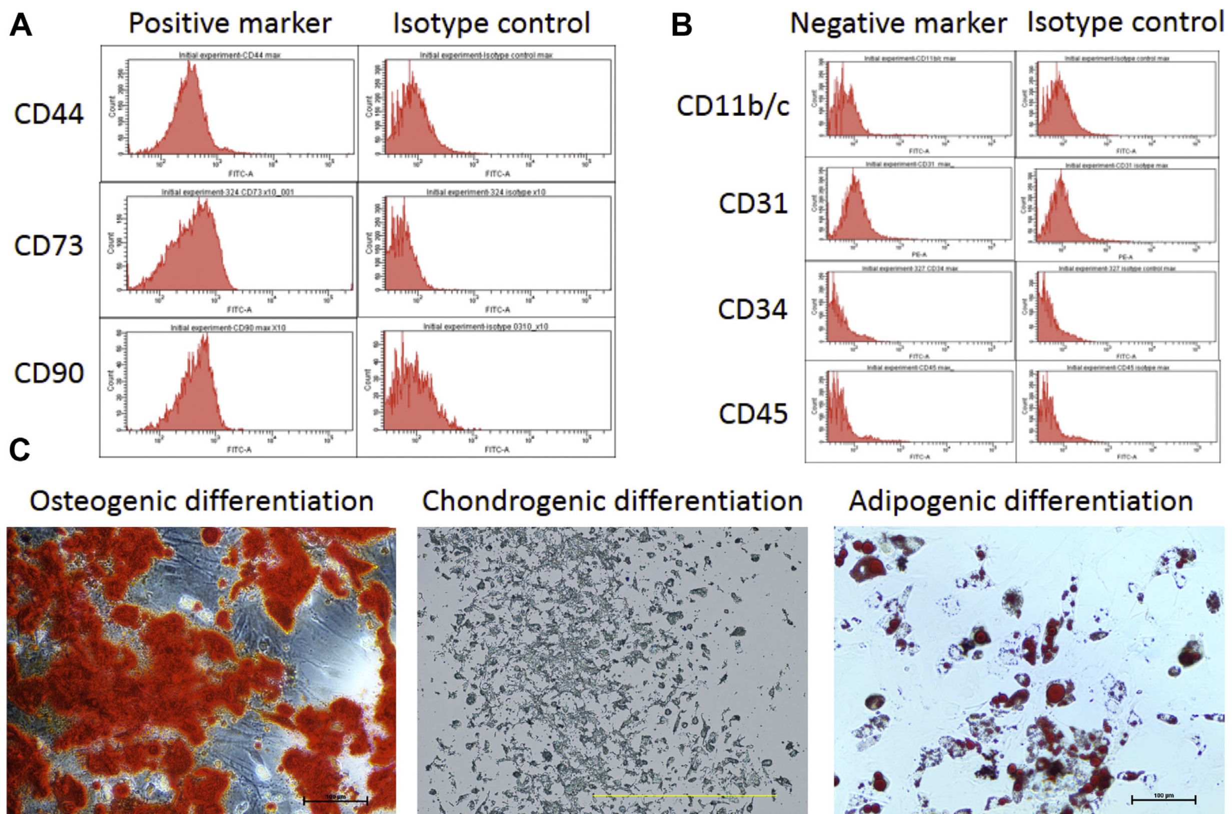

Fig. 1 – Proof that the cultured cells are ADMSCs: flow cytometric analysis of positive and negative markers of ADMSCs, and multilineage differentiation analysis. (A) The cultured cells strongly expressed the ADMSC positive markers CD44, CD73, and CD90 in contrast to isotype controls. (B) Expression of the ADMSC negative cell surface markers CD11b, CD31, CD34, and CD45 did not differ from that of the isotype control. (C) The adipose-derived mesenchymal stromal cells displayed the multilineage differentiation of ADMSCs, which suggested that these cells are ADMSCs. Osteogenic differentiation was confirmed by alizarin red S staining. Chondrogenic differentiation was detected by Alcian blue staining. Adipogenic differentiation was confirmed by oil red O staining.

