Therapeutic effect of lung mixed culture-derived epithelial cells on lung fibrosis

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Cell-based therapy is recognized as one of potential therapeutic options for lung fibrosis. However, preparing stem/progenitor cells is complicated and not always efficient. Here, we show easily prepared cell populations having therapeutic capacity for lung inflammatory disease that are named as 'lung mixed culture-derived epithelial cells' (LMDECs). LMDECs expressed surfactant protein (SP)-C and gave rise to type I alveolar epithelial cells (AECs) in vitro and in vivo that partly satisfied type II AEC-like characteristics. An intratracheal delivery of not HEK 293 cells but LMDECs to the lung ameliorated bleomycin (BLM)-induced lung injury. A comprehensive analysis of bronchoalveolar fluid by western blot array revealed that LMDEC engraftment could improve the microenvironment in the BLM-instilled lung in association with stromal cell-derived factor-1 (SDF-1)/CXC chemokine receptor 4 signaling axis. SDF-1 enhanced both migration activity and differentiating efficiency of LMDECs. Further classification of LMDECs by flow cytometric study showed that a major population of LMDECs (LMDEC^{Maj}, 84% of total LMDECs) was simultaneously SP-C$^+$, CD44$^+$, CD45$^+$, and hematopoietic cell lineage$^+$ and that LMDECs included bronchioalveolar stem cells (BASCs) showing SP-C$^+$ Clara cell secretory protein$^+$ stem cell antigen (Sca)1$^+$ as a small population (1.8% of total LMDECs). CD44$^+$-sorted LMDEC^{Maj} and Sca1$^+$-sorted LMDECs equally ameliorated fibrosis induced by BLM like LMDECs did. However, infiltrated neutrophils were observed in Sca1$^+$-sorted LMDEC-treated alveoli that was not typical in LMDEC^{Maj}- or LMDEC-treated alveoli. These findings suggest that the protective effect of LMDECs against BLM-induced lung injury depends greatly on that of LMDEC^{Maj}. Furthermore, the cells expressing both alveolar epithelial and hematopoietic cell lineage markers (SP-C$^+$ CD45$^+$) that have characteristics corresponding to LMDEC^{Maj} were observed in the alveoli of lung and increased approximately threefold in response to BLM instillation. Taken together, LMDECs newly classified in the present study are easily culture expanded and have a potential role in future regenerative cell therapy for pulmonary fibrosis.

Idiopathic pulmonary fibrosis (IPF) remains an incurable disease with a poor prognosis.[1] Although many therapeutic options have been proposed, corticosteroids and immunosuppressive drugs are still the only key drugs. Recently, stem cell transplantation has shown potential as an alternative therapy. Embryonic stem cells (ESCs), inducible pluripotent stem (iPS) cells, and mesenchymal stem cells (MSCs) have been considered as candidates.[2–10] The use of ESCs to adapt to the injured lung has been attempted, and it has been demonstrated that intratracheal administration of human ESC-derived type II alveolar epithelial cells (AEC II) ameliorated bleomycin (BLM)-induced lung injury.[2] Although the transplantation of ESC-derived AEC II could

prevent tumorigenic side effects, as previously reported,[2] the in vitro differentiation process from ESCs to AEC II is not simple but rather complicated. As for iPS cells, the technology to utilize them is rapidly developing,[3,4] but the way to achieve stable differentiation of iPS cells into cells specific for airway or alveolar epithelium lineage is a hurdle to be overcome. On the other hand, many studies in animal models have shown beneficial effects of MSC instillation into the injured lung.[5–7] In clinical trials, evaluation of the potential role of MSCs in the treatment of patients with IPF is underway.[8] Therapeutic benefit associated with decreased inflammation and improved alveolar structure was also suggested to be triggered by

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immunomodulation in a paracrine manner, using MSC-conditioned medium.[9] However, undesirable malignant transformation of MSCs remains to be considered, and the engraftment and differentiation rate of MSCs in the injured lung are limited.[10–12]

In addition to these stem cells, many groups have reported that certain populations of putative stem/progenitor cells are resident in the mouse lung.[13–16] Bronchioalveolar stem cells (BASCs) characterized by coexpression of Clara cell secretory protein (CCSP/CC10) and surfactant protein (SP)-C have differentiating capacity to both airway and alveolar lineages, and contribute to alveolar epithelial homeostasis and repair after injury.[14,17] On the other hand, a lineage-tracing investigation suggested that CCSP-expressing cells including BASCs do not contribute to either the alveolar epithelial cell population during normal homeostasis or alveolar epithelial regeneration after naphthalene injury.[13] However, another lineage-tracing investigation showed a striking increase of $CCSP^+$ cells-derived AEC II and AEC I in BLM-induced fibrotic pulmonary lesions.[18] In any case, identification and functional evaluation of lung stem/progenitor cells will help elucidate the process of lung repair and their adaptation to the injured lung as therapeutic options. AEC II are generally characterized by expression of SP-C, and are accepted to be the progenitor cell population for the alveolus.[19] Differentiation of various stem cells into lung epithelial lineage-specific cells before administration into the injured lung is considered to enhance engraftment efficiency and the integrity of the alveolar epithelium.[2,20,21] Indeed, it has been reported that isolated AEC II have a potent regenerative effect on BLM-induced lung fibrosis,[22] but one of the problems in using AEC II is their isolation efficiency. Similarly, it is not easy to expand AEC II *in vitro* while maintaining their progenitor capacity.[23]

Here, we report easily and efficiently harvested cell populations, 'lung mixed culture-derived epithelial cells' (LMDECs), with capacity to differentiate to AEC I *in vitro* and *in vivo*. We then showed the antifibrotic effect of LMDECs on the lung of BLM-instilled mice.

MATERIALS AND METHODS
Mice
All animal procedures conformed to the Japanese regulations for animal care and use, following the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science, and were approved by the Animal Care and Use Committee of Chiba University. Male and female C57BL/6J mice were purchased from Clea Japan (Tokyo, Japan). Information of C57BL/6J-SP-C-M2 flag-p38α dominant-negative (d.n.) transgenic (p38α d.n.-TG) mice is shown in Supplementary Figure 1. For construction of the transgene, a 3.7SPC/SV40 vector, provided by Dr Jeffrey A Whitsett, Children's Hospital Medical Center, Division of Pulmonary Biology, Cincinnati, OH, USA, was used.

Antibodies
At our request, a rabbit anti-proSP-C antibody recognizing an epitope within a.a. 11–27 at the N-terminus of mouse proSP-C was produced by Sigma-Aldrich Japan Genosys (Ishikari, Japan). Another rabbit anti-proSP-C was purchased from Millipore (Billerica, MA, USA). In addition, the following primary antibodies were used: anti-M2 flag antibody (Sigma-Aldrich, St Louis, MO, USA); anti-podoplanin/T1α antibody (MBL, Woods Hole, MA, USA); anti-podoplanin/gp36 antibody (Abcam, Cambridge, UK); anti-α smooth muscle actin (SMA) antibody (Abcam); anti-CCSP/CC10 antibody (Santa Cruz Biotech, Santa Cruz, CA, USA); anti-LGR6 antibody (Santa Cruz Biotech); anti-prominin-1 (Miltenyi Biotech, Gladbach, Germany); PerCP-Cy™ 5.5-conjugated lineage antibody cocktail (anti-CD3ε, -CD11b, -CD45R/B220, -Ly-76, and -Ly-6G&6C antibodies; BD Biosciences, San Jose, CA, USA); phycoerythrin (PE)-anti-CD34 antibody; biotin-, PE,- or Alexa Fluor 647-anti-CD44 antibody; biotin-, Brilliant Violet-, or PE-anti-CD45.2 antibody; Alexa Fluor 488-anti-CD90.2 antibody; FITC-anti-c-kit antibody; and biotin- or PE-anti-Sca1 antibody (Biolegend, San Diego, CA, USA). As for fluorescence-conjugated primary antibodies, a fluorescence-conjugated isotype control corresponding to each primary antibody was used as negative control in flow cytometric analysis. In addition, the following secondary reagents and antibodies were used: Alexa Fluor 350-streptavidin, Alexa Fluor 594-streptavidin, Alexa Fluor 594-donkey anti-goat IgG, Alexa Fluor 594-goat anti-hamster IgG, Alexa Fluor 594-donkey anti-rat IgG, Alexa Fluor 594-chicken anti-mouse IgG, Alexa Fluor 350-goat anti-rabbit IgG, Alexa Fluor 488-chicken anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA), and Dylight 680-donkey anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA, USA).