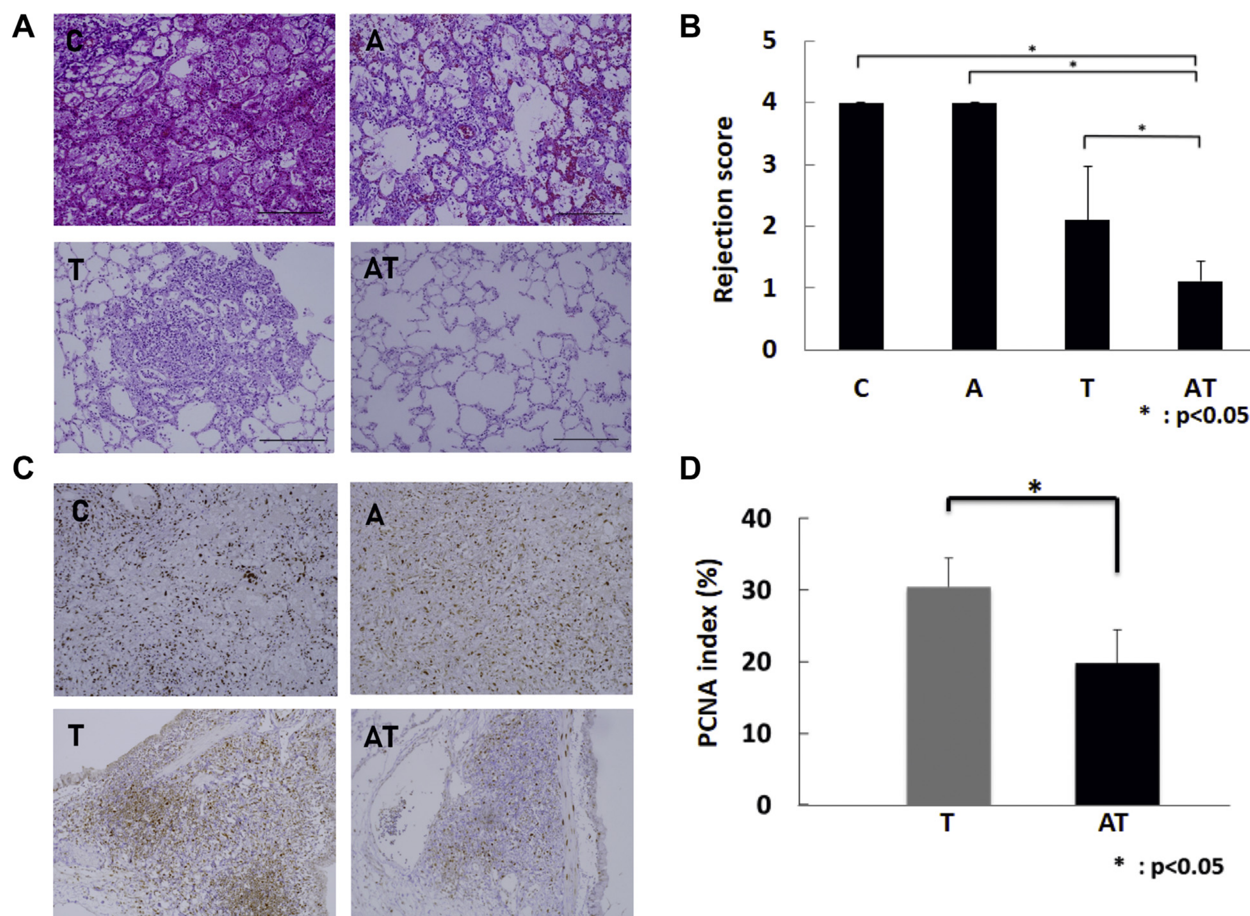

Fig. 2 – Representative pulmonary histological findings of hematoxylin-eosin staining and PCNA assay 7 d after transplantation. (A) Representative H&E staining indicated that infiltration of lymphocytes around small vessels and interstitial spaces was rarely observed in group AT. C, control group; A, ADMSC administration group; T, tacrolimus group; and AT, ADMSCs plus tacrolimus group. (B) The histological rejection grade in groups C, A, T, and AT indicated that the rejection score in group AT was significantly lower than that of the other groups ($n = 7$). P = 0.01 (Kruskal–Wallis test); AT *versus* C: P = 0.006; AT *versus* A: P = 0.001; AT *versus* T: P = 0.043 (Steel-Dwass test). (C) Representative findings of immunohistochemical staining of PCNA in BALT cells of allografts 7 d after transplantation. There was less infiltration of PCNA positive cells in group AT than in group T. (D), The PCNA labeling index in BALT in the allografts 7 d after transplantation. The index in BALT of the AT group was significantly lower than that of group T ($n = 7$). *$P < 0.05$. H&E = hematoxylin-eosin.

(tacrolimus treatment with ADMSC administration) showed a significantly lower grade of histological rejection than those in the other groups (1.11 ± 0.33; AT *versus* C: P = 0.006; AT *versus* A: P = 0.001; AT *versus* T: P = 0.043) (Fig. 2A and B).

PCNA labeling index of BALT

It has been shown that the PCNA labeling index is correlated with rejection grade.[33,39,40] In addition, BALT has been reported to play a major role in alloreactive responses in animal lung allografts.[41] We therefore compared the PCNA labeling index between groups AT and T. PCNA positive cells were mainly observed in lymphocytes in BALT (Fig. 2C). The PCNA labeling index was significantly lower in group AT than that in group T at POD7 (Fig. 2D; 19.88 ± 4.67 *versus* 30.5 ± 3.92, P = 0.037).

In vivo *and* in vitro *HGF analysis*

We first surveyed the HGF levels in the media in which ADMSCs were cultured (incubated for at least 7 d). The HGF level in the media gradually decreased with each passage but was always higher than that in the media alone, which was the negative control (Fig. 3A). This result indicated that HGF was secreted from ADMSCs. We then evaluated the HGF level in serum samples of the experimental groups T and AT. The serum HGF level of group AT was significantly higher than that of group T at PODs 3 and 7 (Fig. 3B; 4.29 ± 1.34 *versus*

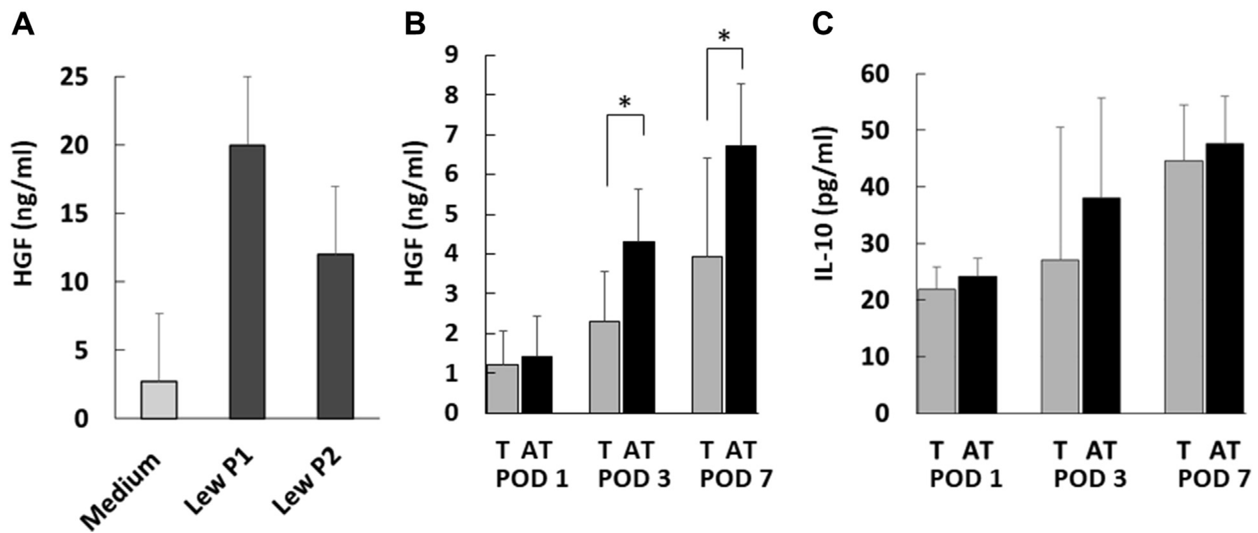

Fig. 3 – The analysis of HGF secreted by ADMSCs, serum levels of HGF, and IL-10. (A). HGF level in the media in which ADMSCs were cultured at the time of cell confluency for each passage was measured using an ELISA. The HGF level in the media from each culture passage (LEW P1, 2) was higher than that in the media alone. (B) The serum HGF levels at POD 1 ($n = 5$), 3 ($n = 5$), and 7 ($n = 7$) after the transplantation were measured using an ELISA. The serum HGF level of the group AT was significantly higher than that of the group T at POD 3 and 7. *$P < 0.05$. C. Serum IL-10 levels were measured in the different groups at POD 1, 3, and 7. Although IL-10 levels were higher in group AT than in the group T at all time points, no significant difference was observed between the groups. ELISA = enzyme-linked immunosorbent assay.