Mixed Culture and Preparation of LMDECs
Male mice (3–4 weeks old) were anesthetized with pentobarbital and intracardially perfused with 25 ml ice-cold phosphate-buffered saline (PBS) to wash out blood cells of the lung thoroughly. Lung lobes separated from the trachea and the main bronchi were minced into small pieces and digested in Dulbecco's modified Eagle's medium (DMEM) (Wako, Tokyo, Japan) supplemented with 2 mg/ml collagenase type I (Worthington, Lakewood, NJ, USA), 1 mg/ml dispase (Life Technologies), 3 U/ml DNase (QIAGEN, Valencia, CA, USA), 0.1 mg/ml streptomycin, and 100 U/ml penicillin for 45 min at 37 °C with gentle shaking. Then, the resulting pieces were well suspended into a single cell suspension, and cells were passed through a 40 μm-cell strainer (BD Biosciences) followed by neutralization with fetal bovine serum (FBS). The cells were washed with DMEM and resuspended in culture medium (DMEM supplemented with 10% FBS, 0.1 mg/ml streptomycin, 100 U/ml penicillin, and 2.5 μg/ml amphotericin B). The cells were plated on culture dishes (cell density, 1.3×10^6 cells/cm²) and incubated at

37 °C in a humidified atmosphere (5% CO_2) as 'mixed culture'. After 2 days in vitro (DIV), the dishes were washed with PBS several times to remove floating cells completely, and then washed once with the culture medium. The cells were further incubated, and round cells floating or loosely attached to the adherent cells were harvested as LMDECs by tapping the dishes at 9 DIV.

Immunofluorescence Study

LMDECs from wild or p38α d.n.-TG were plated on 8-well chamber slides (Thermo Scientific, Waltham, MA, USA). Within 3 h, the cells were washed with ice-cold PBS and fixed with 4% paraformaldehyde (PFA)/0.1 M NaPB. Then, the cells were permeabilized with 0.1% Triton X-100/PBS for 3 min and washed with PBS. After treatment with SuperBlock in PBS (Thermo Scientific) for 15 min, the cells were stained with primary antibodies (anti-proSP-C and/or anti-M2 flag) in combination with appropriate secondary antibodies. In the in vitro differentiation study of LMDECs, LMDECs from wild mice plated on 8-well chamber slides were subjected to double staining with anti-proSP-C and anti-gp36 antibodies in combination with appropriate secondary antibodies at 7 days after seeding. In case of elucidating the effect of stromal cell-derived factor-1 (SDF-1) on the in vitro differentiation capacity of LMDECs, T1α cell-depleted LMDECs prepared by means of anti-T1α plus anti-rat IgG-MicroBeads (Miltenyi Biotech) were plated on 8-well chamber slides (Thermo Scientific). The cells were incubated in the conditioned medium of mixed culture, serum-free Opti-MEM I (Life Technologies), or Opti-MEM I containing 0.3 μg/ml SDF-1α. Then, the cells were subjected to staining with anti-gp36 at 3 and 5 DIV. In the depletion study of $Sca1^+$ cells, a $Sca1^+$ cell-free mixed culture was prepared using an anti-Sca1 MicroBead Kit (Miltenyi Biotech). Then, LMDECs from normal or $Sca1^+$ cell-free mixed culture were subjected to double staining with anti-proSP-C and biotin-anti-CD45.2 in combination with appropriate secondary antibodies. The nuclei were stained with 4',6-diamino-2-phenylindole (DAPI, DOJINDO, Kumamoto, Japan). The stained cells were observed under a fluorescence microscope (Axio Imager A2, Carl Zeiss, Oberkochen, Germany). For tissue investigation, the lung lobes were fixed in 4% PFA/0.1 M NaPB for 24 h and dehydrated in 30% sucrose/PBS for 48 h. Then, the tissues were frozen in Tissue Tek OCT compound (Sakura Finetek, Torrance, CA, USA). Freshly cut lung sections (5 μm in thickness) were placed on poly-L-lysine-coated slides. The sections were treated with 1:10 FcR blocking agent (Miltenyi Biotech) for 15 min, stained with appropriate primary antibodies in combination with secondary antibodies, and observed under a fluorescence microscope.

Flow Cytometric Analysis

Freshly harvested LMDECs and the adherent cells in the mixed culture were treated with 1:10 FcR blocking agent for 15 min and subjected to cell-surface staining for 15 min at 4 °C. After fixing and permeabilizing (Fix/Perm buffer, Biolegend), the cells were further subjected to intracellular staining for 15 min at 4 °C. Primary antibodies and secondary reagents/antibodies were used in a dilution ratio of 1:100 and 1:200, respectively. The resulting cells were filtered through a 70 μm-cell strainer (BD Biosciences) and analyzed with a FACSCantoII (BD Biosciences). Positively stained cells were gated using negative control cells incubated with appropriate isotype controls or secondary reagents/antibodies. Data were collected and analyzed with FACSDiva (BD Biosciences) and FlowJo 9.6.2 software (TreeStar, Ashland, OR, USA).

RT-PCR

Freshly harvested LMDECs were plated on 6-well culture plates and cultured in culture medium. At desirable time points, the cells were washed with PBS and subjected to total RNA preparation with RNAiso Plus (TaKaRa Bio, Seta, Japan) according to the manufacturer's instructions. Single-strand cDNA was synthesized from prepared RNA (3 μg), with Moloney murine leukemia virus reverse transcriptase (Life Technologies) using an oligo(dT) primer (Life Technologies) in a total volume of 20 μl. The resultant cDNA sample (1 μl) was subjected to PCR for amplification of mouse SP-C or T1α using specific primers (for SP-C, sense primer, 5′-TGGACATGAGTAGCAAAGAG-3′; antisense primer, 5′-GTAGCAGTAGGTTCCTGGAG-3′, for T1α, sense primer, 5′-GATCACAGAGAACACGAGAG-3′; antisense primer, 5′-TCTTTCCTTTGGTACTGCTG-3′). As an internal control, mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was amplified using specific primers (sense primer, 5′-GACCACAGTCCATGACATCACT-3′; antisense primer, 5′-TCCACCACCCTGTTGCTGTAG-3′). The settings of the thermal cycler were 35 cycles of 30 s at 98 °C, 30 s at 56 °C, 30 s at 68 °C, and 7 min at 68 °C for SP-C and T1α and 26 cycles of 30 s at 98 °C, 30 s at 60 °C, and 30 s at 68 °C for GAPDH. In case of CXCR4 mRNA detection, freshly harvested LMDECs, LMDECs plated and cultured for 7 days, BALB/c 3T3 cells as a negative control and freshly prepared peritoneal macrophages from WT mice as a positive control were subjected to total RNA preparation followed by RT-PCR using specific primers for CXCR4 (sense primer, 5′-AG-CATGACGGACAAGTAC-3′; antisense primer, 5′-GTGTA-GATGATATGGACAG-3′). The settings of the thermal cycler were 32 cycles of 15 s at 98 °C, 30 s at 58 °C, 45 s at 68 °C, and 7 min at 68 °C. The amplified products were separated in 1.2% agarose gel and visualized with ethidium bromide staining under UV radiation. Specific amplification of mouse SP-C (365 bp), T1α (414 bp), CXCR4 (140 bp), and GAPDH (450 bp) was observed.

BLM-Induced Lung Injury

Mice (10–12 weeks old) under anesthesia were given a single intratracheal injection of BLM hydrochloride (5 mg/kg; Nippon Kayaku, Tokyo, Japan) in PBS using a Microsprayer

atomizer (PennCentury, Philadelphia, PA, USA). Control mice received sham treatment with PBS.