2.29 ± 1.25, P = 0.047; 6.71 ± 1.57 *versus* 3.94 ± 2.46 ng/mL, P = 0.034, respectively).

In vivo *IL-10 analysis*

The serum IL-10 level of each group increased over time, and the levels of group AT at PODs 1, 3, and 7 were higher than those of group T; however, the differences were not significant (Fig. 3C).

Immunostaining and Western blotting of cMet in the transplanted lung

We next surveyed the expression of cMet in BALT. Although cMet positive cells were rarely detected in the BALT of groups AT and T at POD 1, cMet positive cells increased over time in these two groups (Fig. 4A). Western blotting of the cMet protein, which is 145-kDa in molecular weight, revealed that the expression of cMet in group AT was higher than that of group T at PODs 3 and 7 (Fig. 4B)

Immunofluorescent staining of cMet, OX62, and CD40 in the transplanted lung

We also examined the expression of cMet in the lung grafts by immunofluorescent staining of frozen samples. The expression of cMet in the graft lungs were higher in group AT than that in group T on POD 7 (Fig. 5A), which was consistent with the results of Western blotting. In addition, double staining of cMet and OX-62 showed that there were more double positive cells in group AT than group T, which suggested that the expression of cMet on dendritic cells increased in group AT compared with that in group T (Fig. 5B).

CD40 is expressed on antigen-presenting cells such as dendritic cells and works as a costimulatory protein.[23] Immunofluorescent staining of CD40 in the graft lungs showed that CD40 expression in group AT was reduced compared with that in the T group (Fig. 5C).

Localization of administered ADMSCs in the graft lung

In order to prove the implantation of administered ADMSCs, the cells were labeled with red-fluorescent QDs. By analysis of red fluorescence in their cytoplasm, the administered ADMSCs were detected around small vessels and bronchi in graft lungs on POD 7 (Fig. 6A and B). Few ADMSCs were observed in the native lung on the right side (Fig. 6C). ADMSCs were rarely observed in other organs including liver and kidney (data not shown).

Discussion

In this study, we have examined the immunomodulatory effects of administered ADMSCs in a rat lung transplantation model along with co-administration of tacrolimus, which we have demonstrated attenuated acute rejection of the transplanted lung. Furthermore, we have shown that the combined regimen of ADMSCs and tacrolimus administration was the most effective in inhibiting acute rejection of lung grafts, suggesting that ADMSC potentiated the effect of tacrolimus. Histological examination showed that the infiltration of lymphocytes around small vessels and alveolar spaces was lower

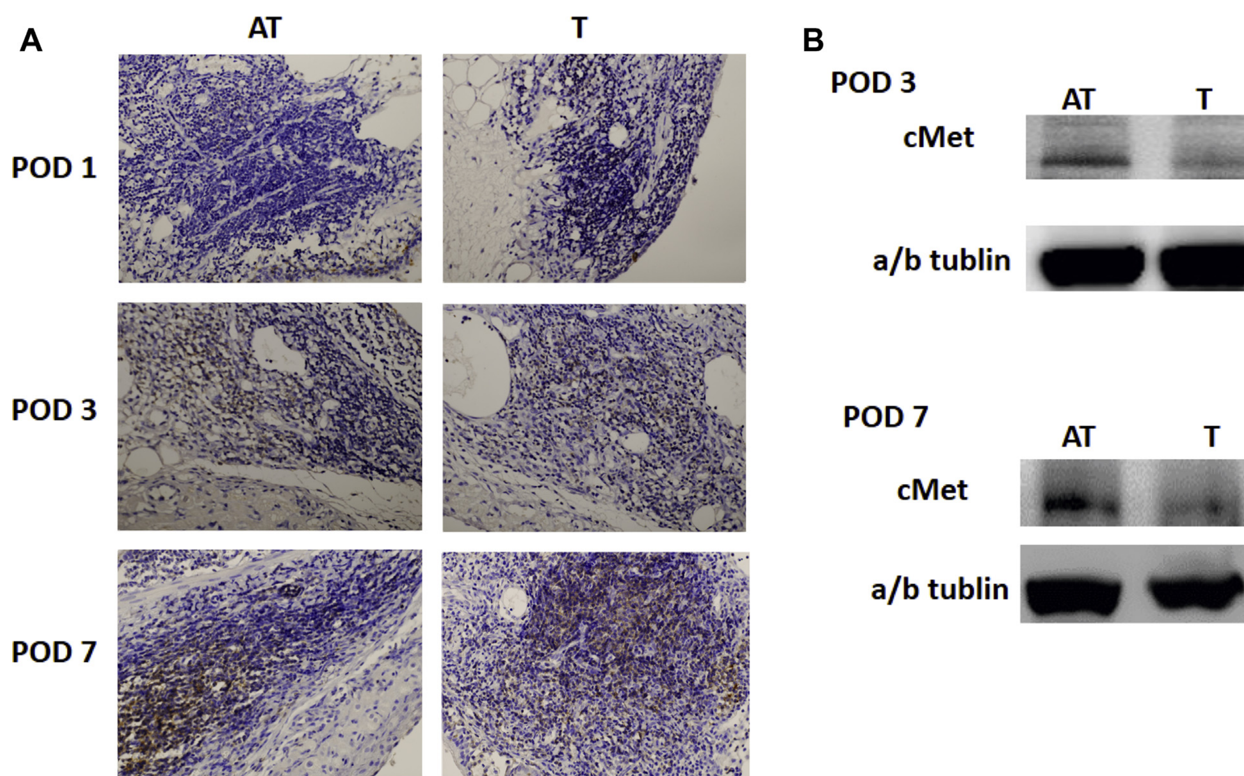

Fig. 4 – Protein expression of cMet in the transplanted lung. (A) Representative findings of immunohistochemical staining of cMet in BALT cells of allografts at POD 1, 3, and 7. (B) Western blot analysis of cMet in the lungs of groups AT and T at POD 3 and 7. cMet expression was higher in group AT than in group T.

in group AT (ADMSC combined with tacrolimus at 0.5 mg/kg) than that in group T (tacrolimus at 0.5 mg/kg alone). In addition, the PCNA labeling index in BALT correlated with rejection, similar to previous reports.[33,39,40]

Previous reports also suggested that ADMSCs secrete various immunomodulating molecules such as HGF, VEGF, and IL-10.[12] Therefore, one suggested mechanism of immunosuppression induced by ADMSCs is that the HGF secreted by ADMSCs activates cMet, which in turn suppresses immune reactions in the transplanted lung. Furthermore, HGF has been described as exerting an immunoregulatory effect by suppressing dendritic cells that function via CD40 down regulation.[23] In the present study, serum HGF was significantly higher, and there was a more obvious increase in cMet expression in the transplanted lungs in group AT than in group T. cMet activation of dendritic cells was also significantly higher in group AT. In addition, CD40 expression was reduced in the lung graft of group AT. These findings suggest that HGF secreted from ADMSCs might potentiate the immunosuppressive effect of tacrolimus through suppression of dendritic cell function. Although immunosuppressive receptors such as PDL-1 and CD44 are candidates for mediating immunosuppression as well,[12,42] the protein expression of PDL-1 as assessed using both immunohistochemistry and Western blot analyses was not different between groups T and AT in our model (data not shown). We also investigated the serum level of IL-10. The serum levels of group AT were slightly higher than those of group T; however, the levels were not significantly different. Because tacrolimus can reduce the production of IL-10,[43] the serum levels of IL-10 might be affected by the administration of tacrolimus in our model. Further studies including analysis of the level of multiple cytokines including VEGF in bronchoalveolar fluids would help to clarify the mechanism of ADMSC function in lung transplantation.

Our data indicated that co-administration of tacrolimus was required for the immunomodulatory effects of ADMSCs on lung grafts. Additive immunosuppressive effects of ADMSCs with immunosuppressive drugs have also been reported in other animal studies.[44,45] Ge *et al.*[44] reported that the combination treatment of MSC and mTOR inhibitor attenuated immunological responses and promoted tolerance in a mouse heart transplantation model. In addition, a short course of cyclosporine A and anti-lymphocyte serum with ADMSCs promoted allograft tolerance in a rat hind-limb transplant model.[45] Although the strengths of immune reactivity are different among organs, combination therapy of MSC administration and immunosuppressive drugs might be a key to the promotion of organ tolerance.