In Vivo Differentiation Assay of LMDECs

Mice (10–12 weeks old) under anesthesia were given a single intratracheal injection of BLM hydrochloride to create a pathological condition of the lungs. After depleting T1α^+ cells from freshly harvested LMDECs by means of anti-T1α plus anti-rat IgG-MicroBeads (Miltenyi Biotech), the resulting LMDECs were labeled with PKH67 using a PKH67 Green Fluorescent Cell Linker Kit (Sigma-Aldrich) according to the manufacturer's instructions. Although we confirmed that T1α^+ or gp36$^+$ cells exhibiting AEC I-like property were hardly observed in freshly harvested LMDECs, the depletion step was performed to enhance reliability of this assay. Then, mice underwent intratracheal transplantation of PKH67-labeled LMDECs (10^5 cells in 100 μl PBS) through a 26G needle 24 h after BLM instillation. After 1 week, lung sections were stained with anti-gp36 antibody.

Engraftment of LMDECs to BLM-Injured Lung

At 24 h after BLM or PBS instillation, mice under anesthesia received an intratracheal injection of LMDECs or cell surface marker-sorted LMDECs through a 26G needle (3×10^5 cells in 100 μl PBS). Control mice received sham treatment with PBS or engraftment of HEK 293 cells. Cell-surface marker-sorted LMDECs, CD44$^+$ LMDECs, and Sca1$^+$ LMDECs were prepared from freshly harvested LMDECs using biotin-anti-CD44 plus streptavidin-MicroBeads (Miltenyi Biotech) and an anti-Sca1 MicroBead Kit, respectively. Mice were killed at 14 days after BLM instillation. The lungs were perfused, dissected out, fixed, sectioned, and stained with HE or Masson's trichrome stain to visualize and score collagen deposition according to the method proposed by Ashcroft *et al.*[24]

Western Blot Array Analysis

Mice receiving each injection (PBS + PBS, BLM + PBS, or BLM + LMDECs) were anesthetized and killed on day 7. Then, the trachea of each mouse was exposed and lavaged with 1 ml ice-cold PBS using a 20-gauge catheter. Collected bronchoalveolar lavage fluid (BALF) from three mice of each group was mixed and centrifuged at 400 g for 10 min. The resulting supernatant was subjected to RayBio® Biotin Label-based Mouse Antibody Array 1 (RayBiotech, Norcross, GA, USA), and changes in expression levels of 308 inflammation-related proteins in the BALF sample were evaluated. The array was performed according to the manufacturer's instructions.

Transmigration Assay

LMDECs (3×10^4 cells) suspended in Opti-MEM I (Life Technologies) were seeded on 24-well cell culture inserts (apical chamber; Falcon) with pore size of 8 μm. All apical chambers were washed with Opti-MEM I twice for removing nonattaching cells 2 h after applying LMDECs to the apical chambers. After incubation of the cells in Opti-MEM I in the presence or absence of 0.3 μg/ml SDF-1α for 12 h, the cells were labeled with CellTracker™ Red CMTPX (Life Technologies) according to the manufacturer's protocol. As a positive control, the cells were incubated in Opti-MEM I containing 10% FBS for 12 h and labeled. Then, the cells were washed with PBS twice and fixed, and the nuclei were stained with DAPI. Nonmigrating cells were removed by scraping the apical side of the apical chambers with a cotton swab. Then, the apical chambers were subjected to the examination of transmigrating LMDECs by a fluorescence microscope (Axio Imager A2).

Proliferative Assay

The cells were loaded with 10 μM 5-ethynyl-2́-deoxyuridine (EdU) for 6 h and washed with PBS followed by fixation with 4% PFA/0.1 M NaPB. EdU incorporated into DNA was detected with Alexa Fluor 488- or 647-azide according to the manufacturer's protocol of Click-iT® EdU Imaging Kits (Life Technologies). Cells with fluorescein-labeled nuclei were examined with a fluorescence microscope and a flow cytometer.

Statistical Analysis

Data are expressed as mean ± s.e.m. Statistical analysis was conducted using Graphpad Prism Version 6 (GraphPad

Figure 1 Isolation and characterization of LMDECs. (**a**) Time-dependent changes of lung homogenate-based crude culture, a mixed culture. Sphere-like cell aggregates (arrowhead) appeared on adherent cells at 5 DIV and were dispersed at 7 DIV, and a large number of round cells floating or loosely attached on the adherent cells were observed at 9 DIV. (**b**) Harvested round cells from the mixed culture were SP-C$^+$ (green). The round cells from p38α d.n.-TG mice (inset) were SP-C$^+$ (green) and M2-tag$^+$ (red). (**c**) Flow cytometric analysis of LMDECs. Histogram shows the percentage of LMDECs positive for proSP-C. Shaded histogram represents LMDECs stained with secondary antibody as negative controls. (**d**) Assay of *in vitro* differentiation of round cells. Most of the cells morphologically changed and turned to be gp36$^+$ (red). Green represents immunoreactivity of proSP-C. An arrowhead shows SP-C$^+$gp36$^+$ cell. (**e**) Time-dependent changes in mRNA expression of SP-C and T1α. Total RNA of plated round cells was prepared at the indicated time points and subjected to PCR for amplification of mouse SP-C or T1α. (**f**) Assay of *in vivo* differentiation of round cells. T1α^+ cell-depleted round cells were labeled with PKH67 dye (green) and injected into BLM-injured lung, and lung sections were stained for gp36 (red) 7 days after BLM instillation. Arrowheads show PKH67 dye$^+$gp36$^+$ cells. Similar results were obtained in three independent experiments. (**g**) Beneficial effects of LMDECs on BLM-induced lung fibrosis. LMDECs and HEK 293 cells were intratracheally administered to the lung 24 h after BLM instillation. As a control experiment, PBS was administered to BLM-induced lung injury. Lung sections were stained with HE 14 days after BLM instillation. We confirmed that administration of LMDECs or HEK 293 cells to the normal lung did not lead to any histopathological changes in the lung 14 days after administration, the profile of which was similar to that of negative control (PBS + PBS). Similar results were obtained in four independent experiments.

Software, San Diego, CA, USA). Statistical significance was determined by Student's *t*-test or analysis of variance (ANOVA) followed by Tukey's test, and *P*-values of <0.05 were considered to be significant.