It has been shown that the most important factor in terms of chronic rejection is the presence of acute rejection. Also, because immunosuppression has such an important impact on the long-term prognosis of the recipient,[2,4] we focused on acute rejection in the present study. Kato *et al.*[21] reported that direct cell contact between ADMSC and recipient cells immediately after transplantation attenuated rejection in

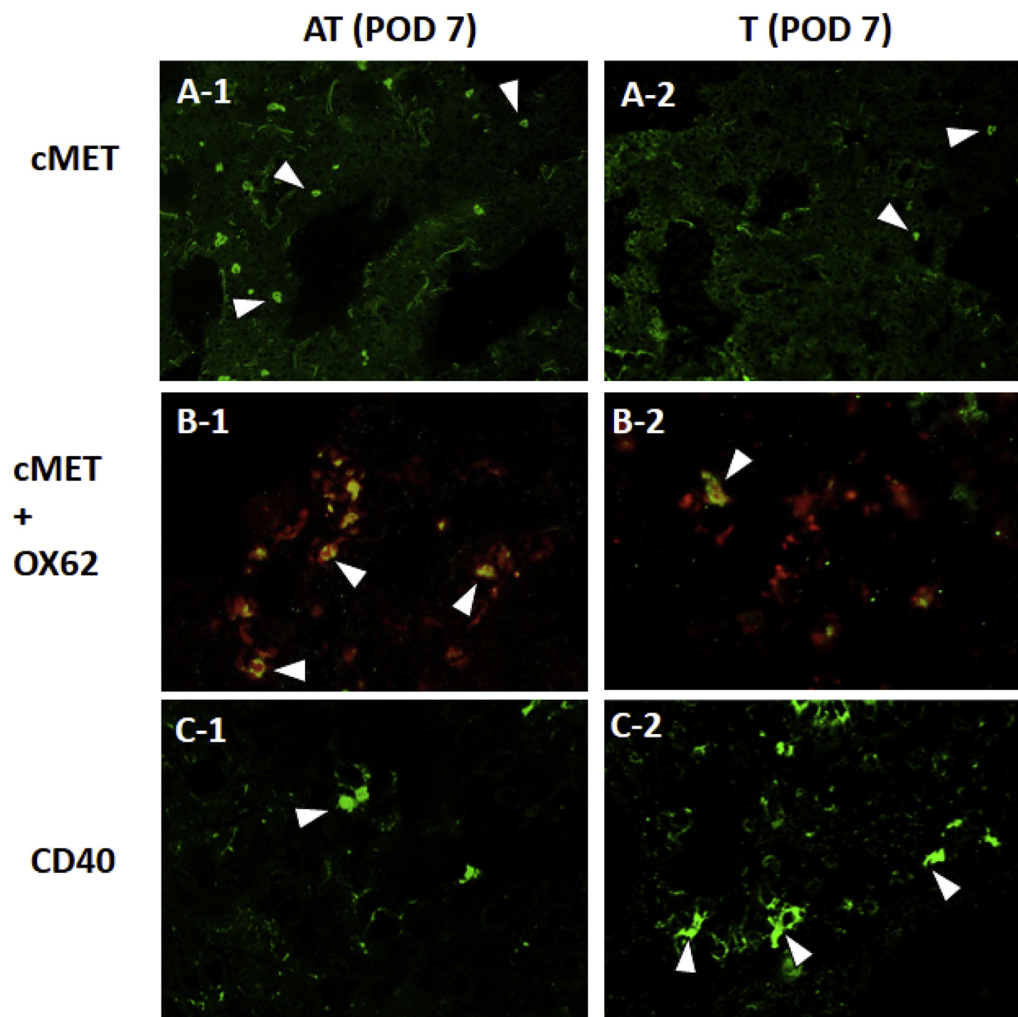

Fig. 5 – The expression of cMet on dendritic cells and CD40 expression in the graft lung. (A) Representative pictures of cMet (green) staining of Groups AT (A-1) and T (A-2). The arrowheads indicate cMet positive cells. The number of cMet positive cells was higher in group AT than in group T (POD 7). (B) Double staining of cMet (green) and OX-62 (red) of groups AT (B-1) and T (B-2). Yellow colored cells indicated by the arrowheads are cells positive for both cMet and OX-62. There were a higher number of cMet and OX-62 double positive cells in group AT than in group T, suggesting that cMet expression on dendritic cells increased more in group AT than in group T. (C) Representative pictures of CD40 staining (green of groups AT (C-1) and T (C-2)). Fewer CD40 positive cells (indicated by arrowheads) were found in group AT than in group T, indicating that CD40 expression was decreased in group AT compared with group T.

their rat kidney model. Also, Ra JC *et al.*[46] demonstrated ADMSCs homing to injured tissue. Because donor lungs tend to be injured by ischemic reperfusion, we administered ADMSC immediately after lung transplantation. Various routes of administration of ADMSCs have been reported such as via tail vein or intraperitoneal injection.[14,19] In our study, we injected ADMSCs via a cervical vein route, which enabled ADMSCs to reach the lung directly via the superior vena-cava. In addition, complications related to fat embolism were reduced by the use of heparin-coated syringes and resuspending the cells appropriately before injection as previously described.[37,38]

Previous studies have shown that a larger dose or repeated injection may enhance the immunomodulatory effects of ADMSCs. Data have shown that triple administration of 2×10^6 ADMSCs, which is six times higher than our single dose of 1×10^6 ADMSCs, significantly alleviated acute rejection in a rat liver transplantation model.[47] Furthermore, Le Blanc *et al.*[48] reported that five doses of allogeneic MSC administration were effective over the course of 60 mo in a patient with graft *versus* host disease after hematopoietic stem cell transplantation. In addition, they were able to successfully discontinue all immunosuppressive drugs in some patients in their study. For repeated administration of ADSMC, survival analysis of the cells is important because the injection timing depends on the half-life of the administrated ADMSCs. In our study, by using cytoplasmic fluorescent QDs, we were able to detect ADMSCs in graft lungs for up to 7 d following lung transplantation, while there were few ADMSCs detected in other organs. In addition, even at 30 d after transplantation,