RESULTS
Isolation, Characterization, and Engraftment of LMDECs
On the basis of the notion that dissection of pulmonary tissue might create a specific pathophysiological micro-environment leading to activation of tissue-resident progenitor cells,[16] we prepared a crude culture of enzymatically digested lung tissue, and observed the time-dependent changes in cell behavior of the mixed culture. As shown in Figure 1a, round cells arose from spindle-shaped adherent cells showing confluence at 5 DIV, and aggregates of sphere-like cells were also observed. The cell aggregates (appearance ratio: 2–3/1.5 mm^2) were larger, loosely attached, and flat at 7 DIV. Thereafter, the number of round cells floating or
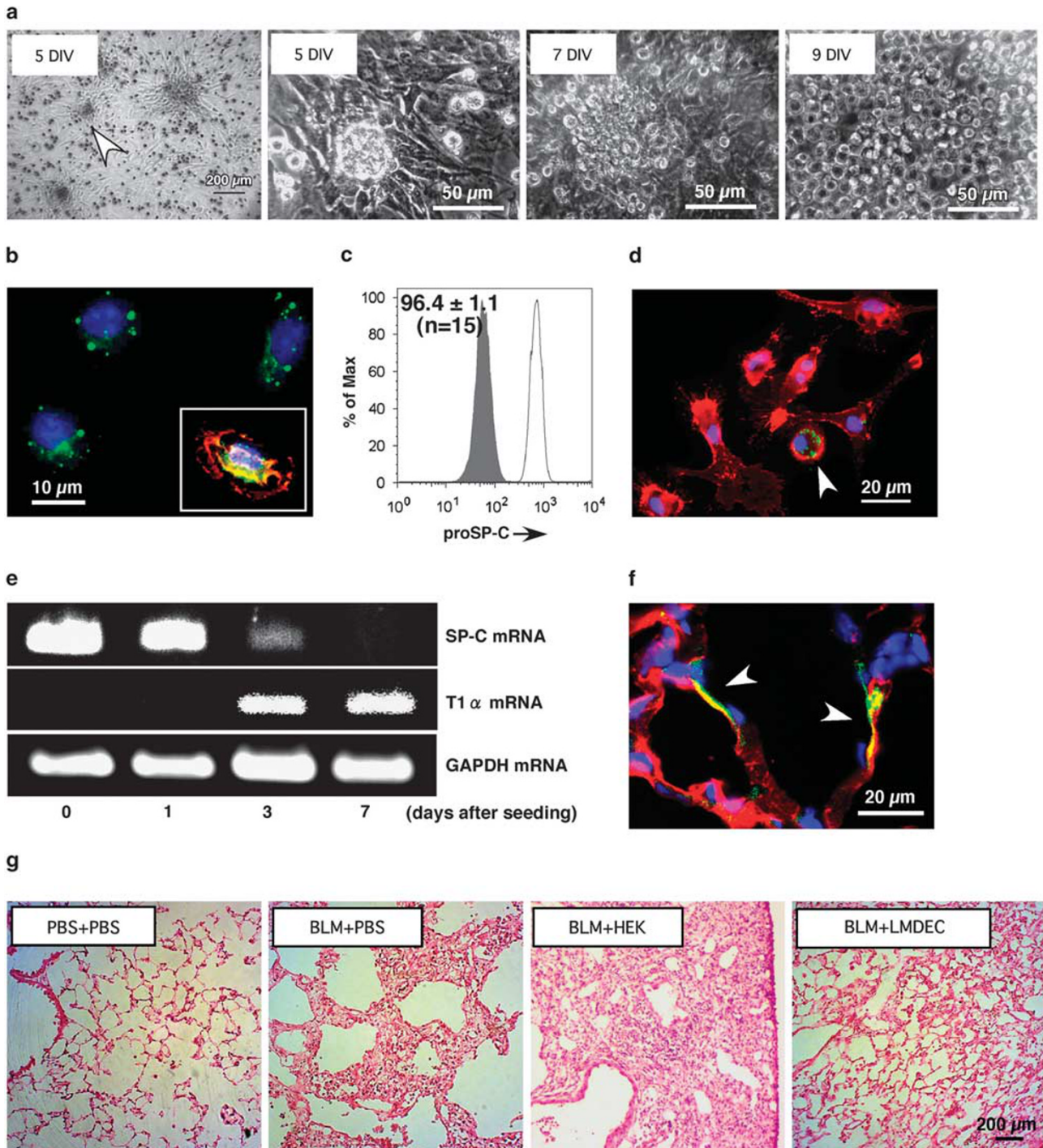
loosely attached to the adherent cells increased, and covered the adherent cells at 9 DIV. The yield of round cells was $2.4 \pm 0.2 \times 10^6$ cells/mouse at 9 DIV.

We investigated whether the round cells could express SP-C, because SP-C-positive cells including AEC II have a regenerative effect on BLM-induced lung fibrosis.[20,22] The round cells harvested at 9 DIV were seeded and subjected to immunofluorescent study with anti-proSP-C antibody within 3 h. As shown in Figure 1b, the round cells had positive immunoreactivity for SP-C. Similarly, the round cells prepared from p38α d.n.-TG mice were SP-C$^+$M2-tag$^+$ (Figure 1b, inset). This finding indicates promoter activity of SP-C in the round cells, because expression of a chimeric protein including M2-tag is controlled under the SP-C promoter (Supplementary Figure 1A and C). Flow cytometric analysis revealed that most of the round cells were SP-C$^+$ (Figure 1c).

Then, we elucidated whether the round cells might function as a progenitor cell for AEC I. After seeding the round cells, their morphology changed to be spindle/fibrous shape with expressing gp36, a marker of AEC I, at 7 DIV. In contrast, the expression of SP-C in the cells was not observed except SP-C$^+$gp36$^+$ cells as a small population at 7 DIV (Figure 1d). Next, after seeding the round cells, the time-dependent changes in mRNA expression of SP-C and T1α, a marker of AEC I, were investigated by RT-PCR. As shown in Figure 1e, there was a decrease of SP-C mRNA expression that was inversely proportional to an increase of T1α mRNA expression that can explain the morphological change-associated cell marker exchange (Figure 1b and d). Furthermore, the cells labeled with PKH dye transplanted into the alveoli became gp36$^+$ under the pathophysiological condition produced by BLM instillation (Figure 1f). These results indicate that SP-C$^+$ round cells can give rise to AEC I *in vitro* and *in vivo*. Thus, we named the round cells LMDECs.

To investigate the reparative effect of LMDECs against lung injury, LMDECs were administered into the lungs of BLM-challenged mice. Representative microscopic findings following HE staining of the lung sections are shown in Figure 1g. The lung tissue exposed to BLM showed a strong accumulation of inflammatory cells, thickening of the alveolar walls, and fibrotic lesions. On the other hand, not HEK 293 cells but LMDEC-engrafted mice revealed successful amelioration of the histopathological changes induced by BLM. Administration of LMDECs and HEK293 cells into the control lung did not induce any histopathological changes in the lung (data not shown).

Effects of LMDEC Engraftment on the Microenvironment in the BLM-Injured Lung

To elucidate the mechanisms underlying the protective effect of LMDECs against BLM-induced lung injury, a comprehensive analysis of BALF by western blot array was performed. BALF from the BLM-instilled lung (BLM + PBS group) simply showed an increase in 71/308 proteins

compared with that from control mice (PBS + PBS group). Among the BLM-induced proteins in BALF, 27 proteins exhibiting over twofold induction ratio (BLM + PBS/PBS + PBS) were picked up. In BALF from the LMDEC-engrafted mice (BLM + LMDEC group), the levels of the 27 proteins showed a tendency to decrease compared with the BLM + PBS group. Then, seven proteins exhibiting a typical reduction (> 25%, BLM + LMDEC *vs* BLM + PBS) are shown in Figure 2a. Among the seven proteins, SDF-1 and T-cell activation (TCA)-3 were soluble factors, and CXCR4, CXCR6, TCCR, TSLPR, and TWEAKR were transmembrane receptors. As the cell-free BALF was subjected to western blot array, signals of those receptors might be reflected by cell debris or mechanically cleaved forms of receptors in BALF. In particular, the levels of SDF-1 and its receptor, CXCR4, in BALF were highly induced by BLM and significantly sensitive to the LMDEC engraftment.

It has been reported that the migration activity of both AEC II and airway epithelial progenitor cells can be regulated by the SDF-1/CXCR4 axis.[25,26] Then, to elucidate whether SDF-1 interacts with LMDECs, we first investigated the expression of CXCR4 in LMDECs. As shown in Figure 2b, the expression of CXCR4 mRNA was clearly observed in freshly harvested LMDECs, indicating that LMDECs could respond to SDF-1. On the other hand, the expression of CXCR4 mRNA was downregulated in LMDECs at 7 DIV, indicating that AEC I differentiated from LMDECs might have a low level of CXCR4 expression. We next investigated the effects of SDF-1 on migratory activity and the *in vitro* differentiating capacity of LMDECs. As shown in Figure 2c, SDF-1 markedly enhanced the transmigration of LMDECs, the potency of which was similar to that of 10% FBS. Serum-free medium induced a time-dependent differentiation of LMDECs to AEC I that was significantly enhanced by SDF-1. In contrast, conditioned medium from the mixed culture inhibited the differentiation of LMDECs to AEC I (Figure 2d).