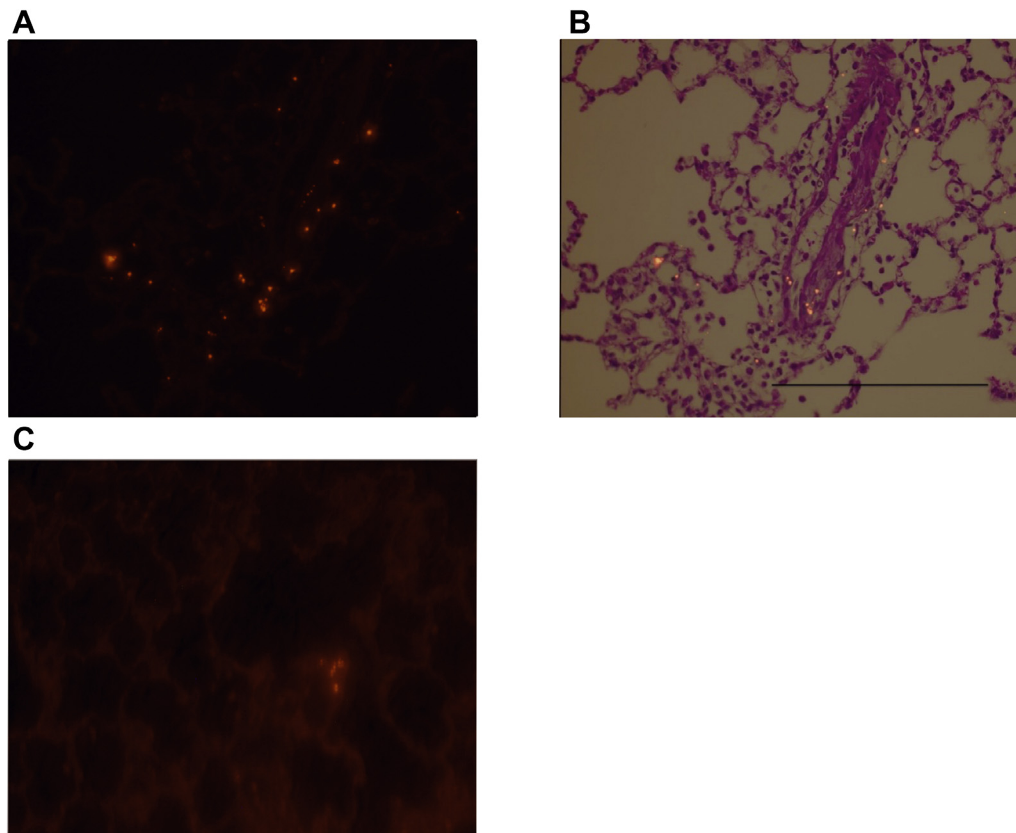

Fig. 6 – Pathological findings of red-fluorescent quantum dot-labeled ADMSCs in the transplanted lung on POD 7. (A) small red-fluorescent quantum dots were present in the cytoplasm of ADMSCs. (B) A hematoxylin-eosin stained image merged with a fluorescent image of the same slice. The quantum dot-labeled cells were observed around small vessel walls and the alveolar septum. (C) Fluorescent image of ADMSCs in the native right lung. In comparison with the transplanted lung, there are few red-fluorescent dot-labeled cells in the native lung.

the administered ADMSCs were detected in the isograft model (i.e., Lewis to Lewis, data not shown). In support of this, there exists much variation in the published literature pertaining to the detection of ADMSCs. Kato *et al.*[21] reported up to day 3 detection of ADMSCs in kidney grafts by utilizing his model. In contrast, Sanz *et al.*[49] reported more than 120 d survival and detection of MSCs in the vascular regeneration model of mice. Determining the optimal timing of injection might be complicated because the biologic environment of the recipient, including immune reactivity, oxygen consumption, and energy supply will affect the life span of administered ADSMCs. Moreover, the source of ADMSCs, including cryopreservation or growing, also affects the immunomodulatory effects of the cells.[50] In the present study, we used freshly harvested and cultured ADMSCs; however, for repeated administration, the usability of cryopreserved cells should be confirmed to avoid repeated harvest from individuals.

We have previously shown that the administration of tacrolimus at 1.0 mg/kg caused severe diarrhea and weight loss in a rat lung transplantation model.[33] In comparison, the administration of tacrolimus at 0.5 mg/kg showed relatively less toxicity. However, mild to moderate rejection was observed in the experimental groups receiving 0.5 mg/kg of tacrolimus alone.[33] Our present study showed similar results to previous studies, where group T (that received 0.5 mg/kg of tacrolimus alone) demonstrated mild to moderate rejection. However, when combination therapy of ADMSCs was administered along with the lesser dose of tacrolimus at 0.5 mg/kg, significantly reduced levels of rejection were observed. This would suggest that ADMSCs have the potential to mediate rejection when used with tacrolimus at the reduced dose. In fact, in their clinical trial, Liang *et al.*[51] showed successful reduction in the dosage of their immunosuppressive regimen, including steroid and cyclophosphamide to minimal dosages in the treatment of systemic lupus erythematosus patients by using MSC administration over a 1 y period. Moreover, they showed stabilized renal function along with decreased occurrences of severe infection in their patient cohort. This reveals a promising area of future study, in which the required dose of ADMSCs could be determined in an effort to minimize the dosage of immunosuppressive drugs. This would be extremely beneficial to patients as this would minimize the well-known adverse side effects of immunosuppressive regimens, without compromising their vulnerability to rejection.

Our study has several limitations, the first of which includes a relatively short follow-up time (7 d). Because MSCs have been known to contribute to both allograft dysfunction and promoting tolerance in others,[52] long-term observation would be required to see many of the effects of the injected

ADMSCs in our model. Due to the short study duration, these long-term effects weren't able to be elucidated beyond the immediate immune response, including hyperacute rejection. Moreover, MSCs have also been reported to have an immunomodulatory effect on NK cells and B cells.[53,54] Further studies aimed at analyzing the effect of ADMSCs on various cell types will clarify the beneficial effect of ADMSCs in an organ transplant model.

In conclusion, we have shown that ADMSC treatment potentiates the effect of tacrolimus in the reduction of allograft immunoreaction in our rat lung transplant model. Although this effect might have been mainly induced by the HGF-cMet signaling cascade, other mechanisms should be investigated further. Administration of ADMSCs might allow for the reduction in the dosing of immunosuppressive drugs and their related complications after lung transplantation. Future long-term studies may determine the optimal dose of ADMSCs based on the cell source (freshly isolated or cultured and stored), and HGF level as they pertain to reducing the dosage of immunosuppressive drugs and their adverse side effect profile.

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Disclosure

The authors reported no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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