Classification of LMDECs
LMDECs showing SP-C-like immunoreactivity and having differentiation capacity to AEC I *in vitro* and *in vivo* are AEC II-like cells. However, the question arose as to whether LMDECs expanded in the mixed culture are simply AEC II and whether LMDECs include another cell population of lung stem/progenitor cells. To address these questions, flow cytometric analysis was performed. It has been reported that the alveolar epithelial cell lineage constitutively expresses CD44 at least in *in vitro* culture.[27] Then, the expression of CD44 on LMDECs was elucidated. As shown in Figure 3a, over 90% of LMDECs (93.1 $\pm$ 1.9%) were CD44 positive. On the other hand, the hematopoietic cell marker CD45 is generally known to be negative in AEC II.[27] Surprisingly, nearly 90% of LMDECs were positive for both CD45 and a hematopoietic cell lineage marker cocktail (91.1 $\pm$ 3.1% and 84.5 $\pm$ 1.7%, respectively; Figure 3b and c). Furthermore, most LMDECs (84% of LMDECs) coexpressed SP-C and
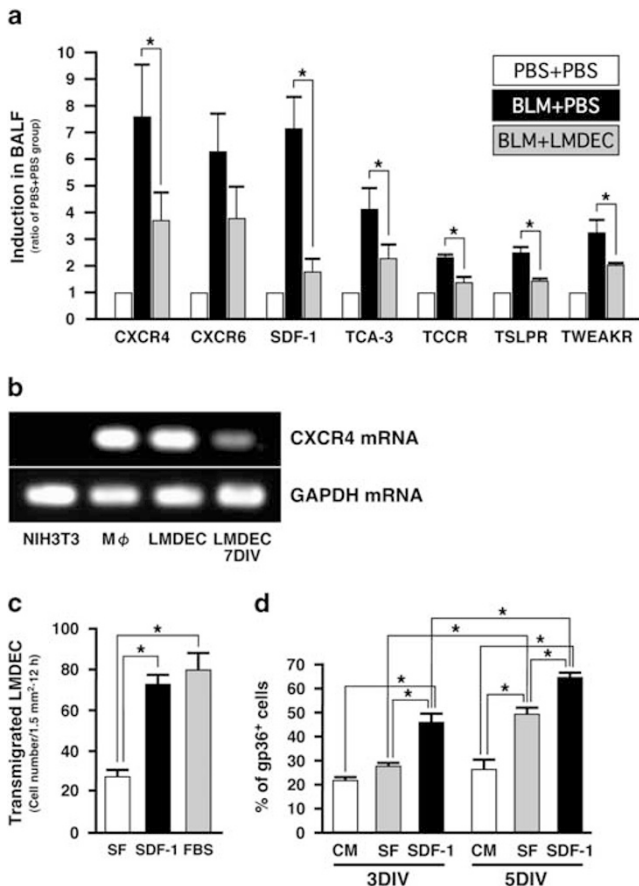
Figure 2 Mechanisms of protective effect of LMDECs against lung injury (a) LMDEC engraftment improves the microenvironment in the lung exposed to BLM. Collected BALF from three mice of each group (PBS + PBS, BLM + PBS, or BLM + LMDEC) was mixed, centrifuged, and subjected to protein array for 308 molecules. Two independent experiments were conducted, and resulting four signals for each protein were evaluated. Then, seven proteins were reproducibly induced in response to BLM (>2-fold) and sensitive to the LMDEC engraftment (>25% reduction, BLM + LMDEC/BLM + PBS). Using a densitometer, each signal was normalized to the positive internal control included in the array membrane, and expressed as induction ratio of the control value (PBS + PBS). Black and shaded bar show mean ± s.e.m. ($n = 4$). The difference between the two groups (BLM + PBS and BLM + LMDEC) was statistically significant (*$P < 0.05$) by Student's t-test for unpaired values. (b) The expression of CXCR4 mRNA in LMDECs. Total RNA of NIH3T3, freshly harvested peritoneal macrophages, freshly harvested LMDECs, and plated LMDECs for 7 days was prepared and subjected to PCR for amplification of mouse CXCR4. (c) Transmigration activity of LMDECs induced by SDF-1. SF means serum-free medium. Data are shown as mean ± s.e.m. ($n = 6$). *$P < 0.05$ (ANOVA followed by Tukey's test). (d) Effect of SDF-1 on differentiating activity of LMDECs to AEC I. T1α+ cell-depleted LMDECs were plated on eight-well chamber slide and stained for gp36 at the indicated time points. Then, the number of gp36+ cells were counted and expressed as the percentages of total cell count. CM and SF mean conditioned medium from mixed culture and serum-free medium, respectively. Data are shown as mean ± s.e.m. ($n = 8$). *$P < 0.05$ (ANOVA followed by Tukey's test).

CD44 that simultaneously identified them as cells expressing both CD45 and hematopoietic lineages (Figure 3d). Thus, the major population of LMDECs (LMDEC^Maj) has a unique profile of cell lineage marker expression that is different from that of AEC II. Similarly, a cell population showing SP-C+ CD45− was rarely observed in LMDECs, indicating that AEC II could not be contained in LMDECs (Figure 3d).

We next investigated the cells using various markers as follows: CD34, a marker of hematopoietic stem/progenitor cells covering from earlier multipotent progenitors to later lineage-restricted progenitors,[28] and an enrichment marker tool for BASCs;[17] CD90, one of the major markers of tissue-resident MSCs;[29] c-kit, a marker for human lung stem cells with ES-like pluripotency;[30] prominin-1 (CD133), a marker of progenitors (PEP) of alveolar epithelial cell lineage;[16] and LGR6, a marker of human alveolar multipotent cells.[31] Expression of CD34, CD90, c-kit, prominin-1, and LGR6 on LMDECs was rarely observed (Supplementary Figure 2). On the other hand, a small cell population showing SP-C+ CCSP+ Sca1+ (1.8% of LMDECs) was observed in LMDECs, indicating that LMDECs include BASCs (Figure 3e). BASCs have differentiating capacity to both airway and alveolar lineages.[17] This indicates the possibility that LMDEC^Maj may be partially derived from BASCs. Then, we prepared a lung mixed culture with or without Sca1+ cells, and investigated the recovery rate of SP-C+ CD45+ LMDECs in each condition. As shown in Figure 3f, Sca1+ cell depletion from the mixed culture did not affect the yield of SP-C+ CD45+ LMDECs, suggesting that BASCs might not differentiate to LMDEC^Maj at least under our culture condition.

Antifibrotic Effects of Classified LMDECs

As LMDECs mostly consisted of SP-C+ CD44+ CD45+ Lin+ cells (LMDEC^Maj) and included BASCs as a small cell population, we further investigated the antifibrotic effects of LMDECs and their selected populations on the BLM-instilled lung. Selection of LMDEC^Maj and BASC-included cell population from LMDECs was performed by using anti-CD44 and anti-Sca1 antibodies, respectively. In accordance with the histopathological changes in Figure 1g, Masson's trichrome staining revealed that LMDECs inhibited BLM-induced lung fibrosis (Figure 4a). In addition, both LMDEC^Maj and Sca1+-sorted LMDECs including BASCs markedly improved BLM-induced lung damage (Figure 4b). Ashcroft analysis showed a significant reduction of fibrosis with each LMDEC treatment to a similar extent compared with PBS treatment (Figure 4c). However, accumulation of segmented neutrophils was observed in the alveoli of Sca1+ LMDEC-treated mice that was significantly greater than that in mice treated with LMDEC^Maj (Figure 4d and e). These results suggest that the protective effect of LMDECs against BLM-induced lung injury depends greatly on that of LMDEC^Maj. Then, we in vitro and in vivo examined the origin of LMDEC^Maj.

Identification of the Cells Having Characteristics Corresponding to LMDEC^Maj

As shown in Figure 1a, LMDECs began to markedly increase as floating or loosely attached cells under the mixed culture
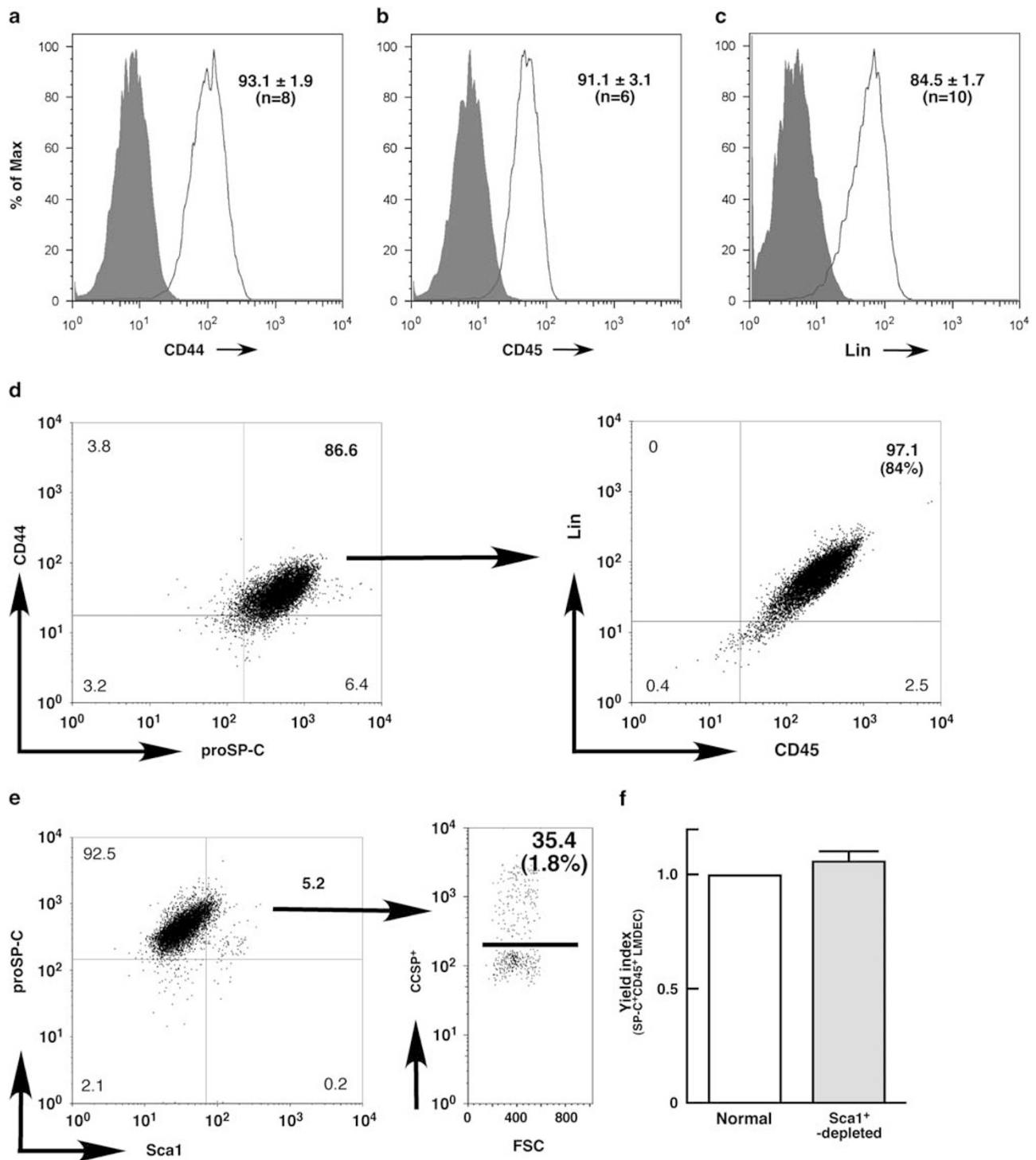
Figure 3 Flow cytometric analysis of LMDECs. Histograms show the percentage of LMDECs positive for CD44 (**a**), CD45 (**b**), and a hematopoietic cell lineage marker, Lin (**c**). Shaded histograms represent LMDECs stained with appropriate isotype controls as negative controls. (**d**) The majority of SP-C$^+$ LMDECs coexpressed CD44, CD45, and Lin (84%). (**e**) A small population of LMDECs (1.8%) was SP-C$^+$Sca1$^+$CCSP$^+$, indicating BASCs. (**f**) Effect of Sca1$^+$ cell depletion on yield of SP-C$^+$CD45$^+$ LMDECs. The yield of SP-C$^+$CD45$^+$ LMDECs from the Sca1$^+$ cell-depleted group is expressed as the ratio of cell count of SP-C$^+$CD45$^+$ LMDECs from the normal group in each trial. Shaded bar shows mean ± s.e.m. ($n = 3$). There was no significant difference between the two groups.
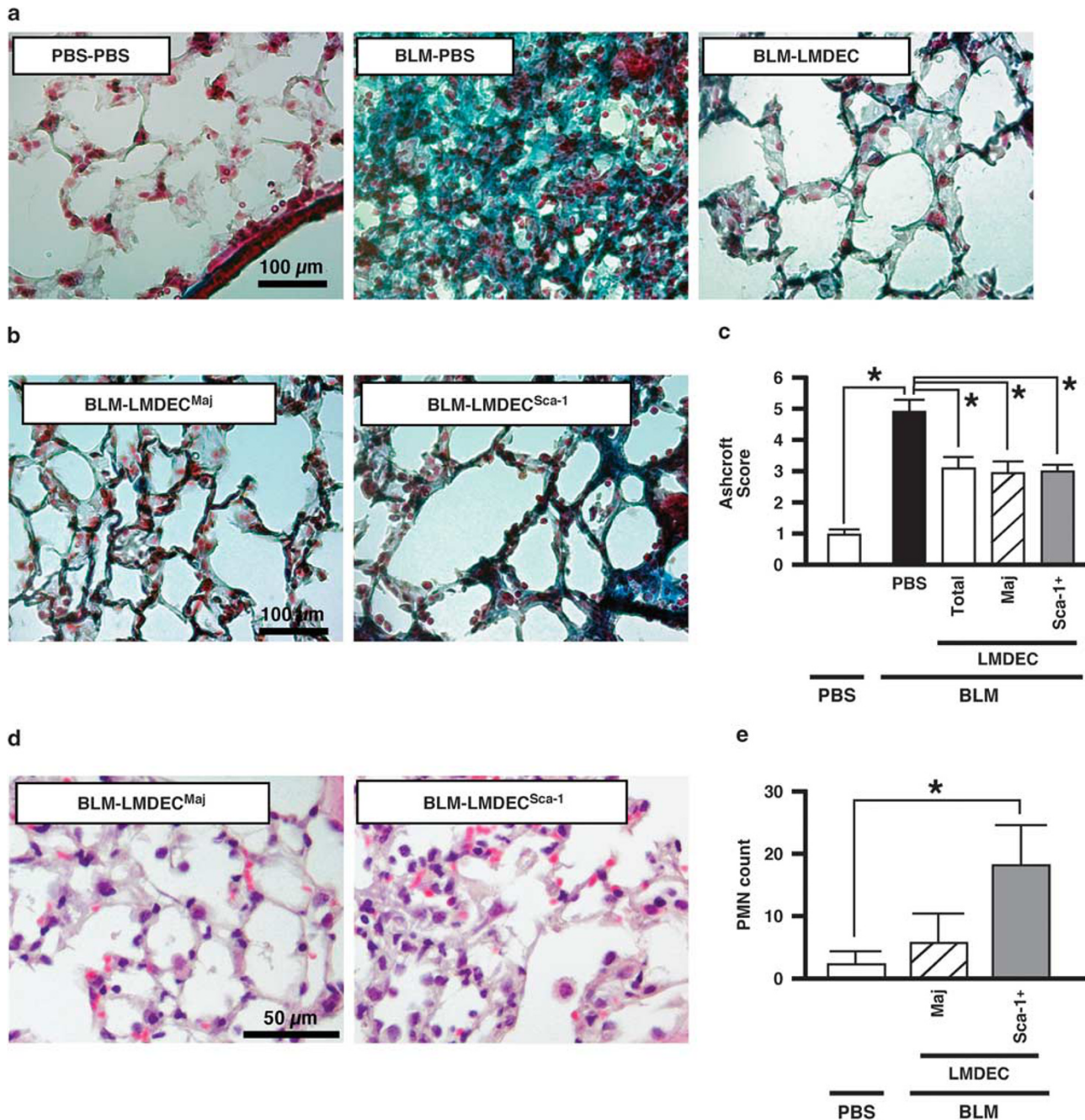
Figure 4 Histological analysis of lung treated with BLM followed by LMDEC intratracheal administration. (**a**, **b**) Engraftment of nonselected (total), CD44^{+}-sorted, or Sca1^{+}-sorted LMDECs beneficially affected BLM-induced lung fibrosis. Lung sections were subjected to Masson's trichrome staining. (**c**) Collagen deposition (six areas) in each section was scored according to the method proposed by Ashcroft *et al.*[24] Data are shown as mean ± s.e.m. of three sections from each individual (*n* = 4). *$P < 0.05$, significantly different from value after BLM treatment without LMDEC engraftment (black bars) (Tukey's method). (**d**) Neutrophilic infiltration in lung-administered CD44^{+} or Sca1^{+}-sorted LMDECs. Lung sections from CD44^{+}- or Sca1^{+}-sorted LMDEC-treated mice were subjected to HE staining. (**e**) Polymorphonuclear leukocyte (PMN) count in lung treated with Sca1^{+}-sorted LMDECs was higher than that with CD44^{+}-sorted LMDECs. Data are shown as mean ± s.e.m. in a high power field (four areas, 0.016 mm²) of three sections from each individual (*n* = 4). *$P < 0.05$ (ANOVA followed by Tukey's test).

condition after 7 DIV. To assess the origin of LMDEC^Maj, we investigated the cell populations of adherent cells in the mixed culture. LMDECs were completely washed out at 7 DIV, and the remaining adherent cells were harvested and subjected to flow cytometric analysis. Notably, SP-C^{+} CD44^{+}CD45^{+}Lin^{+} LMDEC^Maj were included in the adherent cells of the mixed culture as 17.5% of total cells and were EdU^{+} (36.8%). BASCs were also contained in the

Table 1 Cell populations in adherent cells in mixed culture (>5% of total cells)

Nomenclature	Profile	Content (% of total cells)	Trials
LMDEC[Maj]	SP-C$^+$/CD44$^+$/CD45$^+$/Lin$^+$	17.5 ± 0.9 (36.8% EdU^{pos})	$n=6$
BASC	SP-C$^+$/CCSP$^+$/Sca1$^+$	6.6 ± 0.7 (44.4% EdU^{pos})	$n=4$
AEC I	SP-C$^-$/gp36$^+$	7.2 ± 0.5	$n=3$
AEC II	SP-C$^+$/CCSP$^-$/Sca1$^-$/Lin$^-$	13.0 ± 0.6	$n=4$
Clara	CCSP$^+$/SP-C$^-$/Sca1$^-$	9.0 ± 0.7	$n=4$
Fibroblast	SMA$^+$	17.6 ± 0.6	$n=5$
Hematopoietic	SP-C$^-$/CD45$^+$/Lin$^+$	20.0 ± 3.3	$n=6$

Flow cytometric analysis of adherent cells in mixed culture. Expression of markers in each cell population was identified. Proliferative activity of LMDEC[Maj] and BASCs was evaluated as EdU-positive cells.

adherent cells as 6.6% of total cells and were EdU$^+$ (44.4%). However, other lung-resident stem/progenitor cells (prominin-1$^+$ or LGR6$^+$) having capacity to differentiate to SP-C$^+$ alveolar epithelial cells were not observed in the adherent cells of the mixed culture at 7 DIV. Other cell populations observed in the adherent cells (>5% of total cells) were estimated as follows: SP-C$^-$gp36$^+$ AEC I, 7.2%; SP-C$^+$ CCSP$^-$Sca1$^-$Lin$^-$ AEC II, 13%; CCSP$^+$SP-C$^-$Sca1$^-$ Clara cells, 9%; SMA$^+$ fibroblasts, 17.6%; and SP-C$^-$Lin$^+$ CD45$^+$ hematopoietic cells, 20% (Table 1). These findings indicate that the ratio of LMDEC[Maj] in total adherent cells was relatively high compared with other cell populations. Similarly, the proliferative activity of LMDEC[Maj] also showed a similar level to that of BASCs. This fact indicates that the mixed culture is suitable condition for expansion of LMDEC[Maj] and tempts us to consider that LMDEC[Maj] may exist as resident cells in the lung. Then, the existence and pathological response of cells having an LMDEC[Maj]-like profile was investigated.

As expected, the cells having both alveolar epithelial and hematopoietic cell lineages (SP-C$^+$CD45$^+$) were observed in the normal lung (Figure 5a), and the ratio of CD45$^+$ cells/ SP-C$^+$ cells was 0.042 ± 0.015. Similarly, SP-C$^+$CD45$^+$ cells increased in response to BLM treatment (5 days after instillation), although SP-C$^-$CD45$^+$ cells, in addition to alveolar macrophage-like cells, accumulated in the alveoli as infiltrating cells under BLM-induced inflammation (Figure 5a). Statistical analysis of the changes in the number of SP-C$^+$CD45$^-$, SP-C$^-$CD45$^+$, and SP-C$^+$CD45$^+$ cells is shown in Figure 5b. SP-C$^+$CD45$^-$ cells defined as AEC II significantly decreased in response to BLM treatment that was inversely proportional to the significant increase in both SP-C$^-$CD45$^+$ and SP-C$^+$CD45$^+$ cells. Accordingly, the ratio of CD45$^+$ cells/SP-C$^+$ cells significantly increased and was 0.124 ± 0.032 under the pathological condition (*vs* the values in the normal lung ($P=0.014$ by Student's *t*-test for unpaired values)). These results suggest that SP-C$^+$CD45$^+$
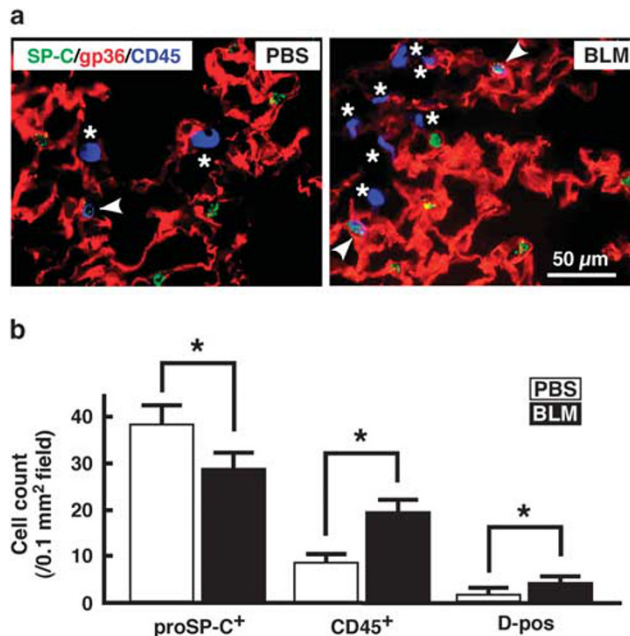
Figure 5 The cells having characteristics corresponding to LMDEC[Maj] in lung. (**a**) Lung sections prepared from PBS-treated lung and BLM-treated lung were stained for proSP-C (green), CD45 (blue), and gp36 (red). Arrowheads and asterisks show SP-C$^+$CD45$^+$ and SP-C$^-$CD45$^+$ cells, respectively. (**b**) Changes in cell numbers of SP-C$^+$, CD45$^+$, and SP-C$^+$CD45$^+$ (D-pos.) in lung in response to BLM injury. Each bar shows mean ± s.e.m. of four sections from each individual ($n=4$). The difference between the two groups (PBS and BLM) was statistically significant (*$P<0.05$) by Student's *t*-test for unpaired values.

cells are expanded in the lung at least during the BLM-induced inflammatory phase.

DISCUSSION

IPF is a specific form of chronic, progressive fibrosing interstitial pneumonia of unknown cause. It is characterized by progressive worsening of dyspnea and lung function and has a poor prognosis.[1] After publication of the first report, in which MSCs were administered in BLM-induced lung fibrosis, resulting in the amelioration of fibrosis, many researchers reported the therapeutic effect of MSC against lung fibrosis.[5,12] In fact, MSCs have been approved for use in clinical trials in patients with IPF.[8] In studies showing beneficial effects of MSCs in the BLM model, however, lung fibrotic injury was significantly reduced by delivery of MSCs during not the fibrotic phase but the early inflammatory phase.[5] Similarly, it has been demonstrated that bone marrow mesenchymal stromal cells systemically injected into mice during the fibrotic phase of radiation-induced lung injury mostly acquire a fibroblast phenotype.[32] These results strongly suggest that delivery of MSCs during the early phase of pulmonary inflammation seems to be important to achieve optimal beneficial effects of MSCs. On the other hand, unlike MSCs, AEC II derived from lung homogenates have beneficial effects even in the established fibrotic phase of

injury, indicating a great advantage of AEC II engraftment in alveolar regeneration.[22] Very recently, genetic lineage-tracing experiments have clearly demonstrated that AEC II contribute to alveolar maintenance and repair as stem cells with long-term self-renewal capacity, and clonal expansion of human AEC II as alveolospheres in 3D culture was successfully established.[33] These findings highlight the potential of a cell therapy strategy with AEC II for fibrotic lung injury.

In the present study, we established an easy, fast, and highly efficient culture system for preparation of a cell population showing beneficial effects on lung fibrosis. In some cases of preparations of pulmonary stem/progenitor cells like tissue-resident MSCs and AEC II, hematopoietic cells marked by anti-CD45 are generally depleted at the first step of preparation to enrich stemness or exclude the hematopoietic cell pool.[29,34] In addition, their culture needs specified condition like a coculture system with feeder cells or 3D culture in bronchial epithelial cell growth medium containing keratinocyte growth factor to maintain the progenitor capacity of those cells. However, our simple and crude culture system without any immunodepletion or sorting of cells successfully provides LMDECs having antifibrotic effect on BLM-injured lung. Similarly, LMDECs can be harvested at least two times from the same dish. After harvesting LMDECs from mixed culture at 9 DIV, the dishes were further incubated for 7–10 days (16–19 DIV), and the round cells floating or loosely attached to the adherent cells appeared again. The yield of round cells was reduced by 10% compared with that at 9 DIV. Although the precise characterization of the second harvested round cells has not been performed, the cells satisfy the profile of LMDECs by the following reasons: most of the cells were positive for both SP-C and CD45 by immunofluorescent study; the cells have an in vitro differentiation capacity to AEC I that was confirmed by anti-gp36 antibody (data not shown); and the engraftment of the cells, like the case of LMDECs harvested at 9 DIV, showed the reparative effect against the lung injury induced by BLM (Ashcroft score, 2nd LMDEC + BLM vs BLM: 3.2 ± 0.6 vs 4.9 ± 0.4 ($n = 4$)). The mixed culture may provide a suitable condition for expansion of LMDECs having the differentiating capacity. Indeed, LMDEC[Maj] showed a high proliferative activity in the adherent cells of the mixed culture (Table 1). In addition, conditioned medium of the mixed culture can maintain the progenitor activity of LMDECs (Figure 2d).

Previous reports have clearly demonstrated that SDF-1/CXCR4 axis critically contributes to the pathogenesis of lung fibrosis induced by BLM via recruiting bone marrow-derived cells called fibrocytes as sources of active fibroblasts.[35,36] A comprehensive analysis of BALF by western blot array suggests that SDF-1 plays an important role in the mechanisms underlying the protective effect of LMDECs against BLM-induced lung injury. LMDECs expressing CXCR4 can respond to SDF-1 and increase their migration

activity and differentiation efficiency to AEC I (Figure 2). On the other hand, SDF-1 induced neither proliferation nor apoptosis in LMDECs (data not shown). Hence, SDF-1 up-regulated in the injured lung may recruit engrafted LMDECs preferentially to the site of injury, and LMDECs efficiently give rise to AEC I to repair the injury. The protective effect of LMDECs against BLM-induced lung injury does not simply attribute to the scavenging of SDF-1 via CXCR4 on LMDECs because HEK 293 cells known to express CXCR4 could not inhibit the histopathological change induced by BLM (Figure 1g).[37] Although LMDECs expressing CXCR4 were administered to the lung, the protein level of CXCR4 in BALF from the BLM + LMDEC group was significantly lower than that from the BLM + PBS group at 7 days after BLM instillation (Figure 2a). As one of plausible explanations, at that time point, LMDECs can in vivo differentiate to AEC I in the BLM-injured lung, the situation of which may lead to the downregulation of CXCR4 expression (Figures 1f and 2b). Taken together, in response to SDF-1 that originally plays a fibrotic inducer in the BLM-injured lung, engrafted LMDECs show a potent countereffect against BLM-induced lung fibrosis. On the other hand, it has been clearly demonstrated that CD44, a receptor for nonsulfated glycosaminoglycan hyaluronan (HA), functions in clearance of low-molecular-weight HA, leading to lung inflammation, and that CD44 deficiency deteriorates the BLM-induced lung fibrosis.[38] The engraftment experiments of classified LMDECs suggest that the protective effect of LMDECs against BLM-induced lung injury depends greatly on that of SP-C^{+}CD44^{+}CD45^{+}Lin^{+} LMDEC[Maj] (Figure 4). Thus, the possibility that CD44 on engrafted LMDEC[Maj] inhibits BLM-induced lung fibrosis by scavenging low-molecular-weight HA cannot be ruled out. Further study is needed for this point.

LMDEC[Maj] showing SP-C-like immunoreactivity are AEC II-like cells, as they show an irreversible fate of differentiating to AEC I in vitro and in vivo (Figure 1). Similarly, the cells having characteristics corresponding to LMDEC[Maj] are large cells at the alveolar-septal junction like AEC II are, but increase in response to the pathophysiological stimuli that decrease the number of total SP-C^{+} cells in the lung (Figure 5). Previous report has demonstrated two subpopulations of SP-C[+] AEC II distinguished by the expression of E-cadherin (E-cad). In addition, most of E-cad[+] AEC II were damaged and quiescent under hypoxic condition. In contrast, E-cad[−] AEC II were undamaged and proliferative with a high telomerase activity under the pathological condition.[39] We have confirmed that LMDEC[Maj] are E-cad[−] by flow cytometric analysis (data not shown). This fact tempts us to consider that LMDEC[Maj] may be a sub-subpopulation of AEC II.

Several reports have clearly demonstrated that p38α is an essential regulator of lung stem/progenitor cells, and that inhibition of p38α enhances self-renewal of stem/progenitor cells.[31,40] Provided that LMDEC[Maj] were committed to alveolar progenitor lineage as a sub-subpopulation of

AEC II, the activity of p38α probably affects the yield efficiency of LMDEC^{Maj} from the mixed culture. Then, we elucidated this hypothesis by means of p38α d.n.-TG mice and a p38 inhibitor, SB203580. Yield of LMDEC^{Maj} from p38α d.n.-TG mice was significantly higher than that from WT mice $(3.01 \pm 0.3 \times 10^6$ vs $2.22 \pm 0.2 \times 10^6$ cells/mouse $(n=4))$. Similarly, application of $10\,\mu M$ SB203580 to the mixed culture from WT mice significantly enhanced yield of LMDEC^{Maj} compared with normal culture condition $(4.86 \pm 0.8 \times 10^6$ vs $2.06 \pm 0.3 \times 10^6$ cells/mouse $(n=6))$. These results suggest that proliferative activity of LMDEC^{Maj} can be negatively regulated by p38α. The fact that the yield efficiency of LMDEC^{Maj} in the case of SB203580 treatment was higher compared with the case of p38α d.n.-TG mice is of interest. In the adherent cells of the mixed culture, the potency of various neighboring cells affecting proliferation of LMDEC^{Maj} may also be regulated by p38.

LMDEC^{Maj} impair several functions specific to AEC II despite the AEC II-like profile. In addition to their unique characteristics of hematopoietic cell lineage, typical lamellar body-like structures were poor by transmission electron microscopy in LMDEC^{Maj}, and the release of SP-D, one of soluble surfactant proteins, was markedly lower in LMDEC^{Maj} than in AEC II prepared according to the usual method (data not shown).[22] To precisely elucidate how LMDEC^{Maj} acquire their unique characteristics, lineage-tracing experiments are needed.

In conclusion, we newly classified a cell population derived from the lung as a new therapeutic option for lung fibrosis. In the case of engraftment of Sca1$^+$ LMDECs, the infiltrated neutrophils induced by BLM may remain in the alveolar space (Figure 4), a concerning finding in translating these findings to human trials. Hence, LMDEC^{Maj} or Sca1$^+$ cells-depleted LMDECs is preferable to nonselected LMDECs as a reparative tool for lung fibrosis.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

AUTHOR CONTRIBUTIONS
K Tanaka and YK developed the concept and designed the experiments. K Tanaka, TF, HU, KN, KY, MH, TS, and YK performed experiments. SK. and K Tatsumi gave conceptual advice. K Tanaka and YK wrote the paper. All authors discussed the results and implications and commented on the manuscript at all stages.

DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

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